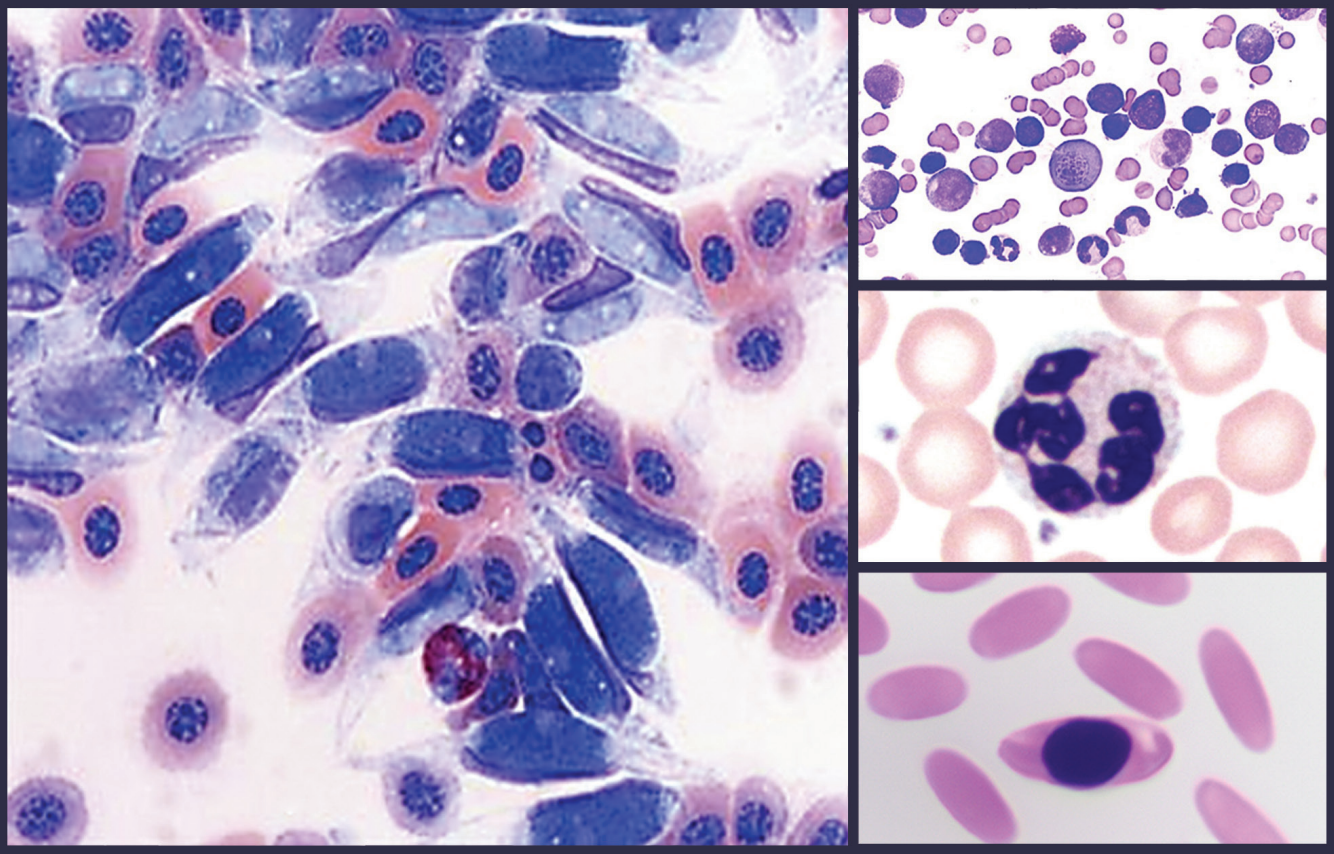
SEVENTH EDITION

SCHALM'S VETERINARY HEMATOLOGY

EDITED BY

MARJORY B. BROOKS | KENDAL E. HARR | DAVIS M. SEELIG

K. JANE WARDROP | DOUGLAS J. WEISS



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EDITED BY

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DEDICATION



A Tribute to Oscar Schalm

Oscar W. Schalm was born in 1909 and reared in Sturgis, Michigan. Upon graduation from Michigan State University, he entered the College of Veterinary Medicine at the urging of fellow students. He married Dorothy Burns, also of Sturgis, in 1930 and after attaining his DVM degree in 1932, he joined the Department of Veterinary Science at Berkeley, California as a research associate. He remained on the Berkeley campus until 1948, earning an M.S. in 1933 and Ph.D. in 1935. In 1948, he relocated to Davis, California, as the Department of Veterinary Medicine became the new School of Veterinary Medicine at the Davis campus.

Early in his career, Oscar established himself as a leader in bovine mastitis research, a subject he continued at Davis. At the founding of the School of Veterinary Medicine, Oscar was assigned the responsibility for clinical pathology, a most fortuitous choice for the field and generations of veterinary students. Oscar focused on the fledgling field of veterinary hematology. With his characteristic energy and productivity, within ten years he had written the first edition of the now classic *Veterinary Hematology*. The original and subsequent early editions represented compilations of materials that he had painstakingly collected and analyzed.

He became an authority in veterinary clinical hematology, and spread the gospel by a prodigious record of publications, national and international seminars, and through his classes at Davis to over 1500 veterinary students. He exemplified the teacher-researcher who can successfully share their expertise and motivate students. As evidence of his dedication to teaching and his students, he was selected three times to receive the School of Veterinary Medicine's Outstanding Teacher Award. He was listed in Educators of America in 1971 and 1973, and in 1973, he received the Davis Division of the Academic Senate's Outstanding Teaching Award.

Oscar's honors, awards, and prizes were many; some among them are the Borden Award in Veterinary Medicine (1964), the Gaines Award for Veterinarian of the Year (1965 and 1972), Alumni Award of Michigan State University (1969), and the Alumni Achievement Award of the School of Veterinary Medicine, University of California, Davis (1980). He was a Fulbright Scholar at the University of Munich in 1959, a visiting scholar and consultant to Israel in 1967, and was named Honorary Life Member of the Israeli Veterinary Medical Association in 1968.

Oscar Schalm retired in 1976, after 44 years of service to the university. He remained an active scholar, speaker, consultant, and contributor to prestigious journals throughout his retirement. At the time of his death, on September 15, 1982, a fourth edition of Schalm's Veterinary Hematology was in preparation. In recognition of Dr. Schalm's fundamental contributions to the field, all subsequent editions of this authoritative textbook have included his name in the title.

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PREFACE

Veterinary clinical pathology has changed considerably since publication of the 6th edition of *Schalm's Veterinary Hematology*. No longer the practitioners of a discipline that deals primarily with the clinical evaluation of dog and cat samples, clinical pathologists are now called on to use their diagnostic skills for a broad variety of animal species. Current specialty areas encompass diverse fields such as pharmaceutical compound and device development and the management of zoo and free-range wildlife. Beyond nontraditional species, clinical pathologists have broadened the scope of their diagnostic expertise to incorporate genomics, proteomics, and metabolomics, and use novel assay platforms such as expression arrays, microfluidic devices, and multiplex immunoassays. Significant advances have also been made in more traditional techniques including flow cytometry, blood typing serology, and platelet function testing.

We have assembled a team of editors capable of covering this exceptional diversity in species and subject areas that now comprise the field of clinical pathology. Dr. Marjory Brooks' career has focused on comparative hemostasis and the development of translational biomarkers for preclinical and diagnostic applications. Dr. Kendal Harr provides expertise in nondomestic species and has devoted the past 15 years to making ASVCP's quality assurance guidelines more robust. Dr. Davis Seelig provides expertise in basic molecular biology, research techniques, and in hematopoietic neoplasia. Dr. Jane Wardrop has expertise in hematological disorders and transfusion medicine. Dr. Douglas Weiss has expertise in bone marrow disorders, infectious disease, and molecular biology.

Specific changes in this edition include a new section titled "Hemolymphatic Tissue" for detailed coverage of basic pathophysiology and disease mechanisms, expanded "Species-Specific Hematology" and "Hematotoxicity" sections, and more in-depth molecular and genetics content. Additionally, we have extensively revised and rearranged chapters to address emerging topics and to provide a more logical sequence for the material. With recognition of the hard work of our contributing authors, we are proud to provide the reader with a comprehensive, coherent, and state of the art presentation of topics in clinical pathology.

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To the teachers and mentors, who set me on the path of discovery, and to the students, clinicians, and colleagues, who continually bring new insights and challenges to the journey.

Marjory Brooks



I would like to express my gratitude to my whole family for their support and patience during the time it has taken to construct this tome. Especially my husband, Bob, who thankfully has fabulous cooking skills (among others) and my daughters, Lily and Maeve, who have, since the start of this work, matured to wonderful, fascinating adults. And, of course, my mom whose voice I still hear and provides guidance to this day. I also appreciate my co-editors, who have helped me become a better editor, reviewer, and author, especially Dr. K. Jane Wardrop.

Kendal E. Harr



To Catherine and Angela, who (in their own unique ways) provided much needed emotional support and relief. To my coworkers and resident trainees, who tolerated the many mornings and afternoons in which I was squirreled away in my office.

Davis Seelig



To my family for their continued support, and to my colleagues around the world, who contributed to the making of this edition. Your work inspires me to be a better author, researcher, teacher, and person.

K. Jane Wardrop



To my parents (Bud and Dorothy) for their strength, moral guidance, and work ethic, my family (Jane, Matthew, Kal) for their love and support, which was essential to my career as well as my well-being, and to Barb for partnering with me in undertaking a whole new vision of how to live in harmony with the earth and all living things.

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SECTION I
Hemolymphatic Tissue

CHAPTER 1

Embryonic and Fetal Hematopoiesis

KELLI L. BOYD and BRAD BOLON

Basic Principles of Hematopoietic Development
  Cell Structure and Function
Primitive Hematopoiesis
  Erythroid Cells
  Other Cells
Definitive Hematopoiesis
Hemoglobin Switching
Molecular Mechanisms Regulating Hematopoietic Development

Acronyms and Abbreviations

AGM, aorta-gonad-mesonephros; Bmp, bone morphogenetic protein; 2,3-DPG, 2,3-diphosphoglycerate; E#, day of embryonic development, where the number indicates age of the embryo in days after conception; EPO, erythropoietin; fL, femtoliter; Gata-1, -2, and -4, GATA-binding proteins 1, 2, and 4; HSC, hematopoietic stem cell; Ihh, Indian hedgehog; IL, interleukin; NK, natural killer; P#, day of postnatal development, where the number indicates age of the neonate in days after delivery; pg, picogram; PU.1, purine box-binding transcription factor 1; Scl/Tal-1, stem cell leukemia/T-cell acute leukemia factor 1.

The complexities of hematopoietic system development have been highly conserved throughout vertebrate evolution. Understanding the embryonic and fetal origins of hematopoiesis provides important insights regarding the function of the adult hematopoietic system. Hematopoiesis in embryonic and fetal animals has been studied intensively for several decades as a model for hematopoietic progression in humans. Recent technical advances have allowed researchers to characterize the spatial and temporal relationships as well as the cellular and molecular mechanisms of hematopoietic development.

This chapter reviews the basic biology of hematopoietic development in the mouse (*Mus musculus*). This appraisal will emphasize hematopoietic events during the embryonic and fetal stages of development, but also will cover selected features of neonatal hematopoiesis.

BASIC PRINCIPLES OF HEMATOPOIETIC DEVELOPMENT

Cell Structure and Function

Blood cells produced at different stages of development differ in morphology and function. Thus, primitive ("fetal") cells fabricated early in gestation have markedly different properties from their definitive ("adult") counterparts produced during late gestation and in postnatal life. This principle has been characterized most completely in erythroid lineage cells. Primitive erythrocytes (RBCs) are formed in the yolk sac, whereas definitive RBCs are produced by the liver and later spleen and bone marrow. Primitive RBCs are nucleated in circulation until approximately day 12.5 (E12.5) of gestation, after which nuclei gradually become condensed before being shed between E14.5 and E16.5.[35]

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Enucleated primitive RBCs retain their large size and can remain in circulation until as late as postnatal day 5 (P5). Both primitive and definitive RBCs are released during most of the latter half of gestation (E10–E18), although the ratio shifts as time progresses from mainly primitive to mainly definitive RBCs.

Primitive and definitive RBCs can be distinguished by their size. The volume of primitive RBCs varies from 465 to 530 femtoliters (fL), which is approximately six times larger than that of definitive RBCs.[35] The hemoglobin content of primitive RBCs, 80–100 picograms (pg)/cell, also is nearly six times the amount found in definitive RBCs.[35] Both primitive and definitive RBCs have basophilic cytoplasm when first produced due to abundant rough endoplasmic reticulum, but basophilia recedes as maximal hemoglobin content is achieved.

Other hematopoietic lineages also differ in cell structure and function during the course of development. Primitive megakaryocytes from the yolk sac contain fewer nuclei of lower ploidy, are about half the size, and respond differently to cytokine stimulation relative to definitive megakaryocytes.[47] Primitive macrophages that originate in the yolk sac[42] lack certain enzyme activities, are capable of division, and survive for extended periods compared to definitive monocyte-derived macrophages. These functional differences are related to the roles that the two cell populations appear to play. Primitive macrophages are the source for many tissue macrophages in embryonic through juvenile stages of development, whereas definitive macrophages are the source for circulating monocytes and resident macrophages characteristic of the adult immune system.

PRIMITIVE HEMATOPOIESIS

The processes that drive primitive and definitive stages of hematopoiesis as well as the events that regulate transition between the two stages are mediated by a constellation of factors.[1,30,45,46] Cell adhesion factors, growth factors, and transcription factors that participate in this process often support differentiation of multiple hematopoietic cell types,[10,29,36] and the dependence of a given cell lineage on any particular molecule may differ between primitive and definitive hematopoiesis.

Erythroid Cells

Hematopoiesis occurs at multiple sites within the embryo and in extraembryonic tissues. The first phase of blood cell production, referred to as primitive hematopoiesis, is responsible for producing blood elements during the earliest stage of embryogenesis. Primitive hematopoiesis takes place in the visceral yolk sac beginning at approximately E7.[15,34] Thus, primitive hematopoietic cells are among the earliest distinct tissues to differentiate in the embryo. Formation of primitive cells declines rapidly after E11. The visceral yolk sac or extraembryonic splanchnopleure (the term for a structure in which mesoderm and endoderm are directly apposed) arises from the migration of extraembryonic mesoderm streaming from the caudal primitive streak along the inner surface of the visceral endoderm. The mesodermal cells committed to initiate and support hematopoiesis have been termed hemangioblasts because the contiguity of primitive hematopoiesis and vasculogenesis in both space and time suggests that primitive hematopoietic and endothelial cells in the yolk sac share a common ancestor.[1,9] Hemangioblasts arise as undifferentiated cells at the primitive-streak stage and commit to producing a particular cell lineage before blood island formation.[34,44] These pluripotent cells also can differentiate into other mesenchyme-derived tissues.

Between E7.5 and E9, hemangioblasts form multiple aggregates termed blood islands.[35] Each blood island contains a central core of unattached inner hemangioblasts (hematopoietic progenitors) surrounded by a rim of spindle-shaped outer hemangioblasts (endothelial progenitors).[15] Nucleated erythroid cells are first recognized in the cores of the blood islands at E8 and are evident circulating in the cardiovascular system starting at E8.25.[18] At this stage embryonic erythroblasts enter the circulation, where they continue to divide until approximately E13.

The majority of cells produced during primitive hematopoiesis are of the erythroid lineage. Committed erythroid colony-forming cells arrive in the yolk sac at approximately E7.25. These cells expand until E8 and then differentiate into primitive erythroblasts; all colony-forming cells have regressed completely by E9,[34] which corresponds approximately to the earliest phase of definitive erythropoiesis. Primitive erythroblasts serve as the sole source of RBCs in the early embryo from E8 to approximately E10.5[34] and remain an important source of RBCs until E13. Thus, embryos with a developmental age between E8 and E11 that are anemic suffer from a defect in primitive erythropoiesis.[26,38] Interestingly, seemingly profound defects in primitive hematopoiesis leading to persistent functional defects in adulthood may not elicit an aberrant hematologic profile in the embryo.

Other Cells

Recent studies suggest that other hematopoietic cell lineages also are generated in the yolk sac during this primitive stage of hematopoietic development. Primitive lymphoid precursors and even some adult stem cells evolve at E7.5 and subsequently seed other sites of hematopoiesis, including the aorta-gonad-mesonephros (AGM) region, umbilical vessels, and liver.[40] Primitive macrophages have been identified in the yolk sac by E8[4]–E9.[34] In vitro experiments have demonstrated that E7.5 yolk sac cells can give rise to functional megakaryocytic precursors by E10.5.[47] Many hemangioblasts actually serve as bipotent or oligopotent progenitors, including those capable of commitment to erythrocytic/myeloid,[4] erythrocytic/megakaryocytic,[27] granulocyte/macrophage,[34] and lymphoid (B cell and T cell)/myeloid

lineages. Stem cells for mast cells have also been reported to arise in the yolk sac during primitive hematopoiesis.[34]

DEFINITIVE HEMATOPOIESIS

The second stage of blood cell production, termed *definitive hematopoiesis*, is thought to arise primarily from the AGM.[3,27] The AGM is an amorphous band of intraembryonic splanchnopleure that encompasses the dorsomedial wall of the abdominal cavity. The AGM domain is the main source of mesenchyme-derived, definitive hematopoietic stem cells (HSCs) that will serve the developing animal during late gestation and postnatal life. Initiation of definitive hematopoiesis ranges between E8.5 and E9.25, with definitive HSCs evident in the AGM by no later than E10. Peak production of HSCs in the AGM occurs between E10.5 and E11.5, at which time they comprise almost 10% of all AGM cells. Although controversial, some AGM-independent HSCs may also arise from the allantois, chorion, definitive placenta, umbilical arteries, and yolk sac. The actual contribution of these secondary sites to the overall HSC population has yet to be defined. However, the placenta appears to serve a particularly important role. The yolk sac also appears to be an essential secondary site because it is a source of multiple progenitor cell lineages and remains for at least a day after the AGM has halted HSC production.[28]

Regardless of their original site of de novo synthesis, HSCs migrate to seed other locations that support definitive hematopoiesis: embryonic liver, followed by embryonic thymus, fetal spleen, and bone marrow (in that order). These latter destinations do not produce HSCs de novo, but rather contain niches suitable for expansion of newly arrived HSCs.[33] The suitability of such niches is controlled by specific characteristics of their stromal support cells.[33] The embryonic liver is colonized first, apparently because it shares many molecular and functional similarities with the yolk sac.[31] It provides the major locus for definitive hematopoiesis from E12 to E16.[39] The HSCs enter the embryonic liver in several succeeding waves between E9/E10 and E12.[12] The first HSCs to enter the liver are pluripotent and can form any type of hematopoietic cell. Their first step in intrahepatic maturation is to commit to a more limited range of lineage options, typically as either an erythromyeloid progenitor or a common myelolymphoid progenitor.[22] Definitive erythroid precursors mature and become enucleated within erythroid islands in the liver before entering the circulation.[27] Liver-derived myelolymphoid progenitors subsequently develop into bipotent cells (B cell and myeloid, or T cell and myeloid) before committing to produce a single-cell lineage.[22] Some T-cell progenitors have a bipotent commitment to natural killer (NK) cell lineage. T-cell precursors destined for transfer to the embryonic thymus are produced even in athymic mice, indicating that the fetal liver may play a role in promoting early T-cell differentiation.[20,21]

Embryonic thymus and fetal spleen are seeded either from the liver or AGM, or both, beginning about E13 for thymus and E15 for spleen. The thymus typically accepts only those HSCs that are committed to make T cells, whereas other multipotent myelolymphoid elements are directed to other sites.[20] The number of T-cell precursors in liver is abundant at E12, but decreases thereafter, whereas the population of intrahepatic B-cell progenitors exhibits a reverse trend.[19] Most types of definitive hematopoietic cells in the spleen arise from precursor cells that commit to a specific lineage before leaving the liver. Multipotent HSCs entering the spleen cease proliferating and differentiate into mature macrophages. These cells may regulate intrasplenic erythropoiesis.

The bone marrow first receives HSCs from hepatic depots at about E16.[39,45] Thereafter, the allocation of colony-forming hematopoietic precursors shifts from a primarily hepatocentric localization at E18 through a more dispersed distribution (bone marrow, liver, and spleen in approximately equal numbers) at P2 to a profile-favoring bone marrow and to a lesser-extent spleen at P4 and after.[49] Thus, the bone marrow, liver, and spleen function cooperatively to regulate definitive hematopoiesis. While cooperating, each organ supports a molecularly distinct subset of hematopoietic progenitors.

Committed hematopoietic progenitors necessary to foster all lineages observed in adult animals arise during definitive hematopoiesis. The AGM-derived HSCs contribute to all major hematopoietic cell lineages. The HSC population from the placenta reportedly supports the genesis of erythroid, lymphoid (both B-cell and T-cell lineages), and myeloid elements. By comparison, the lineages sustained by yolk-sac-derived HSCs are limited to lymphoid and myeloid cells.[40] Whether or not progenitors for a given definitive cell lineage arising from distinct HSC populations exhibit different functional and molecular properties during late fetal and/or postnatal life has yet to be determined.

Late-stage embryos (E13–E15), fetuses (E16 to birth), and neonates which present with anemia are afflicted with a defect in definitive erythropoiesis. Abnormalities associated with this presentation include the total absence of definitive hematopoiesis,[25,41] and an inability of progenitor cells to properly colonize intraembryonic sites of hematopoiesis. Multiple cell lineages may be affected; such a combined effect suggests that the hematopoietic defect occurs in a bipotent or multipotent stem cell rather than in one committed to forming a specific cell lineage.[43] Presentation with late-stage anemia also might result from a general delay in growth and development rather than a focused anomaly in the erythrocytic lineage.[7]

Young animals have circulating blood cell numbers that are different from that of adults.[39] RBC numbers are more than double between birth and young adulthood. Circulating leukocyte counts at birth are approximately 20% of adult levels before increasing to adult numbers by 6–7 weeks of age. Platelet counts in neonates are approximately one-third numbers.

HEMOGLOBIN SWITCHING

Primitive and definitive RBCs bear a battery of seven α- and β-globins, the mix of which varies with the developmental stage. The α-globins are encoded by three genes (ζ, α_1, and α_2), whereas β-globins are encoded by four main genes (εγ, βH1, β*min*, and β*maj*). The globins of a given type (e.g., α- or β-globins) typically exist as a series of closely linked homologous genes and related pseudogenes located on the same chromosome;[16,24] mouse globin genes are carried on chromosomes 7 (β-globins) and 11 (α-globins). All seven mouse globin genes are transcribed during erythroid development, but the production of three—ζ, εγ, and βH1—is limited to primitive RBCs.[23] A consequence of this limitation is that mouse β-globin genes, although closely related to human globins in most respects, do not follow the human pattern of upregulation in the sequence of their chromosomal arrangement.[23,24]

The extent of individual globin gene expression and the blend of globin genes that are expressed vary over time. For example, enucleated primitive RBCs contain relatively more β*min* than do definitive RBCs. At E11.5, β*min* constitutes approximately 80% of the β-globin in circulation. This level is reduced by approximately 60% at birth. Primitive RBCs express increasing levels of adult globins as gestation progresses, whereas definitive RBCs harbor only the adult protein variants. This evolution indicates that the pattern of globin expression switches as the primitive RBCs are replaced by definitive RBCs. Molecular mechanisms which regulate the switching process are complex.[17] The timing of this switch, between E10.5 and E12.5, coincides with the initial escalation in definitive erythropoiesis. Perturbed timing of this switch is a feature of some murine models of hematopoietic disease.[6]

Successful maintenance of the developing conceptus depends on preferential capture of oxygen in embryonic and fetal tissues. Therefore, primitive RBCs generally have a higher affinity for oxygen than do maternal RBCs, although domestic cats are an exception. This sequestration of oxygen is mediated by two primary mechanisms. The mechanism pertinent to the early embryonic period is the greater affinity of embryonic hemoglobin in primitive RBCs for oxygen relative to that of adult hemoglobin.[2] Alternatively, definitive RBCs in the late embryo and fetus possess a lower concentration of 2,3-diphosphoglycerate (2,3-DPG) than do maternal RBCs. Higher levels of 2,3-DPG facilitate oxygen release into tissues. After birth, levels 2,3-DPG content of RBCs rise to adult levels within 10–15 days.

MOLECULAR MECHANISMS REGULATING HEMATOPOIETIC DEVELOPMENT

A wide spectrum of growth factors, hormones, and transcription factors are required to specify the various stages of hematopoietic development in mammals. The entire meshwork responsible for directing any given event has not been completely characterized. Shifting levels of several transcription factors have been shown to modify blood cell production. Insufficiencies in many of these molecules act by forestalling primitive hematopoiesis in the yolk sac. For example, genesis of erythroid precursors is impacted by deficits in GATA-binding protein 1 (Gata-1),[13] shown in vivo to prevent erythroid maturation; Gata-2,[48] demonstrated in vivo to abort precursor expansion; and Gata-4,[5] for which an in vitro shortage thwarts hemangioblast-mediated specification of blood islands and their associated vessels. These effects occur because the GATA consensus elements are critical regulatory regions in many erythroid-specific genes. All cell lineages are affected by stem cell leukemia/T-cell acute leukemia factor 1 (Scl/Tal-1).[38] Abnormal levels of transcription factors can also act later in gestation to disrupt definitive hematopoiesis. For example, purine box-binding transcription factor 1 (PU.1) is required for production of definitive (monocyte-derived) macrophages, but not their primitive (yolk-sac-derived) counterparts. This disparity in response is intriguing in that PU.1 is highly expressed during early hematopoiesis, but fluctuates in various cell lineages as time progresses.[10] Normal genesis of many progenitor cells, including bipotent erythroid/megakaryocytic progenitors as well as B-cell and T-cell progenitors, requires that PU.1 levels be reduced, whereas production of myeloid progenitors necessitates an increase in PU.1.[10]

Secreted molecules also are important regulators of hematopoietic development during gestation. For example, erythropoietin (EPO) sustains both primitive and definitive erythropoiesis by stimulating proliferation and differentiation of immature primitive and definitive RBCs.[25] Reduction in EPO activity within the yolk sac greatly reduces the number of colony-forming cells and erythroblasts via excessive apoptosis. Thrombopoietin fulfills a similar function for megakaryocytes, although other cytokines (interleukin-3 [IL-3], IL-6) and growth factors (granulocyte-colony stimulating factor, stem cell factor) also are required.[47] Other ligand/receptor signaling pathways shown to affect hematopoietic development include the endoderm-derived molecule Indian hedgehog (Ihh)[8] and bone morphogenetic protein 4 (Bmp4),[11] both of which participate in blood island production and vasculogenesis in the yolk sac. In general, secreted molecules act via their interaction with a specific transcription factor.

Cell adhesion molecules of the integrin family are essential for the proper migration of hematopoietic precursors. For instance, β_1-integrins are essential if HSC are to reach the embryonic liver, and later the fetal spleen and bone marrow, at the appropriate developmental stages.[37] A loss of β_1-integrins prevents adhesive interactions between HSCs and endothelial cells, thereby stranding the HSCs within vessels.[32] Some integrins have functions in addition to their targeting role. For example, β_4-integrins are required not only for correct homing but also for expansion and differentiation of erythroid and B-cell precursors in liver, spleen, and bone marrow. As with secreted factors, the activities of some

integrins relate more to late gestation and neonatal stages rather than earlier stages of hematopoietic development. This chronology has been documented for β_4-integrin with respect to lymphoid and myeloid differentiation.[14]

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CHAPTER 2

Stem Cell Biology

DORI L. BORJESSON and JED A. OVERMANN

Defining Stem Cells
  Characteristics
  Types of Stem Cells
  Tests and Markers
    Cluster of Differentiation (CD)34
    Stem Cell Antigen
    Dye Efflux
    c-Kit
    Lin⁻
    Transcription Factors
Bone-Marrow-Derived Stem Cells
Stem Cell Biology
  Regulation of Survival and Pluripotency
    Niche
    Molecular Mechanisms
    Leukemia Inhibitory Factor (LIF)
    Bone Morphogenetic Protein 4 (BMP4) and Basic Fibroblast Growth Factor (FGF)
    Wnt
    Tyrosine Kinase with Immunoglobulin-Like and Endothelial-Growth-Factor-Like Domains 2 (Tie2) and Angiopoietin-1
    Other Cytokines
    Transcription Factors
    Microribonucleic Acids (miRNAs)
  Regulation of Differentiation
Stem-Cell-Associated Diseases
  Stem Cell Failure
  Stem Cells and Proliferative Disorders
Stem Cells in Veterinary Medicine
Stem Cell Utilization in Veterinary Medicine

Acronyms and Abbreviations

ABC transporter, ATP-binding cassette transporter; BMP, bone morphogenetic protein; CD, cluster of differentiation; EPC, endothelial precursor cell; EPO, erythropoietin; ERK, extracellular signal-related kinases; ESC, embryonic stem cell; FGF, fibroblast growth factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; HSC, hematopoietic stem cell; IGF-2, insulin-like growth factor 2; IL, interleukin; iPSC, induced pluripotent stem cells; JAK/STAT, Janus kinase/signal transducers and activators of transcription; LIF, leukemia inhibitory factor; Lin⁻, lineage negative; miRNA, microribonucleic acid; MSC, mesenchymal stem cell; PE, phycoerythrin; PI3K, phosphoinositide 3-kinase; Sca-1, stem cell antigen-1; SCF, stem cell factor; SCID, severe combined immunodeficiency; SP, side population; Tie, tyrosine kinase with immunoglobulin-like and endothelial-growth-factor-like domains; TNK, T cell and natural killer cell progenitor; TPO, thrombopoietin.

DEFINING STEM CELLS

Characteristics

Stem cells are a population of unspecialized precursor cells that have capacity for self-renewal and the ability to differentiate, leading to formation of mature cells and tissues. Hematopoietic stem cells (HSCs) are the reservoir for replacement of blood cells and are present in a frequency of one in every 10,000–100,000 blood cells[3] (Figure 2.1; see Chapters 6–10). Two general functional characteristics are used in defining stem cells. The first of these is the ability of long-term self-renewal. Stem cells have the capability, through mitotic cell division, to maintain a population of undifferentiated cells within the stem cell pool for months to years, and over many cycles of cell division. As stem cells divide, on average, one daughter cell is a replica and remains in an

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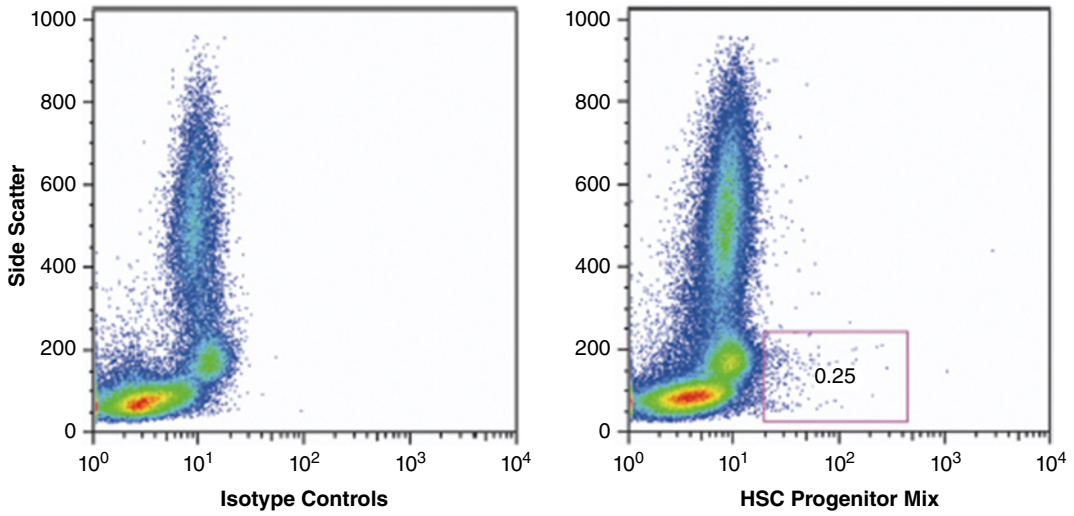


FIGURE 2.1 Hematopoietic progenitor cells in the peripheral circulation. Hematopoietic progenitor cells can be defined by expression of specific cell surface markers such as CD34, c-Kit, and CD133. In this case, such cells are detectable in blood from a normal dog using flow cytometry. Each panel shows a two-dimensional dot plot of FL2 (fluorescence channel-2 set to detect wavelength emission maxima at 575 ± 13 nm) versus right-angle side scatter. Cells were stained using routine protocols; dead cells were excluded using a vital dye. The left panel shows cells stained using an isotype control antibody. The right panel shows cells stained using a mix of antibodies against CD34, c-Kit, and CD133, each labeled with phycoerythrin (PE). In this healthy adult dog, approximately 0.25%, or ~2/1000 viable leukocytes expressed one or more of the progenitor markers. While the frequency is almost 20-fold greater than that seen in most healthy dogs, this case serves to illustrate the presence of hematopoietic progenitor cells in circulation with no associated pathology. (**Source**: Analysis and figure courtesy of Megan Duckett, Masonic Cancer Center, University of Minnesota.)

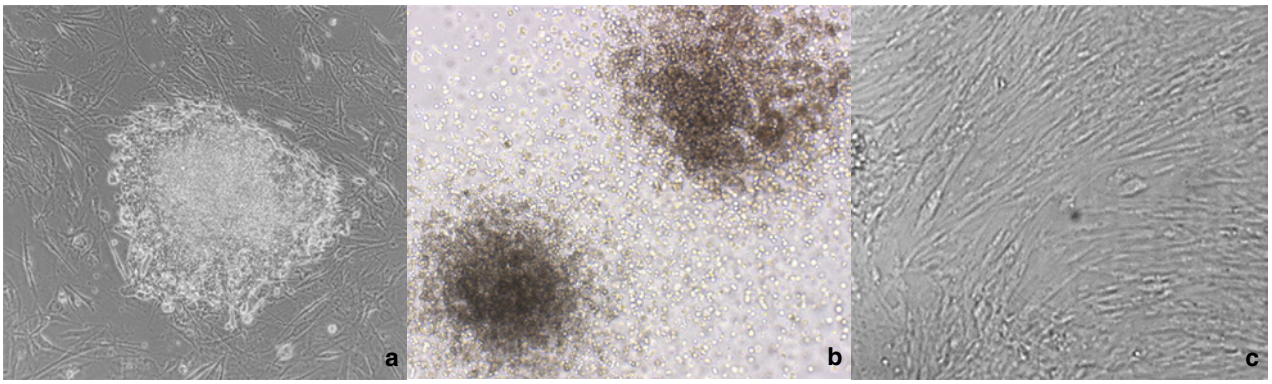


FIGURE 2.2 Light microscopic images depicting the morphology of stem cells in culture. (a) Canine iPSC (passage 12), generated from canine embryonic fibroblasts. Magnification: 100× (**Source**: Figure courtesy of Dr. Amir Kol and Dr. Maria Questa, School of Veterinary Medicine, University of California, Davis); (b) Murine-bone-marrow-derived HSC colonies; each group of cells represents clonal expansion of a single progenitor cell; (c) Equine-bone-marrow-derived MSCs with typical fibroblast morphology.

undifferentiated state, while the second daughter cell is programed to differentiate. This production of two daughter cells with different properties is termed asymmetric cell division. The second characteristic of stem cells is the capacity to form differentiated or specialized cell types.

Types of Stem Cells

Potency is a term that is used to describe the degree or extent to which multiple functional cell lines can be formed. Based on potency, there are four types of stem cells. Totipotent stem cells are those cells that can form entire organisms, including extraembryonic tissues (e.g., placenta). This type of stem cell can be derived from the zygote or early blastomere. Pluripotent stem cells can form all cell types of the body. Embryonic stem cells (ESCs) are the canonical example of pluripotent stem cells (Figure 2.2a). These cells are derived from the inner cell mass of preimplantation blastocysts. ESCs can establish numerous different cells and tissues of the mature organism. Ethical issues surrounding the use of blastocysts for derivation of cell lines drove the development of a novel technique where transcription factors

related to pluripotency are incorporated into the genome of somatic cells to enable reprograming of these cells.[66] This technology enabled differentiated somatic cells to reverse their phenotype to an embryonic state, generating induced pluripotent stem cells (iPSCs, Figure 2.2a). Multipotent stem cells can generate all differentiated cells of a lineage. In the bone marrow (BM), HSCs are the classic example of a multipotent stem cell and much of this chapter will be focused on these cells (Figure 2.2b). HSCs are the reservoir for replacement of blood cells and are present in a frequency of one in every 10,000–100,000 blood cells[10] (Figure 2.1; see Chapters 6–10). Mesenchymal stem cells (MSC) meet the criteria of stem cells for all tissues that are found within bone including the bone tissue itself, cartilage, adipocytes, fibroblasts, and hematopoietic-supporting stroma (Figure 2.2c).[62] Small numbers of stem cells are retained throughout life as adult stem cells and are a reservoir for replacement of short-lived cells or regeneration of damaged tissues (for more information on tissue-resident stem cells, see reviews).[25,67] Finally, unipotent stem cells give rise to only a single-cell line (e.g., spermatogonial stem cells).[38]

Tests and Markers

Stem cells are best defined by functional assays to demonstrate their pluripotent or multipotent nature. For ESCs (and iPSCs), functional potency is demonstrated by the in vitro formation of embryoid bodies and the in vivo generation of teratomas in mice with severe combined immunodeficiency (SCID).[37,54,60] Functional potency for HSCs is demonstrated by repopulation of the hematopoietic system of lethally irradiated mice following transplantation of unpurified bone-marrow-derived cells.[69] Similar to HSCs, functional potency for MSCs was defined through in vivo transplantation assays where an isolated single MSC (skeletal stem cell) could generate a transplantable clonal progeny (ossicle) that included the stromal cells with phenotypes similar to the original explanted cell.[7,8,62]

For practical reasons, the identification and isolation of stem cells now rely largely on the use of a variety of markers such as surface molecules, transcription factors, and dye efflux. Numerous markers are available, and frequently are used in combination, to identify pluripotent and multipotent cells and stem cells within certain types of tissue. While some markers and tests are used more universally to recognize stem cells, special attention will be paid to those used in identifying bone-marrow-derived stem cells.

Cluster of Differentiation (CD)34

CD34 is a cell surface glycoprotein that has traditionally been used in identification and purification of HSCs and progenitor cells.[2] This marker appears to be highly conserved among mammalian species. Experimental evidence suggests that CD34 may be involved in cell adhesion of hematopoietic cells to stromal cells in the bone marrow microenvironment.[30] More recently, however, CD34-negative HSCs called side population (SP) cells were identified. SP cells are thought to be some of the most primitive HSCs because of their high proliferative potential and extreme efficiency at homing to sites of hematopoiesis when injected into recipient mice.[49] CD34 expression on HSCs may thus be related to the degree of activation of these cells, with CD34-negative cells being the most primitive and quiescent.[22]

Stem Cell Antigen

Stem cell antigen-1 (Sca-1) is a cell surface protein often used in identification of murine HSCs. This molecule may play a role in lineage determination.[12]

Dye Efflux

The ability of some primitive HSCs to efflux florescent dye allows for identification of this population, termed SP cells, by flow cytometry.[26,49] This ability appears to be due to increased number or activity of membrane pumps (e.g., ATP-binding cassette transporter [ABC transporter]), a hypothesis supported by the finding of blockage of dye efflux by the drug verapamil, a known inhibitor of these efflux pumps.[49] SP cells lack CD34 expression and have been described in multiple species.[27] As stated earlier, these CD34-negative cells have been proposed to be some of the most primitive HSCs.

c-Kit

c-Kit is a transmembrane tyrosine kinase receptor found on HSCs of multiple species. It binds the ligand stem cell factor (SCF, also called steel factor), and is important in the maintenance, proliferation, and differentiation of HSCs.[79]

Lin$^-$

As an adjunct to the presence of certain markers (e.g., CD34, c-Kit, Sca-1), the absence of markers present on differentiated cells has been used to isolate and purify HSCs. A lineage-negative (Lin$^-$) classification generally indicates that cells are negative for a combination of anywhere from 6 to 14 different lineage markers of mature blood cells.

Transcription Factors

Transcription factors that appear to be important in regulation of stem cell pluripotency and their undifferentiated state have been identified. Most notable are the transcription factors Oct-4, Nanog, and Sox-2, which have been used as markers of embryonic and adult stem cells.[15]

BONE-MARROW-DERIVED STEM CELLS

Within the bone marrow, there appear to be at least three different types of stem cell. HSCs are multipotent stem cells that give rise to the mature cellular elements of the blood (e.g., RBCs, neutrophils, monocytes, platelets,

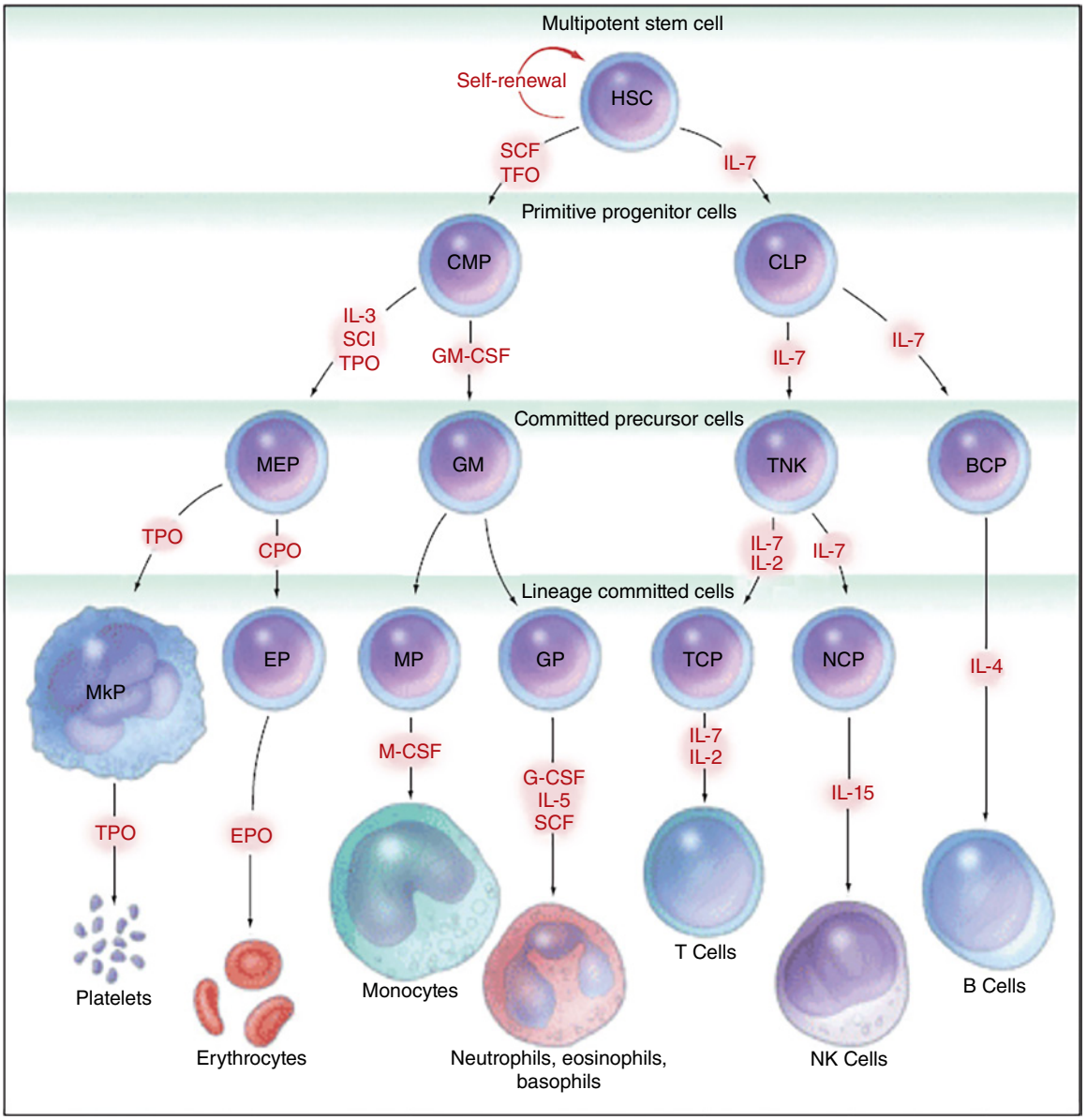


FIGURE 2.3 A general model of hematopoiesis. Blood cell development progresses from a HSC, which can undergo either self-renewal or differentiation into a multilineage committed progenitor cell: a common lymphoid progenitor (CLP) or a common myeloid progenitor (CMP). These cells then give rise to more differentiated progenitors, comprising those committed to two lineages that include T cells and natural killer cells (TNKs), granulocytes and macrophages (GMs), and megakaryocytes and erythroid cells (MEPs). Ultimately, these cells give rise to unilineage committed progenitors for B cells (BCPs), NK cells (NKPs), T cells (TCPs), granulocytes (GPs), monocytes (MPs), erythrocytes (EPs), and megakaryocytes (MkPs). Cytokines and growth factors that support the survival, proliferation, or differentiation of each type of cell are shown in red. For simplicity, the three types of granulocyte progenitor cells are not shown; in reality, distinct progenitors of neutrophils, eosinophils, and basophils or mast cells exist and are supported by distinct transcription factors and cytokines (e.g., interleukin-5 in the case of eosinophils, SCF in the case of basophils or mast cells, and granulocyte colony-stimulating factor (G-CSF) in the case of neutrophils). IL denotes interleukin, TPO thrombopoietin, M-CSF macrophage colony-stimulating factor, GM-CSF granulocyte/macrophage CSF, and EPO erythropoietin. (**Source**: Reprinted from Kaushansky[41], with permission. ©Massachusetts Medical Society 2006.)

etc.; Figure 2.3). The stromal components of bone marrow such as bone, cartilage, fat, and fibrous connective tissue are derived from MSCs, also termed marrow stromal cells. Finally, endothelial precursor cells (EPCs) are a population of bone-marrow-derived cells that function in angiogenesis. EPCs are mobilized from the bone marrow into the peripheral blood, where they home to sites of neovascularization such as those present in areas of inflammation, tumor vascularization, or wound repair (see Chapter 5).

STEM CELL BIOLOGY

Regulation of Survival and Pluripotency

Niche

The niche concept is important to the discussion of stem cell survival and differentiation. Niches are local tissue microenvironments that function to support, maintain, and regulate stem cells. Niches are largely based on the microanatomical organization and structural properties of tissues. These microenvironments are found in various tissues throughout the body; for example, the bulge region of the hair follicle, near the base of crypts in the gastrointestinal tract, and, in the case of HSCs, adjacent to endosteum and bone marrow sinusoids.[50,51,56] Regulation of stem cells by their niche occurs through physical contact and cell–cell interactions with adjacent cells, physical cues through the sympathetic nervous system, as well as elaboration of soluble factors.[50,51,56] Evidence also indicates that stem cells have the ability to influence the cellular elements of their niche. For example, HSCs from mice subjected to an acute hematopoietic stress have an increased ability to direct bone marrow MSCs toward osteoblastic differentiation as a result of HSC-derived bone morphogenetic proteins (BMPs).[40]

Molecular Mechanisms

What are the factors that cause stem cells to go down a pathway of self-renewal and remain undifferentiated versus progression toward lineage differentiation and mature cell phenotypes? The answer to this question is constantly evolving as specific molecular mechanisms are elucidated. Several factors important in the maintenance of stem cell survival, self-renewal, and pluripotency have been revealed in murine ESCs. These factors have been divided into extrinsic factors (e.g., cytokines) and intrinsic factors (e.g., transcription factors).

Leukemia Inhibitory Factor (LIF)

LIF is an interleukin (IL)-6-class cytokine that prevents differentiation of mouse ESCs in culture. Binding of LIF to its membrane receptor results in activation of multiple molecular signaling pathways such as Janus kinase/signal transducers and activators of transcription (JAK/STAT), phosphoinositide 3-kinase (PI3K), and extracellular signal-related kinases (ERK). Although these pathways are common downstream signals of many cytokines, in this context, their activation tends to promote maintenance of self-renewal and pluripotency. Activation of ERK in this example, however, appears to favor differentiation of mouse ESCs. Thus, LIF can activate signals that either promote or inhibit maintenance of an undifferentiated state, and it is the balance between these downstream effects (generally favoring self-renewal and pluripotency) that determines the outcome.[15]

Bone Morphogenetic Protein 4 (BMP4) and Basic Fibroblast Growth Factor (FGF)

BMP4 and basic FGF are additional examples of extrinsic factors that promote self-renewal and pluripotency in mouse ESCs. In the case of BMP4, it appears to work in a synergistic state with LIF.[15] Discovery of additional signaling pathways and factors is likely, as factors important for mouse ESCs are not universal when applied to human ESCs.

Wnt

Specific extrinsic factors involved in self-renewal of HSCs have been identified. The Wnt signaling pathway stimulates self-renewal of HSCs while concurrently inhibiting HSC differentiation. Inducing β-catenin activation, a downstream component of the Wnt signaling pathway, results in increased self-renewal of murine HSCs and limits differentiation of these cells. When inhibitors of the Wnt pathway are added to murine HSCs and growth factors, HSC proliferation is repressed.[61]

Tyrosine Kinase with Immunoglobulin-Like and Endothelial-Growth-Factor-Like Domains 2 (Tie2) and Angiopoietin-1

Tie2/angiopoietin-1 signaling has also been implicated in survival of HSCs. Tie2 is a receptor tyrosine kinase expressed on some HSCs. Angiopoietin-1 is the ligand for the Tie2 receptor and promotes quiescence and increased adhesion of murine HSCs to bone marrow stromal cells. Regulation of the quiescent state and maintenance within the HSC niche is thought to be important in HSC survival through a protective effect against myelosuppresive stresses.[3]

Other Cytokines

Other cytokines important in regulation of HSC survival include SCF, thrombopoietin (TPO), BMP, FGF, insulin-like growth factor 2 (IGF-2), and IL-10. SCF and TPO are common components of most cytokine combinations used in the culture and propagation of HSCs. Although TPO is the primary cytokine involved in megakaryocyte and platelet production, it also has been shown to have significant effects on HSCs. In vitro, TPO promotes survival and expansion of HSCs, and mice that have been genetically altered to lack TPO or its receptor have significantly fewer stem cells.[41,80]

Transcription Factors

The intrinsic factors governing the undifferentiated state of ESCs consist primarily of transcription factors. Most notable are Oct-4, Nanog, and Sox-2. These factors are found in pluripotent cell lines and, in general, downregulation results in differentiation of stem cells. Presence of Oct-4 and Sox-2 appears to be essential for pluripotency; however, the target genes for these transcription factors have not been completely characterized.[15] Several

transcription factors and cell cycle regulators governing self-renewal of HSCs also have been described.

Microribonucleic Acids (miRNAs)

miRNAs are an additional intrinsic molecular mechanism proposed to be involved in maintenance of pluripotent stem cells. miRNAs are short, single-stranded RNA molecules that regulate gene function by suppression of translation through annealing and sometimes degradation of mRNA. Novel miRNAs have been found that appear to be expressed preferentially in undifferentiated ESCs. In addition, evaluation of miRNA expression profiles from ESCs of varying degrees of differentiation as well as cells from mature tissues show repression or loss of specific miRNAs as cells progress to a more differentiated state.[36]

Regulation of Differentiation

Differentiation of stem cells into specific lineages is controlled or directed by factors including cytokines, niche interaction, and regulators of self-renewal and pluripotency. Cytokines influence and guide lineage determination of stem cells and many have been described in the context of HSCs and hematopoietic progenitor cell differentiation (Figure 2.3; see Chapters 6–11).

Stromal cells that constitute the stem cell niche influence differentiation and lineage determination through physical cell–cell interaction and elaboration of soluble or cell-bound factors (e.g., cytokines). Finally, as previously described, there are regulators that promote self-renewal and the pluripotent state of stem cells. For differentiation to occur, these regulators must be inhibited or suppressed. Mechanistically, there may be two general categories by which cells restrict lineage commitment.[68] The first of these involves the spectrum of surface receptors, adhesion proteins, and signaling pathways expressed by a given cell. For example, cytokines play an important role in lineage development. However, if a stem or progenitor cell lacks a cytokine receptor, then that cytokine would have little or no effect on its target. Gene silencing is a second mechanism by which lineage restriction may occur. For cells to differentiate, specific genes are activated or silenced, guiding cells toward a particular lineage. This may be accomplished through mechanisms such as DNA methylation and histone modification, which alter the transcriptional state of the chromatin.

STEM-CELL-ASSOCIATED DISEASES

Stem Cell Failure

The hematopoietic system offers a clear illustration of the effects of stem cell failure. HSCs are responsible for the constant replacement of all cellular components of blood, with HSC failure, cytopenias (e.g., anemia, leukopenia, thrombocytopenia), and their associated clinical manifestations (e.g., lethargy, infection, hemorrhage) ensue. HSC failure can be the result of a number of underlying pathologic processes, including toxic or drug-mediated damage, immune-mediated damage, infectious agents (e.g., parvovirus, feline leukemia virus, and *Ehrlichia* spp.), insufficient stimulation by cytokines and growth factors, and disruption of or damage to the stem cell niche (e.g., myelophthisis, ischemia, inflammation)[11,14,18,74,75] (see Section II).

Stem Cells and Proliferative Disorders

Just as adult stem cells are responsible for replacement of mature cells and tissues, there is strong evidence that cells with stem cell properties underlie the pathology of at least some types of cancer. The hypothesis of cancer stem cells is based on a few basic observations. The first of these is the observation of tumor heterogeneity. Many tumors comprise cells with different morphologies and phenotypes that in some cases loosely resemble the tissue of origin. This suggests a certain degree of differentiation within a population of tumor cells, leading to variability in structure and function. A more primitive precursor cell (i.e., cancer stem cell) could presumably give rise to the different phenotypes within a tumor. The second observation is that transplantation of a tumor required relatively large numbers of cancerous cells, an indication that only small numbers of cells in a given tumor have the ability to form a tumor. Cancer stem cells are present in small numbers within a tumor, and thus relatively large amounts of tissue would be needed to ensure the presence of these cells. Just like normal stem cells, cancer stem cells share the basic functional properties of self-renewal and the ability to differentiate.

In humans, evidence for cancer stem cells has been shown in hematopoietic, brain, breast, colon, prostate, bone, and ovarian cancers, and there is some evidence for the existence of cancer stem cells in animals.[39,43,48,78] Support for the existence of cancer stem cells in humans consists of identification of a subset of tumor cells that express stem cell markers and have exclusive or enhanced ability to form tumors in vitro or in vivo. Evidence exists that cancer stem cells may arise from normal stem cells and/or progenitor cells that have reacquired the ability of self-renewal. The origins of cancer stem cells continue to be explored.

The existence of cancer stem cells has clear implications for understanding cancer biology and treatment in at least certain types of cancers. For example, many chemotherapeutics target rapidly dividing cells. However, cancer stem cells are relatively slowly cycling, thus allowing them to persist with these conventional treatments. Newer therapeutic modalities directed at elimination of cancer stem cells will be important for effective treatment of these types of neoplasia.

STEM CELLS IN VETERINARY MEDICINE

Pluripotent stem cells, including ESCs and iPSCs, have only been variably derived and characterized in veterinary species. For many species, strict criteria for

functional potency have not been met (e.g., teratoma formation). Putative ESC lines have been derived from both companion and farm animals including the dog,[70] cat,[24] rabbit,[72] horse,[46] pig,[53,76] cow,[9,71] goat,[6] and sheep.[53] With most of these lines, there is a lack of appropriate species-specific markers to aid in stem cell identification and there is a loss of pluripotency, over a relatively short number of passages, in vitro and in vivo. Following advances in human and murine stem cell biology, a number of research groups have developed iPSC lines for companion and farm animals from a wide variety of tissue sources including adult and fetal fibroblasts.[20,29,35,47,52,63] New tools and resources, along with the ongoing advances being made in mouse and human stem cell biology focused on identifying factors critical to maintenance of pluripotency, provide promise for further identification and optimization of pluripotent stem cells for veterinary species.

STEM CELL UTILIZATION IN VETERINARY MEDICINE

Pluripotent stem cells are likely decades away from an FDA-approved veterinary product; however, iPSCs hold tremendous promise for the study of disease processes in vitro and the development of patient-specific cell-based regenerative therapies. In farm animals, the development of technologies surrounding ESCs and iPSCs opens the possibility for genomic selection, genome editing, and production of domestic ungulates with high genetic value.[65] These techniques can also be used to develop "tissue in a dish," 3D culture systems (including organoids) that permit novel, physiologically relevant in vitro systems to study cell function, host–pathogen interactions, novel therapeutics, and cell–cell interactions.[28,33,42,57,77] In zoo medicine, technologies are being harnessed to support wildlife conservation with novel resources including the Frozen Zoo® that maintains and stores irreplaceable living cell lines, gametes, and embryos to support conservation and assisted reproduction.[31]

HSCs are the only approved stem cell therapy in the United States. Dogs have been used as models for human hematopoietic cell (BM and HSC) transplantation for decades.[17,21,44] Recently, autologous HSC transplantation in dogs with lymphoma has developed as a viable treatment modality.[19] Dogs with B- and T-cell lymphoma are treated in a clinical setting with autologous peripheral blood progenitor cell transplants, using peripheral blood CD34+ progenitor cells harvested using an apheresis machine.[19,73]

A number of countries have approved human MSCs for a variety of clinical disorders, including graft versus host disease; however, there are no USA FDA-approved veterinary MSC products as of 2019. MSCs were largely viewed as powerful tools for orthopedic repair given their ability to differentiate into lineages including bone, cartilage, and fat. However, data now suggest that some of the most powerful MSC functions are their nonprogenitor functions including their ability to modulate angiogenesis and cells of the immune system.[13,23,45] MSCs also exert strong antimicrobial effects through indirect and direct mechanisms, partially mediated by the secretion of antimicrobial peptides and proteins.[1] Many of the immune modulatory and anti-inflammatory functions of MSC are mediated through a paracrine secretome including exosomes.[59] In veterinary medicine, MSCs have been widely used for a variety of diseases and disorders.[4,5,16,32,34,55,58,64] These clinical trials have largely proven the long-term safety of MSC administration; however, reported efficacy is highly variable. As with any stem-cell-based product, long-term, blinded, controlled prospective clinical trials will be needed to support product development based on strong biologic evidence of function.

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CHAPTER 3

Structure of the Bone Marrow

NICOLE I. STACY and JOHN W. HARVEY

Introduction
Supporting Structures
Vasculature and Sinus Architecture
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Lymphoid Cells (Lymphopoiesis)
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Acronyms and Abbreviations

AGM, aorta-gonad-mesonephros; CMP, common myeloid progenitor; DCP, dendritic cell progenitors; ECM, extracellular matrix; EPO, erythropoietin; FLT3L, (FMS)-like tyrosine kinase 3 ligand; GM-CSF, granulocyte–macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; HSC, hematopoietic stem cell; MDP, monocyte–dendritic cell progenitor; NK, natural killer; PTH, parathyroid hormone; PTHrP, parathyroid hormone related protein; RANK, receptor activator of nuclear factor kappa B; SCF, stem cell factor; TNF, tumor necrosis factor; TNFR, TNF receptor; TPO, thrombopoietin; VCAM, vascular cell adhesion molecule.

INTRODUCTION

Hematopoiesis first occurs during embryonic development in blood islands in the yolk sac, followed shortly thereafter within the aorta-gonad-mesonephros (AGM) region of the embryo in mammals.[26] Hematopoiesis subsequently shifts to liver, spleen, and eventually to the bone marrow during gestation.[17,40] The bone marrow develops in the embryo during the second trimester and becomes the major site of hematopoiesis at time of birth and continues in this function in adult mammals.[50] Bone marrow is a diffuse organ which constitutes approximately 3% of the body mass in rats, 2% in dogs, and 5% in humans.[51] Hematopoietic tissue is highly proliferative, producing billions of cells per kilogram of body weight every day.[17] Differences in locations of active bone marrow by species and changes due to aging need to be considered. Active marrow is mainly present in flat bones, vertebrae, and the proximal ends of the humeri and femurs in most adult mammals. Young mammals have red, active marrow with little fat throughout skeletal bones. Adipose tissue gradually accumulates in bone marrow as animals age to the extent that long bones primarily have yellow, fatty, inactive marrow.[17]

In nonmammalian species, major sites of hematopoiesis vary by taxon, and changes due to aging have not been described (to the authors' knowledge at the time of writing of this chapter). Invertebrates have stem cell niches within various hematopoietic organs that are structurally connected with the open vasculature.[22] Hematopoiesis in bony fish occurs mainly in the anterior kidney (referred to as the head kidney) and also in spleen, liver, intestines, and thymus.[18,20] In elasmobranchs, it is present in the epigonal organ (granulocytes, lymphocytes), spleen (lymphocytes, erythrocytes), organ of Leydig in the submucosa of the alimentary tract (granulocytes, lymphocytes), and thymus (lymphoid).[14,23] Hematopoiesis occurs in amphibians in the spleen and liver, and less frequently in kidney and bone marrow.[2,18] In reptiles, hematopoiesis occurs in bone marrow (e.g., proximal femur or tibia of legged reptiles), spleen, liver, and thymus.[12] In adult birds, hematopoiesis is mostly present in bone marrow (mainly erythropoiesis and thrombopoiesis, with granulopoiesis in

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other tissues such as liver and spleen). While sampling of hematopoietic tissue in invertebrates, amphibians, and fish is performed during postmortem examinations, bone marrow sampling for cytological evaluation in bird patients can be achieved through the proximal tibiotarsus, keel, and most long bones except for pneumatic bones.[7,8] Bone marrow sampling is not recommended in live reptile patients, since it does not exfoliate well due to the fibrous nature of their bones that will even render postmortem tissue imprints of poor diagnostic quality. Therefore, histopathology is needed, but often also has limited diagnostic utility in the assessment of the actual status of hematopoiesis in reptiles, since extramedullary hematopoiesis can be prominent in liver, spleen, and thymus.[49]

Bone marrow consists of hematopoietic cells, vascular structures (including endothelial cells and myocytes), neural elements, supporting connective tissue cells (including adipocytes and reticular cells), extracellular matrix (ECM), and a myriad of soluble factors.[54] These elements are arranged in ways that create intravascular and extravascular spaces. These components create highly specialized environments with cellular communication through cytokines, growth factors, hormones, and interaction with ECM, which create specific niches for maintenance, proliferation, and differentiation of hematopoietic stem cells (HSCs) hematopoietic progenitors, and various progeny.

The complex vasculature and rich innervation of the marrow reflect the plethora of signals and mechanisms contributing to control and regulation of hematopoiesis. Bone marrow is a dynamic organ capable of structural and functional remodeling when responding to physiological changes (e.g., nutritional factors, endocrine signals, and age) or to diseases resulting in varying demands for production of RBCs, WBCs, and platelets. This chapter will review the structure of bone marrow with a brief conceptual framework for structural and functional relationships among the different components of bone marrow. For a more thorough discussion of the biochemical and molecular control of hematopoiesis and the hematopoietic microenvironment, the reader is referred to Chapters 1 and 4.

SUPPORTING STRUCTURES

Hematopoietic tissue is embedded within a rigid bony cortex, and is structurally supported by a meshwork of trabecular bone that serves as a partial scaffold for additional structural components that make up the stroma of the marrow including adipocytes, reticular cells, and ECM. In addition to providing physical support, each of these structural components contributes to the specialized microenvironment of hematopoietic tissue, either directly or via vascular connections. Hematopoiesis in mammals occurs in extravascular hematopoietic spaces which are located between venous sinuses that are composed of a luminal endothelial cell layer and an abluminal layer of adventitial reticular cells (fibroblast type) that provide a scaffold through cytoplasmic processes.[17]

Unlike other tissues that are arranged in stratified layers of developing cells, bone marrow is composed of a seemingly unstructured mixture of cells originating from different cell lineages and of various developmental stages, with unique niches that provide the necessary optimized microenvironment for hematopoiesis.[26] Bone within the marrow cavity is lined by a thin layer of elongated endosteal cells; this layer is punctuated with occasional osteoblasts and osteoclasts, and may be traversed by endosteal blood vessels connecting the hematopoietic space with bone in which osteocytes reside. Osteoblasts contribute to bone production and are derived from multipotent mesenchymal stem cells that also give rise to bone marrow endothelial cells, reticular cells, and adipocytes.[36] Osteoclasts are multinucleated cells derived from fused monocyte–macrophage precursors under the influence of numerous humoral signals, including those from osteoblasts.[36] Osteoblasts and osteoclasts remodel bone within the marrow space, while influencing the endosteal environment and presumably contributing to regulation of HSC proliferation and trafficking.[34] Osteoblasts and osteoclasts produce various cytokines, and interplay between bone and hematopoietic cells can influence bone turnover and remodeling.[36,41] Osteocytes, which originate from osteoblasts, are embedded in the bone matrix. Through their cellular connections with nearby osteocytes, osteoblasts, and cells lining the endosteal surface, they regulate hematopoietic stem cell and progenitor cell mobility and migration into the circulation.[3]

Fine, spindle- to stellate-shaped fibroblast-like reticular cells extend from endosteal regions into the parenchyma of hematopoietic tissue or form adventitial reticular cells supporting the endothelium of venous sinuses.[55] These cells derive from mesenchymal stem cells in the bone marrow, have extensively branched cytoplasmic processes, and form a supporting meshwork. Bone marrow reticular cells, with support from endothelial cells, produce structural fibrils such as collagen fibers, reticulin fibers, laminin, and fibronectin, and ground substance composed of water, salts, glycosaminoglycans (e.g., heparan sulfate, dermatan sulfate, hyaluronic acid), and glycoproteins, which, all together with basal laminae of endothelial cells, are referred to as the ECM.[26,44] Similar to other supporting cellular structures of the marrow, the ECM participates in both the structural and biochemical support of hematopoiesis through entrapping growth factors and through facilitation of cell-to-cell interactions between hematopoietic cells and stromal cell components.[35,42]

Bone marrow contains predominantly types I and III collagen, which take their final form after secretion into the extracellular space and undergoing enzymatic modification. Reticulin fibers are fine, argyrophilic fibers that are composed primarily of type III collagen fibrils surrounding a core of type I collagen embedded in a matrix of glycoproteins and glycosaminoglycans.[35] By contrast, coarse collagen fibers are predominantly type I collagen with less interfibrillar material than reticulin fibers. Although collagen and reticulin fibers are not prominent in routinely processed histology sections,

special stains can enhance their visualization. Coarse collagen fibers can be visualized with Mallory's or Masson's trichrome stains, whereas Gomori's silver stain highlights the presence of reticulin fibers. These stains can be useful in differentiating conditions resulting in reticulin fibrosis versus collagen fibrosis.[26,53]

Adipocytes are the most abundant stromal cells in bone marrow. In health, adipose tissue occupies approximately 25–75% of the bone marrow space, depending on the age of the animal.[26] Adipocytes are interspersed among hematopoietic cells and supporting structures, and present in various proportions depending on sites of active bone marrow in adult mammals. Although the relationships and communication between bone formation and adipose tissue are still not clearly understood, adipocytes and osteoblasts originate from mesenchymal stem cells within the bone marrow, with these compartments holding a reciprocal relationship and the potential for transdifferentiation between both cell types.[39,43] Given their origin from mesenchymal stem cells, adipocytes are believed to share common hematopoietic functions with reticular cells.[19]

Both brown and white adipose tissue are present in bone marrow; differences in biological function of these types of fat are not fully understood. Bone marrow adipose tissue tends to be relatively resistant to lipolysis during starvation compared with adipose tissue elsewhere in the body.[46] However, starvation resulting from many causes can lead to gelatinous transformation of bone marrow (serous atrophy of fat). This condition is characterized by loss of hematopoietic cells, peripheral cytopenias, atrophy of fat, and replacement of fat by acid mucopolysaccharides (mainly hyaluronic acid), which typically can be visualized with Alcian blue stain at pH 2.5.[26]

In addition to providing structural support, adipocytes reportedly participate in the hematopoietic microenvironment, notably as suppressive regulators as shown by lower frequencies of progenitor cells and relatively quiescent stem cells in adipocyte-rich bone marrow in mice.[38] However, cells derived from bone marrow adipose tissue are capable of supporting differentiation of hematopoietic progenitors in vitro.[10,13] Adipose tissue fulfills important endocrine and paracrine functions through production of hundreds of adipokines which are involved in numerous regulatory processes, including hematopoiesis, metabolism, and inflammation.[13,15]

VASCULATURE AND SINUS ARCHITECTURE

Several essential functions highlight the importance of the vasculature to the bone marrow microenvironment, including its contribution to structure, to regulation of movements of hematopoietic cells (e.g., extravascular, transendothelial), and, together with stromal cells, to production of ECM.[26] Bone marrow endothelial cells are specialized cells that support the long-term proliferation of hematopoietic progenitor cells of megakaryocytic and myeloid origin through cytokines, and with HSC trafficking and possibly proliferation through cell-to-cell contact.[30]

Nutrient arteries provide the major blood supply to the bone marrow (Figure 3.1). They enter the medullary cavity via one or more nutrient canals that also may contain one or two nutrient veins (e.g., two for long bones, several for flat bones).[51] Once the vessels have penetrated the cortex, ascending and descending branches bifurcate from the main vessels, coiling around the main venous bone marrow channel and central longitudinal vein. These branches form numerous arterioles and capillaries that penetrate the endosteal surface of the bone to communicate with cortical capillaries derived from arteries that supply surrounding muscle tissue. These interactions facilitate communication and reciprocal regulation between hematopoietic cells and bone.[36] Capillaries derived from the nutrient artery extend as far as the Haversian canals before coursing back to the bone marrow and opening into the venous sinuses. Periosteal arterioles penetrate cortical bone to form a second arterial system for the bone marrow. These vessels form branching networks of medullary venous sinuses that collect into the large central venous sinus; from there, blood enters the systemic circulation via the emissary vein, which exits through the nutrient foramen.[1]

Hematopoiesis occurs in extravascular spaces between venous sinuses in postnatal mammals, with

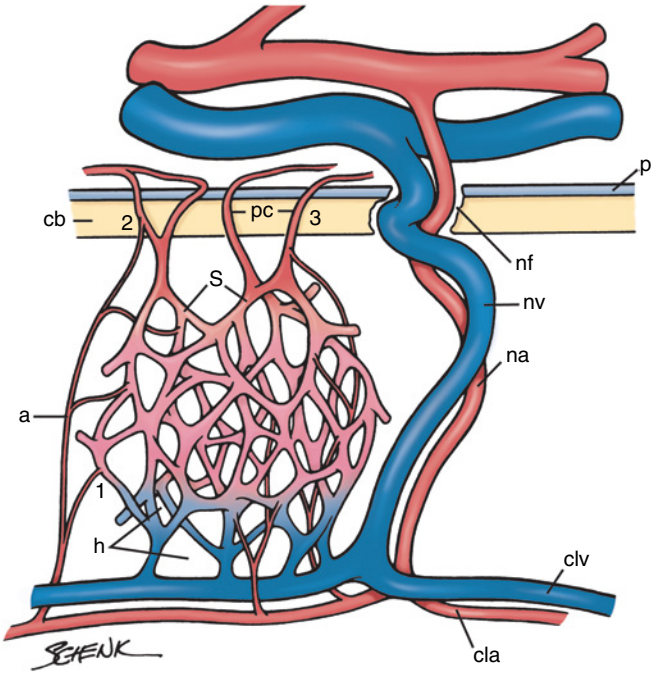


FIGURE 3.1 Anatomy and circulation of the bone marrow. Periosteum (p), cortical bone (cb), nutrient foramen (nf), nutrient artery (na), nutrient vein (nv), central longitudinal artery (cla), central longitudinal vein (clv), periosteal capillaries (pc), arteriole (a), sinuses (s), hematopoietic compartment (h), anastomosis of the nutrient capillaries and sinuses (1), anastomosis of the nutrient artery capillaries and periosteal capillaries (2), anastomosis of the periosteal capillaries and sinuses (3). (**Source:** From Alsaker[60])

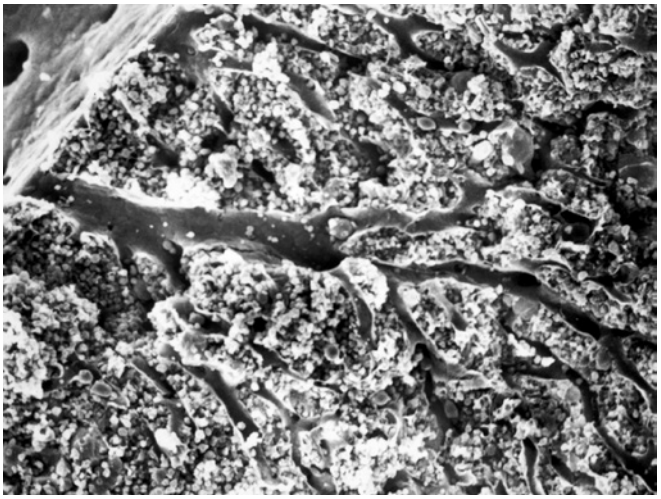


FIGURE 3.2 A scanning electron micrograph of the cut surface of bone marrow showing a system of vascular sinuses originating at the periphery of the marrow (right side of field) and draining into a large vein (upper left corner). The large vein has several apertures in its wall, representing tributary venous sinuses. Hematopoietic tissue lies between the vascular sinuses. (**Source:** From Weiss[54])

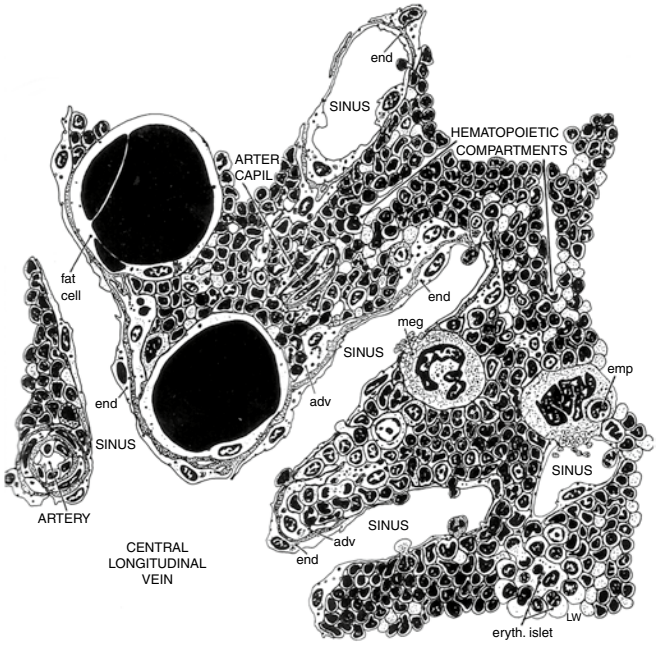


FIGURE 3.3 Schematic view of a cross-section of bone marrow near the central longitudinal vein. Hematopoietic cells lie in the hematopoietic compartment between the vascular sinuses that drain into the central vein. The sinus wall consists of endothelial cells (end), a basement membrane, and, in some areas, adventitial stromal cells (adv). Megakaryocytes (meg) lie against the outside of the vascular sinus wall and discharge proplatelets directly into the vascular lumen through apertures in the sinus wall. Erythroid cells are shown developing in an erythroid islet (eryth islet) around a central macrophage. Emperipolesis (emp), the entry of megakaryocyte cytoplasm by other cells, is occasionally observed. (**Source:** From Weiss[55])

close morphological and functional relationships of cells that line the venous sinuses (Figures 3.2 and 3.3). Reticular cells maintain close physical relationships with hematopoietic cells close to the sinus walls, frequently wrapping around or otherwise contacting hematopoietic precursors.

Sinus endothelial cells produce adhesion molecules, ECM components, and chemokines that not only promote hematopoiesis but also regulate translocation of cells and other substances between the extravascular space and the systemic circulation. Cell egress from the extravascular space into the vascular sinus occurs transcellularly through thin parajunctional zones of endothelial cell cytoplasm in vascular areas lacking basement membrane and adventitial reticular cells.[27]

Endothelial progenitor cells in the bone marrow can be recruited for angiogenesis and directed through surface receptors to healing tissues in adults. These endothelial progenitor cells also give rise to pluripotent angioblasts during fetal vasculogenesis, which can mature into endothelial cells of arteries, veins, or lymphatics, or into pericytes or myocytes surrounding blood vessels.[47]

INNERVATION

Primary innervation of the bone marrow is achieved via myelinated and more numerous nonmyelinated fibers. These fibers originate in spinal nerves entering through the nutrient foramen, although some innervation may originate from the epiphyseal and metaphyseal foramina.[6,51] Once inside the medullary cavity, the mixed myelinated and nonmyelinated nerve bundles, surrounded by a thin perineurium, divide to parallel the arterial vasculature of the bone marrow.[6] The main branches of the arterial vessels are surrounded by several nerve bundles, whereas arterioles and capillaries may be accompanied by only a single fiber, with nerve endings contacting vascular smooth muscle cells or periarterial reticular cells.[56] The sinusoidal system is less richly innervated than the arterial vasculature, with nerve endings frequently contacting the walls of sinusoids. Other nerve fibers appear to terminate within the hematopoietic parenchyma or along the endosteum.[6,16]

Autonomic innervation is mainly responsible for bone marrow regulation, homeostasis, stem cell proliferation and motility, and response to stressors. These effects result from direct interactions with progenitor and stem cells, or indirectly with other cells of the microenvironment via a plethora of receptors, neuropeptides, and neurotransmitters.[5,57] Even nerve-associated nonmyelinating Schwann cells are involved in maintenance of dormant HSCs.[57] Through a myriad of receptors and molecules, neural signaling is involved in hematopoiesis, inflammation, immune functions, and neoplasia.[3,24]

CELLULAR ORGANIZATION

The medullary cavity comprises an intricate three-dimensional complex of hematopoietic cells between vascular sinuses (Figure 3.3). Various cell lineages

localize to specific niches via adhesion molecules (e.g., integrins, lectins, other receptors) that interact with ligands, receptors, and/or ECM components. Lymphocytes, macrophages, and immature granulocytes are concentrated near the endosteum/bony trabeculae and arterioles, and megakaryocytes, erythroid cells, and mature granulocytes are located near venous sinuses.[1,37]

Hematopoietic cells originate from a common, self-renewing pluripotent HSC population which gives rise to committed lymphoid and myeloid progenitor cells.[17] Lymphoid progenitor cells generate lymphocytes, whereas the myeloid progenitor cells generate erythroid cells, megakaryocytes, basophils, eosinophils, and a common granulocyte–macrophage cell that produces neutrophils and macrophages. Hematopoietic cells continue to divide as they mature, resulting in progressively higher numbers (mitotic pool) and continue to mature after they stop dividing (postmitotic pool). The hematopoietic tissue is effectively controlled via local and systemic mechanisms, including numerous cytokines and growth factors, to maintain a steady state of blood cell kinetics in health and to respond rapidly to stimuli or disease.[17]



FIGURE 3.4 Elongated proplatelet processes (center and bottom left) are visible in a venous sinus within bone marrow from a dog. Regional constrictions along the length of the central proplatelet are consistent with platelet formation by fragmentation. (**Source:** From Handagama et al.[61])

Megakaryocytes and Thrombopoiesis

Megakaryocyte development begins with the megakaryoblast, progresses to promegakaryocytes and basophilic megakaryocytes, and results in formation of mature megakaryocytes. Thrombopoiesis is mainly regulated by thrombopoietin (TPO), which is produced primarily in the liver at a relatively constant rate and bound by platelets through their TPO receptor. Thus, in the face of thrombocytopenia, reduced numbers of platelets bind less TPO, which results in higher free TPO in plasma and thus in stimulation of thrombopoiesis.[17]

Megakaryocyte precursors are located near vascular sinuses. Sinus endothelial cells are an essential component of the megakaryocyte microenvironment, supporting megakaryocyte differentiation and maturation.[27] Megakaryocyte precursors progressively enlarge as they mature to become the largest cell in the bone marrow.[4] Their nuclei increase from a single nucleus to a large multilobulated nucleus through a process termed endomitosis (i.e., replication of DNA without cellular division), resulting in a polyploid cell (8–32N).[4,17] The cytoplasm of early megakaryocytic precursors is scant and, as cells continue to mature and increase in size up to 50–200 μm in diameter, it becomes deeply basophilic, more abundant, and then lightly basophilic to eosinophilic, and filled with abundant magenta-staining granules when visualized using Wright and/or Giemsa stains. The location of mature megakaryocytes adjacent to vascular sinuses enables cytoplasmic processes of megakaryocytes to extend through endothelial gaps and extend proplatelets directly into the lumen of vascular sinuses (Figure 3.4). Anucleate platelets are released from proplatelet processes into the peripheral circulation where their life span is about 6 days in dogs.[26] In contrast to mammals, thrombopoiesis of nonmammalian vertebrates begins with the thromboblast and results in production and release of nucleated thrombocytes.[8]

Erythroid Islands and Erythropoiesis

Stages of erythropoiesis include rubriblasts, prorubricytes, rubricytes, metarubricytes, reticulocytes, and non-nucleated mature erythrocytes in mammals (see Chapter 6). Erythropoiesis is regulated by the glycoprotein erythropoietin (EPO) in addition to other cytokines, including interleukin (IL)-3, granulocyte–macrophage colony-stimulating factor (GM-CSF), TPO, and stem cell factor (SCF).[26] Peritubular interstitial cells of kidneys produce EPO in response to hypoxia. EPO promotes proliferation and inhibits apoptosis of erythroid progenitors and early erythroid precursors. It also has a multitude of other effects on endothelial cells.[26] Iron metabolism is intricately linked to erythropoiesis, as it is an essential component of hemoglobin. Other nutrients including amino acids, copper, essential fatty acids, and vitamins are also necessary, and deficiencies can impair adequate erythropoiesis.[26]

As erythroid precursors mature and continue to divide, they become smaller, their nuclear to cytoplasmic ratio reduces, their cytoplasm becomes less basophilic and more polychromatophilic, and the nuclear chromatin becomes condensed. In mammals, the nucleus is extruded at the metarubricyte stage, resulting in formation of the reticulocyte. The reticulocyte retains ribosomes and mitochondria for hemoglobin synthesis, but these are ultimately lost when the cells mature to an erythrocyte.[26] In most mammals, except for horses and presumably closely related ungulates, reticulocytes are released from the bone marrow and continue to mature in the peripheral blood and the spleen. Horses do not

release reticulocytes into the circulation, similar to observations by the authors in some other ungulate species (e.g., rhinoceros, elephant). Nuclei are not extruded as erythrocytes develop in nonmammalian species. However, early nucleated erythroid precursors, with basophilic or polychromatophilic cytoplasm, as well as mitotic cells, may be present in the peripheral blood in response to anemia in these taxa.

Erythroid precursors develop in clusters around central macrophages that can be observed occasionally in bone marrow cytological samples (Figure 3.5). These have been referred to as erythroid (erythroblastic) islands. However, examination of three-dimensional histological sections indicates that most of these cellular aggregates may consist of elongated "erythroblastic cords" that are supported by stromal cells at their axial portion. These erythroblastic cords are reportedly closely connected with each other and interwoven with hematopoietic compartments and vascular sinuses, sometimes forming presumed perivascular structures. Segregated islands can be observed in close connection with sinuses.[37] Central macrophages are surrounded by erythrocyte precursors, whether in islands or cords.

Facilitated through adhesion molecules, including integrin α4β1 on erythroid precursors and vascular cell adhesion molecule 1 (VCAM1) on central macrophages, erythroid precursors proliferate and differentiate around a central macrophage that projects membranous processes in intimate contact with developing erythroid cells.[45] Central macrophages secrete soluble factors that promote proliferation, differentiation, and hemoglobinization of erythroid cells.[33] However, central macrophages can also produce inflammatory cytokines that inhibit erythropoiesis.[28] Central macrophages also phagocytize extruded nuclei that are enclosed in a thin layer of cytoplasm and plasma membrane, also known as pyrenocytes, and defective cells.[9] Maturing erythroid precursor cells are found in concentric circles surrounding the central macrophage with more immature forms closer to the macrophage.[1] The central macrophages are recruited from a subset of resident macrophages that originate from monocyte precursors. Erythroid islands are generally located near venous sinuses because they are capable of migration toward venous sinusoids as they mature.[9,58]

Granulocytes and Granulopoiesis, Monocytes/Macrophages (Monocytopoiesis), and Osteoclasts

Granulocytes and monocytes/macrophages share a common myeloid progenitor (CMP) that originates from an HSC. This CMP produces all nonlymphoid hematopoietic cell lines, including macrophages, dendritic cells, osteoclasts, and mast cells.[32] In granulopoiesis, the myeloblast is the earliest light microscopically recognizable precursor of granulocytes. Each myeloblast is capable of producing 16–32 progeny cells.[17] The mitotic pool is comprised of myeloblasts, promyelocytes, and myelocytes, whereas the postmitotic pool consists of metamyelocytes, bands, and segmented stages.[26] Neutrophils, eosinophils, and basophils develop in a parallel fashion from myeloblasts to mature cells. Granulocytes, similar to RBCs, decrease their cellular size and nuclear to cytoplasmic ratio as they mature. After primary reddish-purple granules lose their capacity to pick up routine cytologic stains (Wright and/or Giemsa), secondary specific granules become visible in the myelocyte stage and allow differentiation of the various granulocytic lineages. At the metamyelocyte stage, the round nucleus elongates and indents to form a bean-shaped nucleus before ultimately forming segmentations at full maturation.[26,46] Mature segmented neutrophils are stored in the bone marrow storage pool of which the size is species-dependent and which has implications for accurate leukogram interpretation.[26]

The monocyte–dendritic cell progenitor (MDP) that originates from the CMP produces monocytes and dendritic cell progenitors (DCP).[59] Monoblasts, the next

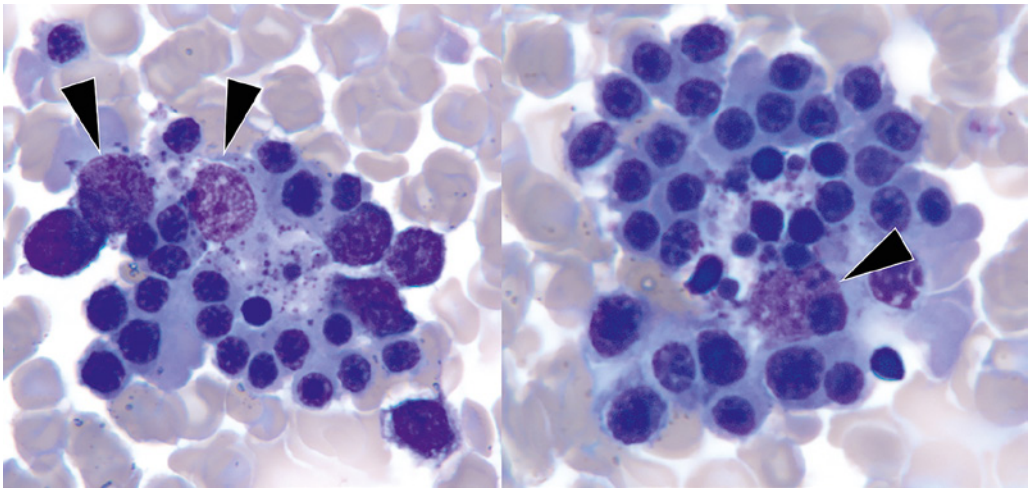


FIGURE 3.5 Erythroid islands in a bone marrow aspirate of a koala (*Phascolarctos cinereus*). Central macrophages (black arrowheads) with intracytoplasmic globular gray-basophilic pigment (presumptive hemosiderin) surrounded by developing erythroid precursors. 100×, Wright–Giemsa stain.

maturation stage after MDPs, are morphologically very similar to myeloblasts except for their nucleus, which is more irregular to convoluted. This morphological distinction can be useful in the cytological differentiation of acute myeloid leukemias.[26] developmental stages after the monoblast include promonocytes (which may be morphologically similar to neutrophilic myelocytes or metamyelocytes) and then finally monocytes.[26] Despite their uniform morphological features (e.g., round-, kidney-, or band-shaped nucleus, fine nuclear chromatin), several functional subsets develop from monocytes. This includes progenies that either leave the circulation and generate macrophages in tissues, or differentiate into inflammatory dendritic cells, depending upon effects of various cytokines in inflammatory or other conditions.[59] In addition, monocyte precursors fuse to produce osteoclasts under the regulation of macrophage-CSF, a family of biologically related tumor necrosis factor (TNF) and TNF receptor (TNFR) superfamilies that consist of receptor activator of nuclear factor kappa B (NF-κB) (RANK), its ligand RANKL, and osteoprotegerin (OPG).[48]

Granulopoiesis and monocytopoiesis are increased by various inflammatory mediators (e.g., interleukins and TNF-alpha) that stimulate fibroblasts, endothelial cells, and macrophages to produce granulocyte colony-stimulating factor (G-CSF) and GM-CSF. The location of granulocytic cells is dependent on their stage of maturity: immature forms are located near arterioles and bony trabeculae; as maturation proceeds, precursors migrate toward venous sinuses where they cross the endothelium and enter the peripheral circulation.[1,26]

Lymphoid Cells (Lymphopoiesis)

Early lymphoid progenitors are produced in the bone marrow under the influence of SCF and feline McDonough sarcoma (FMS)-like tyrosine kinase 3 ligand (FLT3L). These progenitors then give rise to B and T/natural killer (NK) progenitor cells. B lymphocytes develop further in the bone marrow and ileal Peyer's patches, after which they continue maturation in peripheral lymphoid tissues. T/NK precursors leave the bone marrow for further maturation in the thymus and other tissues and subsequently migrate to peripheral lymphoid tissues.[17] Trafficking of lymphocytes to and from these organs is accomplished through various chemokines and adhesion molecules, with recirculation involving both blood and lymphatic vessels. A majority of blood lymphocytes in normal mammals are T lymphocytes, with lesser numbers of B lymphocytes and even lower numbers of NK cells.

Immature lymphoid cells are located near the endosteum and arterioles, whereas mature lymphocytes are relatively uniformly distributed within the bone marrow parenchyma.[1,37] Morphological changes in lymphoid cells during maturation are less obvious than other blood cell lineages and include decreasing cell size, decreasing cytoplasmic basophilia (visualized with Wright and/or Giemsa stains), and increasing condensation of nuclear chromatin.[46]

In contrast to the significance of bone marrow tissue for lymphopoiesis in mammals, major lymphoid organs are thymus, spleen, and kidney in bony fish; epigonal organ, spleen, organ of Leydig, spiral valve, and thymus in elasmobranchs;[18,25] mainly spleen, and to a lesser extent, bone marrow, and kidney in amphibians;[11] thymus and spleen, with aggregates of lymphoid cells occurring in various tissues, e.g., gastrointestinal tract, lung, urinary bladder, kidney, and pancreas in reptiles;[49] and thymus, bone marrow, spleen, and bursa of Fabricius (B lymphocyte development) in birds.[21]

Stem Cell Niches

HSCs produce mammalian blood cells in extravascular spaces of the bone marrow starting mid-gestation and continuing throughout life. HSCs are defined by their long-term capacities for self-renewal and differentiation.[26] As their morphology is similar to small lymphocytes, they are indistinct and evaluation of surface proteins [e.g., cluster of differentiation antigen (CD)[34]] can be used to characterize these cells. HSCs give rise to common lymphoid and common myeloid progenitor cells, which can differentiate into all blood cell lines.

HSCs reside within stem cell niches within the bone marrow which provide an optimized microenvironment for their self-renewal, quiescence, and mobilization.[29,31] Two types of complex, heterogenous stem cell niches (endosteal and vascular) have been recognized. These niches share some common structures and factors, but differ in their function and response to stressors. HSCs in the endosteal niche are generally quiescent and concentrated around arterioles.[52] Osteoblasts have regulatory roles within this niche. HSCs in the vascular niche are located near vascular sinuses where endothelial cells and reticular cells have tropic effects on these cells.[29] These HSCs replicate more often and appear to be more actively involved in maintaining balanced cellular production in steady-state conditions. Factors involved in regulation of hematopoietic activity in niches include intrinsic and extrinsic factors (e.g., parathyroid hormone/parathyroid hormone related protein (PTH/PTHrP) signaling, Wnt/beta-catenin signaling, osteopontin, G-CSF, sympathetic nervous system). The complete difference and degree of interplay between these two niches poorly understood. Proposed theories include redundancy between both compartments, the role of the endosteal niche as a reservoir for the vascular niche in case of damage to central marrow, or that each niche fulfills unique functions that synergize during hematopoiesis.[29]

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CHAPTER 4

The Hematopoietic System

BRUCE D. CAR and DAVIS M. SEELIG

Hematopoietic Stem Cells
Hematopoietic Progenitors and Precursors
Species Specificity of Hematopoiesis
The Bone Marrow Microenvironment
  The Hematopoietic Stem Cell Niche
  The Erythropoietic Niche
Cytokines and Cytokine Signaling in Hematopoiesis
  Negative Regulation of JAK–STAT Signal Transduction
Evaluation of Hematopoietic Function
  Bone Marrow Evaluation in Mice
  Evaluation of Hematopoiesis with Bone Marrow Culture
  Lethal Irradiation and Bone Marrow Transplantation
  Genetically Altered Mice
  Models of Accelerated Hematopoiesis
  Clonal Hematopoiesis

Acronyms and Abbreviations

AML, acute myeloid leukemia; Ang1, angiopoietin 1; BFU-E, erythroid burst-forming unit; CD, cluster of differentiation; CFC-S, splenic colony-forming cell; CFU-E, erythroid colony-forming unit; CFU-GEMM, colony-forming unit granulocyte, erythrocyte monocyte, megakaryocyte; CFU-GM, granulocyte–macrophage colony-forming unit; CHIP, clonal hematopoiesis of indeterminate potential; CLL, chronic lymphocytic leukemia; CLP, common lymphoid progenitor; CXCR4, CXC chemokine receptor 4; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; EPO, erythropoietin; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; GTPase, guanosine triphosphatase; HSC, hematopoietic stem cell; ICAM-4, intercellular adhesion molecule-4; IL, interleukin; JAK, Janus tyrosine kinase; JH, Janus homology; NK cell, natural killer cell; PI3K, phosphatidylinositol 3 kinase; PTH, parathyroid hormone; qPCR, quantitative polymerase chain reaction; Rac1 and Rac2, Ras-related C3 botulinum toxin substrate 1 and 2; SCF, stem cell factor; SCID, severe combined immunodeficiency; SDF-1, stromal-derived factor 1 (CXCL12); SH, src homology; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; TGF-β, transforming growth factor-beta; Tie2, tyrosine kinase with immunoglobulin-like and EGF-like domains 2; TNFα, tumor necrosis factor-alpha; TPO, thrombopoietin; TRAIL, TNF-related apoptosis-inducing ligand; VCAM-1, vascular cellular adhesion molecule 1; VLA-4, very late antigen 4.

Current understanding of the hematopoietic system draws heavily from clinical observations and research in humans and mice. Numerous gene-deleted mice targeting transcription factors, hematopoietic cytokines and their receptors, and extracellular matrix (ECM) components and their receptors are central to our knowledge of hematopoiesis at the molecular level.[8] Recent studies have contributed information about the critical role of the bone marrow microenvironment. In domestic animals, research on spontaneous hematopoietic neoplasia, the roles of retroviruses and viral oncogenes that mimic hematopoietic tyrosine kinases, cyclic neutropenia of Gray Collies, and other species-specific hematopoietic diseases has added to our understanding of hematopoiesis, which is remarkably conserved across mammalian species.[40]

HEMATOPOIETIC STEM CELLS

Hematopoietic stem cells (HSCs), which are considered to have lost their potential for mesenchymal differentiation, arise first in the embryonic yolk sac, then in fetal para-aortic splanchnopleura, from which liver and

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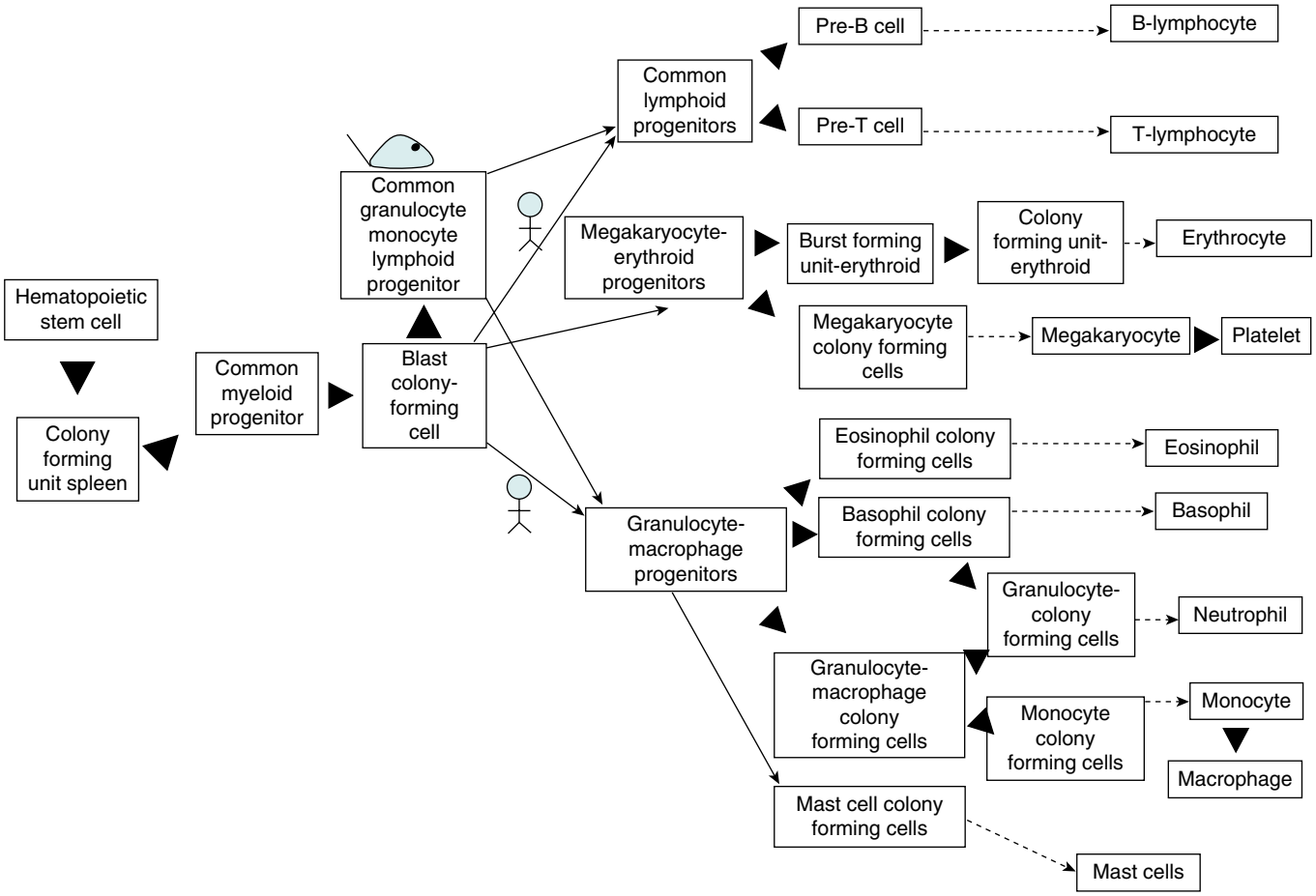


FIGURE 4.1 Hierarchical scheme of hematopoiesis. This figure depicts the conventional view of human and murine hematopoiesis. Larger block arrowheads indicate differentiation from pluripotent to oligopotent hematopoietic stem cells. Smaller block arrowheads indicate lineage commitment of oligopotent stem cells driven by cytokines and the JAK-STAT pathway. Dotted line arrows illustrate the multiple stages of differentiation that can be visually discriminated in Wright-Giemsa-stained bone marrow specimens. The figure has been modified from human and murine illustrations to indicate the mouse-specific GM-lymphoid precursors. (**Source:** Modified from Iwasaki H, Akashi K. Hematopoietic development pathways: on cellular basis. Oncogene 2007;26:6687–6696 and Metcalf D. Hematopoietic stem cells and tissue stem cells: current concepts and unanswered questions. Stem Cells 2007;25:2390–2395.)

finally bone marrow are seeded (see Chapter 1). In the conventional view of hematopoiesis, pluripotential HSCs with unlimited self-generative capacity progressively differentiate to multipotent or oligopotent stem cells with reduced self-replicative capacity, to lineage-committed progenitors with minimal ability to self-renew, to lineage-specific precursors with no self-regenerative ability, and finally to the mature cells of blood (Figure 4.1).[37] As shown in mice, hematopoiesis matures through a common granulocyte–monocyte–lymphoid oligopotent progenitor that has not been identified in higher species.[23] The strict division of common granulocyte–monocyte–erythroid–megakaryocytic progenitors and common natural killer (NK)/B and T-cell progenitors occurs for human hematopoiesis, and likely applies to hematopoiesis in domestic species.

Lineage commitment typically follows expression of lineage-restricted transcription factors (Table 4.1). Induction of these transcription factors occurs through a combination of specific cytokine receptor and ligand interactions, less-specific signal transduction pathways, and an overlay of highly specific, permissive microenvironmental influences from stromal cells, endothelial cells, adipocytes, osteoblasts, ECM proteins, adherent cytokines, and trabecular bone.

Because of the short life span of differentiated hematopoietic cells, mature blood cell production is an ongoing process; estimates suggest production of 1.5×10^6 cells/second in humans. It is unlikely that HSCs supply a continuous flux of cells from left to right as shown in Figure 4.1 because HSCs and multipotent cells are nondividing or very slowly dividing during normal hematopoiesis. Newer evidence suggests that information determined from experiments in lethally irradiated mice may not necessarily recapitulate normal or physiologically accelerated hematopoiesis and that there may be other homeostatic mechanisms that control hematopoiesis.[37]

A somewhat confusing nomenclature has evolved around early committed hematopoietic progenitors based on in vivo experiments in mice. Splenic colonies

TABLE 4.1 Hematopoietic Transcription Factors.[a]

Early hematopoietic development
- SCL (TAL 1) (stem cell leukemia, T-cell acute lymphocytic leukemia-1)
- GATA-2
- LMO2 (Rbtn-2) (Lim finger protein)
- AML-1 (acute myeloid leukemia 1 protein)
- Tel
- Notch 1

Erythropoiesis
- HIF-1 (Hypoxia-inducible factor-1)
- GATA-1
- EKLF-1 (erythroid kruppel-like factor 1)
- p45/NF-E2 (nuclear factor erythroid-2)
- STAT5a, STAT5b
- CBP/p300 (CREB binding protein/p300)
- SP1

Erythropoietic regulators
- FOG-1 (friend of GATA-1)
- TRAP220 (thyroid hormone receptor-associated protein 220)
- BRG1 (Brahma-related gene 1)
- CBP/p300 (CREB binding protein/p300)

Myelopoiesis
- Notch
- GATA-1
- C/EBP_{α} (CCAAT/enhancer binding protein $_{\alpha}$)
- STAT5a, STAT5b

Granulocytopoiesis
- RAR (Retinoic acid receptor)
- C/EBP_{α} and C/EBP_{ε}
- CBF (core binding factor)
- c-Myb
- STAT3
- NFκB (nuclear factor of kappa B) – late precursor
- AML1 – precursor

Monocytopoiesis
- EGR-1, EGR-2 (early growth response genes 1 and 2)
- Vitamin D receptor
- c-Fos, c-Fos
- PU.1
- RAR (retinoic acid receptor)
- C/EBP_{β} and C/EBP_{ε}
- MafB/c
- PU.1

Osteoclast differentation from monocyte precursor
- NFκB

Megakaryocytopoiesis
- Notch
- GATA-1
- PU.1
- Fli-1/NF-E2 (platelet production)
- FOG
- SCF (stem cell factor)
- STAT5a, STAT5b

Lymphoid
- PU.1
- Ikaros
- STAT3, STAT5a, STAT5b, STAT4, STAT6

B-cell
- E2A
- EBF (early B-cell factor)
- Pax 5
- NFκB (progenitors and late maturation)

T-cell
- HEB (TCF12 – T-cell factor 12)
- Notch/HES-1 (Hairy Enhancer of Split-1)
- NFAT (Nuclear Factor of Activated T cells) late maturation
- GATA-3
- TCF-1 (T cell factor 1)
- NFκB (progenitors and late maturation)

Mast cell
- $GATA\text{-}2^{+}$
- Elf-1
- MITF (microphthalmia-associated transcription factor)

Eosinophilopoiesis
- STAT1, STAT3, STAT5a, STAT5b
- GATA-1

[a]All transcription factors are involved in early lineage commitment and exert their effects on committed progenitors unless otherwise stated.

that appear in lethally irradiated mice reconstituted with HSCs are called splenic colony-forming cells (CFC-Ss), which have limited capacity for self-renewal. Whether similar cells occur in other species is unknown, because CFC-Ss are defined by specific experimental conditions in mice.

HEMATOPOIETIC PROGENITORS AND PRECURSORS

The term *progenitors* typically refers to cells whose presence is inferred from cytokine-driven differentiation of colonies in culture, such as granulocyte–macrophage colony-forming units (CFU-GMs) and erythroid colony-forming units (CFU-Es), whereas the term precursors refers to stages of hematopoietic differentiation recognized by cytologic evaluation. HSCs, oligopotent stem cells, and committed progenitors appear like small lymphocytes, although they can be separated by flow cytometry based on expression of surface proteins.

Toward the right of Figure 4.1, clonogenic cell types exist putatively without any capacity for self-generation. However, mature cell types such as macrophages and mast cells may have substantial self-generative properties. The initial model of hematopoiesis did not include the concept of plasticity.[37] In the current model, the concept of plasticity suggests that lineage fidelity

and progressive restriction of proliferative capacity in the traditional model are not absolute. Plasticity is recognized as a property of both proliferation and lineage commitment. How plasticity is regulated under physiologic and pathologic conditions is not well understood. It is clear though that neither HSC nor any of the oligopotent progenitors have the unlimited capacity for self-generation possessed by embryonic stem cells.

SPECIES SPECIFICITY OF HEMATOPOIESIS

In contrast to detailed information about murine hematopoiesis,[45] there are few detailed descriptions about the structure and function of the bone marrow in domestic species. Stem cell function, the biochemical nature of the bone marrow microenvironment, hematopoietic cytokines and their receptors, and lineage-specific and nonspecific transcription factors are assumed to apply to species other than mice or humans.[30,5] Studies have confirmed some common cytokines between domestic species and humans.[18,33–36,39,47,46] Research involving animal models of human diseases, toxicology, or other unique diseases such as cyclic hematopoiesis in dogs has provided additional information about hematopoiesis in domestic animals.[4,15,29,37] The gene sequences for many hematopoietic cytokines, receptors, and transcription factors for multiple species now are available in publicly accessible databases, so they can be assessed specifically by high-density microarray or more broadly by quantitative reverse transcription polymerase chain reaction (qPCR).[14,16,41]

Certain critical differences exist between human and murine hematopoiesis. Human HSCs express Flt3, the tyrosine kinase receptor for FLT3 ligand, whereas murine HSCs do not.[23] However, both murine and human common lymphoid progenitors (CLPs) express Flt3. Such differences are important because certain human acute myelogenous leukemias are associated with constitutive activation of Flt3.[9] Whether other species express similar mutations governs the potential utility of therapy with Flt3-kinase inhibiting drugs. Although the hierarchical relationships observed in murine hematopoiesis are generally preserved in other species (Figure 4.1), expression of a number of surface antigens at each developmental stage is different between mice and humans, and these and other differences may occur in other species. However, important similarities also have been recognized. Based on the recent molecular and genetic understanding of cyclic neutropenia in Gray Collie dogs and cyclic hematopoiesis in humans, some features of hematopoiesis appear similar. For example, allometric scaling correctly predicts granulopoietic cyclicity in mice (3 days), dogs (14 days), humans (19–21 days), and elephants (60 days).[4,37]

THE BONE MARROW MICROENVIRONMENT

The heterogeneous cellular elements in Figure 4.1 are intimately associated with adipocytes, macrophages, endothelial cells, nerves, osteoclasts, osteoblasts, ECM, sinusoids, and cytokines, collectively termed the bone marrow microenvironment. Cell–cell and cell–ECM interactions are important in the regulation of cell proliferation and differentiation of hematopoietic cells.[11] These interactions involve receptors and integrins, which are regulated by growth factors, cytokines, and transcription factors. The ultrastructure of the interactions between hematopoietic cells, stromal cells, and noncellular components of the bone marrow microenvironment was described in detail in 1978,[38] but the molecular mechanisms have more recently been characterized.

The best characterized integrin on HSC is very late antigen 4 (VLA-4 or α4 β1) which binds to fibronectin in the ECM as well as to vascular cellular adhesion molecule 1 (VCAM-1) on adjacent stromal cells. The binding of VLA-4 to fibronectin mediates adhesion of HSC and progenitors to the microenvironment, homing of circulating HSC and progenitors to specific areas within the bone marrow, and signal transduction. The Rho guanosine triphosphatases (GTPases), Ras-related C3 botulinum toxin substrate 1 (Rac1), and Rac2 also are key regulators of adhesion and migration of cells in the hematopoietic microenvironment.

The bone marrow microenvironment has highly specialized and integrated microanatomic functional units called niches. The two most clearly elucidated hematopoietic niches are the HSC niche (see Chapter 3) and the erythroblastic islands (Figures 4.2 and 4.3).[1]

The Hematopoietic Stem Cell Niche

Survival, proliferation, and differentiation of HSCs depend on their spatial and functional relationships with the cells and ECM of the microenvironment.[38] HSCs are enriched at sites adjacent to the endosteal surface of bone. A specialized subset of activated osteoblasts display ligands and receptors that facilitate homing and transient docking of HSC, and regulate slow cycling or rapid mobilization of HSCs as needed.[1] The interaction between stromal-derived factor 1 (SDF-1 or CXCL12) elaborated by osteoblasts and CXC chemokine receptor 4 (CXCR4), its cognate chemokine receptor on HSC, is central to this HSC niche. SDF-1 recruits quiescent progenitors, participates in their cycling and survival, and sensitizes them to further synergistic action of cytokines, thus contributing to hematopoietic homeostasis under both physiologic and stress conditions. HSC function also is regulated by osteoblasts through parathyroid hormone (PTH), the Notch signaling pathway, and interactions between angiopoietin 1 (Ang1) and its tyrosine kinase with immunoglobulin-like and EGF-like domains 2 (Tie2) receptor.[6] Disruption of this HSC niche may result in abnormal cell mobilization and contribute to extramedullary infiltration in leukemia.

The Erythropoietic Niche

Erythropoiesis occurs in distinct niches called erythroblastic islands that consist of a central macrophage surrounded by a ring of developing erythroblasts in

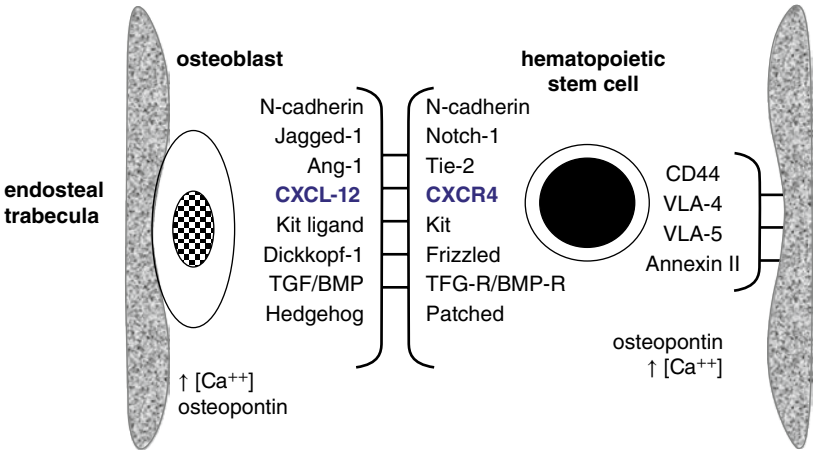


FIGURE 4.2 Hematopoietic stem cell niche (reciprocal molecular interactions). The relationship between a specialized subset of osteoblasts and the hematopoietic stem cell is shown, with reciprocal molecular interactions depicted between the two cell types, and between these cell types and endosteal bone.

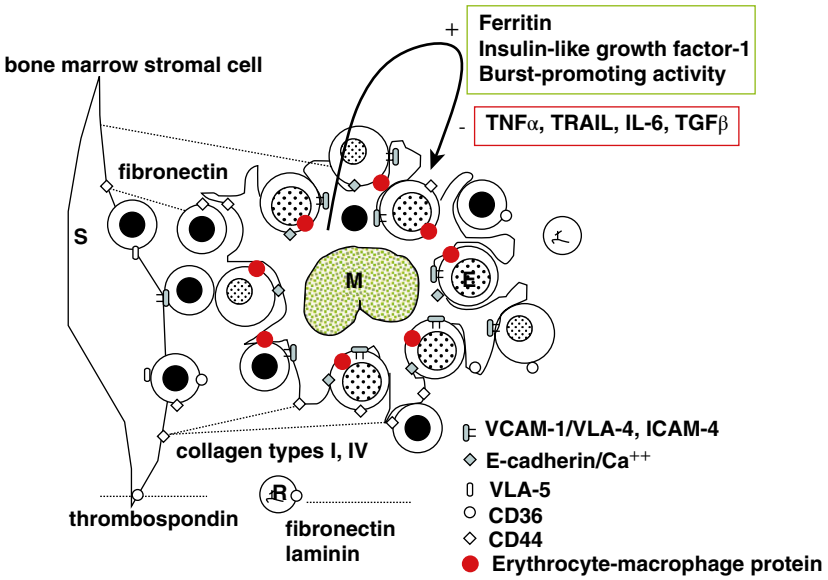


FIGURE 4.3 The erythropoietic niche. The erythroblastic island is illustrated with rubriblasts of graded levels of maturity surrounding macrophage (M) cytoplasm. Specific molecular interactions between macrophages and rubriblasts, between the erythroblastic island and marrow stromal cell(s), and of all these cellular elements with marrow stroma are shown. Autocrine cytokine loops involved in stimulation of erythropoiesis (green bordered box) and in the negative regulation of erythropoiesis (red bordered box) are shown. Dotted lines indicate extracellular matrix components. Arrow indicates factors elaborated by macrophages that influence growth and maturation of rubricytes.

bone marrow, fetal liver, and spleen, and even in long-term marrow cultures.[22] The macrophage contributes important signals to developing rubriblasts, phagocytoses-expelled metarubricyte nuclei, and transfers iron to developing rubriblasts. Unlike megakaryocytes that localize exclusively to marrow sinusoids, erythroblastic islands are located throughout the marrow.[11]

Adhesion molecules mediating important structural and functional interactions between developing erythroid cells and central macrophages include VCAM-1/VLA-4, α4β1/VLA-4, intercellular adhesion molecule-4 (ICAM-4)/αv, and E-cadherin. For example, ICAM-4 is postulated to enable reticulocytes to detach from central macrophages, allowing them to enter the circulation.[11] Laminin and fibronectin and their receptors expressed on late-stage rubriblasts also are key components in the differentiation of reticulocytes. VLA-4 and VLA-5 are involved in binding of erythroid burst-forming units (BFU-Es) to hematopoietic stromal cells. Expression of these adhesion molecules is highest on BFU-Es and CFU-Es, and is progressively lost during erythroid maturation. Hemonectin and collagen type I support binding of BFU-Es in vitro, and a role for tenascin-C has been inferred from knockout mice. Hemonectin is absent from anemic mice with Kit ligand or c-*kit* deficiency.

The surface antigen cluster differentiation 44 (CD44) is highly expressed on almost all hematopoietic cells in bone marrow and is responsible for interaction of these cells with collagen types I and IV, fibronectin, and hyaluronate.[53] Reticulocytes express only low levels of a few surface adhesion receptors, such a CD36 (thrombospondin receptor) and VLA-4. Thrombospondin serves as an adhesive ligand for committed progenitors including colony-forming units granulocyte, erythroid, monocyte, megakaryocyte (CFU-GEMM), and BFU-E. Adhesion molecule interactions of reticulocytes with bone marrow stromal cells and ECM may facilitate their egress into bone marrow sinuses. Mature RBCs do not express adhesion molecules under normal conditions.

CYTOKINES AND CYTOKINE SIGNALING IN HEMATOPOIESIS

Hematopoiesis is the cumulative result of intricately regulated signaling pathways mediated by soluble cytokines and their receptors (Table 4.2).[2] Evaluation of cytokine–receptor interactions in hematopoiesis has largely been achieved through creation of gene-deleted mice and conditional knockout mice, the phenotypes of which range from severe lethal embryonic, fetal, and neonatal defects to redundant null phenotypes.[8] These mice have provided much of our current insight into the regulation of physiologic and pathologic alterations in hematopoiesis.

Most hematopoietic cytokine receptors are multiple subunit complexes, except for those that signal through a single chain, such as erythropoietin (EPO), granulocyte–macrophage colony-stimulating factor (GM-CSF), and thrombopoietin (TPO). Hematopoietic cytokines are approximately 200 amino acids in length and carry a conserved sequence of tryptophan–serine–X–tryptophan–serine (W–S–X–W–S) in their extracellular domain, which functions as part of the ligand-binding domain.

Cytokine receptors contain docking regions for Janus tyrosine kinases (JAK1, JAK2, JAK3, TYK2) in their cytoplasmic termini that, when attached to the ligand-bound form of the cytokine receptor, recruit JAKs which then autophosphorylate. The JAK kinase then phosphorylates tyrosine residues on a specific signal transducer and activator of transcription (STAT) protein, which is STAT1, 3, or 5 for most hematopoietic cytokine receptors. JAKs contain a catalytic Janus homology (JH) 1 domain and a JH2 catalytic-like but inactive domain critical to the ability of JAKs to regulate themselves and to mediate cytokine-induced responses. Src kinase activation of STATs also is important for myeloid cell proliferation.

In unstimulated cells, STATs are present as cytoplasmic monomers in the unphosphorylated state. Phosphorylation by JAK kinases leads to dimerization through reciprocal interactions of SH2 domains with phosphotyrosine residues, and thereby activation of STAT, which then translocates to the nucleus.[20] STATs are transcription factors that prevent apoptosis or positively regulate prosurvival genes of late progenitor and early precursor cells.[20] Although there are numerous STAT responsive genes in many different cell types, STAT regulation of hematopoietic precursors occurs in a cell-type restricted manner.

Lineage-committed colony-forming cells respond to cytokines in a lineage-restricted fashion. The specificity of hematopoietic cytokines is determined by progenitor and precursor cell expression of their cognate receptors. For example, rubriblasts express EPO receptors but myeloblasts do not, which is different from the relatively promiscuous expression of JAK/STAT pathways. Given the importance of JAK-phosphorylation events in driving proliferation of hematopoietic precursors, it is not surprising that mutations leading to constitutively active JAK2 result in myeloproliferative disorders and leukemia. In addition to the prominent role of JAK–STAT interactions in hematopoiesis, the functional involvement of Ras and phosphoinositol 3 kinase (PI3K) pathways also has been shown following interleukin (IL)-3/IL-5 and GM-CSF stimulation of bone marrow cultures in vitro.[2]

TABLE 4.2 Hematopoietic Cytokines.[a]

Lineage/Function	Key Hematopoietic Cytokine
Early hematopoiesis	Stem Cell Factor (SCF) Interleukin 3 (IL-3) Wnt ligands (Jagged) and receptors Kit ligand SDF-1/FGF-4
Common myeloid progenitor/ myelopoiesis	Stem cell factor (SCF) Thrombopoietin (TPO)
Erythropoiesis	Erythropoietin (EPO)
Megakaryocytopoiesis	Thrombopoietin Interleukin-6 (IL-6)
Lymphocytopoiesis T cells NK cells B cells	IL-7 IL-7, IL-2 IL-7, IL-15 IL-4
Regulatory	Transforming growth factor-β (TGF-β) Bone morphogenetic protein (BMP) Activin

[a]Note that this table oversimplifies the actions of these cytokines. With further refined analyses of murine models it has become apparent that even lineage restricted cytokines such as EPO exert pleiotrophic effects within and external to the hematopoietic system.

Negative Regulation of JAK–STAT Signal Transduction

Suppressors of cytokine signaling (SOCSs) are a family of proteins that regulate the strength and duration of the hematopoietic cytokine-driven signaling cascade.[51] They are transcriptionally induced by JAK–STAT signaling. SOCS proteins contain src homology (SH) 2 domains and a SOCS-box which mediate binding to cytokine receptors and associated JAKs, and attenuate signal transduction directly. In addition to their transcriptional induction by hematopoietic cytokines and subsequent self-limiting stimulation of hematopoiesis, other cytokines including tumor necrosis factor-α (TNF-α), IL-1, and toll-like receptor ligands (e.g., lipopolysaccharide) also induce SOCS expression, providing negative regulation for granulocyte colony-stimulating factor (G-CSF) signaling. Another level of regulation is provided by phosphatases. The signaling and subsequent hematopoiesis induced by phosphorylated dimers of STAT proteins is terminated by removal of phosphates from STAT tyrosines by three specific protein tyrosine phosphatases.

Transforming growth factor-β (TGF-β) is perhaps the most potent endogenous negative regulator of hematopoiesis.[28] TGF-β suppresses expression of the stem cell factor (SCF) receptor, the response of progenitors to SCF,

and cell cycle progression of progenitors. The expression of receptors for TGF-β on primitive hematopoietic progenitors and subsequent stages of maturation suggests a broad role for this cytokine. Negative regulation of erythropoiesis by TNF-α, TNF-related apoptosis-inducing ligand (TRAIL), IL-6, and TGF-β occurs when chronic inflammatory disease increases systemic and local bone marrow concentrations of these cytokines (see Chapter 39).[11]

EVALUATION OF HEMATOPOIETIC FUNCTION

Hematopoiesis is studied to gain insight into mechanisms of cytopenias, leukemias, and other pathophysiologic responses. Evaluation of hematopoiesis begins with careful examination of peripheral blood and is complemented by cytologic or histologic assessment of bone marrow. Short- and long-term in vitro culture of bone-marrow-derived cells, genetically modified mice, animal models of retarded and accelerated hematopoiesis, syngeneic and xenogeneic hematopoietic stem cell transplantation, retroviral-mediated gene transfer, and gene therapy also have been used to study hematopoiesis.[52]

Gene-deleted and transgenic mice provide a special challenge to the hematologist because these mice frequently die during the embryonic, fetal, or early neonatal period. Peripheral blood examination may still provide valuable information. A 3 μL volume of heart blood obtained with a fine gauge needle is sufficient to prepare a blood smear; as little as 20 μL of heart blood may be diluted with 2 mg/mL EDTA in saline and analyzed with an electronic counter. Potential artifacts of dilution of small quantities can be overcome by comparing results from treated or genetically altered mice with similarly diluted volumes from control or wild-type mice.

Bone Marrow Evaluation in Mice

Direct examination of bone marrow complements information gained from assessment of blood. In fetal mice, which lack developed medullary hematopoiesis, cytologic examination of liver imprints for hematopoietic precursors is useful in assessing early hematopoietic function.

Evaluation of Hematopoiesis with Bone Marrow Culture

Hematopoietic interactions are best evaluated in vitro where culture systems permit evaluation of effects of individual cytokines, growth factors or their regulators, and combinations of factors. Target gene expression may be transiently induced by gene transfection or inhibited by transfection with antisense RNA. The significance of in vitro studies must be confirmed in vivo. For example, deficiency of hematopoietic cytokines or growth factors which would predictably have severe phenotypes based on in vitro work, such as IL-2 and GM-CSF, has much milder hematologic phenotypes than expected (i.e., failure to develop lymphopenia and neutropenia), underscoring the redundancy and pleiotropy which characterize many of these factors.

Bone marrow cells can be cultured from aspirates or core samples obtained from diseased or healthy animals. These cells are cultured in semisolid methylcellulose-based media with cocktails of cytokines, EPO, transferrin, bovine fetal serum, and albumin. Whereas EPO is active across species and has been cloned from a variety of species, other cytokines have more limited cross-species effects. When a species-specific cytokine is not available, conditioned spleen cell media obtained from phytohemagglutinin, endotoxin, or phorbol-ester-stimulated cells may serve as a useful source of cytokines. Conditions for culture of bone marrow progenitors from dogs, cats, sheep, cattle, horses, chickens, and other species have been described.[17,21,45,49] In general, methodologies applicable to murine and human culture systems apply to those of other species, when care is taken to ensure the quality of collected bone marrow specimens and species-specific reagents or appropriate substitutes are used. Short-term bone marrow cultures from mice, rats, and dogs are routinely performed to assess the potential toxicity of xenobiotics and define hematopoietic phenotypes of genetically altered animals.[45] These culture systems may be used to study hematopoiesis by the addition of individual cytokines, growth factors or their regulators, or combinations thereof. Alternatively, neutralizing antibodies to these components or small molecules (<1000 kDa) produced by medicinal chemistry can be used to dissect regulatory pathways. Similarly, hematopoietic progenitors from clinical cases can be studied in vitro by direct culture following aspiration biopsy. Plasma or plasma components from animals with bone marrow disorders may be added to bone marrow cells of clinically normal animals to study the nature of potential humoral myelosuppressive activities.

Long-term cultures of hematopoietic cells on established stromal cell layers (Dexter cultures) more closely recapitulate the hematopoietic microenvironment and allow long-term survival of pluripotent HSC. These culture systems were used to identify the stromal cell defect (defective SCF production) in Sl/Sl^{d} mouse (Steel anemia) and the complementary defect in W/W^{v} (white spotted) mouse (SCF receptor alternatively known as *c kit*). Long-term bone marrow cultures have been described for dogs[45,44] and sheep.[31]

Lethal Irradiation and Bone Marrow Transplantation

Lethally irradiated mice receiving bone marrow or hematopoietic cells with a capacity for partial or complete renewal of hematopoiesis develop large splenic foci of extramedullary hematopoiesis thought to be initiated by a single pluripotent stem cell. These colonies are grossly visible after 8–14 days, creating hemispherical distortions of the splenic contour which may be visually enumerated.[42] These CFU-S have since been found to represent a multipotent committed progenitor

cell rather than the pluripotent stem cell as originally hypothesized. Irradiation and stem cell transplantation methodologies are described in detail for dogs.[45]

Genetically Altered Mice

Through technology that permits homologous recombination of partially homologous, generally noncoding, genetic sequences in cultured murine stem cells, mice deficient in the protein product of specific target genes can be created. This technology has been extensively exploited to study the function of individual hematopoietic cytokines, growth factors, their cognate receptors, and hematopoietic transcription factors.[41] Without this technology, the study of transcription factors associated with primitive oligopotent progenitors (Tables 4.1 and 4.2) would not have been possible. The hematologic evaluation of mouse embryos or feti is a critical part of the phenotypic assessment of knockout mice. By rendering gene-deleted mice (−/−) transgenic (+/+) for a deficient gene under the control of an inducible or repressible tissue-specific promoter, the function(s) of a gene in adults, for which deletion otherwise results in nonviable feti, can be examined. Using the Cre–loxP system, genes may be removed or reactivated in a tissue-specific manner in the adult mouse.[32]

The development of methods to transplant human hematopoietic cells into severe combined immunodeficient (SCID) mice (SCID-hu) has provided an experimental tool with potential use in modeling leukemias, infectious diseases, autoimmune diseases, and a variety of primary nonneoplastic bone marrow disorders. Potential deficiencies of this model include incomplete cross-species functionality between murine cytokines and cytokine receptors and human receptors and ligands, since the absence of human bone marrow stroma in mice transplanted with human hematopoietic cells creates a chimeric hematopoietic microenvironment. Impaired passage of mature cells into and out of the bone marrow or circulation may occur when adhesion molecules and their receptors on leukocytes, platelets, and endothelial cells fail to recognize their respective murine or human counterparts.

Models of Accelerated Hematopoiesis

Several methods have been used to accelerate hematopoiesis. The intraperitoneal administration of a single 150 mg/kg dose of 5-fluorouracil or appropriate dosing regimen of cyclophosphamide completely depopulates murine bone marrow. Treatment with 5-fluorouracil spares the slowly cycling or noncycling stem cells.[55] Recovery of hematologic parameters is first detectable 5 days post-treatment, with complete recovery occurring after 15–20 days. This model has been used extensively to study accelerated hematopoiesis in gene-deleted mice with apparently normal basal hematopoiesis or in mice treated with agents that do not alter basal hematopoietic function. In this manner, the immunosuppressive rapamycin was shown, by virtue of its ability to inhibit signal transduction of a variety of proliferation-inducing cytokines, to have potential liability when administered in concert with cytotoxic agents.[43] Accelerated erythropoiesis is readily induced in rodents by a single 60 mg/kg intraperitoneal injection of phenylhydrazine. This regimen produces Heinz body hemolytic anemia with approximately 30% decrease in hematocrit after 2 days and a robust reticulocytosis by 5 days post-treatment.[50] Alternatively, a fixed volume of blood (up to 3% of body weight) can be removed from a rodent and replaced with intraperitoneal saline, stimulating erythropoiesis. To mobilize granulocytes and accelerate granulopoiesis, Bacto Tryptone (a potent chemotactic casein digest, 1 mL of a 10% solution) is administered intraperitoneally. A similar effect is achieved with intravenous G-CSF, which generally is species cross-reactive, at a dose of 5 μg/kg.[3]

Lessons learned from the evaluation of hematopoiesis in genetically altered rodents may be extended to the study of hematopoiesis in domestic species and provide a better understanding of dysregulation in primary diseases of bone marrow. The availability of specific reagents for domestic species and accessibility to bone marrow culture, flow cytometry, and PCR technologies permit increasingly sophisticated examination of bone marrow function, providing greater insight into abnormalities recognized by traditional hematologic methods.

Clonal Hematopoiesis

Hematologic malignancies are believed to result from the progressive and gradual accumulation of mutations within HSCs. The identification of common mutations in specific hematologic malignancies allowed for the large-scale genomic analysis of peripheral blood DNA from healthy individuals. These studies have identified age-related, leukemia-associated genetic mutations within healthy individuals, a phenomenon referred to as clonal hematopoiesis or clonal hematopoiesis of indeterminate potential (CHIP).[25]

Early clues to the existence of CHIP were provided by studies using X chromosome inactivation-based assays (see Chapter 62). These studies identified clonally skewed peripheral blood leukocytes (as evident by nonrandom X-allele inactivation) in 20–25% of healthy women greater than 60 years of age, which was much more frequent than younger women.[7,10] The identification of these cells contributed to the development of a stepwise model of leukemogenesis, which was subsequently supported through the identification of preleukemic HSCs (harboring initiating, but not late mutations) in humans with chronic lymphocytic leukemia (CLL) and acute myeloid leukemia (AML).[54,27,13,26,12,48] Subsequent genomic-mining studies have identified numerous mutations, including copy number alterations and deletions, in the peripheral blood of aged people. These mutations are rare in people under the age of 40 (<1%), but affect 10–20% of people in their eighties.[25] Moreover, clonal hematopoiesis is associated with an increased risk of developing a hematologic malignancy, as reflected by hazard ratios of 11.1 and 12.9 in two longitudinal studies of overly 14,000 people.[24,19]

The clinical implications of CHIP have yet to be fully defined. Beyond the gradually evolving risk of development of a hematologic malignancy (which is approximately 0.5–1% per year), patients in which clonal hematopoiesis was identified had an association with non-neoplastic diseases, including cardiovascular disease.[24] In one analysis of over 17,000 individuals, people with CHIP were at a higher risk of coronary heart disease and ischemic stroke.[24] Although the importance of CHIP in veterinary patients is uncertain, particularly given the comparatively shorter life of domestic animals, advances in the hematopoietic neoplasm diagnostic toolkit may open opportunities for comparative research.

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CHAPTER 5

Vasculogenesis and Endothelial Cell Production

JONG HYUK KIM

Endothelial Progenitor Cells
  Phenotypic Determinants
  Mobilization, Recruitment, and Homing
Growth Factors and Signaling Pathways
  Vascular Endothelial Growth Factors
  Transforming Growth Factor-β
  Fibroblast Growth Factors
  Hedgehog Signaling
Experimental Advances and Clinical Relevance

Acronyms and Abbreviations

Ang1, angiopoietin-1; BMP, bone morphogenetic protein; CD, cluster of differentiation; CXCR, CXC chemokine receptors; Dhh, desert hedgehog, EPCs, endothelial progenitor cells; FAK, focal adhesion kinase; FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GDF, growth and differentiation factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; HGF, hepatocyte growth factor; Hh, hedgehog; HUVEC, human umbilical vein endothelial cell; Ihh, Indian hedgehog; iPSC, induced pluripotent stem cell; KDR, kinase insert domain receptor; MAPK, mitogen-activated protein kinase; PBMC, peripheral blood mononuclear cell; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKC, protein kinase C; PKD, protein kinase D; PlGF, placental growth factor; Sca1, stem cell antigen 1; SDF-1α, stromal-derived growth factor-1α; Shh, sonic hedgehog; TGF-β, transforming growth factor-β; TGFβR, transforming growth factor-β receptor; Tie-2, angiopoietin receptor-2; VEGF, vascular endothelial growth factor.

The vasculature is a specialized organ where a number of essential homeostatic functions, including the transport of oxygen and nutrients, are maintained throughout the body. Vasculogenesis is the de novo formation of vessels that establishes the vasculature. It specifically refers the production of new blood and lymphatic vessels via the recruitment of bone-marrow-derived progenitor cells that have an endothelial differentiation capacity (i.e., endothelial progenitor cells; EPCs). By contrast, angiogenesis is the generation of new vessels from endothelial cells through the sprouting of a pre-existing vascular network. Although vasculogenesis and angiogenesis are distinct, vessel formation may occur simultaneously through a combination of both processes. Vasculogenesis is the mechanism of vascular formation in the developmental stage of embryos and in adult tissues, and it is further characterized as prenatal or postnatal vasculogenesis. The first half of this chapter focuses on the roles of bone-marrow-derived EPCs in postnatal vasculogenesis and the remainder addresses the phenotypical identification of EPCs and their signaling pathways.

ENDOTHELIAL PROGENITOR CELLS

Phenotypic Determinants

EPCs are a unique population that is able to differentiate into endothelial cells and contribute to the formation of new vessels. Putative EPCs were first identified as circulating progenitor cells for endothelial lineage differentiation in adult human peripheral blood in the late nineties.[2] Since then, distinct EPC populations have been isolated and the phenotypical and functional characterization of this population has become clearer. EPCs give rise to functional endothelial cells; thus, those progenitor cells are expected to display an upregulation of endothelial cell surface markers and a downregulation of hematopoietic markers during differentiation. However, the definitive identification and characterization of EPCs still remain challenging due to a lack of definite surface markers. To overcome this limitation, a panel of surrogate markers is used to identify cells that show the vasculature-forming properties of EPCs.

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Putative circulating EPCs were first identified as such based upon their expression of cluster of differentiation (CD)34 (Flk-1 in the mouse) and kinase insert domain receptor (KDR) and their capacity to differentiate into cells expressing high levels of endothelial antigens, such as CD31, angiopoietin receptor-2 (Tie-2), and E-selectin.[2] Later, circulating EPCs were further defined as CD45– and CD34+ cells that express either CD133 or KDR.[19] In addition, EPCs appear to express a variety of cell surface markers similar to those that characterize vascular endothelial cells, such as CD31 and Tie-2.[13] Despite these findings, the identification of specific EPC markers has proven challenging because:[9,15] (1) hematopoietic cells and endothelial cells originate from a common progenitor (i.e., hemangioblast); (2) the two share a panel of antigens in both embryonic and adult stages; (3) marker validation is difficult, as in vitro EPC culture is not standardized; and (4) EPCs are dynamic and readily transit between endothelial and hematopoietic progenitors depending on their local niche. Hence, other sophisticated molecular approaches, such as global gene expression profiling, or a combination of in vitro and in vivo functional assays, are necessary for the clear identification of these populations.[26]

Murine EPCs are thought to originate from bone marrow, and the expression of a combination of multiple markers including stem cell antigen 1 (Sca1), cKit, Flk-1, CD31, Tie-2, CXCR4, CD133, and CD144 expression is used to enrich for these cells.[3,10] Circulating murine EPCs might present myeloid and pro-angiogenic properties, and resident EPCs have been isolated from rat lung tissue displaying neovasculogenic capacity. In domestic animals, putative EPCs have been suggested to be isolated from canine peripheral blood mononuclear cells (PBMCs).[14,25] Since there is a variety of antigenic determinants across animal species, determining a panel of specific markers that allows the identification and enrichment of species-specific EPCs remains a focus in the field.

Mobilization, Recruitment, and Homing

EPCs originate from the bone marrow, but can also be found circulating in peripheral blood.[2] EPCs are mobilized from bone marrow to sites where they play an important role in physiological and pathological production of new vessels. This includes vasculogenesis in ischemic tissues, during wound healing and during vascular repair, as well as disease-associated vascuologeneis as occurs in cancer, diabetic retinopathy, and retinopathy of prematurity. EPCs contribute to vasculogenesis through two fundamental mechanisms: they incorporate directly into damaged endothelium to form a functional vessel, and they secrete proangiogenic factors that have a paracrine effect on the residual endothelial cells to recover the vessel. The mobilization of EPCs that reside primarily in the bone marrow is initiated when tissue damage occurs. The affected tissue secretes pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang1), stromal-derived growth factor-1α (SDF-1α), and granulocyte–macrophage colony-stimulating factor (GM-CSF),[7] inducing the recruitment and homing of EPCs. Subsequently, EPCs may continue recruitment via their own release of VEGF, hepatocyte growth factor (HGF), granulocyte colony-stimulating factor (G-CSF), and GM-CSF.

GROWTH FACTORS AND SIGNALING PATHWAYS

Vasculogenesis is a complex process that is mediated by the orchestration of multiple cell types with growth factors and signaling pathways to sustain the vascular system. This section provides a brief introduction to the key signaling molecules that are essential for endothelial cell function and vasculogenesis.

Vascular Endothelial Growth Factors

VEGF is one of the main factors that contributes to vasculogenesis. The VEGF family is composed of a number of secreted polypeptides, including VEGF-A, -B, -C, -D, and placental growth factor (PlGF). The three main receptors that interact with VEGFs are VEGFR-1 (Flt-1), VEGFR-2 (KDR in humans and Flk-1 in mouse), and VEGFR-3 (Flt-4 in mouse). VEGF receptors are tyrosine kinase receptors and are composed of an extracellular domain responsible for VEGF binding and intracellular tyrosine kinase domain. Embryonic lethargy with vascular defects occurs in VEGF-, VEGFR-1-, and VEGFR-2-deficient mice,[6,17,22] confirming that these molecules are necessary for the initiation of vasculogenesis and embryonic development. VEGFR-3 appears to play a major role in lymphatic vessel development; however, its precise role in blood vessel formation is unclear. To activate intracellular signaling, VEGF ligands are required to bind to their respective tyrosine kinase receptors. This interaction induces homodimerization or heterodimerization of the receptors, kinase phosphorylation, and the induction of downstream signaling events that regulate the migration, differentiation, proliferation, and survival of endothelial cells. VEGF-induced signaling may trigger multiple pathways involving mitogen-activated protein kinase (MAPK), protein kinase B (PKB or AKT), protein kinase C (PKC), protein kinase D (PKD), phosphatidylinositol 3-kinase (PI3K), and focal adhesion kinase (FAK).[18] Neuropilin, which is a transmembrane glycoprotein, can also bind to the VEGFs as a coreceptor for the VEGFRs. The binding of VEGFs to neuropilin induces intracellular signal transduction, although it is unclear whether neuropilin is sufficient for independent signaling.[11] Neuropilins are expressed in endothelial cells and participate in vasculogenesis.

Transforming Growth Factor-β

The transforming growth factor-β (TGF-β) family is an enormous group of more than 30 structurally related proteins including TGF-β1, TGF-β2, TGF-β3, bone

morphogenetic protein (BMP), growth and differentiation factor (GDF), and activin. TGF-β receptors (TGFβRs) involved in signal transduction are classified as type I and type II receptors.[12,21] TGF-β signaling is dependent on Smad proteins and can be divided into four steps: (1) ligand binding to TGFβRII, (2) TGFβRI recruitment and formation of a heterotetrameric TGFβRI–TGFβRII complex, (3) phosphorylation of Smad proteins, and (4) translocation of the Smad complex to the nucleus. The TGF-β is expressed in endothelial cells, and TGF-β signaling plays a crucial role in the regulation of endothelial cell differentiation, the establishment of the vascular network, and the maintenance of vessel wall integrity.[20] The functional effects of TGF-β on endothelial cells vary due to the complexity of TGF-β signaling; thus, its precise roles in different contexts remain to be elucidated.

Fibroblast Growth Factors

Fibroblast growth factors (FGFs) are also key factors in vascular development and maintenance. The FGF family of ligands consists of 18 peptide members (FGF1–10 and FGF16–23), including five paracrine subfamilies and one endocrine subfamily. These 18 protein molecules activate FGF receptors (FGFRs) of tyrosine kinases,[4] whereas the FGF homologous factors previously known as FGF11–14 are unable to induce this signal transduction. The majority of FGFs and FGFRs are expressed in endothelial and vascular smooth muscle cells, where activation of FGF signaling stimulates growth and migration of the cells.[1] FGF signaling contributes to vascular homeostasis and vascular permeability by maintaining endothelial barrier function and integrity.[16]

Hedgehog Signaling

The hedgehog family includes sonic hedgehog (Shh), Indian hedgehog (Ihh), and desert hedgehog (Dhh). Hedgehog (Hh) signaling is essential for blood island formation during yolk sac vessel development[8] and for endothelial tube formation at various stages of development in avian and murine embryos.[24] Furthermore, Hh signaling contributes to the production of vascular growth factors by stimulating endothelial cells and cells supporting blood vessel.[5]

EXPERIMENTAL ADVANCES AND CLINICAL RELEVANCE

EPCs and endothelial cells have been isolated from peripheral blood; however, there are several limitations of such isolation. These limitations include the low yield, a low efficiency, difficulty in expansion, and source-dependent heterogeneity. Potential approaches to overcome these challenges include the reprograming of induced pluripotent stem cells (iPSCs). The use of iPSCs offers a powerful tool for multipurpose research and disease modeling, including cancers, immune diseases, and a variety of bone marrow disorders. Recent advances suggest that reprogramed iPSCs are able to differentiate substantial numbers of endothelial cells with consistent outcomes.[23] This approach also benefits from the easy access to source material, such as PBMCs and human umbilical vein endothelial cells (HUVECs). iPSC-derived endothelial cells have similar functional properties to natural endothelial cells and express endothelial markers, such as CD31 and CD144. A better understanding of the precise roles of EPCs and endothelial cells is necessary for the development of therapeutic interventions in vascular disorders. Since EPCs and circulating endothelial cells have been identified in pathological conditions, a better understanding of these cells is also important to define molecular mechanisms that regulate the mobilization, recruitment, and incorporation of EPCs, as well as to develop safe and efficient EPC therapies.

In the veterinary field, lessons learned from the differentiation of EPCs in vitro may be extended to studies on domestic and other species, providing a novel and better strategy to research vascular disorders, such as malignant vascular tumors (e.g., hemangiosarcoma) and cardiovascular diseases. Furthermore, the development of specific reagents for animal species and experimental applications could lead to a sophisticated assessment of the functions of EPCs.

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CHAPTER 6

Cluster of Differentiation (CD) Antigens

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Definition and History
Structure and Integration of Membrane Antigens
Transmembrane Proteins
  Single-Pass Type I Transmembrane Protein (I)
  Single-Pass Type II Transmembrane Protein (II)
  Multipass Transmembrane Protein (III)
  GPI Transmembrane Protein (V)
Tissue Distribution

Acronyms and Abbreviations

BCR, B cell receptor, CD, cluster differentiation; CLAW, Canine Leukocyte Antigen Workshop; ER, endoplasmic reticulum; FIV, feline immunodeficiency virus; GP, glycoprotein; GPI, glycosylphosphatidylinositol; Ig, immunoglobulin; IL, interleukin; LPS, lipopolysaccharide; MAb, monoclonal antibody; MDR-1, multidrug resistance transporter protein-1; MHC, major histocompatibility complex; NK, natural killer; TNFR, tumor necrosis factor receptor, VLA, very late antigen.

DEFINITION AND HISTORY

Cluster differentiation (CD) nomenclature was first introduced at an international conference in Paris (1982) during the boom in monoclonal antibody (MAb) technology.[2] This nomenclature was established to standardize the classification of cell surface antigens and to define the biological functions of molecules expressed by various hematopoietic lineages. Several international workshops have been held to exchange human and other species-specific MAbs and to compare their reactivity with cells and cell proteins from veterinary species.[8,16,19,24-26,29,30]

Based on these workshops, MAbs that have similar reactivity with tissues or cell types are assigned to a cluster group. Therefore, an antigen that is recognized by a cluster of MAbs is assigned a "cluster of differentiation" (CD) number. If MAbs defining a cluster of antigens are derived from the same laboratory, the suffix "w" is appended to the CD designation. Only eight CD antigens are internationally accepted as defined by the 1st Canine Leukocyte Antigen Workshop (CLAW) and include the following homologs to the human system: CD4, CD5, CD8, CD11a/18, CDw41, CD44, CD45, and CD45R.[8] The final meeting of the 8th Human Leukocyte Differentiation Antigen (HLDA) Workshop was held in December 2004, in Adelaide, Australia, and 376 MAbs from various companies, mainly directed against human leukocytes, were tested for their reactivity with cells from 17 different animal species. A special issue of the journal "Veterinary Immunology and Immunopathology" described these efforts.[24] Now, the efforts of the Human Cell Differentiation Molecules (HCDM) organization are focused on understanding the intracellular molecules involved in immune cell reactions with their environment (endothelial cells and stromal cells). Furthermore, the group is working toward aligning CD nomenclature to gene nomenclature. The HCDM website (http://www.hcdm.org/) includes a list of CD molecules and many useful links including new CD antigens defined in the 9th and 10th HLDA workshops.

Today, there are over 371 CD molecules defined (http://www.hcdm.org/index.php/molecule-information). This explosion in CD molecules is the result of the use of molecular biology techniques to identify new molecules. The purpose of this chapter is to provide the most current listing of CD antigens recognized by various antibodies in veterinary species. Table 6.1 summarizes the current knowledge of important veterinary CD antigens having MAb clones that react with dog, cat, horse, cattle, and pig CD antigens;

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TABLE 6.1 Cluster differentiation antigens.

CD antigen	MAb reactivity	Topology	Cellular distribution	Physiology
CD1	Porcine[a] Canine[a] Feline[39]	I	Dendritic cells, macrophages, monocytes, and thymocytes	CD1 is a member of the immunoglobulin superfamily. Presents lipid antigens to T cells
CD2	Bovine[a] Equine[a]	I	T cells, NK cells	Enhances adhesion between T cells and antigen-presenting cells
CD3	Canine[a] Bovine[a] Porcine[a,b]	I	T cells	A family of proteins that forms a signal transduction complex for T-cell receptor when it binds antigen
CD4	Canine[a,b,c] Feline[a,b,c] Equine[a] Bovine[a,b] Porcine[a,b]	I	T cells, canine neutrophil	Receptor for MHC class II; facilitates recognition of peptide antigens
CD5	Canine[a] Feline[a,b] Equine[a,b] Porcine[a]	I	T cells	Receptor for CD72, facilitates signals transduced by T-cell receptor for antigen
CD8	Canine[a,b,c] Feline[a,c] Equine[b] Bovine[a,b] Porcine[a,b]	I	T cells	Receptor for MHC class I; facilitates recognition of peptide antigens
CD8 alpha	Canine[21] Feline[28] Equine[34] Bovine[a] Porcine[a]	I	Thymocytes, T cells	Forms a heterodimer with CD8β receptor for MHC class I; facilitates recognition of peptide antigens
CD8 beta	Canine[21] Feline[28] Equine[34] Porcine[a,b]	I	T cells	Forms a heterodimer with CD8α (see in the earlier text)
CD9	Feline[b] Equine[b] Bovine[b] Porcine[b]	III	Activated lymphocytes, monocytes, lymphocytes, granulocytes, platelets	Important for signal transduction, cell activation, adhesion, aggregation; coreceptor for FIV; associates with tetraspan superfamily (CD63, CD81, CD82)
CD11a	Canine[10] Equine[a] Porcine[a]	I	Monocytes, granulocytes, lymphocytes	Associates with CD18 to form a heterodimner receptor to facilitate adhesion
CD11b	Canine[a,c] Feline[b] Equine[c] Bovine[a]	I	Granulocytes, monocytes, lymphocyte subset	Associates with CD18 to form a heterodimer receptor to facilitate adhesion
CD11c	Canine[a]	I	Dendritic cells, granulocytes, macrophages, lymphocytes	Associates with CD18 to form a heterodimer receptor to facilitate adhesion
CD11d	Canine[a]	I	CD8+ T cells, γδ− T cells, macrophages in splenic red pulp	Associates with CD18 to form a heterodimner receptor to facilitate adhesion
CD13	Equine[a,b] Bovine[a]	II	Granulocytes and monocytes	Aminopeptidase N, a metalopeptidase in humans which removes NH_2 terminal amino acids from peptides
CD14	Canine[a] Feline[b] Equine[c] Bovine[a,b] Porcine[a,b,c]	V	Monocytes, myeloid cells	Receptor for lipopolysaccharide that transduces signals, leading to oxidative burst and proinflammatory cytokine synthesis
CD16	Feline[35] Equine[23] Porcine[a]	V	Granulocyte, monocyte, lymphocyte (natural killer cells)	A low-affinity receptor for the Fc region of aggregated IgG (FcγRIII)
CD18	Canine[a] Feline[a,b] Equine[a] Bovine[a] Porcine[a,b]	I	All leukocytes	Beta subunit of the integrin heterodimer and combines with the alpha subunit of either CD11a, CD11b, CD11c, or CD11d; plays a role in adhesion to endothelium

TABLE 6.1 (Continued)

CD antigen	MAb reactivity	Topology	Cellular distribution	Physiology
CD20	Canine[13] Feline[b]	III	B lymphocytes	Intracellular calcium signaling
CD21	Canine[a] Feline[a,b] Bovine[a,b] Porcine[a,b]	I	B cells, monocytes, follicular dendritic cells	Receptor for C3d fragment and CD23; enhances B cell antigen receptor signal transduction
CD25	Canine[a,c] Bovine[a] Porcine[a]	I	Mitogen stimulated T or B lymphocytes, regulatory T cells, lipopolysaccharide (LPS) stimulated monocytes	IL2-receptor-alpha subunit
CD26	Bovine[a]	II	T cells, dendritic cells	Dipeptidyl peptidase 4, activation molecule for T lymphocytes
CD27	Porcine[a,b]	I	monocytes	Ligand for CD70, costimulatory and proinflammatory
CD28	Canine[38] Bovine[a,b]	I	T cells, thymocytes, NK subset	Ligand for CD80 (B7-1) and CD86 (B7-2), and is a potent costimulator of T cells Signaling through CD28 augments IL-2 and IL-2 receptor expression, as well as cytotoxicity of CD3-activated T cells
CD31	Porcine[a,b,c]	I	Endothelial cells, platelets, leukocytes	Platelet–endothelial adhesion molecule
CD34	Canine[a,c] Porcine[c]	I	Lymphohematopoietic stem cells and progenitors, endothelial cells	Leukocyte–endothelial interactions through binding with CD62L and CD62E
CD40	Bovine[a]	Tumor necrosis factor receptor (TNFR)	B lymphocytes, subset of T lymphocytes	Differentiation of B lymphocytes into effector cells, and is also involved in interactions between T and B lymphocytes
CD41	Canine[a] Feline[a,b]	I	Platelets, megakaryocytes	Integrin αIIB CD41/CD61 complex, receptor for fibrinogen
CD42b	Canine[11] Feline[33]	I	Platelets	GPIb, vWF receptor, platelet activation
CD44	Canine[a,b,c] Feline[b] Equine[a,b,c] Bovine[a] Porcine[a,b,c]	I I	Most cell types including epithelial cells, activated T cells	Receptor for hyaluronate that facilitates lymphocytic binding to high endothelial venules
CD45	Canine[a,b] Bovine[a] Porcine[a]	I	Pan-leukocyte	Membrane-bound tyrosine phosphatase critical for antigen-receptor-mediated activation of leukocytes
CD45RA	Canine[7,8,36] Porcine[a,b]	I	Naïve T/B lymphocytes	Largest of the CD45 isoforms; predominant CD45 isoform expressed on B cells
CD45RB	Bovine[b]	I	Naïve T/B lymphocytes	Predominantly expressed on T cells
CD45RC	Porcine[a]	I	CD4 T cells, gamma delta thymocytes	
CD45RO	Bovine[b]	I	Memory T cells	
CD47	Canine[a] Porcine[a,b]	III	Lymphocytes, macrophages, granulocytes	Associates with CD61 integrins to form receptor for thrombospondin; role in chemotaxis and adhesive interactions with leukocytes and endothelial cells
CD49d	Feline[3,20] Canine Porcine[a]	I	Lymphocytes, macrophages, granulocytes	Very late antigen (VLA) binds with 39CD29; binds fibronectin and mucosal addressin
CD49f	Bovine[c] Porcine[a,b]	I	Macrophages, granulocytes	VLA antigen binds to CD29 and together is the fibronectin receptor and binds to RGD sequence of fibronectin
CD56	Canine[26] Feline[27]	I or V	Subset of lymphocytes	Homotypic adhesion and natural killer cell cytotoxicity
CD61	Canine[a] Feline[a,b] Porcine[a,b]	I	Platelets, megakaryocytes, monocytes	Associates with CD41 to form the GPIIb–IIIa heterodimer that facilitates platelet aggregation
CD62L	Canine[38] Bovine[c]	I	Leukocytes	Cell adhesion molecule L-selectin, homing of T cells to enter high endothelial venules (HEVs) in secondary lymphoid organs

(*Continued*)

TABLE 6.1 (Continued)

CD antigen	MAb reactivity	Topology	Cellular distribution	Physiology
CD62P	Canine[5,37] Feline[a] Equine[4] Porcine[a,b]	I	Activated platelets	Cell adhesion molecule P-selectin, stabilizes initial platelet aggregates in clot formation
CD68	Canine[a] Feline[b]	I	Monocytes, macrophages, and macrophage-derived cells such as osteoclasts	Binds to tissue- and organ-specific lectins or selectins, allowing for homing and migration
CD79a	Canine[a] Feline[b] Equine[b] Bovine[b] Porcine[a,b]	I	B lymphocytes	Cytoplasmic molecule associates with CD79b and heavy chain for surface B cell receptor (BCR) expression and BCR cell transduction
CD79b	Canine[a] Porcine[a]	I	B lymphocytes	Cytoplasmic molecule that associates with CD79a to form a heterodimer mediating B cell receptor signaling
CD80	Canine[b] Bovine[a] Porcine[b]	I	Dendritic cells, activated macrophages, activated B cells	Costimulatory molecule for T cell activation during primary immune response
CD81	Feline[b]	III	Germinal center B cells	Coreceptor for CR2 (CD21). CR2 intracellular domains dimerize with antigen cross-linked BCR to signal B cell activation
CD86	Bovine[a]	I	Dendritic cells, activated macrophages, activated B cells	Costimulatory molecule for T cell activation during primary immune response
CD90	Canine[a, c] Equine[3] Porcine[c]	V	Pro-thymocytes, T cells, monocytes; weak on granulocytes, renal tubular cells	May contribute to formation of neuron memory and to growth regulation of hematopoietic stem cells
CD94	Canine[c]	II	Lymphocyte subset (NK)	Binds to NKG2 and plays a role in recognition of MHC class I molecules by NK cells and some cytotoxic T cells; ligation of CD94 can inhibit or stimulate killing by NK cells
CD117	Canine[31,12] Porcine[a]	I	Hematopoietic stem cells, higher expression in common myeloid progenitor and lower expression in common lymphoid progenitor cells	Receptor tyrosine kinase type III that binds to stem cell factor and promotes cell survival, differentiation, and proliferation
CD133	Canine[b]	III	Hematopoietic stem cells	Function and ligand unknown
CD134	Feline[a]	I	CD4+ activated T cells	Tumor necrosis factor receptor superfamily; regulator of T-cell-dependent immune responses; receptor for FIV in conjunction with CXCR4
CD163	Porcine[a,b]	I	Monocytes, tissue macrophages	Scavenger receptor cysteine-rich family
CD172a	Bovine[a] Porcine[a]	I	Monocytes, granulocytes	Member of the signal regulatory protein family involved in negative regulation of receptor tyrosine-kinase-coupled signaling processes
CD204	Canine[14,15] Porcine[b]	II	Tissue macrophages	Class A scavenger receptor
CD282	Bovine[a]	I	Monocytes	Toll-like receptor (TLR) 2—cell surface pattern recognition receptor (PRR) that responds to a variety of bacterial cell wall components
CD335	Bovine[a] Porcine[a]	I	Natural killer cells	Also known as NKp46, functions as an activating receptor for NK cell activity

Column 1: CD designation of the antigen. Column 2: species of reactivity (letter superscripts are commercially available from [a]Bio-Rad, [b]ThermoFisher, and [c]R&D; number superscripts correspond to references). Column 3: integration of antigen to plasma membrane. Column 4: cell types and tissues known to express a CD. Column 5: proposed or known physiology of a CD antigen based on studies in animals or humans. The original clones and their original characterization can be found in the 6th edition of this chapter.

topology in the membrane; tissue distribution; and known physiology. Most CD antigens listed in Table 6.1 are commercially available; however, selected key references for CD antigens not known to be commercially available are included.

More in-depth information is available for the currently described CD molecules on several websites, including the taxonomic key program maintained by Washington State University, designed to provide information of the specificity of MAbs for intra- and cross-species of leukocyte differentiation molecules (https://apps.vetmed.wsu.edu/tkp/). Biocompare Antibodies is a search tool (https://www.biocompare.com/Antibodies/) where you can query the availability of commercially available

cross-reactive antibodies for a species of interest. Major suppliers of veterinary-specific MAbs are Bio-Rad (https://www.bio-rad-antibodies.com/veterinary-antibodies.html), Thermo Fisher (https://www.ThermoFisher.com/antibody), and R&D Systems (https://www.rndsystems.com/uniquemodels).

STRUCTURE AND INTEGRATION OF MEMBRANE ANTIGENS

CD antigens are principally membrane proteins defined by their location within or at the surface of the phospholipid bilayer. Membrane proteins are classified into three categories: integral, lipid-anchored, and peripheral, depending on the nature of membrane–protein interactions.[17] The CD antigens are grouped as integral membrane proteins or transmembrane proteins and consist of cytosolic, membrane spanning, and exoplasmic (luminal) domains. The cytosolic and exoplasmic domains have hydrophilic exterior surfaces with either C-terminus or N-terminus group endings. The membrane-spanning domains usually contain hydrophobic amino acids and consist of one or more alpha-helix or multiple beta strands.[17] Most integral membrane proteins fall into one of five classes, depending on how they anchor themselves in the membrane (Figure 6.1).[1] Type I and type II proteins have a single transmembrane region, whereas types III and IV have multiple transmembrane regions also referred to as tetraspanins. Type IV proteins (not shown in the figure) are distinguished from type III proteins by the presence of a water-filled transmembrane channel. Type V proteins attach to membranes via lipid. These lipids are either a glycosylphosphatidylinositol (GPI) anchor or lipid moieties such as myristoyl groups, which include cytoplasmic signaling proteins that will not be described in this chapter.[1,17] Each class pertinent to CD antigens used in veterinary medicine will be described briefly.

TRANSMEMBRANE PROTEINS

Single-Pass Type I Transmembrane Protein (I)

Types I and II transmembrane proteins have only one membrane-spanning α-helix containing 20–25 hydrophobic amino acids. Type I proteins have an N-terminal endoplasmic reticulum (ER) signal sequence that is cleaved after the molecule passes into the ER. This is the most common mode of membrane integration among the CD antigens (Figure 6.1). The protein is glycosylated in the Golgi apparatus if the protein has a glycosylation site and then is expressed on the cell surface. Type I proteins are anchored in the membrane with their hydrophilic N-terminal region on the exoplasmic face and their hydrophilic C-terminal region on the cytoplasmic face. These proteins commonly represent cell surface receptors and/or ligands (like CD4), of which many belong to the immunoglobulin (Ig) superfamily.[1]

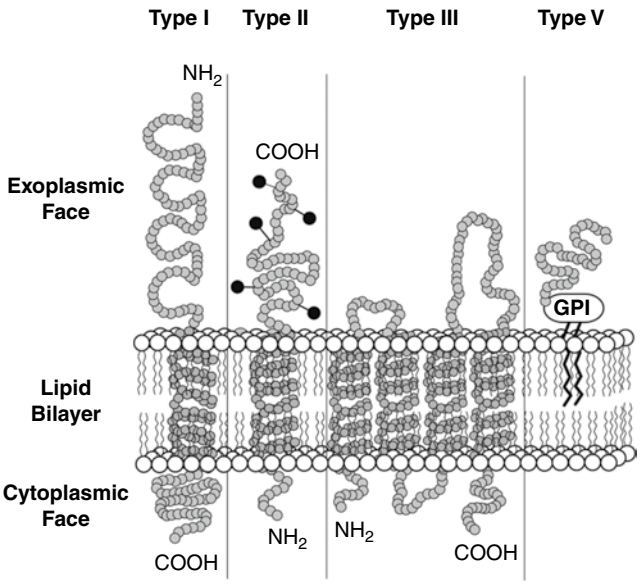


FIGURE 6.1 Major integral membrane proteins and their topology or interaction in the lipid bilayer. The types of membrane proteins are indicated at the top. CD4 is a type I transmembrane protein that passes through the membrane once, has four extracellular immunoglobulin domains, a carboxyl terminus on the cytoplasmic face of the bilayer, and N-terminus (NH_2) on the exoplasmic face. CD13 is a type II transmembrane protein with the N-terminus on the cytoplasmic face and the carboxyl terminus on the exoplasmic face. The extracellular domain of CD13 is heavily N-glycosylated (thin pegs extending from the polypeptide backbone). CD9 is a type III multipass membrane protein with four transmembrane regions, and N- and C-termini on the cytoplasmic face. CD14 is a type V glycosylphosphatidylinositol (GPI)-anchored protein. (**Source:** Courtesy of Mal Rooks Hoover, certified medical illustrator)

Single-Pass Type II Transmembrane Protein (II)

Type II transmembrane proteins lack a cleavable ER signal sequence and are oriented with their hydrophilic C-terminus on the exoplasmic face and the hydrophilic N-terminus on the cytoplasmic face. These proteins have an internal hydrophobic ER signal and membrane anchor sequence. Because these proteins can be released from the cell surface, they can act as plasma proteins with physiologic effect(s) on cells bearing the counter ligands.[1] For example, CD13, a zinc-binding metalloprotease, acts to facilitate antigen presentation by trimming the N-terminal amino acids from major histocompatibility complex (MHC)-class-II-bound peptides.[1]

Multipass Transmembrane Protein (III)

Type III transmembrane proteins cross the membrane multiple (2, 3, 4, 5, 7, or 12) times and are called tetraspanins. The most frequently found tetraspanins include those that span the membrane four and seven times. Structural studies indicate that the transmembrane regions are alpha-helices. If the type III protein has an even number of transmembrane alpha-helices, its N-terminus and C-terminus are oriented toward the

same side of the membrane. Many of these cell surface molecules function as receptors for soluble molecules such as prostaglandins and chemokines. Examples of seven transmembrane type III proteins are interleukin-8 (IL-8) receptor (CD128) and C5a receptor (CD88). CD9 is an example of a four-multipass transmembrane protein.[1] CD47 is an example of type III protein with five transmembrane sequences. The multidrug resistance transporter protein (MDR)-1 (CD243) has 12 transmembrane regions.[1]

GPI Transmembrane Protein (V)

Type V transmembrane proteins utilize GPI anchors attached to the C-terminus residue of the protein (Figure 6.1). GPI-anchored molecules have a secretion signal sequence at their N-terminus and C-terminus that is cleaved and replaced by the GPI anchor after synthesis of the molecule and entry into the ER.[18] CD14 is an example of a type V surface protein involved in the clearance of Gram-negative pathogens that bind to –LPS binding protein. Other examples of GPI-linked glycoproteins include CD56 and CD90.[1]

TISSUE DISTRIBUTION

Cell surface antigens commonly used in immunophenotyping of hematologic neoplasia initially were determined by studies of hematopoietic cell differentiation and maturation.[9,32] Lineage-associated markers can be broadly classified into groups that recognize B cell, T cell, natural killer (NK) cell, myeloid/monocytic, and erythroid, granulocytic, and platelet lineages. Noncommitted hematopoietic stem cells express CD34, a glycosylated surface glycoprotein. This marker is frequently used to differentiate acute immature leukemias of lymphoid or myeloid origin from chronic lymphocytic leukemia or leukemic stages of lymphoma.[36] See Chapter 63 for additional details on applied immunophenotyping. Although the last CLAW was in 1993, it was an important collaborative effort because anomalous expression of the CD4 antigen was identified on canine neutrophils in addition to its predicted expression on helper T cells.[21] Additional examples of CD antigen expression unique to domestic animals are high expression of the γδ T cell receptor in pig and ruminant lymphocytes[6,22] and co-expression of CD4 and CD8 on mature T cells in swine.[32]

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CHAPTER 7

Major Histocompatibility Complex Antigens

PAUL R. HESS

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Acronyms and Abbreviations

BCR, B-cell receptor; CD, cluster of differentiation; DLA, dog leukocyte antigen; DRB, DR beta, DRiP, defective ribosomal product; FLA, feline leukocyte antigen; GAD, glutamic acid decarboxylase; GVHD, graft-versus-host disease; HFE, high Fe; HLA, human leukocyte antigen; HSC, hematopoietic stem cell; IEDB, Immune Epitope Database; IR, immune response; KIR, killer cell immunoglobulin-like receptor; LIR, leukocyte immunoglobulin-like receptor; LMP, low-molecular-mass polypeptide; LT, lymphotoxin; MHC, major histocompatibility complex; MIC, MHC class I chain; NIH, National Institutes of Health; NK, natural killer; pMHC, peptide–MHC complex; TAP, transporters associated with antigen processing; TAPBP, tapasin; TCR, T-cell receptor; TNF, tumor necrosis factor.

INTRODUCTION

Self-/non-self-recognition is an important inherent characteristic of cellular life forms, and the basis of immunity: the process of clearing or compartmentalizing foreign materials and organisms to preserve homeostasis. For large, higher-order life forms, multifaceted, integrated immune systems have been selected to deal with such threats, primarily from much simpler microorganisms. Invasions by equally complex macroscopic

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parasites also trigger patterns of immune defense that can protect the host from harm. For example, more than one-third of humans have been infected with *Toxoplasma gondii*, but most remain asymptomatic.[19] When immunity is impaired, however, such as with HIV/AIDS, then the devastating and sometimes lethal consequences of unrestrained parasitism can be observed.[64,73] Self-/nonself-recognition also occurs between conspecifics, at the level of tissue–tissue interactions, employing some of the same immune mechanisms used to fight off xenogeneic threats. For jawed vertebrates, the cornerstone of this recognition is the major histocompatibility complex, or MHC.

First discovered in the mouse,[25] the mammalian MHC comprises a very large and dense cluster of genes arrayed, generally, but not always, on a single chromosome. Many of the gene products are involved in immune sensing, antigen presentation, and adaptive and innate immune effector action. The MHCs of nonmammalian vertebrates are smaller and simpler, but contain similar elements and overall organization. Not surprisingly, the human MHC, a 4-million-plus base-pair stretch containing more than 220 genes, is the most extensively characterized since its formal identification in 1958,[15] and will serve as one of the standards that we reference in this chapter, to highlight the differences in the MHC of other species of veterinary interest.

MHC CLASS I AND CLASS II GENE DIVERSITIES ASSIST THE ADAPTIVE IMMUNE RESPONSE

Some 30–40% of MHC genes encode proteins related to immunity. The term MHC frequently is used colloquially to refer to two sets of genes within the cluster, class I and class II. More specifically, the reference is to so-called classical class I and class II genes in those regions that encode a number of surface glycoprotein receptors positioned directly at the interface between immune effector cell and foreign threat. Indeed, the rare absence of these classical MHC molecules results in severe immunodeficiency and loss of resistance to infectious agents, demonstrating the importance of the system to survival in an inimical world.[84] The classical genes are well-studied for other noteworthy features. Because MHC genes have and continue to evolve in an unceasing arms race, the tremendous diversity of pathogens (and individual pieces of those pathogens) to which an organism is exposed requires an equally robust level of diversity on the afferent (sensing) and efferent (effector) limbs of the immune system.

B- and T-Cell Responses Depend on MHC Diversity to Provide Robust Host Protection

The adaptive immune response of higher animals is an example of a strategy to counter this diversity problem. The collective business ends of an individual's adaptive effector cells, namely, the repertoires of B-cell receptors (BCR) and T-cell receptors (TCR), have diversity that is generated by switchable genetic recombination machinery operative during lymphocyte ontogeny. The extent of diversity is limited in practical terms only by the number of lymphocytes that the animal can physically hold. The MHC plays the afferent role, initiating, directing, and sustaining TCR-driven responses at a primary level, and BCR-driven responses, at a secondary level, via T-cell help. Diversity on the MHC side, which can range up to two orders of magnitude higher than the genomic average,[21] is maintained in part by extensive polymorphisms in the gene pool, which control the variety of pathogen pieces that are visible to T cells.[57] Not unexpectedly, specific classical MHC class I and class II allelic variants have been shown to correlate with differential resistance or susceptibility to infectious agents,[72,74,81] and in cases where the immune system inadvertently attacks self-tissue, with autoimmune disease risk.[2,22,40,41,97,96,98] As we will see in the following text, MHC polymorphisms also constitute the most significant structural basis for rejection of tissue allografts. Accordingly, the sum of all these features places the MHC at the "center of the immune universe."[91]

STRUCTURE OF THE MHC GENE CLUSTER

The genomic organization of the mouse and human MHCs began with gene mapping studies and was finalized by large-scale sequencing data. In other species, sequencing of orthologous individual MHC genes was the first work undertaken, but now, more complete pictures of their MHCs are emerging.

The MHC Comprises Three Distinct Subregions

The human MHC, located on the short arm of chromosome 6, is divided into three distinct regions of similar function (Figure 7.1), running from centromere to telomere: class II (approximately 800 kb), class III (approximately 1100 kb), and class I (approximately 1800 kb). The class II region contains genes encoding both chains of the previously described classical molecules (called DP, DQ, and DR), nonclassical molecules (DM and DO), immune-related pseudogenes, and discordantly, genes related to processing of antigens destined for class I binding (the transporters associated with antigen processing [TAP]; the inducible proteasome subunits, low-molecular-mass polypeptide [LMP]; the chaperone, tapasin [TAPBP]). Class III is the most conserved, gene-dense region, with few pseudogenes, whose products (e.g., lymphotoxin [LT] and tumor necrosis factor [TNF]; complement factor C4) are related to innate immunity and inflammation, not adaptive responses. The class I region contains classical and nonclassical class I genes, such as human leukocyte antigen (HLA)-E, interspersed among nonimmune "framework" genes. The MHC arbitrarily ends with the last class I gene, but conceptually, the "extended MHC" is considered to run farther to the class-I-like gene high Fe (HFE), which is associated

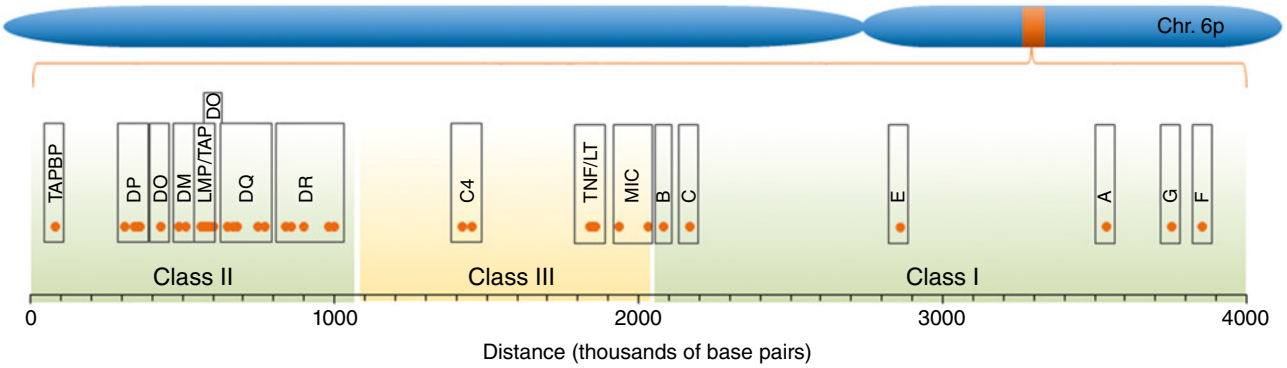


FIGURE 7.1 Overall genomic organization of HLA as a representative MHC. The depiction includes the extended class II (to tapasin [TAPBP]) region, but not extended class I. Boxes enclose a gene cluster; the individual genes are depicted by orange circles. The classical genes in class II are DP, DQ, and DR; nonclassical are DO and DM. The classical genes in class I are B, C, and A; nonclassical are E, G, and F. As described in the text, LMP and TAP are class-I-related antigen processing pathways, and C4, TNF/LT, and MHC class I chain related (MIC) are innate immune genes.

with an HLA-linked disease. It is important to point out that not all MHC haplotypes (i.e., haploid genotype—a particular constellation of MHC alleles) in humans or other animals have the exact same number of genes; this number can vary between individuals.[93] Also, the MHCs of other species are often not so neatly packaged on a single chromosome. Even when that is the case, however, multiple paralogs (i.e., genes that have been duplicated within the species) of many MHC genes can be identified on other chromosomes as well.[18,38] An example of such a paralog is the nonclassical canine MHC class I allele dog leukocyte antigen (DLA)-79.[12]

The Organization of the MHC Varies by Genome

The MHC of the shark, as a cartilaginous fish presumably closest in evolutionary terms to the common ancestor of jawed vertebrates that arose >500 million years ago, can serve as a representative of prototypical genomic organization.[68] Viewed through that lens, there are many differences in MHC organization that have evolved since divergence of particular orders from that ancestor, the most notable of which are listed in Table 7.1. For the interested reader, more detailed genomic information has now been published on the MHC of a number of species, including dogs, cats, rats, horses, pigs, cows, sheep, chickens, reptiles, and fish,[7,20,28,33,35,60,61,77,101,104,105] and broad reviews of comparative MHC structural features are also available.[18,39,45,46]

Comparing Classical Loci across Animal Species is Challenging

In humans, some nonhuman primates, mice, and rats, the classical loci found in the class II and class I regions have been functionally defined, by verifying their ability to direct T-cell responses. The same is not true in most other animals, where only a few have been similarly characterized. Instead, the designation (e.g., "classical class I locus" or "class II DRB") has been assigned by sequence homology. These genes contain the same promoters, exons, splice sites, and structurally important invariant amino acids (e.g., the two cysteine residues that form disulfide linkages between the beta-sheet and alpha-helix in class I molecules) to suggest that a properly folded, full-length, functional molecule will be expressed at the cell surface. That said, because the MHC is rapidly evolving, with genes being gained and lost in a continuous birth-and-death process, it is important to stress that there are limits when looking for orthologous genes between members of orders that are not closely related.[46,94] With the exception of bony fishes, classical class I genes are shorter-lived and more difficult to compare than class II genes, which have greater longevity, allowing orthologous loci to be identified between orders of mammals.[88] Classical class II genes are present in birds, reptiles, amphibians, and fish, but are different than their mammalian counterparts. The bottom line is that, while there are numerous regions of shared synteny that can be illuminating for comparative and evolutionary studies, different MHCs have different genes, order, size, organization, and chromosomal locations that also make the study of each a unique endeavor.

CLASSICAL MHC GENE PRODUCTS REGULATE T-CELL IMMUNITY

Despite these interspecies genetic differences in the MHC, the three-dimensional structures of MHC class I and class II molecules are well-conserved between species, reflecting a common gene ancestor[68] and similar functional demands. That function is to control T-cell activation during adaptive responses by providing (1) a confirmation of self when T cells interact with tissue cells; and (2) to display a fragment of pathogen materials, generally a short peptide (class I, 8–12 amino acids; class II, 13–17). The combination of self and non-self, known as altered self,[106] often, but not always (approximately 60% of the time[5]), has a corresponding TCR

TABLE 7.1 Selected differences in MHC genomic contents and organization.

Species	Chr.[a]	Distinguishing features
Mouse	17	Class II: Nonfunctional DP genes; DR genes functional in only one half of strains. Expanded class I genes in extended class II region. MHC-like MIC genes are absent.
Dog	12	Class II: Single functional DR beta (DRB) gene; inverted DRB gene; nonfunctional DP genes. Class I: split, with 500-kb portion moved to different chromosome (Chr. 35; no classical genes); regions corresponding to HLA-A and HLA-E are absent. "Divergent" class I gene on 3rd chromosome (chr. 18).
Cat	B2	Class II: Lack of DQ genes, nonfunctional DP genes, expansion of DR genes; inverted DRB gene. Class I: split/inversion, with centromere invasion; regions corresponding to HLA-A and HLA-E are absent.
Horse	20	Smaller MHC; nonfunctional DP genes. Five inverted DRB genes.
Cow, sheep	23, 20	Class II separated into two subregions by chromosomal inversion, causing loss of class II DP genes and gain of class II DI/DY genes; inverted DRB gene.
Pig	7	Class II separated from class I and class III by centromere; absence of DP genes.
Chicken	16	Compact MHC divided into two loci, B and Rfp-Y, on same mini-chromosome. Class II alpha chain outside of core MHC region. NK receptors LIR and KIR located within MHC; loss of LMP; only immune gene in class III is C4.
Tuatara	13	Low gene density; high content of repetitive elements. Multiple duplications of class II genes. Intermingling of class I and class II genes. Antigen-processing genes such as TAP and typical framework genes absent from MHC. Nonclassical class I genes (possible paralogs) on 2nd chromosome (chr. 4).
Frog	13[b]	Class I between class II and III regions; only one classical class I locus; some class-III-like genes moved to classical class I region. Duplicate MHC silenced in polyploidy.
Zebrafish	Multiple[c]	Separate chromosomal locations for classical class I (chr. 19) and class II (chr. 4, 8, and 18) genes. Nonclassical or paralogous class I genes on other chromosomes (chr. 1, 3, 8, 22, and 25). Lack of immune genes in class III region.

[a]Major chromosomal location.
[b]In *Xenopus laevis*: Courtet, M., Flajnik, M. & Du Pasquier, L. Major histocompatibility complex and immunoglobulin loci visualized by in situ hybridization on Xenopus chromosomes. Dev Comp Immunol 25, 149–157 (2001).
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available in the animal's T-cell repertoire. When that is the case, and a sufficient number of serial MHC–TCR interactions occur, the T cell is triggered, leading to robust proliferation, followed by a coordinated program of downstream effector functions.

MHC Class I and Class II Gene Products Have Different Distributions and Functions

Well-established differences distinguish MHC class I and class II molecules. The former are found on virtually all cells, except erythrocytes, spermatozoa, and neurons, and regulate CD8+ T cells. The latter are, in most instances, expressed only on professional antigen-presenting cells, such as B cells, dendritic cells, and macrophages, and regulate CD4+ T cells. A depiction of these structures, and their interaction with T cells, is shown in Figure 7.2. Note that there appears to be no differences that distinguish TCRs that recognize MHC class I molecules from those that recognize class II. Instead, this conventional paradigm—class I/CD8; class II/CD4—is dictated by the binding of each coreceptor to conserved sites on their respective MHC molecules during T-cell development.[11,49,95] These simultaneous MHC–coreceptor interactions function to amplify peptide–MHC–TCR signaling.[4] It is possible to identify "rule-breaking" T cells, that is, CD4+ T cells regulated by class I,[10] and CD8+ T cells regulated by class II,[75] but these occur only at very low frequencies and under specific conditions.

MHC PROTEINS ARE ANTIGENS THAT DRIVE ALLOREACTIVITY

In an antigen-naïve animal, T cells corresponding to any given foreign peptide (p)-MHC class I or class II molecule combination are very rare, on the order of 1 in 100,000 T cells,[63,67] even discounting the inherent promiscuity of T-cell recognition.[99] What that rarity means is that observing a given T-cell response (e.g., specific for influenza hemagglutinin) against the enormous background (99.999%) of irrelevant T cells is not a trivial matter. Typically, an initial T-cell expansion in vivo is required (so-called priming, by infection or vaccination), followed 10–14 days later by a second round of antigen exposure ex vivo (restimulation, lasting 5–10 days). Only at that point are specific T cells present at a sufficiently high frequency to be measurable in a functional assay, or by flow cytometry.

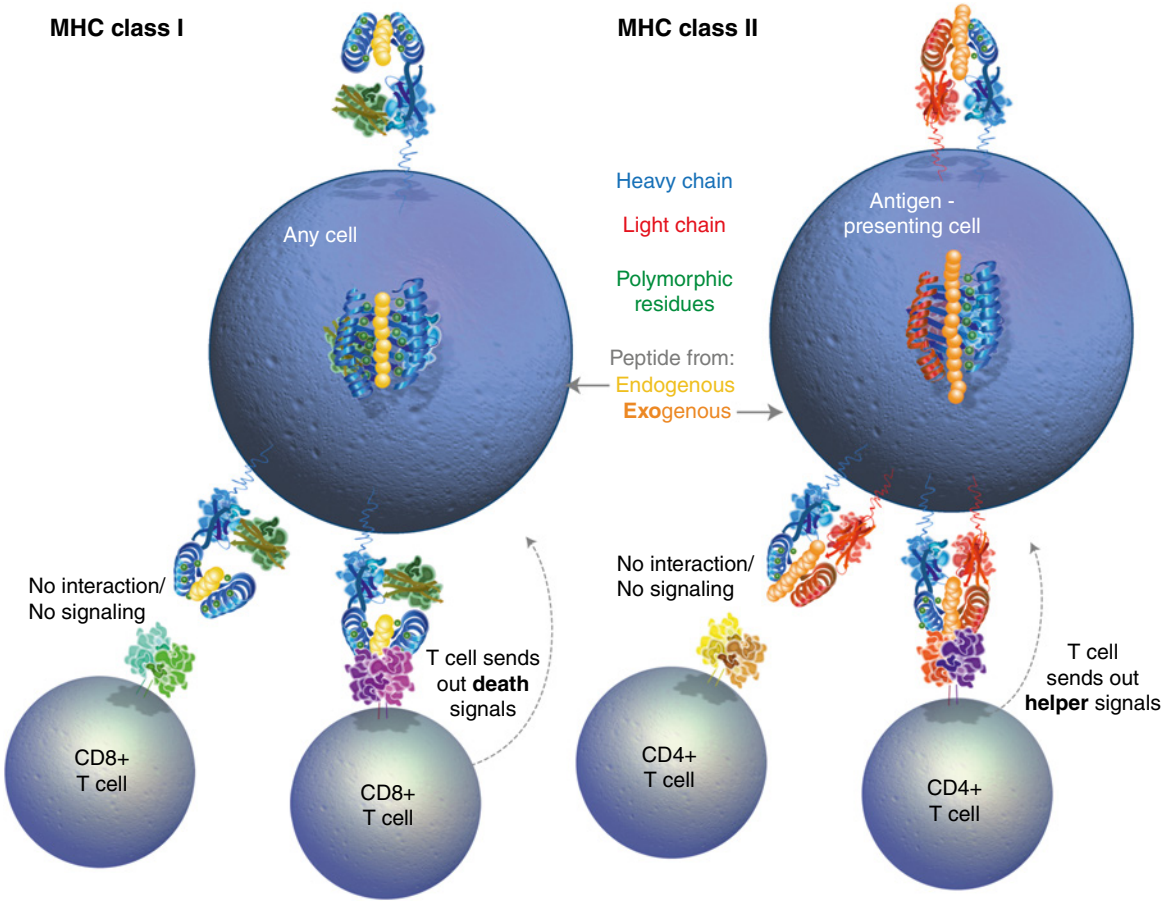


FIGURE 7.2 Left: Shown are three MHC class I molecules on the cell surface, one of which is interacting with the T-cell receptor (TCR) of a cytotoxic CD8+ T cell (bottom of figure). A short, 8–10 amino-acid-long peptide that matches the allomorph's binding preference (dictated by polymorphisms, shown as green circles) is held completely within the binding groove. Recognition by the corresponding ("cognate") TCR of the combination of self-MHC and non-self peptide leads to T-cell triggering and action—in this case, delivery of molecules that leads to death of the target cell. The non-MHC-encoded β2M light chain is shown as the olive/green ribbon structure. Right: Shown are three MHC class II molecules on the cell surface, one of which is interacting with the TCR of a helper CD4+ T cell (bottom of figure). A short, 12–15 amino-acid-long peptide that matches the allomorph's binding preference (dictated by polymorphisms, shown as green circles) is held within the binding groove, but extends beyond either end. Recognition by the cognate TCR of the combination of self-MHC and non-self peptide leads to T-cell triggering and action—in this case, delivery of cytokine and surface ligands that assist the immune function of the antigen-presenting cell ("T-cell help"). Illustration: AM Harvey.

T-Cell Responses to Non-Self-MHC Proteins are a Ubiquitous Phenomenon

Interestingly, T cells capable of recognizing the other form of non-self, that is, a foreign MHC allomorph (allomorph = allele-specific protein product), occur naturally at a high frequency, constituting up to 1 in 10 T cells. A hallmark of the phenomenon, known as alloreactivity, is that it is readily observed, without any need for prior sensitization or expansion. That property is shown by the classic mixed lymphocyte reaction, first described by Bach et al.: MHC-mismatched lymphocytes cultured together proliferate in response to non-self.[6] In fact, it was the observation of alloreactivity, rather than any demonstration of immune defense against microorganisms, that led pioneers such as Peter Gorer, George Snell, and Peter Medawar to the discovery of the MHC (hence the term histocompatibility in the name). Consequentially, polymorphic MHC class I and class II proteins have long been referred to as "antigens," because in early studies, they were detectable serologically, and recognized by T cells of other members of the species (and, of course, as self, they serve as "permissive" antigens, furnishing one half of the primary stimulatory signal for T cells). It is convention therefore to refer to the MHC class I and class II genes by the term "leukocyte antigens," preceded by the species name, such as HLA, or feline leukocyte antigen (FLA).

Anti-MHC Immune Responses Reflect Inherent Properties of T-Cell Populations

It is puzzling that T-cell responses against a rarely encountered foe, foreign tissue, are so much more easily demonstrated than responses against a virtually ubiquitous foe, infectious organisms. At first glance, the vigorous immune reaction against MHC allomorphs might

seem to be a quirk of the T-cell system. Indeed, as a way to explain alloreactivity, some studies have suggested that TCRs have a hardwired affinity for MHC molecules,[82] although others have failed to find support for this notion (reviewed in Felix et al.[17]). More importantly, though, mature T-cell populations have been substantially edited during thymic development, with those bearing TCRs with affinities for self peptide–MHC complex (pMHC) below positive or above negative selection thresholds being eliminated. Self-recognition by T cells is a dose-dependent phenomenon: like Goldilocks, neither too little nor too much is just right. Thus, allorecognition of non-self-MHC polymorphisms can be seen as a consequence of T cells having been chosen for overall increased MHC affinity, but without the restraints imposed by negative selection on too-vigorous binding to some pMHC combinations. In most natural settings, this phenomenon, however robust, might seem to be irrelevant, but there is one important exception: pregnancy. The fetus survives, despite the presence of hostile maternal T cells reactive against paternal MHC allomorphs, because these T cells are transiently rendered tolerant by several mechanisms.[65,87] That alloreactivity persists despite this cost suggests that a fitness advantage is conferred.

Why is Alloreactivity Tolerated?

One early explanation suggested that alloreactivity assisted with inbreeding avoidance.[37] A more recent and instructive model in this regard is provided by transmissible leukemias of mollusks, which lack an MHC system.[58,59] In the last decade or so, five separate lineages of parasitic tumors have been identified in clams, spread across hundreds of miles of coastline, in several far-flung locales, such as New England, northern Spain, and British Columbia. These transmissible tumors are passed horizontally, clam-to-clam, through seawater. In one bed, approximately 50% of clams were found to be afflicted. It seems plausible that MHC-based self-/non-self-recognition of conspecific tissues evolved as a defense against contagious cancers. These risks are evident in mammals, too, when T-cell allorecognition is disabled, as in well-known examples of group transmission of cancer clones in dogs and Tasmanian devils,[13,30] as well as in isolated human cases of vertical cancer spread.[34]

Alloreactivity Has Consequences for Clinical Medicine

Of course, alloreactivity against MHC antigens has taken on greater significance in humans since technological advances have made it possible to transfer solid and liquid (hematopoietic) tissues between species members. The consequences of HLA mismatch include graft rejection and graft-versus-host disease (GVHD). Newer molecular methods have facilitated histocompatibility typing, but, ironically, have also revealed far more HLA polymorphisms than recognized by older serologic-based methods, making exact matching difficult.[17] Nonetheless, recipients of mismatched and matched solid organ grafts can now enjoy similarly good outcomes, thanks to the development of powerful immunosuppressant agents capable of inhibiting alloreactive T cells. The same is not true for transplantation of hematopoietic stem cells (HSC), where matching remains critical to prevent the lethal consequences of GVHD. Presently, clinical allotransplantation is rare in veterinary patients, and, consequently, information on MHC typing and matching is limited. Appropriate protocols will likely need to be established and shared by individual investigators working in the field, on a per-species basis, so will not be reviewed here. Sequence-based typing of polymorphic DLA genes is available through the Fred Hutchinson Cancer Research Center, and is performed in preparation for allogeneic HSC transplantation following total body irradiation for treating lymphoid neoplasia.[53,85]

MHC PROTEINS PRESENT ANTIGENIC PEPTIDES TO T CELLS TO DIRECT IMMUNE RESPONSES

The demonstration that classical MHC gene function extended beyond transplanted tissue histocompatibility, and regulated immune responses to exogenous antigens, was not actually made until the late 1960s. Even then, the relationship between the two types of responses was unclear.[55,56] At that time, studies showed that some mouse strains carrying particular MHC genes made antibodies to weak synthetic polymeric antigens, while other strains were unable to do so. Now we understand that the differences in responsiveness were artifacts resulting from inbreeding and MHC homozygosity, which limited the diversity of the T-cell responses. More than a decade would then pass until Doherty and Zinkernagel showed that T-cell recognition of non-self viral antigens was dependent on the simultaneous presence of self-MHC polymorphisms. Hence, T-cell reactivity is said to be "MHC-restricted."[107] That the specific targets of T-cell recognition were peptide fragments of larger foreign proteins would emerge in the mid-1980s,[83,90] as did the structural basis of MHC class I presentation of those peptides.[8,9]

Classical MHC Molecules Bind Antigenic Peptides for T-Cell Recognition

What became clear from these and other studies is that antigenic peptides (also known as epitopes) bind to MHC class I and class II molecules via the interaction of amino acids at defined "anchor positions" in the peptide with certain residues contained in specific pockets of the peptide-binding groove. Most importantly, the amino acids in these peptide-binding pockets are encoded by gene regions where the extensive polymorphisms of classical MHC genes are concentrated (Figure 7.2, green circles). With different pocket chemistries, individual MHC allomorphs are seen to have

distinct and discernable preferences for peptides that vary at their anchor positions. These preferences constitute the binding motif, which can be worked out by sequencing large numbers of peptides eluted from the binding groove of a selected allomorph to form a consensus picture. For example, in the dog, the DLA-88 (dominant class I gene) allomorph *508:01 prefers 9- and 11-mer length peptides with hydrophobic residues at positions 2 and 3, and a basic residue at the C-terminus, while *034:01 prefers 9-mers with a basic residue at position 1, hydrophobic at position 2, an acidic at position 4, and a phenylalanine at the C-terminus.[66,79] The motif be quite constraining for MHC class I molecules, but less so for class II molecules, which are more accommodating of peptide variability. Catalogs of such peptides for a variety of species can be found at Immune Epitope Database (IEDB; see: https://www.iedb.org/home_v3.php), a resource funded by the National Institutes of Health (NIH). If the motif of a given class I or class II allomorph is established, then T-cell epitopes can also be predicted using online algorithms designed for this purpose.[50] Flow-cytometry-based binding assays using synthesized peptides can subsequently be used to confirm or refute such predictions for MHC class I molecules, as demonstrated for DLA.[80]

Diversity in Classical MHC Molecules Stems from Multiple Sources

Because of their binding fussiness, MHC molecules only present to T cells a very small percentage of possible peptides contained within a pathogen's proteome, estimated at just 2–3% for MHC class I molecules.[5] While this characteristic would seem to be a vulnerability in the host's armor, that is, pathogen mutants lacking motif-matching peptides would quickly be selected for, the MHC system has built-in countermeasures to reduce the likelihood of such immunoevasion. Most notably, at the population level, polymorphisms collectively increase the overall diversity of peptide coverage of a pathogen (e.g., different allomorphs = different motifs = different peptides). Thousands of MHC class I and class II alleles are found in humans (see the IPD-IMGT/HLA database at https://www.ebi.ac.uk/ipd/imgt/hla/ for the latest tally). In many species, the rich sequence diversity of the MHC appears to have arisen from multiple mechanisms, including single nucleotide mutations, insertions and deletions, and intralocus and interlocus gene conversion. Erosion of this genetic diversity is thought to be counterbalanced by parasite-mediated selection. There are two principal forces behind this process: negative frequency-dependent selection (rare alleles confer an advantage) and overdominant selection (heterozygosity confers an advantage).[3,14,32,44] Other mechanisms have also been postulated.[51] Moreover, there is evidence in multiple species that MHC-dependent sexual selection—selective fertilization or abortion, and disassortative matings—assists in maintaining polymorphisms.[23,48,52,78] Further, individual animals increase their MHC diversity by expressing both parental copies of multiple class I and class II genes (polygeny), for which they are typically heterozygous. Finally, some species use alternatives to allelic polymorphisms to achieve MHC diversity, such as the variable numbers and combinations of expressed class I genes, a strategy employed by rhesus macaques, for example.[69]

MHC-Presented Peptides are Generated by Several Cellular Mechanisms

The peptides presented to T cells by MHC class I and class II molecules have distinct origins. Classically, class I peptides come from degraded proteins, as well as defective ribosomal products (DRiPs), in the cytosol.[103] On the other hand, class II peptides are extracellular in origin, passing through the intracellular vesicular system of endosomes, phagolysosomes, endoplasmic reticulum, and Golgi apparatus on their way to the cell surface. The elegant cellular machinery for processing and loading peptides onto MHC molecules of either stripe has been well described in basic immunology texts, and will not be reviewed here. Many of these components responsible for antigen processing and presentation are encoded in the MHC, potentially to facilitate advantageous coevolution.[92] And, as we have seen before, classical pathways usually have alternatives in place to prevent pathogen evasion of immune mechanisms. Accordingly, extracellular antigens can be captured and "cross-presented" on MHC class I molecules by some dendritic cells, and, conversely, cytosolic antigens can be processed during autophagy for MHC class II presentation.[1,27,29,70]

SUBVERSION OF THE MHC TO EVADE T-CELL RECOGNITION

If the MHC protects from foreign invaders, then an obvious corollary is that such invaders, whether microorganism or transformed cell, should try to avoid this system to survive. A variety of examples of evasive activity can be seen in infectious diseases and cancer, particularly in the subversion of MHC class I, which constitutes the protection system that alerts patrolling cytotoxic CD8+ T cells to the presence of intracellular non-self antigens. For example, bovine herpes virus produces the immunoevasin UL49.5, which interferes with normal peptide loading, and hence stable surface expression, of class I molecules.[42,43] Similar phenomena are induced by herpes virus in other species, such as the cat, horse, and chicken.[36,54,62] Downregulation of class I expression is observed in canine and feline mammary gland tumors.[16,89] Naturally, a complementary surveillance system is in place to guard against this phenomenon of "missing self." Killer cell and leukocyte immunoglobulin-like receptors (KIRs and LIRs) expressed on natural killer (NK) cells normally bind MHC class I molecules, inhibiting cell activation. Should MHC I expression be reduced or absent, inhibition is removed and the NK cell lyses the immunoevasive

target cell. An additional self-sensor is provided by the nonclassical MHC class I gene product HLA-E, which presents peptides derived from conserved leader sequences of classical MHC class I molecules. This peptide–MHC complex signals the normal presence of self via interaction with an inhibitory NK cell receptor known as CD94/NKG2A.

BASIC UNDERSTANDING OF THE MHC: WHAT MATTERS, AND WHY?

Beyond the functional understanding of the role of the MHC in self-/non-self-recognition, and in the control of T cells, NK T cells, and NK cells, the study of highly polymorphic MHC class I and II genes has been useful in providing insights into the molecular evolution of vertebrates. But what should the reader interested in veterinary hematology know about MHC antigens, particularly in a comparative sense? Basic knowledge usually focuses on a handful of characteristics: (1) overall genomic organization; (2) identification and description of the classical loci; (3) catalog of known alleles of the classical loci, and their prevalence; or, if appropriate, other descriptors of diversity; (4) the peptide-binding motif of class I and class II allomorphs, and a catalog of immunologically significant peptides that they restrict; (5) associations of specific alleles or haplotypes with risks of particular diseases; and (6) information describing nonclassical loci and the types of lymphocytes, such as unconventional T cells and NK T cells, that they regulate. For those working outside the MHC field, it is only necessary to be conversant with general principles along those lines, as there are a lot of details and exceptions that vary with species and are not worthy of committing to memory, since they can be searched for when needed.

Systematic Naming Conventions are Necessary to Study the MHC

That said, it will be helpful for the reader to have some nodding acquaintance with the details of MHC nomenclature. Because polygeny and polymorphisms characterize MHC class I and class II genes, it became necessary to have a robust and easily expandable naming system in place to keep track of all the players as they are discovered. Some MHC names have arisen because of historical precedent and have been preserved in their now-idiosyncratic form. For example, the murine MHC is called H-2, named by Snell in honor of Gorer's discovery of histocompatibility antigen II in mice. But other vague, descriptive terms, such as the immune response (Ir)-1 locus, or overtly confusing terms, such as I-A, which designates a murine class II locus but sounds deceptively like a member of class I, have thankfully been jettisoned. The now-systematic naming convention began with the first International HLA Workshop meeting in Durham, North Carolina in 1964, with periodic issuing of Nomenclature Reports since then, the last in 2010, with additional sequence updates and corrections published on a monthly basis. The current convention is illustrated in Figure 7.3, using a cat MHC class I allele as an example. Of course, the reader is urged to consult the online IPD-HLA database for the most current naming guidelines. Allele numbers are usually assigned sequentially, in the order of discovery. As a note, shorter designations (Fields 1 and 2 only) are generally used in referencing an allele; that is, HLA-A*02:01 is preferred over HLA-A*02:01:01:01. This preference is because (1) the nucleotide substitutions specified in Fields 2 and 3 generally have no effect on peptide binding or TCR recognition, so the variants are functionally identical; (2) genotyping is often performed by partial-length sequencing of polymorphic regions, so information outside this area is unknown; and (3) expression status of

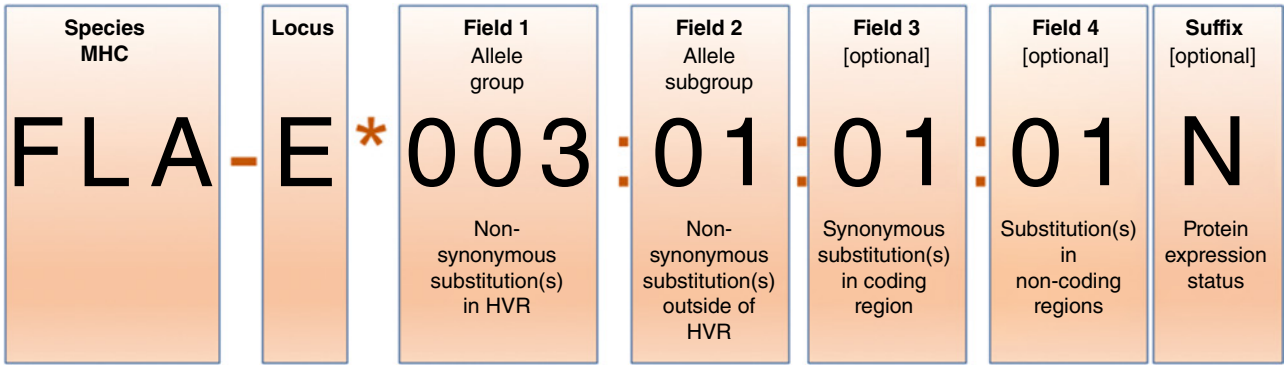


FIGURE 7.3 Nomenclature guidelines for classical MHC genes and their products, using feline leukocyte antigen (FLA) as an example. Separator marks are shown in dark orange. In Fields 1 and 2, the term HVR indicates the hypervariable regions in the exons that encode the functionally relevant peptide-binding residues in the class I and class II binding grooves that confer the allele group and subgroup name. Fields 3 and 4 are optional and often omitted, except for formal naming purposes. Letter suffixes can be appended to indicate protein expression status, if known; N: null; L: low; A: aberrant; Q: questionable; C: cytoplasm only; S: secreted. Note that other designations are sometimes used for a species' MHC (e.g., Feca, for *Felis catus*), as assigned by the Comparative MHC Nomenclature Committee of the International Society for Animal Genetics (ISAG); see: https://www.ebi.ac.uk/ipd/mhc/taxonomy/.

the allele (the letter designation) typically has not been formally investigated. In many cases, only names indicating the allele group (e.g., HLA-A*02) carried by the populations of individuals under study are used as shorthand. Finally, despite being surface antigens on leukocytes, MHC class I and class II molecules are not given cluster of differentiation (CD) antigen designations.

CHARACTERISTICS OF THE MHC IN SELECTED ANIMAL SPECIES

Table 7.1 summarized the distinctive genomic features of the MHC of various species. A brief description of some additional salient features of classical MHC genes is shown in Table 7.2. A catalog of MHC class I and class II genes and their products has also been compiled for some nonhuman animals, and is available at the IPD-MHC (https://www.ebi.ac.uk/ipd/mhc/), which was developed by the Anthony Nolan Research Institute, in collaboration with the European Bioinformatics Institute (EMBL-EBI), or the International Immunogenetics Information system (IMGT, www.imgt.org; mouse only).

HOW INVESTIGATING THE MHC AIDS THE STUDY OF T-CELL IMMUNITY

Precise information about the MHC has great significance to immunologists, because understanding T-cell immune responses depends on careful characterizations of the MHC genes and allomorphs controlling these responses. Despite the collective contributions of diversity inherent in the pathogen proteome, MHC polymorphisms, and the TCR repertoire, T-cell responses are surprisingly limited in number and predictable, with specific responses to specific pathogens or antigens shared across individuals that carry the same classical MHC allele. This phenomenon is known as immundominance.[102] Because of this restriction, which arises in part from the stringent binding preferences of MHC molecules, the study of T-cell responses in groups of individual animals is feasible and orderly.

Specific T-Cell Responses Can be Named by Their Cognate Peptide/MHC Molecule

Importantly, the particular peptide that a given T cell recognizes and the MHC allomorph that restricts the response can often be identified with some effort, and then be used to refer to that T-cell specificity. For example, HLA-A*02:01-carrying humans infected with influenza A virus frequently develop GILGFVFTL/HLA-A*02:01-specific T-cell responses against matrix protein (using single-letter code for the amino acids of the peptide).[26] Those with melanoma treated with immune checkpoint blockade may share newly primed QLSLLMWIT/HLA-A*02:01-specific T-cell responses against the cancer-testis antigen NY-ESO-1.[47] Those with type I diabetes mellitus may have in common HLVEALYLVT/HLA-A*02:01-specific T-cell responses against insulin B chain associated with rejection of their islet grafts.[71] One can be equally precise with CD4+ T cells. For example, type I diabetic patients can have autoimmune responses against glutamic acid decarboxylase (GAD)65 in the form of circulating NFIRMVISNPAAT/HLA-DRB1*04:01-specific T cells.[76] Defining T cells at this level allows the magnitude, kinetics, avidity, and other features of responses to be precisely studied, which is helpful in understanding immunity induced or modulated by infections, cancer, vaccination, transplantation, checkpoint blockade therapy, and in other settings.

Specific T-Cell Responses Can be Visualized by Their Cognate Peptide/MHC Molecule

There is another, more tangible benefit of achieving this level of specificity, too. It is possible to produce recombinant MHC class I and class II molecules, and mix them with synthetic copies of a peptide that they bind, to produce a correctly folded, soluble, functional pMHC complex. While the avidity of this pMHC complex for its corresponding TCR is too low to be of direct use, when these pMHC molecules are assembled into multimeric form (e.g., dimers, tetramers, pentamers, dextramers, and dodecamers[31]), and coupled to fluorescent or other functional molecules, they can be powerful tools for visualizing and manipulating epitope-specific T cells in polyclonal populations.[24,100] It is important to note that, for the most part, epitope-level T-cell responses are known in primates and rodents, and to a far lesser extent in other veterinary species, such as companion or food-source animals (Table 7.2). Reflecting that dearth, pMHC multimers are also rarely employed in T-cell studies in the latter populations, with scattered exceptions.[86]

CONCLUSION

The survival of complex animal life forms depends on preventing the invasion and colonization by parasitic agents, namely, prions, viruses, free-living microscopic and macroscopic organisms, and cancer cells. The key to this resistance is self-/non-self-recognition. One of the most important players in this defense network is the MHC, a large, dense cluster of genes involved in immune surveillance and regulation—most notably, the processing, transport, molecular loading, and presentation of self and foreign antigens. In order to counter almost any conceivable external threat, the MHC has evolved into a complex, diverse system, which varies in particulars across species, and even across individuals in these populations. However, there are many commonalities that unite MHC characteristics across these groups, too, reflecting the ancestry of the system, and common defense needs of animals in a hostile world.

TABLE 7.2 Features of classical MHC class I and II genes and their products in selected species of veterinary interest.

Species (MHC)	# of loci (class I) or subregions (class II)[a]	Locus name(s)	# of alleles reported[b]	Peptide-binding motif known?	Minimal peptide-reactive T cells?[c] Restricting element known?	MHC-disease susceptibility association?	Specifics for this species
Class I							
Mouse (H-2)	3	-D, -K, -L	46	Y—many	Y—many	Y—many	Inbred strains only; many null at L. Non-numerical allele naming scheme (b, d, etc.). # includes minor polymorphisms d2, k2, q2, and s2 listed at IMGT
Rabbit (RLA)	2–4	-A1,?	19	Y	N	Y—virus	Copy number variation may generate diversity
Guinea pig (GPLA)	2	-B, -S?	5	N	Y, but RE[d] unknown	N	Serologic and protein analyses only; classical status based on alloreactivity
Dog (DLA)	2	-88, -88L/-12	108	Y	Y, but RE not established	N	Two structural Hps: DLA-88/DLA-12 (80% freq.); DLA-88/DLA-88L (20% freq.) 22 of the 108 alleles at the DLA-12/DLA-88L locus. DLA-64 is nonclassical
Cat (FLA)	3	-E, -H, -K	206	Y	Y, but RE unknown	N	# includes unpublished data from Holmes & Hess
Ferret (Mufu)	?	-?	77	N	N	N	No named loci. # from two reports (48, 29 alleles) from same lab, but redundancy and/or originating loci unknown—probably mixture of classical & nonclassical
Horse (Eqca)	5–7	-1, -2, -3, -4, -16, -17?, -18?	30	Y	Y—several	N	Expression of loci varies with Hp. Peptide–MHC class I tetramers made
Cow (BoLA)	6	-1, -2, -3, -4, -5, -6	264	Y	Y—several	Y—virus, bacteria (mastitis), endoparasites, and ectoparasites	Expression of loci varies with Hp. Peptide–MHC class I tetramers made
Goat (Cahi)	1?	-N	1	N	N	N	Cahi-NC4 aligns with nonclassical BoLA class I, but status in goat is unknown
Pig (SLA)	3–4	-1, -2, -3, -12?	229	Y	Y	Y—diarrhea; endoparasite; melanoma (Hp-level only)	Expression of loci varies with Hp. Peptide–MHC class I tetramers made
Tuatara (Sppu)	2–3	-UA, -UB	49	N	N	N	Fewer expressed class I genes than other reptile orders. Subpopulations can have two or three genes
Chicken (Gaga)	2	-BF1, -BF2	50	Y	Y	Y—viruses, bacteria, protozoa, endoparasites, and ectoparasites (Hp-level only)	Only BF2 may have classical role in peptide presentation

(*Continued*)

TABLE 7.2 (Continued)

Species (MHC)	# of loci (class I) or subregions (class II)[a]	Locus name(s)	# of alleles reported[b]	Peptide-binding motif known?	Minimal peptide-reactive T cells?[c] Restricting element known?	MHC-disease susceptibility association?	Specifics for this species
Frog (Rana, Raja, etc.)	3?	-UA, -?	60	N	N	Y—fungus, virus, bacteria	# from three Rana species. Single locus in *R. temporaria* (129 alleles), Xenopus. Six other anuran species (three families) have been characterized. Susceptibility data from multiple species
Zebrafish (mhc1)	12	-uba to -uma	Few?	N	N	N	Predicted to bind β2M, CD8, and peptide; polymorphic. Genes vary with Hp. Expression consistent within, but varies across, Hps; also consistent across tissues in individual fish
Class II							
Mouse (H-2)	2	-A, -E	62	Y—many	Y—many	Y—many	Inbred strains only; many null at E. Non-numerical allele naming scheme (b, d, etc.). # includes minor polymorphisms d2, k2, q2, and s2 listed at IMGT. H2-Aa polymorphic but -Ea monomorphic
Rabbit (RLA)	3	-DP, -DQ, -DR	40	N	N	Y—virus, parasite	
Guinea pig (GPLA)	3	Ia group 1 (Ia1); Ia2; Ia3	9	N	Y, but RE unknown	Y—parasite	Serologic and protein analyses only; classical status based on alloreactivity
Dog (DLA)	2	-DQ, -DR	345	N	Y, but RE unknown	Y—protozoal, autoimmune, endocrine	Alleles can be grouped into functional DQB ($n = 9$) and DRB ($n = 5$) supertypes
Cat (FLA)	1	-DR	84	N	Y, but RE unknown	N	Expanded # of DR genes: DRA1, DRA2, DRA3 (all have different signal peptides); DRB1, DRB3, DRB4, and DRB5 (DRB2 is pseudogene)
Ferret (Mufu)	1	-DR	N/A	N	N	N	Serologic determination of DR only; SMRT sequencing of the ferret MHC reportedly completed (2018) but not yet available
Horse (Eqca)	2	-DQ, -DR	53	N	Y	Y—atopic dermatitis; sarcoid?	# from IPD-MHC database
Cow (BoLA)	2	-DQ, -DR	499	Y	Y	Y—virus, bacteria (mastitis), cancer	# from IPD-MHC database. T-cell responses against foot-and-mouth disease virus epitopes presented by DRB3 have been found
Goat (Cahi)	2	-DQ, -DR	71	N	N	Y—mycobacteria	Domestic goat, but includes other species (e.g., wild goat, *Capra aegagrus*) and strains
Pig (SLA)	2	-DQ, -DR	204	Y	Y, but RE unknown	Y—diarrheal disease; endoparasite; melanoma (Hp-level only)	# from IPD-MHC database

Tuatara (Sppu)	2?	-DA, -DB, -DC	6	N	N	N	DB may be nonclassical
Chicken (Gaga)	1	-BL	21	Y	N	Y—viruses, bacteria, protozoa, endoparasites, and ectoparasites (Hp-level only)	Two beta chain genes, BLB1 and BLB2. Allele assignment challenging. BLB1 expressed primarily in intestine. Monomorphic BLA gene outside B subregion
Frog (Rana, Raja, etc.)	2	-D1, -D2	82	N	N	Y—fungus	Other references call II or DA. # from multiple Rana sp. Fungal susceptibility in multiple non-Rana species
Zebrafish (mhc1)	1	-DA	N/A	N	N	N	Polymorphisms yet to be described

[a]For class II, subregion refers to specific cluster, e.g., DR, in which there are usually multiple loci/genes. For example, cats have three DRA and four DRB genes. Because the DQ subregion is lacking, and DPA and DPB are pseudogenes, the number of active class II subregions is counted as one (DR). For many of the species listed, class II gene-level details are not yet available.
[b]Allele numbers are provided to illustrate genetic diversity at the classical MHC loci in various animal species, and represent current (2020) estimates based on authors' claims in the literature; totals could thus include redundant alleles. These numbers are imprecise because, in most species, the number and identity of classical loci are ill-defined, sequence assignments are tentative. It should also be noted that different numbers of individuals have been studied, and that new alleles are constantly being reported.
[c]T cell recognizing a specific approximately 9-mer (class I) or approximately 15-mer (class II) peptide.
[d]Abbreviations: RE, restriction element; Hp, haplotype; N/A, not available.

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CHAPTER 8

Lymphocyte Biology and Functions

IAN TIZARD

General Properties
- Lymphocyte Structure
- Lymphocyte Populations
- Lymphocyte Origins
- Lymphocyte Circulation
- Lymphocyte Mitogens

Lymphocyte Surface Molecules
- Antigen Receptor Complexes
- Regulatory Receptors
- Adherence Molecules
- WC1 Molecules
- Changes in Phenotype
- Species Differences

B Cells
- The Response of B Cells to Antigen
- B-Cell Antigen Receptors
- Antigen Presentation by B Cells
- Costimulation of B Cells
- B-Cell Responses
- Plasma Cells
- Memory B Cells
- B-Cell Subpopulations

Helper T Cells
- The Response of Helper T Cells to Antigen
- T-Cell Antigen Receptors
  - Costimulation of T Cells
- Helper T-Cell Subpopulations
- γ/δ T Cells

Cytotoxic T Cells
- The Response of Cytotoxic T Cells to Antigen
- Cytotoxic T-Cell Subpopulations
- Memory T Cells

Innate Lymphoid Cells
- Innate Helper Cells
- Innate Cytotoxic Cells

NKT Cells

Acronyms and Abbreviations

APC, antigen-presenting cell; BCR, B-cell antigen receptor; CpG, cytosine-phosphate-guanine; CR, complement receptor; CTLA, cytotoxic T-lymphocyte-associated protein; FcR, Fc (immunoglobulin) receptor; HEV, high endothelial venule; ICAM, intercellular cell adhesion molecule; IFN, interferon; IL, interleukin; ILC, innate lymphoid cell; MHC, major histocompatibility complex; NF-AT, nuclear factor of activated T cells; NF-κB, nuclear factor κB; NK cell, natural killer cell; NKT cell, natural killer T cell; SWC-1, swine workshop cluster-1; TCR, T-cell antigen receptor; Th1 cells, type 1 helper T cell; Th2 cells, type 2 helper T cell; TLR, toll-like receptors; TNF, tumor necrosis factor; WC1, workshop cluster 1.

Lymphocytes are the morphologically bland, small round leukocytes that predominate in the peripheral blood of herbivores and are a major leukocyte population in other mammals. They range in concentration from 4500–13,000 cells/μL in pigs to 1000–4800/μL in dogs. Their morphology is however no guide to their critical role in the defense of the body. There are three major populations of these cells; T cells, B cells, and innate lymphoid cells (ILCs).

All lymphocyte populations are engaged in the defense of the body. Given the diversity of potential microbial pathogens, it is unsurprising that lymphocytes also show considerable diversity. Immunologists classify the adaptive immune responses into two types. Type 1 immunity is mediated by type 1 helper (Th1) cells. Type 1 responses are responsible for immunity to bacteria, viruses, protozoa, and fungi. They generate some antibodies, cytotoxic T-cell responses and activate

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macrophages. Type 2 immunity in contrast is mediated by type 2 helper (Th2) cells. Type 2 immunity is primarily antibody-mediated. Type 2 responses are responsible for the destruction of extracellular bacteria and viruses, helminths, and arthropods, as well as for allergic responses. This functional division is directly related to the activities of different lymphocyte populations.

GENERAL PROPERTIES

Lymphocyte Structure

Lymphocytes are spherical cells with a round nucleus (that almost fills the cell), a thin rim of cytoplasm, and a smooth surface that may develop multiple villous projections when activated. Resting G1 lymphocytes are about 6–7 μm in diameter and contain a few mitochondria, vesicles, ribosomes, and a small Golgi (Figure 8.1). Natural killer (NK) cells, a cytotoxic population of innate lymphocytes, may be larger than the T or B cells, and may contain obvious magenta granules. Lymphocytes have historically been classified as either small or large; however, their size distribution is more heterogeneous. Since these cells are often dividing, a lymphocyte in M phase will be twice the size of its two progeny cells and can reach approximately 10–12 μm in diameter.

Lymphocyte Populations

Lymphocytes are found in peripheral blood, in lymphoid organs, and throughout many nonlymphoid tissues, especially the mucosa of the gastrointestinal tract.[24] As might be expected, changes in the circulating lymphocyte concentration reflect absolute alterations in population numbers or changes in distribution among tissue compartments, including the peripheral blood, lymphoid organs, and nonlymphoid tissues. Despite their morphologic overlap, lymphocytes represent cells that are functionally and phenotypically diverse. Their phenotype also includes the set of cell surface molecules, receptors, and secreted proteins that regulate their functions and reflect the activation and suppression of selected genes and their expressed products. Lymphocyte populations are characterized by their characteristic cell surface proteins and glycoproteins, especially their antigen-binding receptors (see Chapters 6 and 63 for more information of lymphocyte phenotyping). Further separation classifies B cells according to their location and function, divides T cells into helper (Th) or regulatory (Treg) cell types, and classifies ILCs as helper cells or NK cells (Table 8.1).

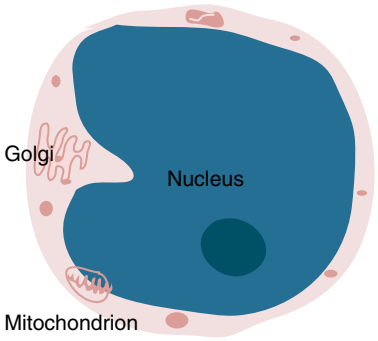


FIGURE 8.1 The structure of a lymphocyte. The nucleus is usually surrounded by a small amount of cytoplasm that contains the cellular organelles.

TABLE 8.1 Published peripheral blood lymphocyte percentages.

CELLS	DOG	CAT	BOVINE	SHEEP	PIG	HORSE
T cells	46–84	31–89	45–53	56–64	45–57	38–66
B cells	7–30	6–50	16–21	11–50	13–38	17–38
CD4+	27–58	19–49	8–31	8–22	23–43	56
CD8+	17–18	6–39	11–30	4–22	17–39	20–37
CD4/CD8	4.7	1.9	1.53	1.55	1.4	4.75
T cells						
α/β	92		5–30	5–30	14–34	
γ/δ	7.6		5–50	22–68	31–66	
WC1			5–44	15–70	40	

Lymphocyte Origins

Lymphocytes originate in the fetal omentum and yolk sac, then in the fetal liver, and finally in the bone marrow where they are derived from lymphoid stem cells. Some newly formed cells leave the bone marrow and migrate to the thymus. Following positive and negative selection, which eliminates many self-reactive T cells and may result in the loss of 75–95% of the entering cells, thymic lymphocytes exit as T cells and migrate to secondary lymphoid organs. It takes 4–5 days for these cells to pass through thymic processing before they emigrate as functional T cells. Although the thymus shrinks once it has succeeded in colonizing the secondary lymphoid organs, it remains functional throughout life.

In some mammals, most notably sheep, B-cell precursors migrate from the bone marrow to ileal Peyer's patches. These are sites of apoptosis for presumably faulty or self-reactive B cells and a site of microbiota-influenced B-cell maturation and diversification.[9,42] In other mammals, the bone marrow serves as both a primary lymphoid organ and the source of B-cell maturation.[43] Once they diversify and mature, B and T cells migrate to secondary lymphoid organs (e.g., lymph node and spleen) where they are optimally positioned to encounter invading organisms and foreign antigens.

Lymphocyte Circulation

Lymphocytes are highly mobile cells. They originate in the bone marrow, migrate to the thymus or Peyer's patches, move to secondary lymphoid organs, and readily traffic between these (and other) organs. However, their movement patterns vary according to phenotype. For example, effector ($CD8^+$) T cells survey the body, interrogate nucleated cells for abnormal or foreign proteins, and, if found, engage in cell killing. By contrast, B cells remain relatively static waiting for antigens to

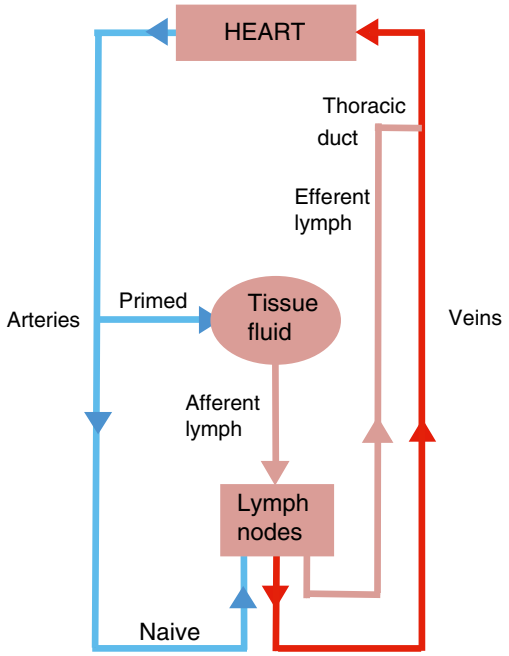


FIGURE 8.2 Lymphocyte circulation. Naïve lymphocytes enter lymph nodes from the bloodstream through high endothelial venules. Once primed they enter tissue fluid and are carried to lymph nodes through afferent lymph. They all return to the bloodstream by way of major lymphatic vessels such as the thoracic duct.

come to them. Additionally, lymphocyte circulation patterns differ between naïve and antigen primed cells. For example, naïve T cells leave the bloodstream and emigrate into the lymph node paracortex via specialized high endothelial venules (HEVs).[21] By contrast, memory T cells leave the bloodstream through conventional blood vessels and are carried to lymph nodes through afferent lymph. Both types of T cells leave lymph nodes through efferent lymphatics. Sheep afferent lymphatics contain approximately 85% T cells, 10% B cells, and 5% dendritic cells, whereas their efferent lymph contains approximately 75% T cells and 25% B cells. These T cells are continually patrolling the body, while the T cells migrate between lymphoid organs. Efferent lymphocytes pass through increasingly larger lymph vessels, including the thoracic duct, before eventually rejoining the bloodstream and, ultimately, circulating back to lymph nodes (Figure 8.2). Lymphocytes circulate differently in the pig.[5] In this species, lymphocytes enter and exit lymph nodes through HEVs. This results in a comparatively lower concentration of lymphocytes in the efferent lymph, but a correspondingly higher concentration of lymphocytes in the peripheral blood (i.e., 4500–13,000 cells/μL).

Lymphocyte Mitogens

To quantitate lymphocyte responsiveness or characterize chromosome structure and karyotype, lymphocytes can be stimulated to divide by exposure to lectins (proteins that bind to carbohydrate side chains on glycoproteins).[4] By measuring the magnitude of lymphocyte division in response to these mitogens, they can provide a quantitative measure of lymphocyte functionality. The most widely employed lectin is phytohemagglutinin, which is a plant protein that binds to cell surface glycoproteins and stimulates T-cell division. Additional, commonly used mitogens include the T-cell mitogen concanavalin A and the T- and B-cell mitogen, derived from the pokeweed plant.

LYMPHOCYTE SURFACE MOLECULES

All external cell surfaces are covered by a dense and diverse array of proteins.[37] These cell surface proteins are classified by the cluster of differentiation (CD) system. The CD system has primarily been established for mouse and human cells. While most of these proteins are homologous with those in domestic species, there are some distinctive and important molecules found only in domestic animals (see Chapters 6, 63, and 64 for more information).

Antigen Receptor Complexes

The most important lymphocyte surface molecules are the receptors for foreign antigens, namely, the B–cell antigen receptor (BCR) and the T–cell antigen receptor (TCR). Both receptors consist of glycoprotein complexes formed by multiple peptide chains, which for B cells consist of immunoglobulin molecules, whereas T cells use a specialized multipeptide antigen receptor (Figure 8.3).[50] Some of these chains form the antigen-binding site, while others play a role in signal transduction. Across T and B cells, there are subpopulations that utilize different peptide chains in their antigen receptors. B cells use five different immunoglobulin heavy chains (e.g., α, δ, γ, ε, and μ), whereas the TCR complex consists of either of the paired antigen-binding α/β or γ/δ chains plus a signal transduction complex collectively called CD3.[22] Notably, innate lymphoid cells do not possess specific antigen receptors, but rather they possess receptors that recognize surface molecules expressed on healthy normal cells (e.g., major histocompatibility complex [MHC] class I). When these lymphocytes encounter a cell lacking these surface molecules (a common feature of virus-infected cells), they will kill it.

T cells recognize and respond to two different kinds of antigens, namely, foreign antigens (bound to MHC class II molecules on the surface of antigen-processing cells [APCs]) or endogenous antigens (bound to MHC class I molecules). To bind to MHC class II, specialized T cells (helper cells) use a receptor called CD4. Alternately, endogenous antigens, which are generated in virus-infected cells, bind to MHC class I molecules. These MHC class I bind to CD8 on the T-cell surface. Thus, both CD8 and CD4 serve to bind the T cell tightly to the APC and so facilitate the exchange of signals and their activation. T-cell subsets in peripheral blood and tissues vary across species (e.g., in human blood, 65% of T cells are CD4+, whereas

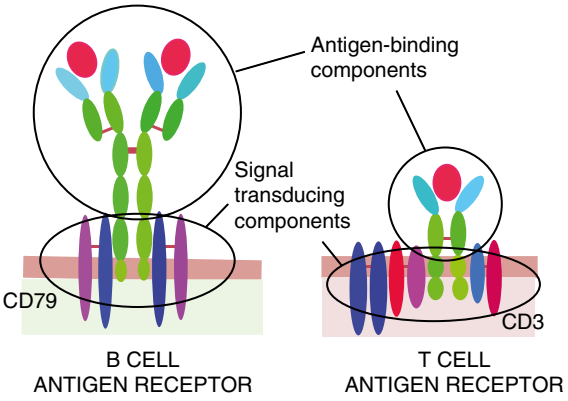


FIGURE 8.3 T and B cell antigen receptors. Both consist of an antigen-binding component and a signal transduction component. The signal transduction component of the TCR consists of six different peptide chains collectively called CD3. The signal transduction component of the BCR consists of two dimers called CD79.

30% are CD8+). In humans, these numerical relationships have significance, but their utility in animal species is largely unproven.

Regulatory Receptors

Regulatory proteins, including chemokines, cytokines, antibodies, and complement proteins and their corresponding receptors, regulate both B- and T-cell activation and function. For example, Th1 cells are regulated by specific receptors to interleukin (IL)-1, IL-12, and interferon (IFN)-γ Th2 cells are regulated by IL-4, IL-13, and IL-18; while Th17 cells are regulated by IL-23 (Figure 8.4).

Similarly, antibodies signal lymphocytes through specific receptors. Since these receptors bind to the Fc regions of immunoglobulins, they are called Fc receptors (FcR). One such receptor is CD32 (FcγR2), which is found on B cells. In the case of B cells, this receptor provides a negative feedback loop that regulates antibody production. Alternately, CD16 is a low-affinity IgG receptor (FcγRIII) found on NK cells. This receptor binds and allows the NK cell to destroy antibody-coated targets. In addition, complement proteins regulate lymphocyte activity through specific receptors. There are four of these, CR1–CR4. B cells express CR1 and CR2. NK cells express CR3 and CR4.

Adherence Molecules

Lymphocytes must interact with other lymphocytes, blood vessel walls, and APCs. As a result, they express intercellular adhesion molecules such as integrins and selectins. Integrins are heterodimeric glycoproteins that bind lymphocytes to extracellular matrix (e.g., laminin and fibronectin), to vascular endothelial cells, and to other lymphocytes. Selectins are also cell adherence molecules. For example, L-selectin is expressed on lymphocytes and binds them to high endothelial venules in the lymph node paracortex. Other adherence molecules include intercellular cell adhesion molecules (ICAM-1) (which is expressed on B cells and T cells, binds integrins, and is required for the emigration of circulating lymphocytes into tissues) and CD2 (which is expressed on T cells, binds to CD58 on antigen-processing macrophages and dendritic cells, and is essential for T-cell cytotoxicity).[40,41]

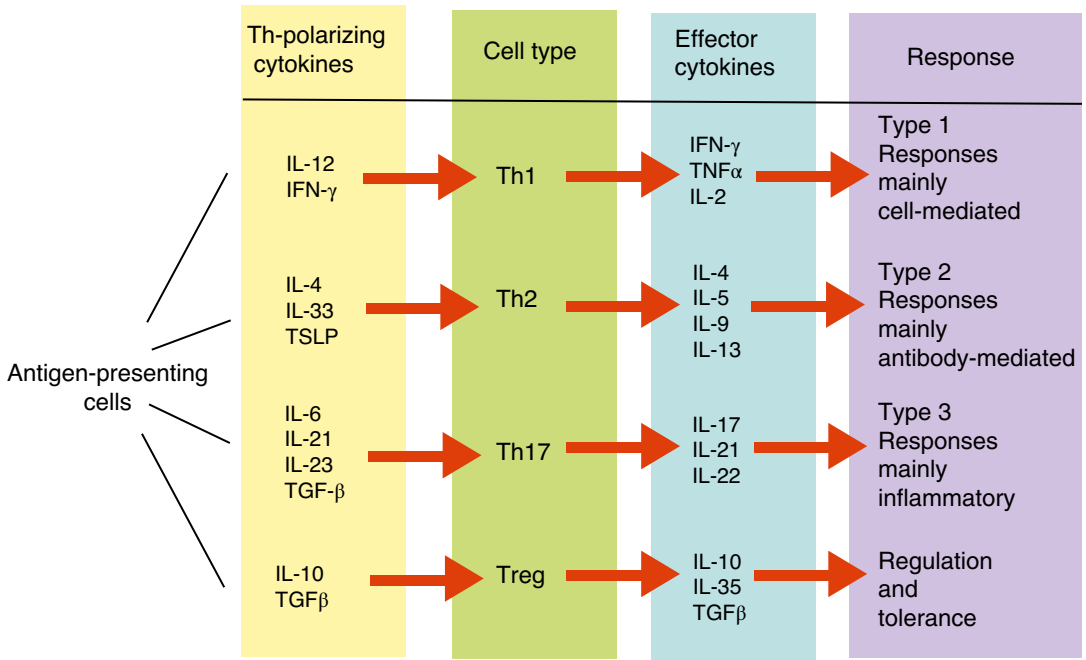


FIGURE 8.4 T-cell diversity. The development of specific T cell subpopulations is determined by the mixture of cytokines generated by antigen presenting cells and depends in large part on the properties of the initiating antigen. Once differentiated, each T cell subpopulation secretes its own unique mixture of cytokines. These in turn trigger specific types of immune response or tolerance.

WC1 Molecules

Workshop cluster 1 (WC1) molecules are a family of lymphocyte surface proteins found in many mammals.[26] WC1, while not found in humans, is a major cell surface protein on γ/δ T cells in cattle and sheep.[26,33,53] These WC1$^+$ cells are found in large numbers under the skin and mucus membranes. They have homologs in other domestic species, primarily herbivores.[49] The function of the WC1 family is unclear, but they likely bind T cells to macrophages and dendritic cells and are involved in T-cell activation.[25,54]

Changes in Phenotype

Lymphocytes do not present a stable phenotype.[56] As genes are turned on or off or epigenetic mechanisms come into play, their phenotype may change.[16,48] Thus, B cells switch the immunoglobulin class they produce and can turn into plasma cells and eventually into memory cells.[48] T cells also switch functions to become helper, regulatory, effector, or memory T cells.

Species Differences

There are notable, species-specific differences in circulating lymphocyte subsets. For example, B cells comprise 17–38% of total blood lymphocytes in the horse, 16–21% in cattle, 13–38% in pigs, and 7–30% in dogs. Similar differences in circulating T cells are seen, as they constitute 38–66% of blood lymphocytes in the horse, 45–53% in cattle, 45–57% in pigs, and 46–84% in dogs. In addition, individual species express unique lymphocyte subsets. For example, horse lymphocytes express two unique antigens of uncertain function, EqWC1 and EqWC2. EqWC1 is expressed on 70% of T cells and on 30% of B cells, whereas EqWC2 is found on most horse T cells. Cattle, sheep, and pigs are characterized by the presence of comparatively high levels of γ/δ T cells when compared to other veterinary species, humans, and laboratory rodents.[23] In adult cattle, γ/δ cells account for 10–15% of circulating T cells and up to 40% in young calves. These percentages are highly variable and are affected by stress and management. Most of these γ/δ T cells are also WC1-positive and, as a result, may be activated from signals from either the TCR or through WC1. When activated, they secrete a mixture of cytokines associated with type 1 immune responses. CD4$^+$ cells account for about 20–30% of T cells in calves. Double negative cells may account for 80% of lymphocytes in newborn calves, but this proportion drops significantly as they age, eventually stabilizing around 15–30%. These double negative cells are mainly γ/δ$^+$ WC1$^+$.

Sheep similarly demonstrate an age-dependent profile of γ/δ T-cell expression. In newborns, γ/δ cells constitute about 60% of circulating T cells, but this drops to 30% by 1 year and as low as 5% in aged sheep. They also express a unique antigen OvWC1 (T 19) that is structurally different in α/β T cells when compared to γ/δ cells.[52] Pigs also express proportionally high levels of γ/δ T cells.[6] Neonatal piglets have up to 66% circulating γ/δ-positive T cells, which drops to 25–50% of cells as they age. Pigs currently have nine unique WC molecules, although this number may drop if homologies with mice and humans are identified.[7] For example, swine workshop cluster-1 (SWC-1) is homologous to CD27 and occurs on T and NK cells. Notably, up to 60% of pig T cells are CD4/8 double positives, while the remainder are double negative.[15,31,38] In contrast to these species, less than 10% of their circulating T cells are γ/δ-positive.

B CELLS

The Response of B Cells to Antigen

Although B cells can be found circulating in peripheral blood, they predominantly reside in lymphoid organs, including the cortex and germinal centers of lymph nodes, Peyer's patches, bone marrow, and splenic white pulp, where they wait for antigens, both soluble and particulate, to be brought to them by lymph or blood flow. Each B cell possesses a unique antigen receptor that is, when activated, shed as an antibody molecule.

B-Cell Antigen Receptors

Each B cell is covered by up to 500,000 identical antigen receptor molecules. The BCR consists of two components, an immunoglobulin molecule that acts as an antigen-binding component and CD79, the signal-transducing component. Structurally, the antigen-binding component of the BCR consists of four immunoglobulin chains—two light chains and two heavy chains, bound by disulfide bonds. This immunoglobulin structure binds antigens through its Fab region, which is itself composed of regions of both the heavy and light chains. The signal-transducing CD79 is also composed of two chains, CD79-α and -β. The CD79-α chain differs according to the heavy chain class, while the CD79-β chain is homogeneous across all heavy chains. Functionally, when an antigen cross-links two BCRs, a signal is generated that activates transcription factors such as nuclear factor κB (NF-κB) and nuclear factor of activated T cells (NF-AT) leading to repeated cell division and gradual differentiation into a plasma cell. These resultant plasma cells are capable of secreting many thousands of immunoglobulin molecules every second into the bloodstream.

Antigen Presentation by B Cells

In addition to acting as antibody secretors, B cells can function as efficient antigen-presenting cells for CD4$^+$ helper T cells. In this pathway, activation of the B-cell receptor by antigen results in endocytosis and intracellular processing of that antigen and concludes with cell-surface expression of antigen bound to MHC class II molecules. That pathway only works in primed animals when these specific B cells have expanded their numbers.

Costimulation of B Cells

Binding of specific antigen to BCRs alone is usually insufficient to completely activate B cells and trigger an antibody response. B cells often require additional stimuli from T cells through cytokines, cell contact, complement, and toll-like receptors (TLR) ligands. Helper T cells serve as one such costimulant through the delivery of cytokines that not only activate B cells but also determine B-cell development and function. More specifically, Th2 T helper cells secrete IL-4, IL-5, IL-6, IL-13, and IL-21. Of these cytokines, IL-4 and IL-13 promote expression of Fc and MHC receptors, B-cell class switching, and the production of IgA and IgE. The other cytokines (IL-5 and IL-6) promote the differentiation of B cells into plasma cells, while IL-21 induces the differentiation of B cells into plasma cells and memory cells. The costimulatory effects of CD4 T cells on B cells are initiated by the binding CD154 (on T cells) to CD40 (on B cells). This binding triggers B-cell division and upregulation of B-cell cytokine receptors. Additional B-cell costimulants include the complement component C3d and pathogen-associated molecular patterns (PAMPs), provided by lipopolysaccharide, flagellin, or cytosine-phosphate-guanine (CpG) dinucleotides, that bind to B-cell toll-like receptors. These signals can partially replace T cell help and account for the activities of many vaccine adjuvants.[36]

B-Cell Responses

In newborn mammals, the earliest B cells express IgM and IgD BCRs. Once appropriately stimulated, B cells divide asymmetrically.[3] The side of the B cell that attaches to the helper cell receives a full dose of stimuli and progressively differentiates into a plasma cell, whereas the opposite pole receives less stimulus and differentiates into a memory B cell. The B cell destined to become a plasma cell develops an extensive endoplasmic reticulum as well as a large Golgi apparatus, both of which are needed for the synthesis and secretion of enormous quantities of immunoglobulins. Once differentiated, plasma cells switch from making IgM to another immunoglobulin class depending upon their signals they receive and their anatomic location. For example, B cells on the body surfaces primarily produce IgA or IgE, whereas those in the spleen primarily produce IgG. Simultaneous with division, B cells randomly change the genes encoding their immunoglobulin variable region. As a result, the B cell either increases or decreases its affinity for antigen. If the affinity decreases, the antigen provides less stimulus and the B cell dies. If the affinity increases, then that B cell is preferentially stimulated and that clone of cells expands. As a result, antibody affinity for antigen gradually increases over the course of an antibody response.

Plasma Cells

Plasma cells develop as B cells differentiate. They first appear in the B cell areas of secondary lymphoid organs and subsequently migrate to colonize all the secondary lymphoid organs. Plasma cells have a characteristic morphology and staining pattern that distinguishes them from other forms of lymphocyte. They are 10–12 µm diameter, ovoid cells that contain a round, paracentrally positioned nucleus with "clock-face" pattern chromatin and a prominent nucleolus. Because of the dense concentration of ribosomes, the cytoplasm is basophilic and pyroninophilic, but the very large Golgi is obvious as a pale area adjacent to the nucleus (Figure 8.5). Plasma cells are specialized immunoglobulin producers, and it is claimed that they can synthesize up to 10,000 immunoglobulin molecules/second.

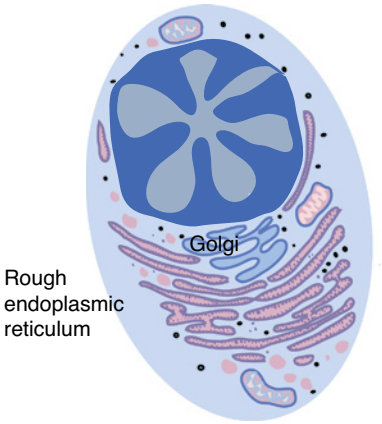


FIGURE 8.5 The structure of a plasma cell. A plasma cell is simply a B cell that has been activated so that it will produce an enormous quantity of antibody molecules. As a result, its cytoplasm expands to accommodate large quantities of rough endoplasmic reticulum and an enlarged Golgi apparatus.

Memory B Cells

As an antibody response progresses, short-lived plasma cells die but memory B cells survive. These are indistinguishable in morphology from other B cells. Their survival is, in part, mediated by helper T cells, which upregulate expression of the anti-apoptotic protein *Bcl*-2 in memory B cells. Although several different populations of memory B cells have been identified in humans and mice, a similar diversity has not been found in domestic species. This diversity is represented by long-lived lymphocytes that can be thought of as adult stem cells and will respond very rapidly when they next encounter antigen and long-lived plasma cells that continue to produce antibodies in response to persistent antigens. This latter population is the source of low levels of antibodies in older animals that have not been recently vaccinated.

B-Cell Subpopulations

Two distinct subpopulations of B cells have been identified in mice. One of the populations (B1 cells) originate early in fetal life and are phagocytic and can kill ingested bacteria. B1 cells have been further divided into B1a cells, which produce most "natural" IgM, and B1b cells, which are needed for antiparasitic immunity ("natural" antibodies are the low level of antibodies found in the

bloodstream of normal, unimmunized animals). B2 cells constitute the majority of B cells found in lymphoid organs and have slightly different phenotype.

HELPER T CELLS

The Response of Helper T Cells to Antigen

Foreign antigens trapped by APCs or expressed by virus-infected cells are bound to MHC molecules and presented to helper T cells. Upon receiving suitable costimulation, the helper T cells respond to the antigen by secreting a complex mixture of cytokines.[8] These cytokines then serve to determine the developmental pathway and magnitude of the subsequent immune response. Because of their central role in the immune system, helper T cells must be carefully regulated by cell–cell contact and cytokine exposure.

T-Cell Antigen Receptors

As noted in the earlier text, each T cell is covered with a large number (approximately 30,000 molecules) of identical antigen-binding TCRs. Analogous to the BCR, the TCR is structurally characterized by an antigen-binding component and a signal transduction component. The antigen-binding component consists of two disulfide-linked chains, the specifics of which vary according to species. In most nonruminants, α and β chains account for over 90% of the TCRs with the remaining containing γ and δ chains. These four antigen-binding chains all contain an N terminal variable domain, which is involved in antigen binding, and a set of C terminal constant domains, which links to the signal transduction component. Antigen peptides bind in the groove formed between the paired chains.

The signal transduction component of the TCR consists of six peptide chains in the form of three dimers. Collectively, this is called CD3 and its presence is a characteristic feature of T cells. Associated with the CD3 complex are two other peptide chains, CD4 and CD8. As described in the earlier text, T cells may have one, both, or neither. CD4 molecules bind T cells to MHC class II molecules and thus link the T cells to antigen-presenting cells. CD8, on the other hand, binds T cells to MHC class I molecules associated with abnormal cells of any type. This interaction between CD8 and MHC class I locks the cells together and facilitates cytotoxicity. As with B cells, the area of contact between the antigen-presenting cell and the T cell forms an immunological synapse. In the case of helper cells, the circular contact area has a target-like structure with the TCR complex at the center surrounded by a ring of costimulators and on the outside surrounded by a "gasket" of adherence molecules.[35]

Costimulation of T Cells

For a T cell to maximally respond to an antigen, it must receive multiple signals from the APC (Figure 8.6).

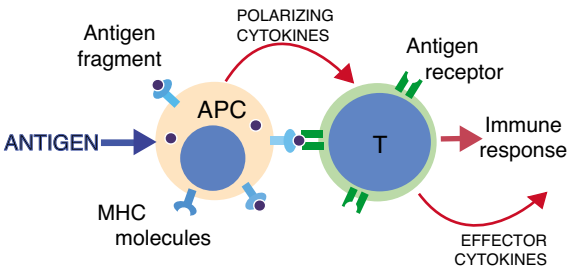


FIGURE 8.6 Development of an adaptive immune response. The key step in triggering an adaptive immune response is the presentation of processed antigen to T cells. The T cell antigen receptor will only recognize peptide fragments bound to MHC molecules on the surface of antigen-presenting cells such as dendritic cells.

This additional signaling is provided by costimulatory receptors, cytokines, and adherence molecules. Costimulatory receptor pairs include CD40 ([on] the APC) and CD154 (on the helper T cell), which, when bound to each other, induce the T cell to express CD28. CD28 then binds to CD80 on the APC and, as a result, the T cell becomes activated. A few days after being activated, the T cell turns on the genes encoding another T-cell receptor called cytotoxic T-lymphocyte-associated protein (CTLA)-4 (CD162). Binding to CTLA-4 suppresses T-cell cytotoxicity and its upregulation has been identified in many cancers. Monoclonal antibody-based blocking of CTLA-4 activity permits T-cell activation and antitumor immune activity. T-cell function is thus regulated by a balance between the suppressive effect of CTLA-4 and the stimulatory effect of CD28.

Helper T cells are also regulated by complex cytokine mixtures secreted by APCs.[55]

For example, IL-12, along with IFN-γ and IL-18, from dendritic cells causes undifferentiated T cells to become Th1 cells. In the absence of IL-12, and in the presence of cytokines such as IL-4, IL-13, IL-33, and IL-25, T cells differentiate into Th2 cells. Finally, T cell costimulation is provided by adherence [molecules], which bind the helper T cell to the APC so that signals can pass more freely between them. These cell adherence molecules include the CD11/CD18 integrin complex that is found on the surface of T cell and binds to CD54 on the APC.

Helper T-Cell Subpopulations

As $CD4^+$ T cells differentiate, they eventually form four discrete populations determined by their exposure to specific cytokine mixtures. These cytokine mixtures activate selected genes, which causes the T cells to secrete other cytokine mixtures that determine their biological activity and functions. Two of these cell populations, Th1 and Th2 cells, have been described in the earlier text. They are responsible for the generation of type 1 and type 2 immune responses, respectively (Figure 8.7).

Th1 cell differentiation is driven by interleukin-12 with the assistance of IFN-γ and IL-18. When

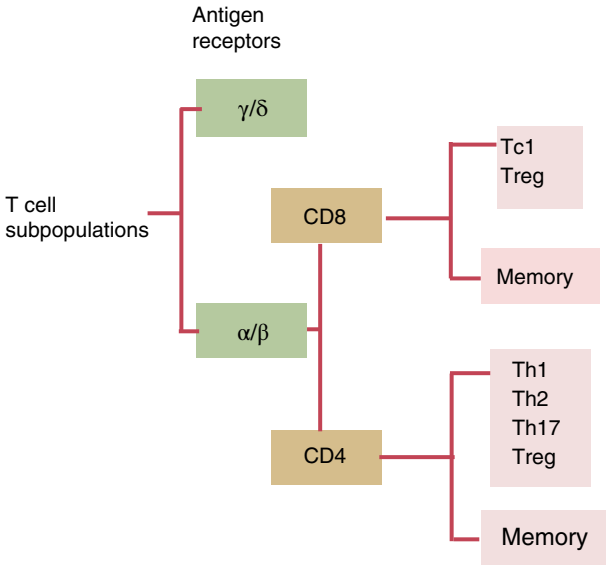


FIGURE 8.7 T-cell differentiation. As T cells develop, they turn genes on or off and eventually differentiate into multiple subpopulations. Some use alpha and beta chains in their TCTs, others use gamma and delta chains. The proportion of these two cell types differs between different domestic species.

they are activated, Th1 cells generate IL-2, IFN-γ, tumor necrosis factor (TNF)-α, and lymphotoxin (TNF-β). These cells promote type 1 responses that are primarily cell-mediated immune responses. Th2 cell differentiation is driven by IL-25 and IL-33. Once differentiated, they secrete IL-4, IL-5, IL-9, and IL-13. These cytokines stimulate B-cell proliferation and the production of immunoglobulins. While the B cells make predominantly IgG, under appropriate circumstances such as worm infestations, they may also promote the development of allergies by enhancing IgE synthesis. Th17 cells are a population of $CD4^+$ T cells that produce IL-17. This production is mainly stimulated by IL-23 with help from IL-1, IL-6, and IL-21. IL-17 and related molecules such as IL-21 and IL-22 promote inflammation. They play an important role in protection against extracellular bacteria. Treg cells are also $CD4^+$ cells that characteristically also express CD25. They produce immunosuppressive cytokines such as TGF-β and IL-10. Treg cells play an important role in controlling immune responses and preventing allergic and autoimmune disease.[18]

γ/δ T Cells

As described earlier, the relative proportion of γ/δ T cells to α/β T cells varies enormously between the different domestic animal species. Pigs and ruminants are γ/δ-high, while other mammals including the carnivores, rodents, and primates are γ/δ-low.[23] In γ/δ-low species, two subsets of γ/δ cells have been identified. One subset shows limited TCR diversity and probably functions as innate cells. These are mainly located in the genital tract and skin where they bind microbial products and lipids. They are activated by IL-23. The second γ/δ subset possesses highly variable TCRs and probably functions in adaptive immunity either as helper or effector cells. In γ/δ-high species, these cells predominantly are involved in adaptive immunity. They constitute the major T-cell population on surfaces such as the skin and mammary gland, the intestinal tract, and the genital tract. In ruminant blood, almost all are $WC1^+$ and are of the innate immunity type.[22] They may also have subpopulations that have both effector and regulatory functions. A similar situation applies to pigs where some γ/δ subsets have Th1 functions, while others have Th17-like activity.[45]

CYTOTOXIC T CELLS

When the body needs to rid itself of unwanted, damaged, or surplus cells, it may utilize apoptosis. Cytotoxic $CD8+$ T cells make use of this pathway to destroy virus-infected or otherwise abnormal cells. To affect this process, cytotoxic T cells bind to their target using a synapse that contains "two centers." One center consists of the molecules signaling *from* the target (i.e., antigen-MHC I signaling to the T cell), whereas the second consists of molecules signaling *to* the target (i.e., pro-apoptotic signaling).

The Response of Cytotoxic T Cells to Antigen

Cytotoxic $CD8^+$ T cells are activated and expanded by exposure to IL-12 from APCs together with IL-2 and IFN-γ from Th1 cells. Once activated, the T cells begin to divide. Their numbers increase very rapidly, as they can divide every 4–6 hours and do so up to 20 times. They reach maximal numbers 5–7 days after the onset of infection. These effector cells are however short-lived and die once the invaders are eliminated. Almost all undergo apoptosis within a few weeks.

Cytotoxic T-Cell Subpopulations

Mice have two subsets of cytotoxic T cells. Tc1 secrete IL-2 and IFN-γ, while Tc2 cells secrete IL-4 and IL-5. Many of these cells that do not even secrete cytokines are called Tc0 cells.

Memory T Cells

When a T-cell response occurs, there is a massive expansion of short-lived effector T cells that die once their job is done. However, about 5–10% of the responding cells evade apoptosis to become memory T cells. These are long-lived cells that accumulate over an animal's lifetime and, thus, the number of memory cells is much greater in older versus younger animals. Memory T cells, like memory B cells, are derived by asymmetric cell division.[12] These memory cells are $CD44^+$ and require IL-15 to survive. In mice, three types of memory T cells have been identified. Central memory T cells circulate with other lymphocytes and mount a very rapid

effector response upon re-encountering their original antigen. Effector memory T cells are attracted to sites of microbial invaders where they can immediately attack and destroy them. The third population, tissue-resident memory T cells, is located beneath body surfaces.[39] They do not recirculate but serve as a line of defense against invaders. All memory T cells express CD4 or CD8. They are in effect adult stem cells that slowly divide to replenish their numbers.

INNATE LYMPHOID CELLS

T and B cells express an enormous number of randomly generated antigen receptors that are derived from a limited number of germline genes through somatic mutation and gene recombination. Their recruitment and activation require a significant amount of time, and, thus, there is a lag before adaptive immunity develops. To partially fill this gap, there are populations of innate lymphocytes. These cells, which possess a limited array of germline-encoded receptors, circulate throughout the body and attack invading organisms as soon as they are recognized.[44] Innate lymphocytes include both innate helper cells and innate cytotoxic cells (i.e., NK cells) (Figure 8.8).[2,13]

Innate Helper Cells

There are three populations of ILCs: ILC1, ILC2, and ILC3 cells. ILC1 cells are the innate counterparts to Th1 cells and play an immediate role in the defense against invading bacteria, viruses, and parasites. They are mainly located under body surfaces and, like Th1 cells, can produce large amounts of IFN-γ and TNF-α in response to IL-12. ILC2 cells, by contrast, are found in many organs, including the lung, skin, and bone marrow. They are the innate counterparts of Th2 cells. They produce large amounts of IL-5 and IL-13 and are required for the early innate response to parasitic helminths.[32] They also play a major role in the development of allergies, since these cytokines control eosinophil production. ILC3 cells are located in the intestinal mucosa where they serve a function similar to Th17 cells. They produce IL-17 and IL-22 in response to IL-23. They are important in protecting mucosal surfaces against bacterial and fungal invasion and defend against intracellular bacteria. ILC3 cells are also essential for the development of lymphoid organs and in the generation of intestinal IgA-producing B cells.

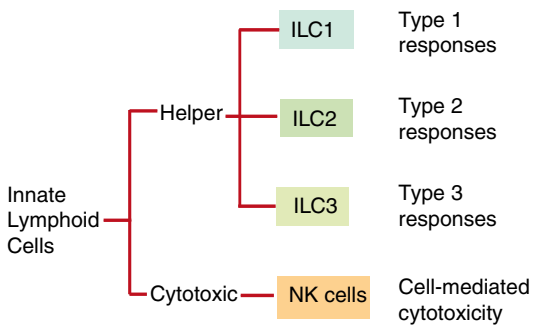


FIGURE 8.8 T-cell subpopulations. It is now recognized that there are several different innate lymphoid cell subpopulations that have equivalent functions to the T cell subpopulations. Thus ILC1 cells have similar functions to Th1 cells and produce cytokines that promote type 1 responses. The other innate populations also have T cell equivalents.

Innate Cytotoxic Cells

NK cells were first identified by their ability to kill abnormal cells, including tumor- and virus-infected cells, without previous priming. Morphologically, in most mammals, NK cells appear as large granular lymphocytes.[51] In cattle, they may be large lymphocytes without obvious granules, while in pigs there is a debate about their morphology with some asserting that they are small, lack granules, and thus indistinguishable from other lymphocytes.[20] NK cells are produced by bone marrow stem cells and are found throughout the secondary lymphoid organs. They may account for up to 15% of circulating lymphocytes in humans and 2–4% of circulating lymphocytes in cattle.[7] They have a characteristic phenotype of CD3−, CD56+, and NKp46+.[34]

Unlike T and B cells, NK cells do not possess an array of highly diverse antigen receptors. Instead, they have a small number of germline-encoded receptors that can detect one of two different signals, either: (1) the presence of MHC class I molecules on the target cell's surface or (2) the expression of stress-related molecules on cancer- or virus-infected cells. NK cells employ a "missing-self" strategy when evaluating cells for MHC class I expression. Recall that cytotoxic T cells rely upon the detection of antigen bound to MHC class I to identify and destroy a virus-infected cell. Thus, to evade destruction, a virus may downregulate cellular MHC I expression. To counter this, NK cells attack and destroy any cell *not* expressing MHC I. NK cells also possess receptors that detect the expression of "stress" proteins on cells which, when detected, send a positive signal stimulating NK cytotoxicity.

In different mammalian species, NK cells fall into three different types based on their critical receptors.[10] For example, in cattle and humans, the NK cells express killer cell immunoglobulin-like receptors (KIR), in rodents and horses, they express a C-type lectin called Ly49, whereas in rodents and primates, they express a receptor called NKG2D.[14,15,19,20,29,46,47] Each of these receptor types belongs to a family with both inhibitory and stimulatory members. NK cells also possess Fc receptors that allow them to bind and kill antibody-coated targets. Upon activation by contact with a target cell, NK cells kill their targets either by inducing their apoptosis through the CD95/95L pathway or by secreting toxic NK-lysins through the perforin pathway.[11] Unlike T and B cells that need to be activated before killing their targets, NK cells can immediately attack any virus-infected and tumor cells they encounter. Thus, they actively participate in the immune defense of the body.[30] NK cell numbers expand when needed and decline once the stimulus is removed. However, in a process termed

"trained immunity," small numbers of "memory" NK cells may persist to facilitate a more efficient response should the original stimulus be reencountered.[1]

NKT CELLS

Some [lymphocytes] express both T-cell and NK-cell markers and are accordingly called *NKT cells*.[17] There are two distinct subsets of these cells. Some, called *NKT1 cells*, use a TCR with an invariant α chain and a variable β chain. These cells recognize lipids and glycolipid antigens presented to them by cells with a specialized MHC molecule called *CD1*.[27] NKT1 cells are found in lymphoid tissues and the liver, and they constitute about 1% of human blood mononuclear cells. Type II NKT cells use a conventional TCR and also recognize lipids. They are likely primarily regulatory cells. Equine NKT cells may play a key role in recognizing the cell wall lipids of *Rhodococcus equi*. Cattle and other ruminants may not even possess classical NKT cells, but they have other cell types with similar functions.[28]

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CHAPTER 9

Structure and Function of Primary and Secondary Lymphoid Tissue

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Hematopoietic System Cells and Organs
Functional Anatomy of the Hematopoietic System
  Thymus
  Spleen
  Lymph Nodes
  Lymphoid Systems of Body Surfaces

Acronyms and Abbreviations

BLV, bovine leukemia virus; BALT, bronchus-associated lymphoid tissue; FeLV, feline leukemia virus; Ig, immunoglobulin; MALT, mucosa-associated lymphoid tissue; MHC, major histocompatibility complex; TCR, T-cell receptor genes.

HEMATOPOIETIC SYSTEM CELLS AND ORGANS

The hematopoietic system consists of the cascade of cells produced by the bone marrow and their specialized conducting and supporting systems. These systems include vascular endothelium, the connective tissue cells of the marrow, lymph nodes, and spleen, and organ-specific specialized cell populations.[5,15,20,21,25]

The hematopoietic cells consist of all differentiated products of pluripotent stem cells, including monocyte–macrophage and granulocytic cells, as well as RBC, platelet, and lymphocyte precursors. The latter, which include the thymic- and bone-marrow-dependent arms of the lymphoid system, are respectively responsible for cellular and humoral immunity.[15]

The vascular endothelium of the hematopoietic system includes the regionally undifferentiated cells lining the lymphatics and regionally differentiated endothelial cells of the blood vascular system.[14]

The specialized circulating blood cells and their conducting and supporting structures are uniquely packaged in a series of separate organs—that is, the thymus, lymph nodes, and spleen—or are incorporated into other organs, as with the bone marrow. The interrelation and integration of the cells and organs of the vascular system have important consequences in health and disease.

FUNCTIONAL ANATOMY OF THE HEMATOPOIETIC SYSTEM

Thymus

The thymus is cytologically simple, but architecturally complex. Cytologically, the thymus contains lymphocytes and epithelial cells; architecturally, it consists of lobules differentiated into cortical and medullary areas (Figures 9.1 and 9.2).

Thymic epithelial cells are derived from the third and fourth pharyngeal pouches that, in the embryo, migrate in two streams to form paired thymic lobes within the anterior mediastinum. This migration occurs very early in embryological development and is immediately followed by seeding of the thymus with progenitor cells from the blood islands of the yolk sac.[15]

The first stream of epithelial migration forms the isolated reticular epithelial cells of the cortex and medulla. Early reticular epithelial cells form loose cuffs around

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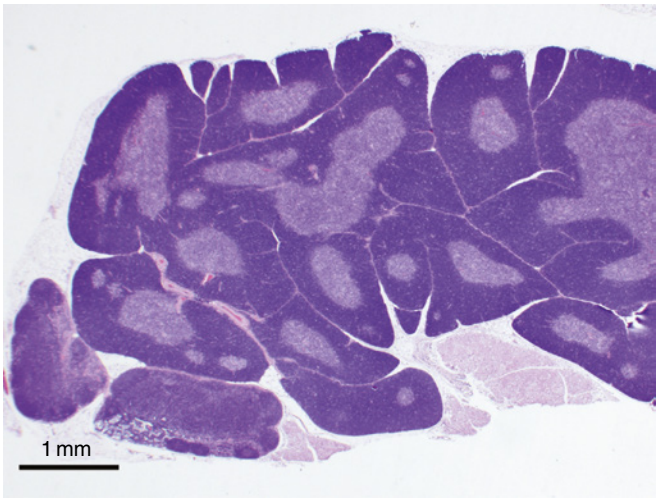


FIGURE 9.1 Thymus from a young adult male rat. The organ is composed of closely faceted lobules with a sharp distinction between the darker cortex and the lighter medullary areas of each lobule. H&E stain; bar = 1 mm.

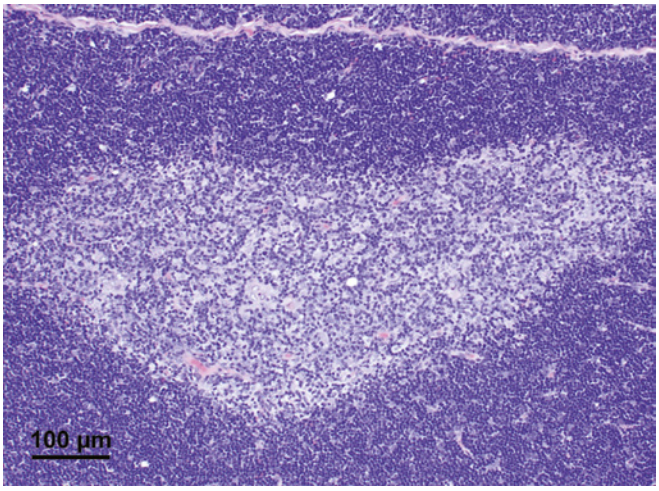


FIGURE 9.2 Mammalian thymus. In young healthy mammals, the medulla and cortex are sharply delineated; the width of the cortices and medulla equals approximately one-third the width of the entire lobe. H&E stain; bar = 100 µm.

small vessels that persist in adult life and become obvious in conditions of lymphoid atrophy. These epithelial cuffs of thymic vessels are a barrier to blood-borne antigens and the sole provider of immunologic training to naïve bone marrow lymphocytes.

The second epithelial migration forms the thymic duct epithelium and later the Hassall's corpuscles of the thymic medulla (Figure 9.3).[21] In early development, this ductular epithelium forms the branching system that communicates between the lobules of a single thymic lobe. The medullary epithelium produces trophic hormones that assist lymphocytic colonization and are the source of cysts lined by ciliated epithelium that frequently develop in adult life. The concentrically arranged, laminar squamous epithelial cells of the

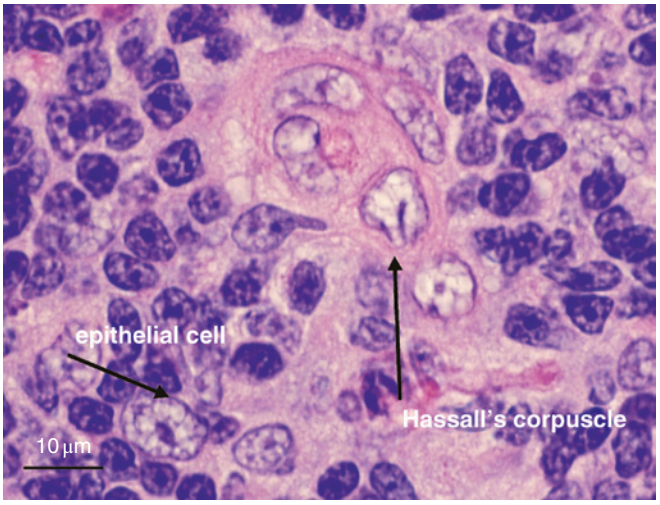


FIGURE 9.3 A Hassall's corpuscle in the thymic medulla consists of concentric laminations of squamous epithelial cells. The surrounding cells are a mix of small and medium lymphocytes and epithelial cells with large pale nuclei (arrow). These epithelial cells, with moderately abundant cytoplasm separating the nuclei, give the medulla a less-dense histologic appearance than the cortex. H&E stain; bar = 10 µm.

medullary Hassall's corpuscles are surrounded by myoid cells. These myoid cells are of uncertain histogenesis, but may have shared epitopes with thymic epithelial cells and are important in the pathogenesis of myasthenia gravis.[21]

The embryological relationship of these cells and structures is relevant in the diagnosis of adult thymic lesions. For example, a thymic lesion with loss of corticomedullary distinction that may be either medullary hyperplasia or a thymoma can be differentiated by the presence of reticular cuffs around the vessels, a finding that indicates thymoma.

The thymus is vital for the training and development of T cells. Uncommitted lymphocytes, originating in the bone marrow, have cell surface molecules to selectively home to thymic cortical vascular endothelium. Consequently, these uncommitted lymphocytes stream continuously into the thymic cortex. In the cortex, reticular epithelial cells form thin-walled pouches (caveolae) within which these lymphocytes undergo immunological selection for tolerance to self-antigens (see Chapter 51). These thymic cortical lymphocytes (thymocytes) have small, densely stained nuclei without apparent nucleoli, despite their intense cellular proliferation. The vast majority of these thymocyte progeny fail immunologic selection or successful T-cell receptor gene (TCR) rearrangement, die by apoptosis, and are removed by tingible body macrophages (Figure 9.4).[5] These macrophages become more prominent in conditions causing cortical lympholysis, such as certain viral infections, irradiation, or corticosteroid therapy.

Medullary lymphocytes are larger than cortical lymphocytes, with bigger, more vesicular nuclei and increased volumes of cytoplasm. As a consequence of this larger lymphocyte size, the thymic medulla is less

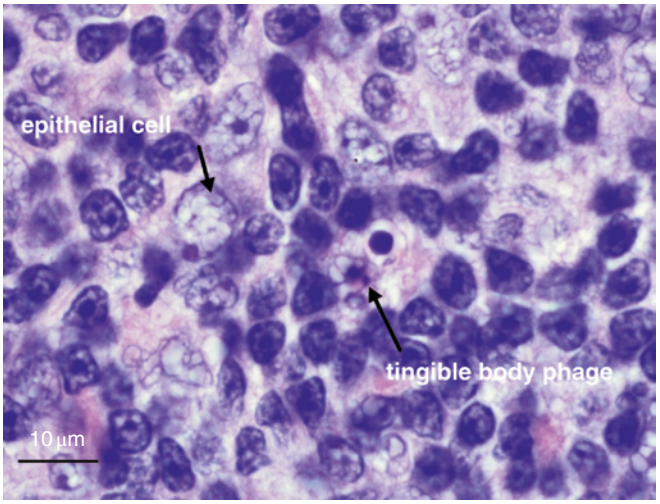


FIGURE 9.4 Thymic cortex composed of densely packed small lymphocytes (thymocytes). The cytoplasm of a tingible body macrophage (arrow) contains several pyknotic nuclear fragments (tingible bodies) of apoptotic thymocytes. The larger vesicular nuclei (arrow) are probably reticular epithelial cells involved in the immunologic training of developing T cells. H&E stain; bar = 10 µm.

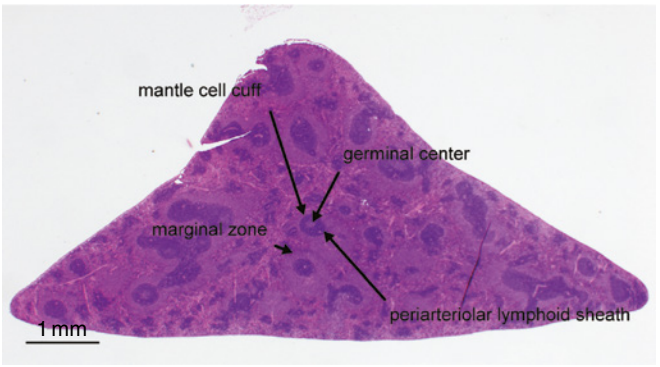


FIGURE 9.5 Cross-section of spleen from a young adult male rat. At the architectural level, the spleen is composed of multiple, dispersed round dark lymphoid nodules (white pulp) with the intervening lighter areas consisting of vascular sinuses (red pulp). The lymphoid areas may have a germinal center surrounded by a mantle cell cuff (arrows). The lighter mantle and marginal zones surrounded this mantle cell cuff. H&E stain; bar = 1 mm.

histologically dense. Within the medulla, some of the small fraction of mature T cells that survived both positive and negative selection undergo additional training that results in preferential homing to the intestinal mucosa and Peyer's patches.

Recent work suggests that terminal maturation of T cells may occur in the intestinal tract as well as in the thymus.[13] It would not be surprising if similar activity was found in both the lung and skin, which would provide a more comprehensive explanation for aberrant lymphoid reactions that can occur in benign and malignant states.

In addition to epithelial cells and lymphocytes, other cell types can be found within the thymus. In young rabbits, heterophils are frequently found in thymic tissue, particularly in lobular connective tissue. Eosinophils may occasionally be found in thymic connective tissues of other species. Mast cells are present in the thymic capsule of most species and are particularly frequent in rats.

In most species, the thymus reaches its maximal physiologic development about the time of puberty. An unusual antigenic stimulation during adolescence may cause benign thymic hyperplasia, which—in the calf—can result in an exaggerated chain of thymic lobules extending from the rami of the mandibles to the heart base. This thymic hyperplasia occurs largely through an increased number of lobules rather than increased lobule size. After adolescence, the thymus slowly decreases in size as a result of age-associated atrophy. Thymic atrophy, whether physiologic or pathologic, tends to result in a blurring of the corticomedullary distinction and fatty infiltration.

Thymic function—facilitating T-cell development and education—does not necessarily vary in relation to thymic size. Consequently, even a small thymic remnant may be responsible for persistent autoimmune disease in the adult. Unlike secondary lymphoid tissues (e.g., lymph nodes) that respond to antigenic stimulation, the thymus does not form lymphoid germinal centers. However, nonpathologic germinal centers can develop within the thymic vascular epithelial sheaths, as this unique location is immunologically "outside" the thymus.

Spleen

The spleen is cytologically and architecturally complex. It forms early in embryonic life and is a site of active erythropoiesis during the fetal period. Postnatally, it contains a wide variety of lymphoid and hematopoietic cells whose proportions vary in reactive and disease states. Diverse, dynamic regional anatomy is the result of a complex vascular system and expansile venous sinus system.

The spleen is composed of two primary tissues—red pulp and white pulp. The red pulp is comprised of many vascular sinusoids and serves as the spleen's filtering system to remove hematogenous antigens, microorganisms, and abnormal or effete blood cells. The white pulp is composed of lymphoid tissue and functions in lymphopoiesis and antibody production. These functional tissues are supported by internal trabeculae and an external capsule.

The spleen is unique in having efferent, but no afferent, lymphatics. Thus, all antigens enter the spleen through the blood vascular system. Both the major arterial supply and venous outflow enter through the spleen hilum and arborize together throughout its length. This interior arborization is relatively random in mammals, but reptiles have a major central venous sinus that is somewhat analogous to the bone marrow.

The white pulp consists of lymphoid tissue with unique histologic organization. At the level of small- and medium-sized arterioles, the blood vessels are sheathed in a cuff of small thymic-derived lymphocytes. These cuffs of lymphocytes are known as periarteriolar lymphoid sheaths (Figure 9.5). Very small branches from

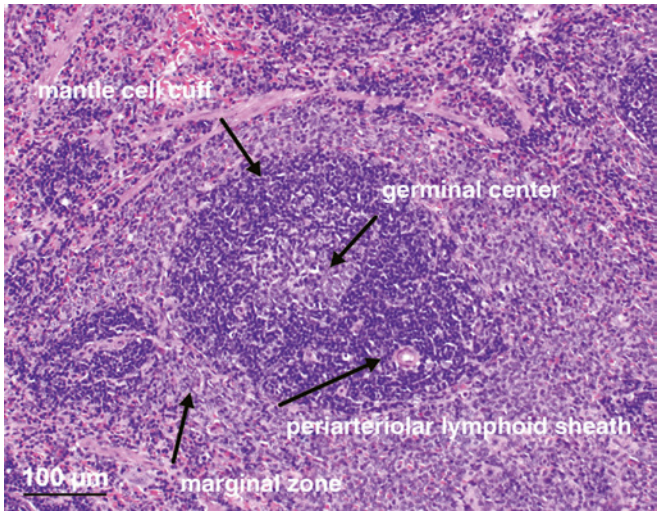


FIGURE 9.6 Details of Figure 9.5. Unlike larger mammals, rats tend to have less defined splenic germinal centers (shown). In the image center, a lighter zone constitutes the dendritic cell bed of a germinal center that abuts an eccentric small muscular arteriole (arrow). The germinal center is surrounded by cuffs of darker mantle lymphocytes and lighter marginal zone lymphocytes. The outermost marginal zone interfaces with the surrounding splenic red pulp. H&E stain; bar = 100 µm.

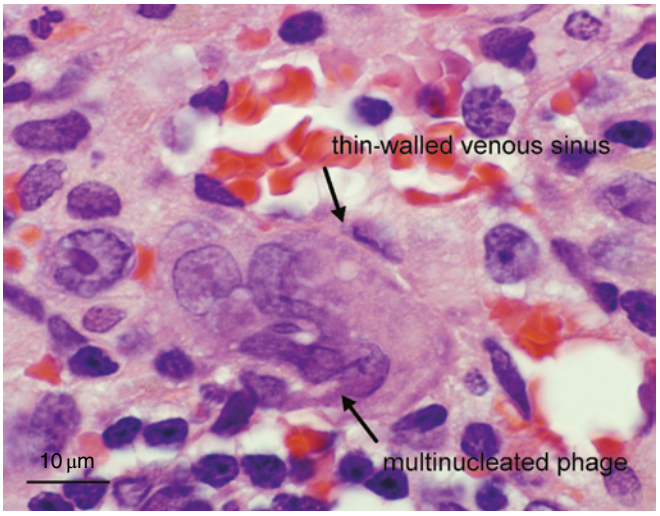


FIGURE 9.7 The interposition of macrophages, a multinucleated form in this field, and thin-walled vascular sinuses act as the filtering system of the spleen. H&E stain; bar = 10 µm.

these sheathed arteries give rise to germinal centers that are foci of B-cell proliferation. Surrounding the densely cellular periarteriolar lymphoid sheaths and germinal centers is a more loosely aggregated area of mixed T- and B-cell lymphocytes called the mantle cell layer. The rat and mouse have a clear stromal-appearing boundary around the mantle cells and germinal center that is not present in humans or domestic animals. Outside the mantle cells is a marginal zone of B cells (Figure 9.6). The marginal zone's width varies with immune activity and is itself surrounded by a trough or sinus area that receives blood passing through the germinal center. This trough or sinus area may appear redder in histological section and contains cells in the general circulation. In humans and the cat, this peripheral trough or sinus lies between the mantle cell cuff and the marginal zone layer.

While the cells of the periarteriolar sheath and germinal center are composed of resident cells whose movement occurs primarily through negative selection and replenishment, the lymphocytes of the mantle cell layer interchange with germinal center cells.

Small branches of the sheathed central arteriole may terminate in germinal centers, as described, or pass through the mantle and marginal zones to terminate in a penicillary array of small branches feeding directly into the sinusoids. These penicillary vessels are ensheathed by a few plump reticular cells that form a contractile ellipsoid. These ellipsoids are under neural and hormonal control to adjust the level of blood entering venous sinuses.

Under normal circumstances, 97% of the arteriolar blood entering the spleen exits directly via the penicillary vessels into a major sinus, which then quickly re-enters the central circulation. The remaining 3% undergoes filtration in the red pulp sinusoids. The walls of these sinusoids are unique. They consist of elongated endothelial cells arranged like the staves of a barrel, without junctions to their neighbors, which are maintained in alignment by a discontinuous encirclement of fine reticular fibers (Figure 9.7). Any filtered blood passes into the extrasinusoidal area—consisting of macrophages suspended in a loose reticular supporting framework—and then returns to central circulation by traversing between the sinusoidal endothelial cells. Reticulocytes, normal RBCs, and leukocytes easily pass between these endothelial cells to re-enter circulation, while senescent RBCs, those containing Howell–Jolly bodies, effete leukocytes, and foreign material cannot, so are phagocytosed by the extrasinusoidal macrophages. Cells that successfully re-enter the sinusoidal lumen exit to systemic circulation via the splenic vein. By this process, the entire blood volume is filtered by the spleen once each day.

Species with the genetic potential for sustained aerobic activity (i.e., humans, dogs, cats, and horses) have spleens with a contractile muscular capsule supported by equally contractile internal trabeculae. Contraction of these supporting structures permits maximal utilization of blood cells in systemic circulation. By contrast, species such as ruminants that group together for protection against their more agile predators tend to have spleens whose capsules are largely connective tissue with less capability for this contraction. In a muscular, contracted spleen, all of the blood passes directly from the arterial to venous system, thereby bypassing filtration. This "closed" circulation permits maximal utilization of blood cells. By contrast, any influence that causes a smooth muscle-rich splenic capsule to dilate (e.g., anesthesia, portal hypertension due to hepatic fibrosis, or marked increase in extrasinusoidal macrophages)

results in an expansion of the venous sinuses and a greater portion of blood passing through the extrasinusoidal filtering system.

The flow of blood through the red pulp is slow, thus cells are exposed to lower glucose and cholesterol concentrations and a lower pH than in systemic circulation. This biochemical environment is less hospitable to RBCs and platelets, so contributes to their premature aging and destruction. Thus, an enlarged spleen, regardless of cause, can decrease RBC and platelet life spans.[1]

A variety of changes occur in the spleen in response to systemic states such as toxicity, inflammation, starvation, immune-mediated disease, infarction, or neoplasia.

Acute toxic disease will result in lympholysis of the splenic germinal centers and replacement by residual proteinaceous debris. This process is referred to as follicular hyalinosis. While the nuclear debris resulting from acute lympholysis is cleared within 24 hours, the proteinaceous hyalin debris may remain for many months. In systemic amyloidosis, the germinal centers may become sites of amyloid deposition.

Atrophic changes due to starvation, aging, cancer, or chemotherapy may impact the thymic-dependent arteriolar sheaths and/or bone-marrow-dependent germinal center systems.

In chronic hemolytic anemias—particularly in old dogs—a marked increase in hemosiderin-bearing macrophages in sinus areas and iron deposition in the connective tissues can occur.

In dogs, hemorrhagic splenic infarction occurs in focal areas of hemangiosarcoma and can cause clinical anorexia or resistance to activity, even without hemorrhagic effusion. Ischemic infarction can be a consequence of myeloma-related blood hyperviscosity.

Painful splenic torsions, which are surprisingly compatible with life, occur primarily in dogs and humans. They also occur in adult pigs, but are rare.

Given the spleen's role in blood filtration, it is unsurprising that the major hazard to life after splenectomy appears to be septicemia.

Lymph Nodes

Lymph nodes are designed and strategically located to mount rapid and effective immune responses to foreign antigen.

The organization of lymph nodes and lymphatic vasculature is established during embryological development. During this early development, lymph nodes form at the confluence of an afferent lymphatic sprig and dilated sheath of blood vascular serosa. The blood vessel within the dilated serosal sheath produces a fine arborization of vessels that forms an architectural framework for future lymphocytic colonization.

Structurally, fully developed lymph nodes are composed of an outer cortex and inner medulla that are surrounded by a fibrous capsule and subcapsular sinus. The cortex consists primarily of B cells arranged as follicles, which may develop a germinal center upon antigen challenge. The deeper cortex, called the paracortex, is composed primarily of T cells. The medulla is composed of cords and sinuses. These medullary cords consist of plasma cells, macrophages, and B cells, while the medullary sinuses are vessel-like spaces between cords containing histiocytes and reticular cells.

The lymph node blood supply is highly organized with the arteriolar and venous branches arborizing through the medullary cords to form microcirculatory units. These circulatory units become the functional basis for germinal center formation. Specialized high endothelial venules form in the paracortical areas between–but never within–the germinal centers and present specific luminal adhesion molecules that regulate transmural lymphocyte traffic from the blood to the paracortex.[3]

The afferent lymphatics, peripheral capsule, and subcapsular sinus are the delivery system, whereby lymphatic fluid and antigens are delivered to the interior of the lymph node (Figure 9.8). The fibrous peripheral capsule will be thin and taut with a narrow subcapsular sinus when the lymph node has enlarged rapidly, while lymph node atrophy results in a thickened and wavy-appearing capsule with a wide subcapsular sinus. Chronic antigenic stimulation of a node, whether iatrogenic or pathologic, results in a thickened, sclerotic capsule with thick fibrous raphe that spans the cortex and terminates in a dense collagenous medullary fibrovascular network.[19] In cattle, and likely in other species, the lymph node capsule is contractile and under neural control, thus assisting lymph flow.[12]

Channels extend from the inner lining of the subcapsular sinus, through the cortex's lymphoid follicles and germinal centers, to the vascular microcirculatory units. These lymphoid follicles and germinal centers therefore act as the first order of antigenic filtration.

A fully developed germinal center is an organized structure consisting of a progression of cell types. The proliferative gradient created by this cellular progression provides the germinal center with an easily recognized polarity (Figures 9.9 and 9.10).[16] This polarity is largely due to an outer mantle cuff of small lymphocytes that is eccentrically thickened at the superficial or capsular pole, just beneath the inner lining of the subcapsular sinus, and thinned at the base or deep pole in the paracortex. Morphologically, the lymphocytes of the superficial pole have small dense nuclei with abundant lightly stained cytoplasm, while those in the deep pole have large nuclei with multiple prominent, usually peripheral, nucleoli and only a narrow rim of very basophilic cytoplasm. Antigen-presenting dendritic reticular cells and tingible body macrophages are located in the middle of the germinal center.

Dendritic reticular cells are derived from a variety of sources including myeloid or lymphoid precursors.[4,9,10,17] Consequently, dendritic cells at various anatomic sites have different phenotypic characteristics.[10] Ultrastructurally, dendritic cells are characterized by cytoplasmic tendrils that are joined between cells by tight junctions. In conditions of lymphoid depletion, these cells become exposed in germinal centers as an eosinophilic background of large pale cells.

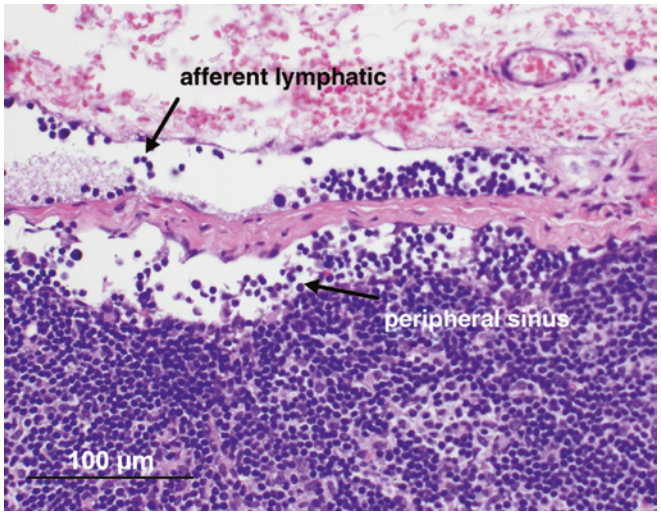


FIGURE 9.8 Lymph node outer cortex from a healthy dog. An afferent lymphatic (arrow) leads into the subcapsular sinus. The sinus is open and contains a few cells in transit on the left, but is compressed on the right by developing germinal centers. H&E stain; bar = 100 µm.

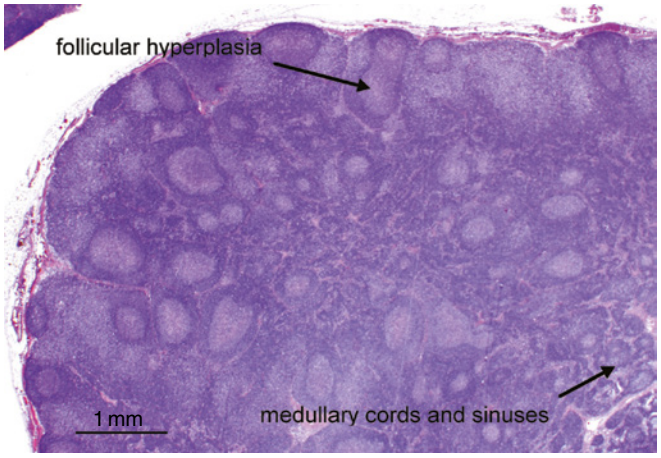


FIGURE 9.9 Architecture of the lymph node in Figure 9.8 showing follicular hyperplasia and densely cellular medullary cords and sinuses (arrows). H&E stain; bar = 1 mm.

One of the more difficult decisions when examining lymph nodes is the distinction between florid hyperplasia and lymphoma.[24] The neoplastic transformation of germinal centers to malignant disease is described in humans and it would appear that chronic lymphoid proliferation of any cause in animals can be a risk factor for lymphoma—examples include feline leukemia virus (FeLV) or bovine leukemia virus (BLV) infections and immunoproliferative small intestinal disease (e.g., inflammatory bowel disease).[6,14] The recognition of normal germinal center structure and orderly lymphoid proliferation is essential to distinguish benign and atypical follicular hyperplasia from follicular lymphoma. To this end, histiologic sections are made, so the germinal centers beneath the node capsule are in a plane that allows recognition of their antigen orientation, but those

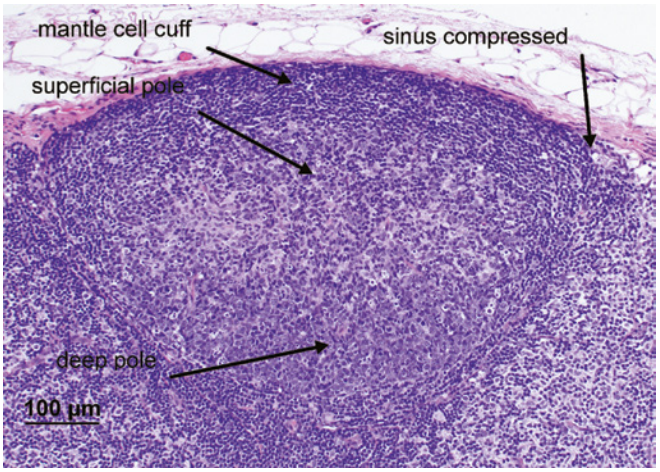


FIGURE 9.10 Lymph node cortex of Figure 9.9. The polarity of a subcapsular germinal center is directed toward the antigen being delivered from the subcapsular sinus into the superficial pole of small lymphocytes. The pale middle of the germinal center is composed of small and large lymphocytes with tingible body macrophages removing apoptotic lymphocyte nuclei. The deep pole area consists of lymphocytes with large nuclei and narrow rims of highly basophilic cytoplasm. H&E stain; bar = 100 µm.

in the medulla are oriented in a variety of planes, so all levels of the proliferative cellular progression can be viewed.[8] Follicular lymphomas, whether of small cleaved centrocytes, large centroblasts, or more often a mixture of both, can completely lack a mantle cell cuff and may instead be surrounded by a few residual small benign lymphocytes and aggregations of high endothelial venules. A particular type of follicular lymphoma that arises from the corona of cells surrounding the germinal center is known as mantle zone lymphoma. In this condition, coalescing nodular proliferations of moderate-sized lymphocytes surround fading germinal centers. Other distinguishing characteristics of follicular lymphoma include a conspicuous lack of cellular heterogeneity within the germinal center due to a clonal neoplastic expansion and a scarcity or absence of tingible body macrophages, since the tumor cells are not undergoing apoptosis.

Distinction between true benign or malignant follicular lesions and pseudofollicular lesions can be based on the location of the high endothelial venules. These specialized vessels are always between follicular proliferations and never within them, as may be seen with "pseudofollicular" lesions.

In the paracortex of some lymph nodes, particularly those in the mesentery of adult rats, there are semicircular or oval nodular structures distinct from germinal centers.[3,24] The main significance of these deep cortical nodules is to recognize their normalcy and not mistake them for a malignant lymphoid proliferation.[3] These deep cortical nodules, whose cellular input is from a distinct afferent lymphatic branch, form as a result of antigenic stimulation (Figure 9.11). They lie beneath the germinal centers of the outer cortex and are most easily visualized in lymph nodes with some degree of

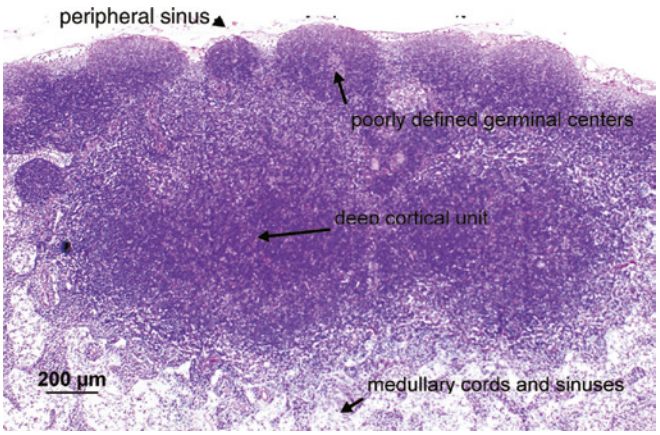


FIGURE 9.11 Cross-section of rat mesenteric lymph node. The superficial cortex is occupied by poorly defined germinal centers. The medullary cords and sinuses lie along the bottom of the image and abut a large, uniformly dense, oval deep cortical nodule. The deep cortical nodule is outlined by a paler rim of less-dense cells. H&E stain; bar = 200 µm.

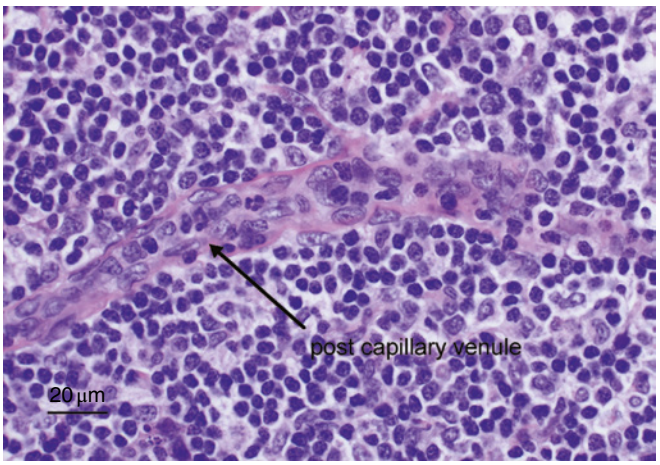


FIGURE 9.12 Paracortex of rat mesenteric lymph node. A high endothelial venule crosses this field (arrow). Several lymphocytes within the venule wall are in transmural transit—exiting the blood and entering the nodal cortex (near arrow). H&E stain; bar = 20 µm.

paracortical atrophy. The central area of these nodules is somewhat mottled due to varying proportions of large and small lymphocytes, some macrophages, and post-capillary venules (Figure 9.12). Edges of these deep cortical nodules gradually blend with the surrounding medullary cords and sinuses and are apparently the egress for cells entering the efferent lymphatic channels.[3]

The medullary cords and medullary sinuses are best considered together, as they form an interwoven matrix that waxes and wanes in unison or at the expense of one other (Figure 9.13). A medullary cord is composed of a branch of vascular structures—an artery, vein, and lymphatic—from the node hilum surrounded by a variable amount of connective tissue and covered by thin endothelium. The sinus area constitutes the space between the endothelial wall of the vascular structures and the similar endothelial wall of the medullary cords.

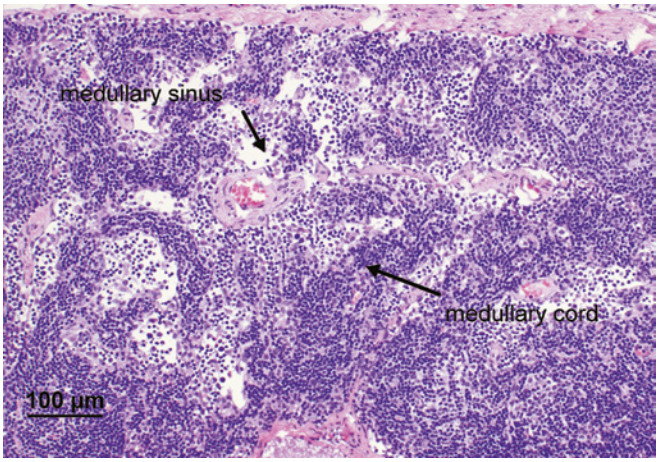


FIGURE 9.13 Medulla of a lymph node from a dog with normal cords and sinuses. The cords are densely cellular and surrounded by a thin endothelial membrane. The sinuses contain a few fixed macrophages, cells in transit, and lymphatic fluid. H&E stain; bar = 100 µm.

The medullary sinuses can extend all the way up to the subcapsular sinus in nodes with severe paracortical and follicular atrophy, such as the mesenteric nodes of cattle dying of acute bovine viral diarrhea virus infection. In this particular condition, massive B-cell lysis occurs and the sinuses tend to be filled with macrophages (sinus histiocytosis).

The cellularity and composition of the medullary sinuses vary widely. In diseases characterized by chronic immune stimulation, there are often high numbers of sinus macrophages suspended in a syncytial network. In nodes-draining areas of chronic dermatitis, the macrophages of the superficial cortex and medullary sinuses frequently contain abundant normal-appearing melanin granules.[18] Lymph-nodes-draining areas of hemorrhage have numerous hemosiderin-bearing medullary macrophages. Since the transit of RBCs to the lymph nodes and RBC intracellular degradation by medullary macrophages is rapid, the observation of erythrophagocytosis should be interpreted as a very recent event.

The medullary cords contain activated lymphocytes that encountered an antigen-presenting cell in the outer cortex expressing a major histocompatibility complex (MHC)/antigen to which they successfully bound. Antibody-producing plasma cells are typical of the medullary cords, but small or medium lymphocytes—rather than plasma cells—may predominate in reactions that have persisted for many weeks.

Nonlymphoid or nonmacrophagic cells can also be found in the medullary cords, as the fine reticular network forms a fertile microenvironment for extramedullary hematopoiesis. Malignant cells in myeloid leukemia can also colonize the medullary cords and may coexist with benign hematopoietic cells.

Naïve (nonactivated) lymphocytes circulate randomly in the blood and lymphatic fluid. However, their future migration becomes altered once activated by antigen recognition in the lymph node cortex. Activated B

cells migrate preferentially to mucosa-associated lymphoid tissues (MALT), while T cells migrate to peripheral lymph nodes.[5,7] This organ-specific lymphocyte homing occurs in both neoplastic and inflammatory conditions. The spread of malignant lymphocytes is therefore limited by their inherent capacity to migrate into various tissues. Thus, the spread of lymphoma to lymphoid structures is not a function of the tumor cells being able to enter the peripheral blood, but rather evidence of their successful binding to high endothelial venules and migration out of the blood and into additional lymphoid structures.[15] By this understanding, all lymphoid neoplasms circulate in the blood, but their degree of dissemination is limited by their binding capability. Therefore, differentiation between lymphoid leukemia and lymphoma is based simply on identification of the tissue with the greatest volume of tumor.

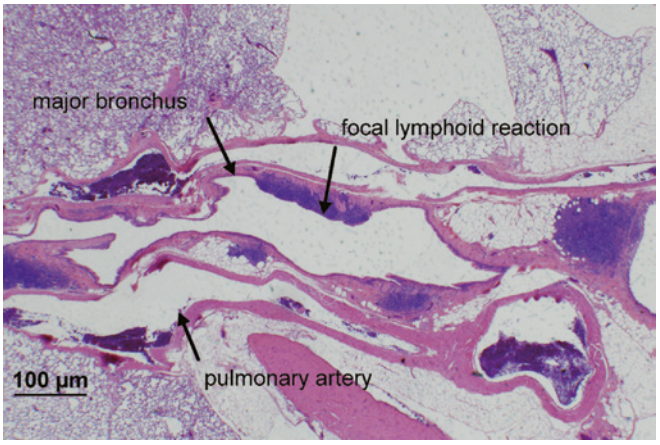


FIGURE 9.14 Lung from a normal rat with a longitudinal section of a major bronchus. The focal areas of lymphoid proliferation are peribronchiolar and subepithelial. H&E stain; bar = 100 µm.

LYMPHOID SYSTEMS OF BODY SURFACES

Body surfaces that interface with the "outside world," including the skin, respiratory tract, and enteric system, have resident lymphoid populations and antigen-presenting cells vital in immune defense. Intraepithelial lymphocytes in these locations–those admixed within the epithelial layer–are cells in training, which return to deeper lymphoid structures following their specific sensitization to perform specific functions in differentiation and proliferation.

The skin's epidermis is 85% keratinocytes and 15% melanocytes, Langerhans cells, Merkel cells, and dendritic cells of uncertain type. The Langerhans cells arise from a variety of precursors and constitute the major antigen-presenting cells of the epidermis.[1] In the skin of humans and cattle, the Langerhans cells contain a typical cytoplasmic organelle visible on electron microscopy that is rod-shaped with a unipolar bulb called the Birbeck granule. The presence of Birbeck granules permits identification of Langerhans cells in the paracortex of lymph-nodes-draining skin. These cells phagocytose foreign molecules without degrading them, migrate to the local lymph nodes, and present these antigens in their specific MHC context to naïve lymphocytes. Both T and B cells are residents of the skin where their function is largely unknown, although a fairly consistent proportion of cutaneous lymphocytes are suppressor T cells that may be responsible for dampening cutaneous immune sensitization.[2]

The mucosa-associated lymphoid tissue of the respiratory and enteric systems is shared in the tonsillar and pharyngeal areas. In humans, these MALTs are collectively known as Waldeyer's ring. In the respiratory system, the epithelium of the nasal mucosa, larynx, trachea, and bronchi is infiltrated by variable numbers of lymphocytes with fewer neutrophils and macrophages. Mucosal lymphoid infiltration is more prominent above areas of submucosal lymphoid proliferation, such as the tonsil and bronchiolar-associated lymphoid tissues (BALT).[2] Lymphoid proliferation in the tonsil is usually characterized by prominent germinal center formation; while in the healthy lower lung, the BALT may be minimal and restricted to diffuse accumulations of cells between the bronchial epithelium and the supporting smooth muscle and cartilaginous plates (Figure 9.14).[20] In general, the degree of lymphoid reactivity in these areas is proportional to the level of antigenic stimulation. The impact of ambient air quality—reflected in the amount of dust and airborne infectious agents—is apparent in the lung histology of laboratory animals. Animals maintained in facilities with well-filtered air have minimal BALT and few intraepithelial lymphocytes. By contrast, animals exposed to poorer air quality have diffuse lymphoid proliferation in the proximal trachea and laryngeal areas with heavy, mixed cellular infiltration of the surface epithelium. In the lower lung, the reactive lymphoid proliferations may contain germinal centers, but are usually diffuse and may be of sufficient volume to partially occlude airways. The lymph nodes at the heart base and in the caudal mediastinum receive lymphatic fluid from the lung and consequently bear evidence of past environmental exposures, such as smoke or particulate inhalation.

The gut-associated lymphoid tissue forms a relatively continuous chain from the oral cavity to the anus with characteristic areas of proliferation. There are typically only a few lymphocytes in the epithelium and mucosa of the esophagus, whereas germinal center formation is relatively common, but not necessarily normal, beneath the gastric epithelium. In recent years, an association between *Helicobacter* sp. bacterial organisms and human gastric ulceration has been discovered. The gastric lymphoid tissue present in such cases appears antigen-dependent as it recedes with long-term antibiotic therapy targeting these organisms. Even more remarkably, the early stages of human gastric lymphoma appear antigen-dependent and the disease may regress with antibiotic treatment alone.

The amount of organized lymphoid tissue in the lower small intestine–including the Peyer's patches–is

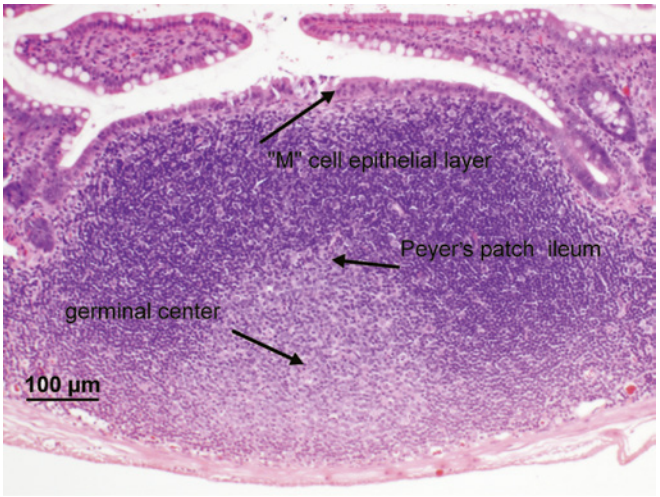


FIGURE 9.15 Lower small intestine of a normal rat. The epithelium overlying a focal, dome-shaped submucosal proliferation of lymphocytes lacks villi and is modified to facilitate antigen recognition and sorting. H&E stain; bar = 100 µm.

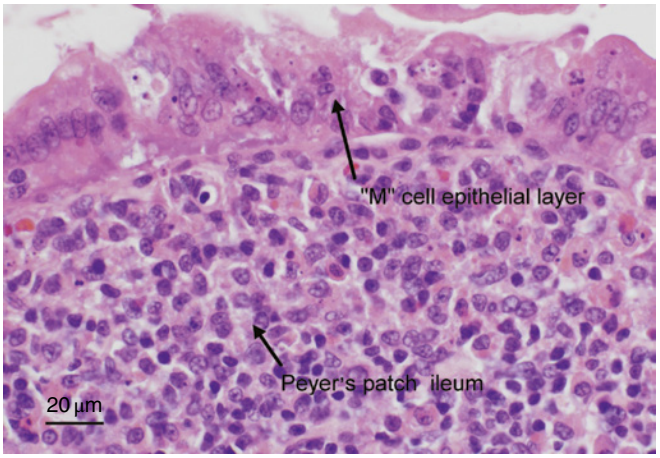


FIGURE 9.16 Details of Figure 9.15. The epithelium above the lymphoid follicle contains M cells with deeply folded cellular membranes. The M cells are capable of antigen uptake from the intestinal lumen and transport to secondary lymphoid tissues. H&E stain; bar = 20 µm.

roughly equivalent to the thymus gland in young animals (Figure 9.15). The intestinal epithelium overlying germinal centers is typically devoid of goblet cells or villi and has no glandular crypts (Figure 9.16). Instead, this distinctive epithelium has unique epithelial cells called microfold or M cells. These specialized cells, involved in the initiation of mucosal immunity responses, have complex apical membrane microfolding, hence their name.[23] M cells take up antigen from the intestinal lumen via selective endocytosis and deliver it to antigen-presenting dendritic cells or B cells in regional lymphoid tissues.

Much of the immunoglobulin (Ig) produced in BALT and MALT is IgA-type with luminal and mucosal concentrations significantly higher than in blood. The structure of immunoglobulin-A facilitates its crucial role in the immune function and protection of mucous membranes.

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SECTION II
Hematotoxicity

CHAPTER 10

Design and Methods of Nonclinical Hematotoxicity Studies

WILLIAM J. REAGAN and ARMANDO R. IRIZARRY ROVIRA

Regulatory Guidelines and Best Practices
Preanalytical Variables
  General
  Fasted State
  Timing of Blood Collection
  Method of Blood Collection
  Amount of Blood Collected
Hematologic Analysis and Parameters
Bone Marrow Histopathology
Bone Marrow Cytology
Flow Cytometry
Other Techniques

Acronyms and Abbreviations

CBC, complete blood count; CBER, Center for Biologics Evaluation and Research; CDER, Center for Drug Evaluation and Research; CO_2, carbon dioxide; EDTA ethylenediaminetetraacetic acid; EMA, European Medicines Agency; FDA, Food and Drug Administration; HCT, hematocrit; HGB, hemoglobin concentration; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; MHLW, Ministry of Health, Labor and Welfare; O_2, oxygen; PCV, packed cell volume; PLT, platelet count; PMDA, Pharmaceutical and Medical Devices Agency; RBC, red blood cell count; RDW, red cell distribution width; WBC, white blood cell count.

The CBC is the foundation of the evaluation of the hematopoietic system in nonclinical toxicology studies and clinical trials in humans. Microscopic evaluation of the bone marrow in nonclinical studies, together with concurrent evaluation of the CBC, provides a thorough evaluation of the health of the hematopoietic system and is often predictive of what will be seen in humans. The goal of this chapter is to briefly review the regulatory guidelines and best practices pertaining to the evaluation of the hematopoietic system in nonclinical toxicology studies, how and when to obtain samples, and the general approach to integration of the different hematologic tests.

REGULATORY GUIDELINES AND BEST PRACTICES

Governmental agencies responsible for ensuring the safety and efficacy of drug products, such as the United States Food and Drug Administration (FDA), European Medicines Agency (EMA), and Japan's Ministry of Health, Labor and Welfare (MHLW), have guidance documents for nonclinical toxicology studies that contain recommendations for hematologic testing, as well as bone marrow histologic and cytologic evaluation. The FDA has two main centers that are involved in human drug safety, including the Center for Drug Evaluation and Research (CDER) and the Center for Biologics Evaluation and Research (CBER). The Pharmaceutical and Medical Devices Agency (PMDA) partners with MHLW and has the responsibility for assessing human drug safety.

The types of toxicology studies that are needed for approval of new drugs consist of acute, subacute (or subchronic), chronic, and carcinogenicity toxicity studies. In stand-alone acute toxicity studies, the test article is administered once at different doses followed by an observation period of 14 days.[15] It is now recommended that the assessment of acute toxicity be done as part of other short-duration dose-range-finding studies that

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will help to define the maximal tolerated dose.[14] Subacute and chronic toxicity studies are repeat-dose studies where the test article is administered multiple times at different doses for at least 2–4 weeks and for 6 or more months, respectively. Carcinogenicity studies are repeat-dose studies where the test article is administered for most of the lifetime of a rodent.

Hematologic testing is routinely performed in subchronic and chronic studies. The EMA gives very broad guidelines for clinical pathology testing.[12,13] Pharmaceuticals should be tested in two mammalian species, one of which must be a non-rodent, and both sexes should be evaluated in both species. Development of a pharmaceutical in a single mammalian species may be acceptable with appropriate justification. For rodents and non-rodents, historical control data should be available. Pre-study data should be generated in studies using non-rodents. The evaluation should be done at relevant time points to detect changes over time. Although EMA does not stipulate specific hematological parameters to evaluate, or timing of sampling, during the in-life and terminal phases of a study, it refers to the recommendations of the Joint Scientific Committee for International Harmonization of Clinical Pathology Testing for guidance.[32] EMA suggests that evaluation during the study should be done in all animals in studies with low numbers of animals. In rodent studies, specialized examinations may be performed in a subset of animals at all dose levels. Evaluations performed on animals to which test article is administered should also be performed on animals from the control group. Necropsy must be performed in all animals in the study. Blood smears are not specifically requested in the most recent guidance document.[13] Bone marrow smears and bone marrow for histopathologic evaluation (sternebrae, femur, or rib, depending on species) should be collected. Microscopic examination of bone marrow smears is to occur "if treatment-related changes are suspected in tissues/organs or in peripheral blood." If bone marrow smears are evaluated, EMA recommends a visual assessment of cellularity, distribution, morphology, and myeloid to erythroid ratio. However, the International Conference on Harmonization (ICH) S8 guideline, which the EMA adopted, is less definitive about when bone marrow smear evaluation is to occur, since it indicates that unexplained alterations in the peripheral blood or histology of the bone marrow "suggest" that microscopic evaluation of the bone marrow smears "would be appropriate."[12,20] The ICH S8 recommendation is more in line with the recommendations by the Bone Marrow Working Group of the American Society for Veterinary Clinical Pathology/Society of Toxicologic Pathology (ASVCP/STP), which indicated that bone marrow smears should be evaluated if characterization of the changes in the hematopoietic system were inadequate using hematology and histopathology results.[27] The EMA also recommends histopathologic evaluation of recommended tissues from all non-rodents, but in rodent studies histopathologic evaluation is performed first on animals in control and high-dose groups.[13] If microscopic changes are identified in the high-dose group, additional dose groups should be examined to clarify the dose relationship.

The MHLW guidelines for repeat-dose testing are very similar to EMA's guidelines.[33] They recommend blood samples be taken before necropsy in rodents, including interim sacrifice, and in non-rodents pretreatment samples should be evaluated. Although they recommend hematologic testing be done at the time of interim sacrifice of rodents, if these animals are in poor condition, many of the changes in the parameters may reflect secondary changes and thus may not be particularly useful in assessing the primary compound effect. In non-rodent studies of 1 month or longer duration, at least one interim blood sampling should be considered. If a 2-week exploratory study is done before the 1-month study, then hematologic evaluation at an interim time period may not be necessary. Hematological parameters to assess include RBC count, WBC count, WBC differential cell count, platelet (PLT) count, hemoglobin concentration (HGB), hematocrit (HCT), and reticulocyte count. Histopathology recommendations include bone marrow (sternum, femur). There is no mention of bone marrow cytologic examination.

CDER and CBER have not published their own recent guidelines for hematologic testing in repeated-dose toxicity studies. However, FDA had adopted ICH guidelines, some of which refer to, or provide general guidance about, the evaluation of the hematopoietic system.[16,20]

The guidelines governing the clinicopathologic evaluation of carcinogenicity studies vary between regulatory agencies. FDA and MHLW, both of which have adopted ICH guidelines for drugs intended for humans, do not explicitly indicate that hematological assessment is mandatory.[16,17,21,33] However, MHLW suggests that blood samples should be taken, if appropriate, to measure RBCs and WBCs, and to make blood smears in animals that are sacrificed before or at the end of the study. Blood smears may be useful to assess anemia or changes in lymph nodes, liver and spleen that may be present.[33] By contrast, the EMA suggests that monitoring hematologic parameters should be considered during the study and should be performed at study termination.[11] This recommendation is in contrast to the one made by the Joint Scientific Committee for International Harmonization of Clinical Pathology Testing that stated that blood smears at unscheduled and scheduled terminations should be made to help in the detection and differentiation of hematopoietic neoplasia, but other hematologic testing is not recommended because of the variability of the data.[32] Histopathologic evaluation of bone marrow is expected in carcinogenicity studies.[11,33]

There are now several new key position papers with recommendations of hematologic parameters to use in nonclinical toxicity testing.[27,29,34] The recommendations within these papers are very similar and overall align well with previous position papers as well as with most of the regulatory guidelines outlined in the preceding text. The most recent and comprehensive publication, "Best Practices for Veterinary Toxicologic Clinical Pathology, with Emphasis on the Pharmaceutical and Biotechnology Industries," was endorsed by the

Regulatory Affairs Committee of the ASVCP and STP and recommended these core parameters: RBC count, HGB and HCT, RBC indices (mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), red cell distribution width (RDW)), reticulocyte count, WBC count, absolute WBC differential counts, PLT count, and blood smear. Blood smears do not need to be reviewed routinely, but the criteria for review should be defined in standard operating procedures. Bone marrow cytology smears should also be prepared from each animal at termination in regulated studies, but only need to be reviewed on a case-by-case basis. This is typically done if the histologic and hematologic evaluation did not adequately characterize the bone marrow abnormality.[27] For carcinogenicity studies, the recent best practice paper recommends to follow the recommendations of the Carcinogenicity Study Working Group (STP/ASVCP).[34] Briefly, they recommend that blood smears be routinely collected from all animals that are euthanized (unscheduled and scheduled), since these slides may be useful in assessing hematologic neoplasia, but these should only be examined if necessary following histologic evaluation. They do not recommend routine hematology (or other standard testing) on these animals. Bone marrow smears should only be collected on a case-by-case basis.

PREANALYTICAL VARIABLES

General

Several preanalytical variables, including age, sex, source of the animals, fasting state, randomization and time of bleeding, anesthesia, diet, housing, and sample handling, should all be considered; controlling these variables will help ensure robust hematology data.[28] First, animals of similar age should be utilized, especially in rats, since hematologic parameters change with animal age.[18] Obtaining nonhuman primates of similar age can be more challenging, especially if wild-caught animals are used, or if sexual maturity is required. Animals should also be obtained from the same commercial and geographical source because this can affect hematological parameters. For example, cynomolgus monkeys (*Macaca fascicularis*) from Mauritius, the Philippines, or Indonesia have smaller RBCs than those from China or Vietnam.[19]

Randomization of the order of blood collection in toxicology studies also increases the uniformity of the data. This is especially true in rodents. If hematology sampling occurs on the same day as collection of a toxicokinetic sample, the hematology specimen should ideally be taken first to minimize the effect of changes in hematology parameters due to repeated phlebotomy.

Inconsistencies in how the animals are handled can have a major effect on hematologic parameters, especially for nonhuman primates where epinephrine-induced excitement responses readily occur (see Chapter 11). It has also been shown that transportation of mice within a facility to mimic what may be done prior to collection of blood can increase plasma corticosterone and lead to decreases in lymphocyte counts.[10] Therefore, it is important to treat all animals in the study in a similar manner to minimize stress. All these preanalytical variables will be minimized by a well-planned study that is carried out in a well-managed facility with appropriately trained personnel.

Fasted State

An important preanalytical variable to control is fasting.[32] The in-life samples typically are collected from rats and large laboratory animals with an overnight fast (15–16 hours). Collecting samples in the fasted state will help increase the homogeneity of the data as well as minimize chances of potentially inaccurate analysis due to postprandial lipemia. Thirty percent of non-fasted rat serum samples were found to be lipemic.[30] Fasting of rats can have significant effects on hematologic parameters, mainly caused by hemoconcentration resulting in increases in RBC count, HCT, HGB, and PLT count, but also decreases in WBC count and prolongation of prothrombin time and activated partial thromboplastin time.[1,24] In contrast to large laboratory animals and rats, mice should not be fasted overnight, as they tend to dehydrate rapidly; however, some facilities will use a three to five-hour fast.[18]

Timing of Blood Collection

The timing of sample acquisition from the peripheral blood is somewhat dependent on regulatory requirements, but also on the goal of the study, the animal species, the blood volume of the test animal, the number of additional samples needed to assess other parameters, the dosing time, and the results of previous studies. For repeat-dose studies in non-rodent species, hematologic testing is recommended at study termination and at least once at an earlier interval. For studies of 2–6 weeks' duration in non-rodent species, testing is also recommended within 7 days of dosing initiation, unless it compromises the health of the animals.[31] Additional sampling time points are included depending on the design of the study and should include the recovery period if present. In dogs and nonhuman primates, one or two prestudy baseline samples should always be taken. Due to the small number of animals per dose group and the variability of the hematological results, the baseline sampling is critical to accurately assess any changes that occur during the study. The comparison of the in-life and end of study hematologic results to the baseline sample(s) will allow for the most accurate assessment of the relevant changes in the hematologic parameters. Hematologic testing is necessary at study termination in rodent repeat-dose studies. Interim study testing may not be necessary in long-duration studies if it has been done in short-duration studies using similar dose levels.[32] Testing should be done at the end of the recovery period, if included.[32]

In rodent studies, baseline sampling is not typically done. This is in part due to less variability in the

hematological parameters of rats versus large animals. In addition, the number of rats used per dose group is greater (typically 10 rats per sex per group versus three or four per sex per group in large animal studies), which increases the confidence of the mean data in this species. The mean data of each dose group are then compared to the respective vehicle control, although individual animal data should also be evaluated. In addition, in longer-term studies, the hematologic parameters will change with age, which make comparison to pretreatment values difficult.

Method of Blood Collection

In dogs, interim in vivo samples are taken from the jugular vein with manual restraint of the animals. Other sites including the cephalic and saphenous veins could be used. In nonhuman primates, the animals are either manually restrained, typically with help of specialized chairs or cages, or anesthetized. The femoral vein is frequently used, but cephalic and saphenous veins can also be used. If chair restraint is used without anesthesia, it is critical to have a good acclimatization period for these animals before the start of the study to minimize the excitement of the animals during sampling.

Samples can be obtained terminally, under anesthesia, from large laboratory animals and are collected from the caudal vena cava, aorta, or cardiac puncture. There are differences in hematological parameters depending on which vessel is sampled, so having the site of sampling consistent throughout the study is ideal.

Sites of interim in vivo sampling in rats are the jugular vein, lateral tail vein, tail artery, and sublingual vein, and these procedures can be done with either manual restraint or with anesthesia.

In mice, limited amounts of blood can be obtained from the lateral tail vein or retro-orbital sinus. Some facilities are able to obtain a larger volume from the jugular vein with manual restraint or anesthesia. Terminal samples, under anesthesia, are taken at the time of necropsy from the caudal vena cava, aorta, or via cardiac puncture, and due to the greater volume these samples are easier to use for hematological analysis.

The use of anesthesia will have an effect on the hematologic results. Anesthesia is not typically used in dogs and may be used in nonhuman primates for in-life sampling. If anesthesia is utilized in nonhuman primates, ketamine is typically used and will decrease WBC counts, mainly due to decreased lymphocytes.[22] Historically, two of the more commonly used anesthetics for rats include isoflurane and CO_2/O_2.[8]

Amount of Blood Collected

The amount of blood that can be taken will be dictated by recommendations of the Animal Care and Use Committee at each facility. A good review on blood sampling has been published and makes recommendations on how much blood volume can be safely withdrawn.[9] The blood volume of a 10-kg dog is estimated to be 850 mL (85 mL/kg). Of this volume, 85 mL (10%) of the blood can be removed in a single sample or with multiple samplings over 24 hours if animals are allowed to recover for 2 weeks.[9] Blood volume is more of an issue in monkeys due to their small size. A 5-kg cynomolgus macaque would have 325 mL (65 mL/kg) of blood. Therefore, a total of 32 mL of blood (10%) can be removed in a single sample or with multiple samples over 24 hours if the animals are allowed to recover for 2 weeks.[9] Although these volumes are adequate for routine hematological assessment, multiple blood samples taken for other analyses such as toxicokinetic, cytokine, and immunophenotyping analyses frequently result in reaching maximal allowable amounts, and can lead to iatrogenic decreases in RBC mass.

Interim sampling can be done in rats, although the overall amount of blood and frequency of the bleeding will be limited. A 250-g rat has approximately 16 mL (64 mL/kg) of blood. Of this volume, a total of 1.6 mL (10%) can be safely removed in a single sample or with multiple samples over a 24-hour period if animals are allowed to recover for 2 weeks.[9]

Interim sampling in mice is infrequently done due the limited blood volume. A 25-g mouse has 1.8 mL (72 mL/kg) of blood. A total of 0.2 mL (10.0%) can be removed in a single sample or with multiple samples over 24 hours if the animals are allowed to recover for 2 weeks.[9]

HEMATOLOGIC ANALYSIS AND PARAMETERS

A few types of automated hematology analyzers work well for the laboratory animal samples used in nonclinical toxicity studies. These analyzers should have multispecies software to ensure proper analysis. It is recommended that the blood be analyzed within a few hours after collection in all species. In addition, blood smears from all animals should be made soon after collection for possible review. In dogs and nonhuman primates, making the blood smears within two hours after collection is sufficient for retention of good cellular morphology, although the sooner the slides are made after collection, the better. In some strains of rats, if blood smears are not made immediately after collection, there may be secondary artifacts of preparation, such as crenation of the RBCs.

Each laboratory needs proper review criteria in place to ensure good quality hematologic results from hematologic analyzers. Although not clearly defined in regulatory guidelines, it is the authors' opinion that blood smears should be reviewed in all good laboratory-practice-regulated studies. This review would include assessment of RBC, WBC, and PLT morphology, as well as identification of other rare cell types. At a minimum, blood smears should be made from all animals in a study for potential review, if warranted. Most laboratories that review blood smears will review all blood smears from the study. An alternative is to review blood smears from all large animals, and have a tiered response for rat studies where high-dose male and female rats as well as control samples are evaluated.

If there are significant changes in morphology, then additional dose groups are evaluated.

The hematologic parameters that should be measured are those recommended by the most recent best practices paper and include RBC count, HGB and HCT, RBC indices (MCV, MCHC, MCH, RDW), absolute reticulocyte count, WBC count, absolute differential WBC count, and PLT count.[29] Blood smears should be made for potential evaluation. Other parameters such as mean platelet volume may sometimes be useful to assess changes in PLTs, but was not recommended for routine use. The CBC should be evaluated in conjunction with the bone marrow histopathologic analysis.

BONE MARROW HISTOPATHOLOGY

Histopathologic evaluation of bone marrow is greatly dependent on prompt and proper preservation, collection, handling, processing, and staining.[27] In large animals, the sternebrae or ribs are the preferred sites for histologic analysis because these sites will be hematopoietically active relative to other sites. By contrast, sternebrae and long bones of rodents, such as the femur, are frequently used. An overall assessment of bone marrow cellularity can be obtained with histopathology (see Chapters 16 and 17). The cellularity of bone marrow is dependent on the species, the site of collection, and the age of the animal. In addition, food restriction, or treatment-induced decrements in food consumption, will affect bone marrow cellularity and blood cell counts.[23,25] Concurrent vehicle controls and historical knowledge of what is the normal range of cellularity of bone marrow for that age animal and species are necessary to make an accurate assessment. If there are changes in cellularity, one should try to differentiate the cell types that are changed. In large animals, relative changes in the myeloid population compared to the erythroid population should be assessed if possible, but in rodents, due to the high percentage of lymphoid cells, differentiation of erythroid changes from lymphoid changes is difficult.

BONE MARROW CYTOLOGY

Bone marrow cytologic examination should be considered if there are significant or unexplained changes in the CBC and/or in bone marrow histopathology results that need further clarification. Ideally, bone marrow smears should be made immediately or soon after euthanasia. Smears can be made by several techniques, including push smears, pull smears, and paintbrush technique (see Chapter 141). The slides made with any of the abovementioned techniques are air-dried and stained with a Romanowsky-type stain, such as Wright's or Wright–Giemsa stain.

Cytologic analysis may consist of qualitative evaluation of the marrow smears alone and/or enumeration of the relative percentages of cells, typically by doing a 500-cell differential cell count. Morphologic abnormalities should be noted during qualitative assessments or when enumerating cell types. Enumeration of hematopoietic cells can be done by detailed classification of the different cell types and stages of maturation or by classifying hematopoietic cells into the major categories of myeloid, erythroid, and lymphoid lineages. If the latter technique is used, a qualitative assessment can then be made of the maturation of the different lineages. Megakaryocytes should be noted as present or absent, but not enumerated. These cytological results will then be used in conjunction with the CBC and histopathologic results to make an accurate assessment of the effect of xenobiotics on the hematopoietic system.

FLOW CYTOMETRY

Bone marrow flow cytometric examination could be considered if there are significant or unexplained changes in the CBC and/or bone marrow histopathology results that need further clarification (see Chapter 142). This technique is an alternative to counting hematopoietic cells manually, although does not replace the cytologic morphologic assessment of the bone marrow.[2–7] Percentages of the different cell lineages, and with some techniques a subdivision into proliferating and nonproliferating populations, can be assessed.[3,5–7] In addition to these semi-quantitative assessments, a total nucleated cell count can be determined, and absolute counts of the different cell populations can be calculated. In contrast to only determining relative percentages of the different cell populations, absolute counts provide more definitive data to allow accurate assessment of which cell populations are truly affected. The results on any flow cytometric assessment need to be interpreted in conjunction with the CBC, cytomorphology, and histopathology of the bone marrow to determine which population is affected.

OTHER TECHNIQUES

Rarely, other techniques such as bone marrow clonogenic assays or electron microscopy may be useful during the development phase of a drug. Bone marrow clonogenic assays, also known as colony-forming-unit assays, are in vitro assays that are utilized to evaluate the potential effect of xenobiotics on the differentiation of bone marrow cells.[26] These assays use different mixtures of growth factors to drive differentiation of pluripotent cells into different hematopoietic lineages and thus allow an assessment of differential sensitivity of the various bone marrow precursors to the effects of xenobiotics. In vitro assays are most useful, and utilized, early in the drug discovery process to screen the evolving chemical space for potential hematotoxicity and help identify compounds that have less or no hematotoxicity. Electron microscopy, mainly transmission electron microscopy, is rarely used when additional morphologic characterization of in vivo findings, in blood or bone marrow, is needed.

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CHAPTER 11

Interpretation of Hematologic Data in Nonclinical Studies

JEFFREY McCARTNEY

Data Collection and Presentation
  Statistical Analysis
Good Laboratory Practice
Reference Ranges
Overview of Data Interpretation
Differences in Hematology Parameters
Red Blood Cells
White Blood Cells
  Platelet Count
  Assessment of Recovery Studies
  Bone Marrow Evaluation
  Correlation with Other Toxicology Study Data
Toxicokinetic Data
Report Writing
Conclusion

Acronyms and Abbreviations

EPO, erythropoietin; GLP, good laboratory practice; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MPV, mean platelet volume; N, number of individuals per group; M, male; NOAEL, no-observed-adverse-effect level; NOEL, no-observed-effect level; PLT, platelet; RBC, red blood cell; RETIC, reticulocyte count; RDW, range distribution width; SOP, standard operating procedures; SD, standard deviation; WBC, white blood cell.

Safety assessment toxicological studies are designed to determine the potential toxicity of novel pharmaceutical compounds and chemicals. Many synthesized compounds are screened each year through the drug development process, but very few reach the consumer market. Short-term studies with limited number of animals are initiated early in the drug development process to identify compounds to move forward for continued safety and efficacy investigation. Typically, only basic clinical pathology measurements are applied to these studies. Longer-term studies with new compounds are part of the development process and can identify findings of toxicity in all aspects of study design including clinical pathology, anatomic pathology, immunology, and other biomarkers. These studies typically involve one rodent species (mouse or rat) and one non-rodent species (e.g., dog, minipig, or nonhuman primate). They are regulated by government regulatory health agencies in various geographic locations (see Chapter 10).[22] Basic hematology, clinical chemistry, and coagulation measurements are also applied to these studies, and bone marrow histopathological evaluation is performed in at least one of these studies.[1,12,15,32,36] Further investigations, such as bone marrow cytology, are optional depending on the initial findings and evaluation of the hematology data.

Therefore, interpretation of hematological data is important at all stages of drug discovery and development. Final interpretation and conclusion should differentiate test-item-related effects and biological variations. These conclusions are part of an interpretative report, which is a subsection of the overall preclinical study data package. These are written in collaboration with pathologists and study toxicologists in order to provide not only the difference in the hematology parameters but also a scientific interpretation and the significance of the changes.[2,30]

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DATA COLLECTION AND PRESENTATION

Recommendations for the core clinical pathology parameters in nonclinical toxicity studies have been published.[33,34] The standard panel for hematology includes white and red blood cell parameters with differential counts and blood and bone marrow smears prepared for each animal (see Chapter 10). The tabulation of the hematology data will typically involve both group mean and individual data for studies with adequate numbers of animals per group. For studies in rodents, the comparison of groups given the test item is with the control group typically given a reference item or drug vehicle. For studies involving larger animals, there are usually blood sampling time points prior to study start and comparison is done with both control and pre-study data.

Statistical Analysis

In nonclinical studies, there is typically a significant amount of laboratory data generated especially with larger numbers of animals or longer-term studies with multiple sampling time points. Statistical analysis of the data will frequently identify significant differences between control groups and groups to which a test item has been administered. These differences should be interpreted with caution, and not be used as a substitute for a complete evaluation of the data. A change may be statistically significant but have little biologic or toxicologic significance.[5] Conversely, compound-related changes that are not statistically significant may be considered biologically meaningful. Both parametric and nonparametric statistical models are used in nonclinical studies. Most parametric statistical models are suitable for data that are normally distributed and exhibit homogeneous variance. Nonparametric procedures are used when data are not normally distributed. The power of a statistical test is proportional to the number of animals per group. As the number of animals increase, differences of small magnitude become statistically significant, although they may not be toxicologically relevant. In studies with lower numbers of animals, there tend to be fewer statistically significant differences.

The group mean or average is another less sophisticated tool that is appropriate for normally distributed data. Standard deviation describes the variability around the mean and should be carefully evaluated as an indication of variability with that group of animals.

GOOD LABORATORY PRACTICE

Evaluation of hematology data should be done with knowledge of the inherent precision and variability of the assays used (see Chapters 137–139). Analytical data must be reliable, and the laboratory should validate all methods and operate internal and external control procedures. Agencies responsible for the investigation of the safety and efficacy of human health products regulate the conduct of nonclinical laboratory studies under good laboratory practice (GLP) regulations. GLP regulations must be applied to all nonclinical health and environmental safety studies required for registering or licensing pharmaceuticals for human use. Some countries may have additional GLP regulations, but differences in regulations are minor. There are memoranda of understanding with several countries that accept study data from different geographical locations.[6]

The GLP clinical laboratory must have written approved standard operating procedures (SOPs), record systems, and quality assurance for sample handling, analysis, and reporting. Quality assurance personnel audit the study protocol and perform in-process audits of study conduct, including generation of clinical pathology data and report.

REFERENCE RANGES

Reference ranges are also referred to as historical control data and are generally available from the laboratory generating the hematology data from the nonclinical study (see Chapter 140). Guidelines for generation of reference intervals in veterinary species are available.[11] Reference ranges can be calculated from naive control animals in toxicological studies. They are usually determined by the smallest and largest values if 20 or fewer animals are sampled, or by the 2.5th and 97.5th percentile range if more than 20 animals are sampled. Consequently, reference ranges display values observed in 95% of a healthy population. Values outside the reference range do not necessarily indicate an abnormal condition and, conversely, values within the reference range do not imply a normal condition.[21]

Reference ranges are influenced by many variables, including species, strain, sex, age, animal supplier, animal husbandry or handling practices, diet and fasting, anesthesia, sample matrix, instrumentation, and test methodology. It is important to use reference ranges produced by the laboratory performing the study rather than published reference ranges, which would be generated using animals and samples with different conditions than those used in a particular study. Therefore, there is no substitute for age- and sex-matched control animals from the same supplier undergoing the same procedures as the animals in a nonclinical study.[13,28] Reference ranges, however, are adequate to evaluate an individual animal, data prior to study start, data from controls, and to understand analyte variability. When referring to reference ranges in a nonclinical study report, the range is typically included as an appendix and will need to meet certain guidelines if the study is conducted under GLP conditions. Reference ranges should not be relied upon to determine potential test-item-related effects on hematology data, which in some scenarios are minimal and post-treatment values fall within the reference range. Comparison of hematologic data to the appropriate control group is best practice for data evaluation.

OVERVIEW OF DATA INTERPRETATION

Veterinary clinical pathologists evaluating clinical pathology data in nonclinical studies evaluate groups of animals and compare results with a vehicle-treated control group and/or pre-study values rather than evaluating individual animals. For most rodent toxicity studies, pretreatment testing is not done. In non-rodents studies (e.g., monkey and dog), one or two sets of pre-study values are usually collected. When comparing data to pre-study values, comparison to the second set of pretreatment data is usually preferred because these data are less likely to be influenced by stress, food intake, and other variables in the laboratory setting. In longer-term studies, comparison with a concurrent control group is also important.[35] For example, if multiple blood samplings are performed during a study, RBC parameters of treated groups might vary if they are compared to their pretreatment values, but differences in RBC values will not be observed if compared to control group values.

Biological variation is due to interanimal components (i.e., differences between individuals) and intraanimal components (i.e., changes within an individual over time).[5] Multiple physiological and procedural variables may influence hematology measurements, such as age, sex,[26] diet, fasting, restraint, circadian effects, blood sampling site,[8,24,27] collection technique, order of collection, transport time, and sample preparation (see Chapter 10).[14] Sources of preanalytical variations should be avoided, as these could potentially interfere with results and interpretation.

When evaluating nonclinical data, the overall objectives include determining if noted differences in the data are due to administration of the test item or related to analytical or biological variation or related to study procedures. If differences are test-item-related, then any relationship to dose response is also determined.[14] Identification of test-item-related effects in hematology data with subtle differences can be difficult and points to consider include: known class effects of the compound, presence of dose response, correlating findings in clinical pathology data and histopathology. Data interpretation should always include evaluation of both group mean and individual data. Individual data can identify outliers, which are values (low or high) widely divergent from the mean of a group of data. These values may be due to a technical error, or directly related to another cause or disease considered non-test-item-related.[18] For example, a rodent with inflammation in certain tissues, which is incidental, may have increases in white blood cell count and neutrophil count. In these situations, the group mean is affected and the standard deviation of the group will be increased.

Data interpretation has a subjective component.[18,20] If a difference is small, it is often interpreted as not important, and within pre-analytical or biological variability. As the change becomes larger, it is more likely to be relevant and compound-related. However, there is frequently a range of results that are difficult to classify, and not possible to clearly differentiate as compound-related effects or biological effects. Therefore, these changes may be reported as unknown significance to test item administration. Simplistic approaches to data interpretation such as statistics and comparison to reference ranges using a cutoff point decision method, with no consideration of a *gray zone*, may be a source of inappropriate decision-making in the drug development process.[4,9]

DIFFERENCES IN HEMATOLOGY PARAMETERS

Test-item-related differences in hematology parameters in nonclinical studies are typically described using the following qualitative terms: increased, higher, decreased, lower. Terminology typically used in clinical medicine (e.g., anemia, leukopenia, leukocytosis) is not used to describe differences in the data in an interpretative report. Typically, differences in hematology parameters are also reported with quantitative findings (fold or percent difference), and when several parameters are affected, a text table is used within the report to summarize the findings.

RED BLOOD CELLS

Lower red blood cell parameters (RBC count, HGB, HCT) can be observed in nonclinical studies. The reticulocyte count is considered a reliable and early indicator of RBC regeneration when sufficient time has elapsed in species with circulating reticulocytes.[3] Laboratory animals normally have low (dogs and nonhuman primates) to moderate (rodents) numbers of circulating reticulocytes. Decreases in RBC parameters with evidence of regeneration are either hemorrhagic or hemolytic, while decreases in RBC in the absence of regeneration indicate suppressed erythropoiesis. Slightly lower RBC parameters with no evidence of regeneration is not an uncommon observation and animals may exhibit mild reductions in body mass and food consumption. Decreased food intake may have a major impact on the hematopoietic system and has been shown to decrease white blood cell, platelet, and reticulocyte counts (see Chapter 12).[14,19,25] There are several compounds that impair erythropoiesis, either directly by injuring pluripotent hematopoietic stem cells, or their stromal microenvironment, or indirectly by inhibiting erythropoiesis (e.g., chronic inflammatory diseases).[16]

Higher red blood cell parameters are observed with subclinical or clinical dehydration in nonclinical studies, especially in animals with decreased food consumption or in rodents with prolonged fasting. Increases in RBC parameters (RBC, HGB, HCT) are often associated with increases in total protein levels or albumin and/or urea. Absolute erythrocytosis resulting from a persistent increase in RBC mass and associated expansion of blood volume is rarely observed in toxicity studies, but may be

seen when testing compounds with known pharmacologic activity of increasing erythropoiesis.

WHITE BLOOD CELLS

Increases in neutrophil and/or monocyte counts are observed with inflammation, excitement, steroid or stress response, or when testing pro-inflammatory compounds.[26] Physiological leukocytosis in response to excitement can increase counts in minutes. In laboratory primates, it is evenly distributed between neutrophils and lymphocytes. Increased neutrophil counts are most obvious in dogs, and increased lymphocyte counts are most evident in rats. Neutrophil counts increase in response to a variety of inflammatory conditions. Monocyte counts increase secondary to any condition with substantial tissue destruction, including inflammation or necrosis. Increases in lymphocyte counts may be observed with excitement in dogs and monkeys, and with chronic inflammatory lesions, especially in rodents, or with administration of antigenic compounds that elicit an immune response. Increases in eosinophil and/or basophil counts are not commonly observed in toxicological studies unless the test item specifically targets the production of these cell types. Leukemias involving any of the major white cell lineages can be observed in carcinogenicity studies using rodents.[14,31]

Leukocytes are frequently the first target of cytotoxic drugs because they have a shorter life span than platelets and red blood cells. In dogs and primates, marked acute inflammation and endotoxin-mediated pro-inflammatory cytokine release produce marked decreases in neutrophil counts, which are usually followed by a rebound neutrophilia. Recognition of decreased neutrophil counts is more difficult in mice and young rats that normally have low neutrophil counts. Immunotoxic drugs may trigger alterations in blood lymphocyte counts. Involution of the thymus is often interpreted as stress-related and correlates frequently with decreased lymphocyte counts. The significance of decreases in circulating monocytes, eosinophils, and basophils can be difficult to determine because these cells are normally present in low numbers and should not be overinterpreted. Decreases in eosinophils or monocytes may be observed with chemotherapeutic agents, which typically affect multiple white blood cell types, and decreases in eosinophils are sometimes observed with stress or steroid-related compounds.

Platelet Count

Lower platelet counts are frequently associated with difficult venipuncture resulting in platelet aggregation especially in dogs and rodents. Blood smear evaluation is important to confirm any low platelet counts and processes within the laboratory should be in place to trigger examination of the blood smear to confirm any decreased count. Compound-related decreases in platelet counts can also result from impaired production or accelerated destruction or utilization. Administration of a test item that reduces erythroid and myeloid cell lineage production can also inhibit platelet production. An example of hematology data interpretation from a nonclinical study is presented in Tables 11.1 and 11.2.

Physiologic increases in platelet counts are usually transient, and result from increased mobilization of platelets from storage pools during mild muscular activity or excitement. Healthy rodents have high platelet counts compared to other animal species. Compound-related increases in platelet counts may be associated with blood loss, iron deficiency, or inflammatory conditions. Test items occasionally induce platelet dysfunction, characterized by normal platelet count, increased bleeding time, and normal coagulation times (prothrombin time and activated partial thromboplastin time). When this profile is observed, platelet function testing is warranted.

TABLE 11.1 Group mean RBC parameters for a 13-week study with daily subcutaneous injection of human recombinant erythropoietin (EPO-R) in the Sprague–Dawley rat.[a]

Group	Summary information	RBC ($\times 10^6/\mu L$)	HGB (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RDW (%)	PLT ($\times 10^3/\mu L$)	MPV (fL)	RETIC (%)	RETIC ($\times 10^9/\mu L$)
1	Mean	8.64	15.54	47.14	54.59	18	32.97	12.42	1126.2	7.54	2.68	230.16
	SD	0.42	0.84	2.56	1.03	0.47	0.6	0.54	107.1	0.27	0.34	32.18
	N	16	16	16	16	16	16	16	16	16	16	16
2	Mean	13.01	23.28	71.96	55.08	17.85	32.46	19.89	851.7	10.80	3.91	531.79
	SD	2.17	4.10	13.2	2.07	0.68	0.95	2.03	261.4	2.11	1.37	187.26
	N	10	10	10	10	10	10	10	10	10	10	10
3	Mean	15.04	22.43	75.44	50.32	14.98	29.78	23.01	1046.7	14.46	5.47	818.56F
	SD	1.379	1.88	5.62	2.88	1.04	0.83	0.99	361.4	4.93	0.26	68.98
	N	9	9	9	9	9	9	9	9	9	9	9
4	Mean	11.72	17.41	56.72	47.77	14	27.33	22.16	1400.97	16.09	6.48	964.92
	SD	6.16	8.53	33	2.44	0.95	2.94	4.19	728.32	8.71	4.84	714.28
	N	10	10	10	10	10	10	10	10	10	10	10

[a] Group 1 is the control group given vehicle control. Groups 2, 3, and 4 were given low, medium, and high doses of EPO-R, respectively. Interpretation of data: A treatment-related increase was observed in RBC, HGB, HCT, and reticulocytes (RETIC) in rats of Groups 2 and 3 compared to control group. A treatment-related increase was also observed in RBC, HGB, HCT, RETIC, and PLT in high-dose group (4) compared to control group. High standard deviations in Group 4 were indicative of large individual variations within the high-dose group (see Table 11.2).

TABLE 11.2 Individual RBC measurements from high-dose group Sprague–Dawley rats given human recombinant erythropoietin.[a]

Group/ sex	Animal number	RBC (×10⁶/μL)	HGB (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RDW (%)	PLT (×10³/μL)	MPV (fL)	RETIC (%)	RETIC (×10⁹/μL)
4/m	4001	15.10	22	77.5	51.3	14.6	28.4	22.2	1784	26.3	8.2	1242
	4002	3	5	14.3	47.7	16.7	35	17.2	1153	8.1	0.7	19.6
	4003	2.61	4.2	12.7	48.5	15.7	32.3	18.6	1260	8.3	0.7	17.8
	4004	12.95	19	71	55	14.5	26.4	30.5	260	9.6	14.4	1866
	4005	15.20	22	18	51.4	14.5	28.3	23.4	1615	24.9	8.8	1328.2
	4006	15.30	20.5	75	49.1	13.5	27.4	22.7	2520	25.2	7.8	1205
	4007	16.10	23	81	50.3	14.3	28.4	24.4	2620	27.2	8.1	1308.4
	4008	2.6	4.1	12.5	48.1	15.6	32.5	17.9	1115	8.6	0.4	10.3
	4009	14.96	21.5	79	53	14.5	27.4	25.2	1470	20	9.4	1403.4
	4010	15.70	21.5	75.5	48.2	13.8	28.7	23.5	2700	23.8	6.6	1035.4

[a] Large individual variations were observed in RBC, HGB, HCT, and RETIC. The majority of individuals had marked increases in RBC, HGB, HCT, and RETIC compared with control group, consistent with erythrocytosis and the pharmacological action of the compound. Some individuals (nos. 4002, 4003, and 4008) had a marked non-regenerative anemia due to anti-erythropoietin antibodies inducing an immune-mediated anemia in these individual animals.

Assessment of Recovery Studies

There are no specific study design recommendations available by the Food and Drug Administration to detail which comparisons should be made to evaluate the reversibility of findings in a nonclinical study. Best practice guidelines for the evaluation of clinical pathology data in the recovery phase of nonclinical studies have been established.[33,34] Standard clinical pathology parameters are commonly included in the recovery phase and the number of recovery groups may be similar to the dosing phase groups or limited to control and high-dose groups. In rodents, evaluation of different cohorts at the end of the dosing and recovery phases may be required preventing evaluation of recovery in the same individual animal. The number of animals in the recovery phase is typically smaller than in the dosing phase. Consultation with the clinical pathologist is important when determining the recovery phase strategy with respect to number of animals per group and length of the recovery phase.

Bone Marrow Evaluation

Bone marrow smears are typically collected from all animals in a nonclinical study as per best practice recommendations.[33,34] The decision to perform a bone marrow smear evaluation is based on interpretation of hematology data, histopathology findings, and all other study data to determine if cytologic evaluation is warranted.[29] Evaluation typically consists of both qualitative assessment of cell maturation and morphology or a quantitative evaluation (see Chapter 10). A quantitative approach involves a 500-cell differential count with classification of cells into the myeloid, erythroid, and lymphoid cell lineages and calculation of a myeloid:erythroid (M:E) ratio. Bone marrow cytologic and histopathologic assessment provides a complete evaluation of the hematopoietic system in a nonclinical study and an integrated report includes bone marrow cytologic observations and correlations with hematology data.

Correlation with Other Toxicology Study Data

Test-item-related hematology findings should be described with any association with other nonclinical study data including clinical signs, other clinical pathology data, organ weights, gross pathology and histopathologic findings, and toxicokinetic data.[10] The correlation of compound-related hematology differences and noteworthy pathology findings should be evaluated by an expert in veterinary hematology (veterinary clinical pathologist) and captured in the report. Decreases in red blood cell parameters should be correlated with histopathologic evidence of hemorrhage, extramedullary hematopoiesis, and bile pigment deposits and/or hemosiderin deposits in liver and/or spleen. Lymphoid decreases often correlate with histologic atrophy of lymphoid organs. Increases in leukocytes may be correlated with microscopic evidence of inflammation in tissues and bone marrow increased myeloid cellularity. Decreases in red blood cells should be correlated with any bone marrow histopathology observations such as decreased cellularity.

TOXICOKINETIC DATA

Toxicokinetics is a subdiscipline of pharmacokinetics concerning the absorption, distribution, metabolism, and elimination at doses higher than those expected to produce therapeutic effects.[7] Evaluations are determined directly from animals involved in nonclinical safety studies or from a designated toxicokinetic group of animals and determine systemic exposure, any dose–response relationship, and degree of interanimal variability.

When interpreting hematology data, the level of exposure to the test item must be considered. Knowledge of the bioavailability of a drug and the kinetics of absorption, distribution, and elimination are important to consider when interpreting hematology data.[17] In some studies, there may be unexpected differences in the level of drug exposure and this may influence the interpretation of the differences observed in the hematology data.

REPORT WRITING

An interpretative report is typically produced to capture all test-item-related findings in all clinical pathology data in the nonclinical study.[23] The test-item-related findings are evaluated in the context of other preclinical study data and are judged if considered adverse or non-adverse to help in determining the no-observed-effect level (NOEL) or no-observed-adverse-effect level (NOAEL). Some criteria to determine the adversity of observed differences can include adverse impact on function of life of the animal, magnitude and direction of change, and monitorability in nonclinical species and human patients.[33]

Data presentation can vary between companies, but as a general rule, data are tabulated to include group and individual values for each dose group. Group values include mean, standard deviation, and statistical analysis applied to each group. Group and individual values are generally presented in a conventional order: control group given vehicle followed by groups given increasing order of dosage (e.g., Table 11.1). When reporting the data, mean percentage differences or fold differences between treated and control groups can be used, and significance of the changes can be qualified by usage of modifiers: minimal, mild, moderate, or marked. When referring to fold difference, the method of comparison (e.g., pre-test values, second pre-test value, control group mean) should be stated. The US Food and Drug Association now requires all study data in nonclinical studies to be submitted in consistent harmonized format using the Standard for Exchange of Nonclinical Data (SEND). The data in this format are collected and presented in a standard and consistent format. The interpretative report should concisely report all relevant test-item-related findings, as this section is typically carried forward into an overall summary of the complete nonclinical study report and is often reviewed by nonpathologists in the entire drug development process.

CONCLUSION

Principles of hematologic interpretation used to clinical veterinary medicine apply in nonclinical toxicological studies. The main differences between these practices result from the evaluation of groups of animals rather than a single patient. Interpretation is based on comparison to values obtained prior to study or from animals given a nondrug vehicle, utilization of reference values and statistical analysis, correlation of hematology data with chemistry and urinalysis data, clinical observations, toxicokinetics, necropsy, and histopathological results. Clinical pathology data interpretation will help define the safety assessment profile for a new drug candidate moving through the drug development process.

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CHAPTER 12

Nonclinical Evaluation of Compound-Related Cytopenias

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Compound-Related Cytopenias in Nonclinical Studies
  Predicting Hematotoxicity
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Mechanistic Classification of Drug-Induced Cytopenias
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    Anemia Secondary to Induced Suicidal Erythrocyte Death
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Acronyms and Abbreviations

AIHA, autoimmune hemolytic anemia; AITP, autoimmune thrombocytopenia; BFU-E, burst-forming unit-erythroid; BM, bone marrow; CFU-E, colony-forming unit-erythroid; CFU-GM, colony-forming unit-granulocytic monocytic; DIA, drug-induced agranulocytosis; DIIMHA, drug-induced immune-mediated hemolytic anemia; DIMT, drug-induced immune-mediated thrombocytopenia; GP, glycoprotein; IMHA, immune-mediated hemolytic anemia; IMT, immune-mediated thrombocytopenia; IgG, immunoglobulin G; IL, interleukin; PRCA, pure red cell aplasia; RBC, red blood cell; RDW, red cell distribution width; REDOX, reduction–oxidation reaction; TNF-α, tumor necrosis factor-alpha; TNFR, TNF-α receptor.

Exposure to xenobiotics (synthetic, pharmacologically active molecules, further defined as large or small molecular entities and biotherapeutics, which include monoclonal antibodies, recombinant proteins, cytokines, hormones, and nucleic acids) or their metabolites subjects the hematopoietic system to potential toxic insult. Bone marrow (BM) consists of a remarkably heterogeneous population of cells of mixed ontogenic origin capable of continuous replacement of circulating leukocytes, erythrocytes, and platelets and reserve ability to respond immediately to physiological and pathological demands. Rapid metabolism, high rate of proliferation, marked cross talk within and between BM niches and other organ systems make BM one of the most sensitive target organs evaluated in nonclinical toxicology studies. Unlike other target organs, the hematopoietic system can be easily and frequently biopsied via a blood sample before, during, and after exposure, making it one of the most critically evaluated organs of the risk assessment process.

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COMPOUND-RELATED CYTOPENIAS IN NONCLINICAL STUDIES

Predicting Hematotoxicity

Toxicologic injury can occur via oxidative damage (see Chapter 32), nonoxidant mechanisms (see Chapters 15–17), immune-mediated damage (see Chapters 35–38, 40, and 80), and, very rarely, induced apoptosis. The intended purpose of the test molecule determines the acceptability of any impact on the hematopoietic system. For example, marketed antihypertensive drugs are safe and effective when used properly, but have been infrequently associated with adverse effects on the hematopoietic system.[27] A potential new antihypertensive must be less toxic and more efficacious than anything currently marketed to warrant development; therefore, any evidence of hematopoietic toxicity is unacceptable. However, the risk/benefit ratio associated with hematopoietic toxicity produced by a molecule being developed as a second-line anticancer drug might be acceptable. Highly targeted biotherapeutics that produce unexpected hematologic toxicity in nonclinical testing may offer therapeutic outcomes that warrant clinical use, with the caveat that monitoring, dose management, drug withdrawal, and/or therapeutic intervention become part of the drug protocol.

Knowledge of the bioavailability of a compound is helpful when evaluating compound-related effects.[14] In particular, distribution, binding properties, metabolism, and $t_{1/2}$ of parent or active metabolites can aid in predicting both nadir and recovery of circulating cell counts before initiation of safety studies.

Compound-related effects can be directly or indirectly induced by the parent molecule and/or its metabolites. Secondary effects are consequences of direct or indirect toxic effect, such as renal failure due to nephrotoxicity. Less well-understood or predictable are results termed as hyperpharmacology. This adverse effect is frequently observed with recombinant molecules that directly target key biochemical pathways, altering specific protein synthesis, secretion, or catabolism, blocking key receptors, or gene regulation, often producing unpredictable consequences associated with induction of endogenous signaling molecules. Adverse effects associated with biotherapeutic pharmaceuticals are most often hyperpharmacologic, immune-mediated, and/or idiosyncratic reactions.[15]

All cells that are found within the BM microenvironment are susceptible to compound-related effects. Chemists use in silico (computational) three-dimensional mock-ups to characterize common structural features of highly myelotoxic compounds for screening and modeling to design and modify molecules to eliminate offending portions while retaining the active moiety.[13,17,21] In vitro toxicity colony-forming unit-granulocytic monocytic (CFU-GM) assay testing, using fresh or cryopreserved primary target stem cells (hematopoietic and stromal/mesenchymal) from nonclinical test species and humans, has greatly expanded our ability to assess comparative myelotoxicity and evaluate compound toxicity relative to other known molecules by using maximum tolerated dose (MTD) and inhibitory concentration (IC).[26,33,40] In vivo mechanistic models, based on pharmacokinetic–pharmacodynamic data, are used to study the entire time course of myelosuppression and separate drug-specific parameters from system-related parameters, with the goal of optimizing dosing in clinical studies.[17] The complexity of cytokine and hormonal effects on BM, peripheral cell counts, and cell function are the most unpredictable.[3,16,49] Cytokines may directly or indirectly mediate cellular changes in hematopoietic and/or stromal/mesenchymal cells, by upregulation or downregulation. As changes are highly dynamic, sequential peripheral blood and BM collection relative to dosing become invaluable for interpretation and prognostication of toxicity and reversibility. In vitro and in vivo studies using transgenic test species may predict cytokine release and establish safety margins for clinical safety studies in healthy human cohorts.[3,33] While prescreening has greatly reduced hematopoietic toxicity, study data must be critically reviewed for effects that suggest toxicity related to the parent compound and/or metabolites, species specificity, hyperpharmacologic responses, immune-mediated effects, or idiosyncratic changes.[36]

Route of Exposure and Vehicles

Each route of exposure may introduce variables that can alter the effect of the compound on the hematopoietic system. Parenteral administration may prolong exposure. Intravascular administration introduces the molecule directly to circulating cells. Vehicles used may have no effect when tested separately, but may enhance or alter exposure to the test molecule. Such an event occurred in Europe when treatment with recombinant human erythropoietin was linked to an upsurge in the incidence of antibody-mediated pure red cell aplasia (PRCA) in dialysis patients. The increase in PRCA coincided with a change in formulation of erythropoietin in which serum albumin was replaced by glycine and polysorbate 80.[13,42] Variables that may have increased immunogenicity were investigated: (1) formation of epoetin-containing micelles, (2) interaction with leachates released from uncoated rubber stoppers of prefilled syringes, and (3) denaturation or aggregate formation due to improper storage and handling. The incidence of PRCA returned to very low levels following replacement of the uncoated rubber stoppers with coated stoppers.

Test Species

Mouse, rat, dog, marmoset, and macaque are used most extensively for toxicity studies; baboon and chimpanzee are used infrequently (use of nonhuman primates, in particular great apes, has been outlawed in some countries). Familiarity with the test species is important, as differences in response may occur between species (primates) and strains (rodents), and within species and strains based on vendor source.[39] Although dose-related

small-molecule-induced hematotoxicity may have a high concordance between in vitro and in vivo studies and between species, hematotoxicity findings in nonclinical safety studies that are low-incidence or idiosyncratic or that are high-incidence and species-specific are unpredictable across species.[15] Some strains of rodents are genetically prone to hematopoietic abnormalities or have increased sensitivity to certain compounds, particularly knockout and transgenic animals.

MECHANISTIC CLASSIFICATION OF DRUG-INDUCED CYTOPENIAS

Drug-Induced Immune-(Mediated) Cytopenias

Nonclinical observations are typically proprietary and rarely reported in the public domain; thus, no estimate is available for the incidence of drug-induced immune cytopenias identified at toxicological exposures. Only 6–10% of all adverse drug reactions identified in clinical trials or postmarketing are recognized as idiosyncratic.[17] Frequently, the mechanisms of these reactions are unknown, but the majority are believed to be immune-mediated. The incidence of reported drug-induced immune-mediated hemolytic anemia (DIIMHA) in the human population is estimated to be one per million individuals per year.[5] Drug-induced agranulocytosis (DIA), defined as $\leq 0.5 \times 10^9/L$, is estimated at an annual incidence of 2–15 per million people and the mechanism may be direct toxicity, immune-mediated, or both.[4] Drug-induced immune-mediated thrombocytopenia (DIMT), in contrast, is more frequent.[7,41] Immune-mediated reactions to xenobiotics may be classified as Type I (immediate, IgE-mediated), Type II (IgG- or IgM-mediated), Type III (IgG/IgM immune-complex-mediated), or Type IV (delayed or cell-mediated).[18,35] Nonimmune hypersensitivity arises from direct compliment activation. Adverse drug reactions identified in human subjects during clinical trials or postmarketing are reported as (1) Type A (more common; usually dose-dependent and predictable; readily reversible by dose reduction or drug withdrawal; result of primary or secondary pharmacology; may be problematic when a drug has a narrow therapeutic index); (2) Type B (unrelated to known pharmacologic actions of drug; often serious and associated with high mortality; thought to be of immunologic and pharmacogenetic mechanisms); (3) additional categories Type C (chronic), Type D (delayed), and Type E (end of use); and (4) drug interactions.[29]

Idiosyncratic reactions appear to arise from a combination of immune-mediated genetic predisposition and environmental factors in susceptible individuals, and are frequently non-dose-dependent and unpredictable. A minimum of four events have been suggested as necessary for the initiation of an idiosyncratic reaction:

1. Adequate amount of reactive metabolite(s).
2. Binding with a protein with high epitope density.
3. Upregulation of costimulatory signals on antigen-presenting cells to activate T cells.
4. Failure of immune system to downregulate a harmful immune response.[21]

Sites of reactive metabolite formation usually correlate with the target of idiosyncratic reactions.[23] For example, drugs oxidized by myeloperoxidase to reactive metabolites may induce immune-mediated agranulocytosis.[21] It has also been suggested that glucuronide conjugation of metabolites may increase immunogenicity.[11]

There are two types of drug-induced antibodies: drug-independent antibodies and drug-dependent antibodies.[5] No drug is required in the test system for the detection of drug-independent antibodies, which may be "true" autoantibodies or antibodies that react like autoantibodies in vitro and can be found in combination with drug-dependent antibodies. The mechanism of drug-independent antibody production is unknown, but is hypothesized to be the result of a direct effect by the drug or its metabolites on the immune system that induces an autoimmune disease. Reactions attributed to "true" autoantibodies are detectable by direct antiglobulin test (Coombs test). Well-documented cases of autoimmune hemolytic anemia (AIHA) have been induced by α-methyldopa and procaine amide.[6] Heavy metals are also incriminated, and depot injections of gold salts have frequently induced chronic autoimmune thrombocytopenia (AITP).[48] Drug-dependent antibodies require the presence of the drug in the test system for their detection and are frequently described as "hapten-type."[6] Hemolytic anemias induced by this type of antibody response are associated with a high IgG titer and extravascular RBC destruction. This mechanism may also be responsible for some types of drug-induced thrombocytopenia.[7] A second type of drug-dependent antibody, associated with complement activation and acute, severe intravascular hemolysis, is referred to as "immune complex type." A proposed mechanism is that drug and/or metabolites interact with the cell membrane (without covalent bonding) and lead to a polyclonal response to several epitopes. Antibodies that react with epitopes shared with the drug and cell membrane produce immune complexes that trigger the reaction.[5] The fibrinogen receptor, glycoprotein IIb/IIIa, von Willebrand factor receptor, or GPIb/IX may become selected epitopes, but the mechanism is not hapten-specific.[6] Immunoglobulins only react with these platelet membrane glycoproteins when drug or metabolite is present in soluble form.

Drugs developed as ligand-mimetic GPIIb/IIIa inhibitors have produced acute and often severe thrombocytopenia in a subset of patients during clinical trials.[10,41] Naturally occurring antibodies present in otherwise normal patients recognize GPIIb/IIIa complexed with the drug on the surface of platelets and produce DIMT.

Drug-specific antibodies may be naturally occurring or induced. The cephalosporins are particularly prone to

producing this response, and the problem has become more frequently observed with newer generations of the drug. These DIIMHA can be very severe, with hemoglobinemia and hemoglobinuria. Differences in response to these drugs are found between age groups, with children often developing severe hemolysis within minutes after administration, while similar problems occur in adults only after several days.[5]

Drug-induced immune disease against granulocytes has been associated with:

1. no significant effect on circulating numbers of neutrophils,
2. moderate to severe neutropenia with normocellular to hypercellular BM, or
3. neutropenia with BM hypoplasia or maturation arrest.[12]

Identification of anti-neutrophil antibodies using various detection methods is difficult because they tend to be present in low titers and bind with low avidity. Autoantibodies that have been characterized in humans are directed against various membrane glycoproteins, including adhesion molecules. Anti-neutrophil antibodies can produce neutrophil dysfunction resulting in defects in adhesion, aggregation, chemotaxis, phagocytosis, or killing.

Aplastic anemia may be a predictable or idiosyncratic reaction that presents as pancytopenia (leukopenia and thrombocytopenia will be detected before anemia) due to BM failure (see Chapter 40). Most drug-related cases are believed to be immune-mediated.[9]

PRCA is an infrequent idiosyncratic drug reaction in which BM suppression is limited to the erythroid lineage. An immune-mediated process has been speculated as the mechanism behind PRCA.

Drug-Induced Marrow Suppression

In addition to direct cellular injury, hematopoiesis may be suppressed or enhanced by alterations in the cytokine network. For example, based on in vitro observation that interleukin-12 (IL-12) works synergistically with the c-kit ligand to promote hematopoietic stem cell proliferation, mice dosed with recombinant mouse IL-12 were expected to develop accelerated myelopoiesis.[46] Instead, treatment with IL-12 produced BM myelosuppression while enhancing splenic extramedullary hematopoiesis. The complex cross talk within the hematopoietic microenvironment is not restricted to compounds designed to impact hematopoiesis or regulate inflammation.[22] Cytokines, growth factors, and receptors in tissues outside the hematopoietic system that are seen as potential drug targets may impact BM as well. Blockade of neurokinin receptors has been pursued as a potential anti-anxiety target by allowing accumulation of substance P. However, the same receptors exist in the BM and play a critical regulatory role in hematopoiesis.[37] Depending on the receptor and the cross talk, substance P can either inhibit or stimulate hematopoiesis.

ANEMIA

Causes of anemia include decreased circulating lifespan, hemolysis, hemorrhage, decreased or ineffective production, or a combination of processes. The mechanism(s) of toxic effect(s) determines the type of anemia, time to onset, duration of effect, and whether persistence of the inciting agent is required for anemia to be maintained or progress.

Hypoproliferative Anemia

Mild normocytic normochromic anemia that is poorly regenerative and reverses when administration of the compound is discontinued is frequently seen in repeat-dose toxicity studies. Usually, histological and cytological BM evaluation from study animals is unremarkable and no impairment of leukocyte or platelet production is identified. Decreased erythropoiesis may be secondary to inanition associated with inappetence and other systemic toxicological effects of the administered compound.[25]

Compounds with toxic effects on proliferating BM cells or stromal cells may produce nonregenerative or hypoproliferative anemia. Examples include anti-neoplastic drugs that target DNA and drugs that interfere with hemoglobin synthesis.

Hypoproliferative anemia may occur secondary to decreased availability of iron, and may be induced by chronic administration of xenobiotics (e.g., recombinant cytokines or cytokine receptor inhibitors) that induce chronic inflammatory changes or renal toxicity (see Chapters 27 and 39).[20,28]

Hemorrhagic Anemia

Hemorrhagic anemia, caused by blood loss, may be a direct or indirect effect of a xenobiotic. Acute hemorrhagic anemia, occurring over 2–3 days, will present with a decrease in the erythron and no evidence of regeneration. Thereafter, a regenerative response (polychromasia, anisocytosis, increased red cell distribution width (RDW), reticulocytosis) is generally observed. More protracted hemorrhage may present as regenerative or nonregenerative, or with characteristics consistent with iron-deficiency anemia. Changes in platelet counts and plasma protein concentrations will vary depending upon cause, duration, severity, and whether blood loss is internal or external. Mechanisms of compound-related hemorrhage include enhanced fibrinolysis, impaired platelet function, single or multiple coagulation factor deficiencies, and/or coagulation factor inhibition.[28] Secondary or indirect compound-related hemorrhage may occur as a result of compound-related gastrointestinal lesions, impaired vitamin K absorption, or disseminated intravascular coagulopathy. Examples of compound classes that may be associated with hemorrhage include nonsteroidal anti-inflammatory drugs (secondary to gastrointestinal erosion, ulceration secondary to local irritation, inhibition of prostaglandin synthesis), vitamin K antagonists, platelet aggregation inhibitors, and gene therapy molecules.

Anemia Secondary to Induced Suicidal Erythrocyte Death

Wear and tear on RBCs expose them to osmotic shock, oxidative stress, and energy depletion until the erythrocyte, through the process of senescence, is no longer able to perform adequately and is removed from circulation by the mononuclear phagocyte system.[24] RBCs are also able to initiate programmed cell death, termed eryptosis, as it mimics apoptosis.[23] Hallmarks of eryptosis and the triggers that initiate it are similar to senescence, and include cell shrinkage, cell membrane blebbing, and translocation of phosphatidylserine to the extracellular face of the RBC membrane. Eryptosis contributes to decreased RBC life span and anemia found in a number of genetic and acquired disease conditions (e.g., sickle cell anemia, thalassemia, iron deficiency, renal failure, hepatic failure, malaria).[34,38] Eryptosis's complex regulation can be overridden by the presence of a foreign substance in the blood (i.e., a xenobiotic, which can produce oxidative stress, hyperosmolarity, and/or energy depletion in RBC) leading to activation of eryptosis mechanisms. Using flow cytometry and confocal microscopy, various endogenous molecules (e.g., ceramide, prostaglandin E2, platelet-activating factor) and xenobiotics (paclitaxel, azathioprine, amantadine, amiodarone, chlorpromazine, cyclosporine, methyldopa, retinoic acid, cisplatin, and zidovudine) tested in vitro have induced eryptosis activation mechanisms through Ca^{2+} ion entry, ceramide formation, or ATP depletion.[24,34] Finding that therapeutic concentrations of chlorpromazine caused eryptosis by triggering multiple mechanisms suggests that shifts in eryptosis probably occur in nonclinical studies, but are masked by the robust ability of the BM reserve to increase production.[38] Compound-induced eryptosis may occur simultaneously with other toxic effects and might be mechanistically responsible for minimal decreases in the erythron relative to baseline and concurrent controls or evidence of reduced RBC life span often seen in dosed animals. It may, therefore, be important to determine if a decrease in the erythron is a result of increased eryptosis rather than dismissing it as a secondary effect.

Hemolytic Anemia

Direct effects on circulating RBCs can produce acute or chronic hemolytic anemia. Hemolysis may be intravascular, extravascular, or both. Intravascular hemolysis may be accompanied by hemoglobinemia and hemoglobinuria. When damage is due to direct contact of a compound or its metabolite with the RBC, hemolysis is immediate and often severe. Examples of compounds capable of producing direct hemolysis include saponin, phenylhydrazine, arsine, or naphthalene.[43]

Drug-Induced Nonimmunologic Adsorption of Protein

Nonimmunologic protein adsorption onto the RBC membrane is a possible mechanism of hemolytic anemia.[5,6] Some drugs may be able to modify the RBC membrane, allowing adsorption of plasma proteins (e.g., immunoglobulins, albumin, complement, fibrinogen).[45] Unfortunately, diagnostic tests are not available to differentiate this mechanism from immune-mediated mechanisms.[5]

Oxidative Damage

Strong oxidants are able to overwhelm the capacity of RBCs to remain in a reduced state, resulting in production of methemoglobin and/or denatured hemoglobin (Heinz body formation) (see Chapters 15 and 32). Heinz body formation and altered ion transport lead to intravascular hemolysis and RBC removal by the mononuclear phagocyte system.[9,44] Many species form Heinz bodies when exposed to aniline, nitrobenzene, and related homologs. A blood film should be examined for evidence of Heinz bodies, eccentrocytes, and keratocytes whenever oxidative damage is suspected. Unless there is continual exposure, Heinz bodies may be difficult to detect in some species due to rapid removal by the mononuclear phagocyte system. The ability of a molecule to function as an oxidant depends upon the concentration of exposure, the reducing ability of RBCs, and the susceptibility of the hemoglobin molecule. Obtaining an accurate measurement of methemoglobin is difficult due to the constant reduction–oxidation reaction (REDOX) activity in the RBC, particularly in rodents. Some species are more susceptible to methemoglobin formation, while others produce more Heinz bodies. Human RBCs have a comparatively slow rate of methemoglobin reductase activity relative to laboratory species, and are particularly susceptible to Heinz body formation;[44] therefore, detection of either or both in a nonclinical study is a major toxicological finding that must be carefully weighed against potential benefits of the compound.

Leukopenia

Drug-induced leukopenias may be transient or persistent. Transient depressions in leukocyte counts that return to baseline or are comparable to concurrent control data despite continued exposure to the compound may represent various compensatory mechanisms. Leukocyte depressions that persist throughout the dosing period of the study but return to baseline at the end of the study represent reversible suppressions. Drug-induced neutropenia may be due to direct damage to BM progenitor cells, or damage to stromal cells that support granulopoiesis.[19] BM damage may be limited to granulopoiesis, or affect all cell lineages.[47] Time of evaluation can greatly influence interpretation. Neutropenia can occur after prolonged exposure, and may be due to decreased production secondary to hypoplastic BM.[29] Immune-mediated granulocytopenia may be directed against precursor cells and/or against circulating cells. Some drugs, such as clozapine, may interact with intracellular myeloperoxidase or NADPH oxidase to produce highly reactive products that are toxic to granulocytes.[30]

Criteria established to define idiosyncratic drug-induced agranulocytosis include:

1. neutropenia ($\leq 0.5 \times 10^9$/L);
2. onset during treatment or within 7 days of exposure with recovery within 1 month of discontinuation;
3. recurrence upon re-exposure.[4,8,43]

A recent review of nonchemotherapy drug-induced agranulocytosis found the median duration of exposure before onset ranged from 19 to 60 days, though one drug (dipyrone) had a median exposure of only 2 days.[2] Agranulocytosis caused by nonimmunological mechanisms appeared to have a later onset than when attributed to immune-mediated mechanism. Impaired granulopoiesis was responsible for 67% of the cases reviewed. This finding contrasts significantly with drug-induced thrombocytopenia or drug-induced hemolytic anemia, where circulating cells are the main target of the immune reaction.[5,7]

Thrombocytopenia

Thrombocytopenia in nonclinical studies is rarely associated with gross or microscopic hemorrhage unless there is concurrent platelet dysfunction. Thrombocytopenia is not a common finding in nonclinical studies evaluating toxicity of traditional chemical entities.[5,9,44] This may be a somewhat more common finding when the test article is a biotherapeutic.[15,16,41] Thrombocytopenia in nonclinical studies is always highly significant and requires full investigation. Compounds that indiscriminately attack actively dividing cells produce BM panhypoplasia. Selective megakaryocytic toxicity, either through toxicity or an immune-mediated mechanism, is associated with a paucity of BM megakaryocytes. Conversely, with platelet destruction or consumption, as happens with DIMT, BM will show a marked increase in megakaryocyte numbers. Because the majority of reported drug-related thrombocytopenias are idiosyncratic and occur postmarketing, nonclinical predictability remains underestimated. Any signal in the data from nonclinical studies that suggests that platelets are a target should be carefully and fully evaluated.

DISPARITY BETWEEN NONCLINICAL AND CLINICAL SAFETY DATA

Nonclinical studies may unfairly incriminate a compound or overestimate or underestimate the risk of exposure in humans receiving pharmacological doses. For example, during development of omalizumab (XOLAIR®), a recombinant humanized anti-IgE monoclonal antibody that neutralizes circulating IgE, thrombocytopenia was identified and reproduced in nonclinical safety studies in monkeys receiving the drug at 5–27 times the maximum clinical dose.[1] No petechiae, ecchymosis, or hemorrhage was identified in study animals. No effect on circulating platelet numbers was identified in clinical studies. After a temporary hold on development, the observation was determined likely to be species-specific and platelet counts in further clinical trials were intensely monitored. To date, this has not materialized as an adverse effect in humans.[50]

Conversely, nonclinical testing can fail to predict toxicity at intended exposure. A low incidence (<0.3%) of agranulocytosis was detected in the late phase of clinical trials with prinomide, a pyrroleprioprionitrile, nonsteroidal anti-inflammatory drug, resulting in immediate termination of further development of the drug.[24] No effect on hematopoiesis or circulating numbers of cells was evident in nonclinical studies (rodents, dogs, monkeys). The unique difference between the individuals enrolled in late phase studies who developed agranulocytosis was that they were experiencing active inflammatory conditions such as chronic arthritis. Mass spectroscopy revealed that one reactive metabolite, 1,4-benzoquinone, a compound implicated in myelotoxicity associated with benzene and produced under conditions of increased hydrogen peroxide (e.g., active inflammation), might explain the agranulocytosis identified in the late phase studies.[31,32]

Different abilities to detoxify, by conjugation or reductive properties between test species and humans, may not be identified until the first human studies are conducted. For example, a potential new drug administered to healthy adults unexpectedly produced methemoglobinemia during dose escalation studies, but had not produced similar effects in test species (L. G. O'Rourke, personal observation). Again, full review of all nonclinical data failed to predict this occurrence.

These examples are presented to first illustrate that classic animal models and nonclinical studies, despite their rigors, may fail to predict life-threatening adverse effects on the hematopoietic system in the intended species, or may overestimate risk of exposure. They also serve as reminders of the importance of understanding both differences and similarities between species. Clinical safety studies are a second line of defense, where discrepancies with nonclinical studies can usually be detected. However, when toxicity occurs only in certain individuals within the human population, it becomes difficult to determine if the responsible mechanism is due to a property of the drug or to the unique biology of that individual. The incidence of idiosyncrasy within the test population may be such that it is not detected in the premarketing phase of development. Many case reports present instances of drug-induced hematotoxicity that were either not predicted in nonclinical safety studies or were not identified until enough individuals were exposed postmarketing.

INVESTIGATIVE WORK

Cytopenia in individual or treatment group data needs to be addressed. All clinical observations, treatments, and handling must be reviewed to differentiate a direct compound-related effect from a secondary effect, such

as blood loss (e.g., excess phlebotomy; gastrointestinal bleeding) or illness. Actions that follow identification of a cytopenia in a safety study include:

- no change in protocol;
- temporary suspension of dosing;
- dose reduction;
- cessation of dosing and initiation of a monitoring period;
- humane termination;

or a combination of the abovementioned actions. If dosing is suspended and findings resolve only to return when dosing is reinstated, the compound or its metabolites are clearly implicated.

It is difficult to determine if an effect is idiosyncratic when the incidence of an adverse finding is extremely low and is only found in one species. However, a hematotoxicologic finding in a nonclinical study must be taken seriously and critically assessed. Depending on the gravity of the finding and mechanism(s) involved, molecular redesign or project termination may ensue. Conversely, findings limited to one species may be interpreted as species-specific. In these instances, further development of the compound may not be impacted, although all clinical trials should be carefully monitored.

CONCLUSIONS

Nonclinical safety studies in animals and clinical safety studies in humans are designed to rigorously test new chemical entities for evidence of toxicity based on their relative risk/benefit. Because the number of animals exposed is relatively small, any adverse effect on the hematopoietic system must be fully evaluated. When evidence of hematotoxicity occurs in human studies, either premarketing or postmarketing, both in vivo and in vitro studies specifically designed to define the mechanism(s) are indicated. Many of these mechanisms remain a mystery. As the tools of pharmacogenomics and pharmacogenetics are advanced, our abilities to examine and understand interspecies drug-, class-, and organ-specific pathways underlying toxicity, and to explore portions of the genome that may detect potential unanticipated impacts, will improve, further enhancing our ability to select useful new xenobiotics that are not associated with hematotoxicity or to address concerns detected through pharmacovigilance when potential adverse drug reactions targeting the hematopoietic system are identified.

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CHAPTER 13

Nonclinical Evaluation of Compound-Related Alterations in Hemostasis

F. POITOUT-BELISSENT

Regulatory Requirements
Evaluation of Coagulation Times: PT and APTT
  Laboratory Analyses and Species Consideration
  Consideration of Blood Volumes for Laboratory Animals
  Interpretation of Changes in PT and APTT
    Preanalytical Changes
    Selective Increases in PT or APTT
    Combined Increases in PT and APTT
    Compounds Affecting Vitamin K
    FIIa and FXa Inhibitors
    Decreases in Coagulation Times
Evaluation of Specific Coagulation or Anticoagulant Factors
  Fibrinogen
  Individual Coagulation Factors
    Coagulation Factors Assay Using Human Deficient Plasma
    Chromogenic Factor Assays
    FDP and D-dimers
    ATIII and TAT
Global Coagulation Assays
  Thrombin Generation
  Thromboelastography
  Bleeding Time
  Microparticles
  Whole Blood Clotting Assays
Evaluation of Platelets
  Disseminated Intravascular Coagulation
  Immune-Mediated Platelet Decreases
  Platelet Dysfunction
Conclusion

Acronyms and Abbreviations

ATIII, antithrombin III; AA, arachidonic acid; ADP, adenosine diphosphate; APTT, activated partial thromboplastin time; CYP, cytochrome isozyme; ELISA, enzyme-linked immunosorbent assay; EMA, European Medicines Agency; DIC, disseminated intravascular coagulation; FDA, Food and Drug Administration; FDP, fibrin degradation products; INR, international normalized ratio; ISI, international sensitivity index; MHLW, Ministry of Health, Labour and Welfare of Japan; MPs, microparticles; NHP, nonhuman primate; NSAID, nonsteroidal anti-inflammatory drug; OECD, Organization for Economic Cooperation and Development; PT, prothrombin time; TEG, thromboelastography; TF, tissue factor; TAT, thrombin–antithrombin complexes; TT, thrombin time.

Hemostasis is a normal physiological response and a protective mechanism preventing blood loss after alteration of the vascular system. It is comprised of the formation and stabilization of a blood clot after a vessel injury, and the dissolution of the blood clot after repair of the endothelium (see Chapter 87). Hemostasis is maintained by the equilibrium between coagulation and anticoagulant factors, and the presence and function of platelets.

Evaluation of compound-related alterations on hemostasis is done during nonclinical studies in laboratory animals, and is essential to avoid off-target hemostatic toxicity once the compound is administered to humans in clinical studies. Although the evaluation of hemostasis is common and well known in humans, multiple aspects remain difficult to evaluate in laboratory animals because of the lack of species-specific assays. In nonclinical studies, hemostasis testing focuses primarily

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on the risk of bleeding, but the evaluation of the risk of thrombosis is particularly challenging and in constant development.[38] It is also difficult to anticipate whether the effects observed in laboratory animals will occur in humans. This chapter provides an overview of routine testing in nonclinical laboratories, reviews specific compound effects on hemostasis, and presents additional assays used in confirmatory investigational studies.

REGULATORY REQUIREMENTS

The agencies responsible for the investigation of safety and efficacy of human health products include the United States Food and Drug Administration (FDA), the European Medicines Agency (EMA), the Japan's Ministry of Health, Labour and Welfare (MHLW), and the Organization for Economic Cooperation and Development (OECD). These agencies provide regulatory guidelines and recommendations for testing during nonclinical toxicity evaluations, although these are often quite vague and infrequently updated. They include the evaluation of coagulation times—prothrombin time (PT) and activated partial thromboplastin time (APTT)—and platelet counts, in nonclinical studies with rodent (rat, mouse) and non-rodent (dog, nonhuman primate (NHP)) species. Treated animals and concurrent controls should be evaluated together at least once during chronic studies. In addition, fibrinogen is frequently measured as an acute phase protein.[17] Guidelines often refer to a previous publication recommending the minimum evaluation of PT, APTT, and platelet count, but also advocate for a flexible approach depending on the species, the observed or expected effects of a given substance, and states that additional testing should be considered if necessary for the investigation of toxic effects.[9,10,13,30,42] In this regard, the Regulatory Affairs Committee of the American Society for Veterinary Clinical Pathology (ASVCP) has recently recommended that PT, APTT, platelet count, and fibrinogen concentration be evaluated routinely, and has added more recommendations for the evaluation of additional hemostatic endpoints based on previous experience with the compound, or known class effects. These include the measurements of D-dimer, fibrinolytic factors, individual coagulation factors, platelet function and aggregation, thromboelastography, and flow cytometry assays, which should be included as part of a "fit-for-purpose" approach.[41]

EVALUATION OF COAGULATION TIMES: PT AND APTT

Laboratory Analyses and Species Consideration

Automated coagulation analyzers allow high throughput determination of PT and APTT for all laboratory species, using reagents approved for human diagnostic evaluation by regulatory agencies (see Chapter 88). In laboratory species (rat, mouse, rabbit, minipig, NHP, and dog), all coagulation factors, except for Ca^{++}, are synthesized in the liver, and all factors except for tissue factor (TF) are present in the circulation. However, their concentrations vary between species and strains, and even between sexes (see Table 13.1). Life spans of these coagulation factors vary from a few hours to 10 days.[11]

Coagulation times depend not only on the concentration of coagulation factors, but also on the reagents and method employed. Published reference ranges can only be used as a guide for method development and should not be used as a reference in nonclinical study evaluation (see Table 13.2). It is necessary to establish species-specific reference ranges for each laboratory and use a control group for each study.

PT time may be affected by the source and sensitivity of the commercially available thromboplastin reagents used for its determination, derived either from rabbit brain tissues or from human recombinant thromboplastin (see Chapter 88). In human medicine, the PT is measured and expressed as an international normalized ratio (INR). The international sensitivity index (ISI) value of the thromboplastin reagent refers to international reference plasma and is assigned by the manufacturer. ISI values vary among manufacturers between approximately 1.2 and 2.2. There is no validated reference material in animal hematology to determine the INR, but ISI values are important to understand the variability that can be observed in the PT assay within a given species. In rats, PT can vary by several seconds depending on PT reagents, whereas less variability is observed in dogs and humans (author's unpublished data).[7,14,27]

TABLE 13.1 Individual coagulation factor activity of laboratory animals compared to humans.

Species	Higher	Similar	Lower
Rat	VIII, antithrombin III	II, V, VII, XI, XII fibrinogen	X, VIIIR:vWf, and IX
Mouse	V, VII, X	fibrinogen	II
Dog	V, VII, VIII, IX, X, XI, XIII	fibrinogen	II
Rabbit	IV, VII, VIII, IX, X, XI, XIII	fibrinogen	II
NHP		All factors including fibrinogen	
Guinea pig	II, IX, XI, fibrinogen		VII, X

Sources: Lewis et al.[25]; Evans[11]

TABLE 13.2 Reference intervals for coagulation times in adult laboratory animals (in seconds).

Species	Method/reagent	PT	APTT	TT
Rat (Sprague–Dawley)	a	12.3–16.1	12.6–24.3	–
	b	14.2–16.3		14–20.1
Rat (Wistar)	a	13.6–16	13–20.4	12–16.5
Mouse (CD1)[a]	a	9.2–11.4	26.6–34.8	8.7–17.2
	b	10.4–11.7	14.4–43.4	–
Dog (Beagle)	a	5.8–7.9	9.1–17.8	5.9–6.4
	b	7.2–9.3	11.7–15	–
Rabbit (New Zealand White)	a	5.2–7.1	13.7–26.9	–
	b	8.4–14.2	16.6–23.1	–
NHP (Cynomolgus monkey)	a	8.3–12.7	16.7–25.4	–
	b	11.3–24.7	21.5–39.8	22.4–27.3
Minipig (Göttingen)	a	10.5–12.8	13.5–26.2	
	b	12.5–15.4	10.2–17.6	17.9–23.9
Guinea pig	a	26.8–53.2	9.5–25.4	–
Human	a	9.9–11.2	25.2–33.2	13.7–17.8
	b	12.1–13.9	25.9–33.4	–

a ACL advance® coagulation analyzer, Instrument laboratory reagents: APTT synthasil®, PT Fibrinogen®, TT HemosIL®. b STA Compact® coagulation analyzer, Stago reagents: APTT STA-Neoplastine® CL Plus 10, for PT, STA-C.K. Prest®.

In vitro APTT measurement evaluates the intrinsic coagulation pathway (see Chapter 87). Clotting is initiated by addition of a contact particle to citrate plasma: celite, kaolin, or ellagic acid with calcium and phospholipids, which can impact assay sensitivity (see Chapter 88).[14] Assays using synthetic phospholipids are highly reproducible, especially in NHPs, compared to assays using phospholipids extracted from rabbit brain (author's unpublished data).

Both PT and APTT assays lead to the generation of thrombin, which transforms fibrinogen into fibrin resulting in clot formation. Their sensitivity remains limited because the clot formation occurs when only 5% of the thrombin potentially present in plasma is produced.

In vitro thrombin time (TT) assesses the common pathway. Prolongation of TT is indicative of a deficiency of fibrinogen or an inhibition of thrombin.

Consideration of Blood Volumes for Laboratory Animals

Blood volume limitation is always of concern in laboratory animals, especially in rodents. In mice, coagulation times are rarely evaluated because of the small blood volume available in this species. In rat studies, as unexpected changes in coagulation times are quite unusual, assessment of coagulation is often deferred to the end of the studies. Small commercialized citrated tubes are preferred (0.5, 1, and 1.3 mL) and the 9:1 ratio of blood:citrate (3.2% or 3.8% sodium citrate) must be respected. It is possible to prepare smaller citrated tubes by adjusting the quantity of citrate, but these are prone to clotting and not recommended. Plasma should not be diluted for the evaluation of coagulation times, as these assays do not demonstrate linearity with dilution. The use of small non-automated coagulation analyzers can be advantageous because the whole plasma volume in the tubes can be utilized, and there is no dead volume remaining unlike with large automated analyzers.

Interpretation of Changes in PT and APTT

Preanalytical Changes

Preanalytical effects can cause either prolongation or shortening of PT and APTT. A difficult venipuncture may trigger the release of TF and collagen and the formation of small clots in the tube, increasing coagulation times. Overfilling citrate tubes increases the volume of plasma; hence, the sample concentrations of coagulation factors and calcium are increased and there is a deficient calcium–citrate binding, both coagulation times tend to decrease.[29] Likewise, underfilling of citrated tubes will result in increases of coagulation times.[1] In nonclinical studies, it is recommended not to analyze underfilled or overfilled tubes.

Selective Increases in PT or APTT

Increase in PT with no concurrent increase in APTT is an expected compound effect observed with administration of FVII inhibitors. Increases in APTT with no concurrent increase in PT are expected with administration of FVIII or FIX inhibitors. The first generation of antisense oligonucleotides has been reported to bind and inhibit coagulation factors VIIIa, IXa, X, and II and increase APTT.[26]

Combined Increases in PT and APTT

Combined increases in PT and APTT are more frequently observed and changes in APTT are most often of larger magnitude compared to PT. Increased red cell mass in dehydrated animals decreases the volume of plasma, consequently the proportion of coagulation

factors to citrate is decreased, and both coagulation times tend to increase.[29]

On a general basis in nonclinical studies, mild increases of group mean coagulation times (15–20%) are considered to be compound-related, but these criteria will change between laboratories based on assay variability. However, laboratory animals will not show clinical signs before APTT or PT increases by approximately 200%. Indeed, laboratory animals depleted of coagulation factors rarely form hematomas because they are maintained in cages and often isolated from their counterparts when considered at risk.

Compounds Affecting Vitamin K

Compound-related vitamin K deficiency can be suspected when PT and APTT are increased simultaneously with decreased availability of vitamin-K-dependent factors. Vitamin-K-dependent coagulation factors may be affected during nonclinical studies by anticoagulant compounds with coumarin moieties, such as warfarin, or by off-target compound effects.[40] Vitamin K is required for the synthesis of functional factors II, VII, IX, and X and proteins C and S. Unexpected vitamin K deficiencies can be observed in studies conducted in neonatal animals because of the immaturity of the liver, or with compounds causing gastrointestinal or pancreatic toxicity, which affects the absorption of fat-soluble vitamins.[7,11]

Vitamin Kconcentrations in liver, serum, or urine are measured by mass spectrometry and liquid chromatography, but measurement of vitamin-K-dependent factors remains an easier option.

Decreased activity in vitamin-K-dependent factors (II, VII, X, and X) without changes in factors that are vitamin-K-independent (VIII) is strongly supportive of vitamin K deficiency or antagonism.[7] PT is frequently increased before APTT because FVII has the shortest half-life of all factors, and animals may not show hemorrhages until FII (prothrombin) is deficient.[6] Changes in coagulation times may be different between male and female rats, because of a gender difference in the metabolism of vitamin K, and in activities of clotting factors.[23] Estrogens are reported to protect from vitamin K deficiency in rats, while testosterone is reported to aggravate it.[18,24,40] Compounds may have an impact of the hydroxylation of vitamin K by induction of hepatic cytochrome isozymes (CYP), and species differences in CYP induction could explain compound-related species differences observed in vitamin K deficiency.[8,40]

FIIa and FXa Inhibitors

Heparins and vitamin K antagonists have been used for many years for the treatment and prevention of thromboembolic disease, but pose a risk of bleeding. In recent years, a large number of direct thrombin and factor Xa inhibitors have been developed and accepted for clinical usage for the prophylaxis and treatment of venous thromboembolism and acute coronary syndromes. Direct thrombin inhibitors block both free and fibrin-bound thrombin in opposition to heparin that can form a bridge between thrombin and fibrin and enhance thrombin affinity for fibrin.[22] APTT and PT are both sensitive to the inhibition of FXa and thrombin, although this sensitivity varies between species. When evaluating heparins, APTT increases are proportional to the concentrations of heparins, but the PT assay remains unchanged because of the presence of a heparin inhibitor in the PT reagent.

Decreased production of coagulation factors secondary to compound-induced hepatic lesions are rarely encountered in nonclinical studies. Although the liver is often the target for compound-related toxicity, chronic hepatic lesions are rarely observed because of the short duration of nonclinical studies, and the animals suffering important hepatic lesions are not kept on studies for ethical reasons.

Decreases in Coagulation Times

Shortened APTT and/or PT are rarely encountered, and do not indicate hypercoagulability. These coagulation times are not very sensitive to procoagulant factors or the effects of fibrinolysis. Increased concentrations of fibrinogen up to 800 mg/dL do not impact APTT and PT but shorten TT in rats, and shorten APTT, PT, and TT in dogs.[20]

EVALUATION OF SPECIFIC COAGULATION OR ANTICOAGULANT FACTORS

Fibrinogen

Fibrinogen concentration is routinely evaluated during nonclinical studies. It is an acute-phase protein produced by the liver. Among all species, there is considerable similarity in the fibrinogen molecule.[14] Fibrinogen is most often determined by the modified thrombin time but an ELISA-based method can be used. Decreased fibrinogen concentration is commonly observed in poor-quality samples containing fibrin strands and in disseminated intravascular coagulation (DIC). Increased fibrinogen is a reliable biomarker of inflammation in all laboratory animal species.

Individual Coagulation Factors

Individual coagulation factors are infrequently measured, but may be evaluated to identify a mechanism of action for off-target effects.

Coagulation Factors Assay Using Human Deficient Plasma

Human-coagulation-factor-deficient plasmas are used for the quantification of specific coagulation factors. The assay is based on the ability of the tested plasma to correct the clotting time of the deficient plasma (see Chapter 88). In animal species, it is necessary to generate a standard curve for each species and reagent, using a pool of healthy nontreated animals, in order to

accommodate species differences. Coagulation factor activities vary with species, sex, age, and health status. In a rat model of diabetes, age-related changes consisted in increases in coagulation factor activities and decreases in fibrinolytic factors.[28]

Chromogenic Factor Assays

When absolute factor activities are required, chromogenic ELISA using a colorimetric detection can be adapted on automated coagulation analyzers (see Chapter 88). However, these quantitative immunoassays are designed with antibodies reacting specifically with human or mouse proteins and may be limited by the absence of cross-reactivity between species. Several chromogenic assays were successfully validated with laboratory animal samples. The FXa chromogenic assay was used to measure the efficacy of anti FXa inhibitors in rats and dogs, and the efficacy of heparins in rabbits.[2,7,32] Chromogenic FVIII and FIX assays were used for the evaluation of nonhuman primate models of hemophilia.[15]

FDP and D-dimers

Fibrin degradation products (FDP) result from the degradation of fibrinogen or fibrin fragments. Increased FDP is an indication of accelerated fibrinolysis, but is less specific than D-dimers concentration that is indicative of increased production of thrombin and activation of plasmin. D-dimers can be measured in NHPs and dogs using an immunoturbidimetric method (human reagent kit) adapted to an automated chemistry analyzer. Determination of D-dimers in rats was reported using an ELISA assay, although the variability of this assay was found to limit its reliability (author's unpublished data). D-dimers tend to vary in nonhuman primates and are elevated in prothrombotic conditions, and highly elevated with the formation of thrombi.

ATIII and TAT

There are several intrinsic coagulation inhibitors: antithrombin III (ATIII), protein C, protein S, heparin, heparin cofactor II, tissue factor pathway inhibitors, and annexin V. Antithrombin activity is most commonly evaluated in NHP and dogs, and is decreased in conditions of DIC. It is measured indirectly by its ability to inhibit thrombin and FXa using a chromogenic substrate or directly with an ELISA.[6,39]

Thrombin–antithrombin (TAT) complexes are also sensitive and specific indicators of thrombin activation. A high concentration of TAT indicates an earlier generation of thrombin and suggests hypercoagulability. In a rat model of inflammation, activation of coagulation by administration of lipopolysaccharide (LPS) resulted in an increase in TAT 3 hours postdose.[34] TAT complexes have also been determined in mice, rats, rabbits, dogs, pigs, and baboons using an ELISA kit for humans.[35]

GLOBAL COAGULATION ASSAYS

Thrombin Generation

Thrombin generation is a kinetic assay that is gaining interest in nonclinical studies, as it can be initiated on frozen plasma after analyses of first-tier markers and can detect both hypocoagulable and hypercoagulable conditions (see Chapter 88). Indeed, it is among the most interesting and sensitive assays that can detect an early compound-induced prothrombotic state. This assay can easily be validated in all species, as the processes of thrombin generation and fibrin clot formation are similar among all.

Thromboelastography

Whole blood viscoelastic assays, such as thromboelastography (TEG), are now used as investigative assays to provide a more comprehensive assessment of clot integrity (see Chapter 79). These assays should be considered in replacement of bleeding times and whole blood clotting times. A standard protocol should be used to ensure the reliability of viscoelastic assays.

In rodents, TEG is performed on citrate whole blood sampled from the femoral artery or abdominal aorta, stored 15–30 minutes before analysis, and measured within 2 hours of collection. Citrated blood is recalcified with calcium chloride to initiate the reaction. Addition of kaolin or TF is not necessary.[43]

Bleeding Time

Assessment of bleeding time is poorly reproducible because of large individual variability and lack of sensitivity (see Chapter 79). Hence, it is rarely performed, but is still considered advantageous to reflect the effects of anticoagulants or compounds affecting platelet function. In rats, the incision cut is made on the tail or pedal artery on anesthesized animals. In NHP, the incision is done on the forearm, and BT is typically less than 200 seconds.

Microparticles

Microparticles (MPs) have different hemostatic properties depending on their origin. The majority of circulating MPs are derived from platelets, and a lesser portion originates from endothelial cells and leukocytes. MPs generated after platelet activation have high pro-coagulant properties; leukocyte-derived MPs can inhibit coagulation, and calcium phosphate vesicles prevent coagulation. MPs, measured by flow cytometry, are infrequently evaluated in nonclinical studies.[3]

Whole Blood Clotting Assays

Whole blood clotting time consists of adding whole blood to glass tubes at 37 °C and measuring the time taken for the blood to clot. A contact activator is added in the activated clotting time (see Chapter 88). Such

assays offer a crude and limited assessment of the intrinsic system, and have limited interest, however they are mentioned in regulatory guidelines.[31]

EVALUATION OF PLATELETS

Platelets are the first line of defense against vascular bleeding and are major contributors to thrombosis and inflammation. Changes in platelet counts and/or platelet function are important findings in regulatory nonclinical studies. Although some drugs are developed specifically for their antiplatelet effects, others may affect platelet count or function as off-target effects, including effects that could lead to risks of thrombosis and increased bleeding.

Disseminated intravascular coagulation

DIC is a lethal syndrome characterized by multiorgan microvascular thrombosis (see Chapter 92). It represents an unbalanced response leading to the systemic release of proinflammatory cytokines. In toxicological studies, it may occur secondary to an inflammatory event or administration of an immunomodulator which increases cytokines, activates monocytes, and triggers activation of coagulation factors.[16] Overt DIC is recognized by clinical criteria of thrombocytopenia, prolonged clotting times, depletion of fibrinogen and antithrombin, and elevated D-dimers. By contrast, early subclinical or non-overt DIC has no specific predictive tests to differentiate patients who re-establish effective hemostatic balance from those who progress to overt DIC and death.[3] An endotoxin-induced inflammatory state was studied in rats. Rapid and transient increases in circulating concentrations of D-dimer, intercellular adhesion molecule-1 (sICAM-1), PAI-1, procoagulant extracellular vesicles and decreases in antithrombin III were observed within 4 hours, denoting early cellular activation and dysregulated coagulation and fibrinolysis that promote thrombus formation.[4] In a similar rat study, TAT were increased 3 hours postendotoxin injection, while thrombin generation was decreased, suggesting a rapid consumption of coagulation factors.[34]

Immune-Mediated Platelet Decreases

Compound-induced immune-mediated platelet decreases have been associated with several drug classes including sulfonamides, nonsteroidal anti-inflammatory drugs (NSAIDs), anticonvulsants, and diuretics (see Chapters 12 and 15). Small molecular compounds uncommonly generate immune responses. Alternatively, biologics such as monoclonal antibodies, growth factors, cytokines, enzymes, and peptides are more prone to induce immune reactions. [12,21,36,37]

Immunogenicity in animals is not considered predictive of immunogenicity in humans, therefore it is important to determine if findings observed in nonclinical studies are related to the pharmacology of the compound or to the immunogenicity. Possible assays for investigating immune-related toxicity include: measurement of circulating immune complexes (ICs), complement split products C3a and C5b9, and CH50 by ELISA.[21] Platelet aggregometry and platelet activation (by flow cytometry) may help to differentiate a direct effect on platelets or an anti-drug antibody-related effect.[33] Immunohistochemistry or electron microscopy can be used to visualize the presence of immune complexes in kidney, lungs, or other damaged organs.

Platelet Dysfunction

Altered platelet function should be suspected in nonclinical studies in animals with bruises, hematomas, or hemorrhages (often only observed histopathologically). Platelet dysfunction is typically assessed by platelet aggregation assays by adding aggregating agents or agonists to platelet-rich plasma or whole blood (see Chapter 79). There is a large variability of response to aggregating reagents and agonist concentration among animal species (Table 13.3).

Several drug classes, including antiplatelet agents, anticoagulants, and NSAIDs, interfere with platelet function disrupting distinct pathways of platelet function. Therefore, a new compound can be evaluated by combining it with drugs that are known to inhibit distinct pathways in order to assess potential synergistic or

TABLE 13.3 Platelet aggregation reactivity among species with different agonists (Chrono-log® reagents) (author's non published data). Aggregation was performed in whole blood using an impedance method or in PRP using an optical method.

Species	Platelet Aggregation
Dog	Whole blood: Large individual variability with collagen up to 10 µg/mL PRP: Large individual reactivity with collagen, ADP, arachidonic acid (AA), epinephrine, ristocetin. Many dogs do not show platelet reactivity, due in part to genetic variability in the response in thromboxane A2
NHP	Whole blood: Poor reactivity with ADP up to 20 µM, low reactivity with collagen 10 µg/mL and thrombin 0.2 µg/mL **PRP:** Good response with ADP 10 µM and collagen 2 µg/mL. Good reactivity with ristocetin ATP release is not detected with ADP, but with collagen
Guinea pig	PRP: Good responses with collagen, ADP, AA, and platelet-activating factor (PAF). Variable to poor reactivity with ristocetin
Rat	Whole blood: Poor reactivity PRP: Good response to ADP 1–5 µM, collagen 0.5–1 µg/mL. No response to AA in citrated PRP, and to PAF (lack of receptor). No reactivity with ristocetin
Mice	Whole blood: Poor reactivity PRP: Good response to ADP 10 µM
Human	PRP: Good reactivity with ADP, collagen, AA, thrombin receptor activating peptide (TRAP) and ristocetin

Sources: Kurata and Horii[19]; Nobukata et al.[28]

inhibitory effects on platelet function. Such assays are done first in vitro on human or nonhuman primate samples, and then in vivo.[5] For in vitro assay, the compound is spiked at various concentrations in whole blood or platelet-rich plasma, and platelet aggregation is evaluated after addition of an agonist. Platelet aggregation is generally measured using two agonists, ADP and collagen, because they use different membrane receptors and pathways for platelet activation.

Flow cytometric evaluation of platelet function is commonly used, often in parallel with platelet aggregation assays, in in vivo and ex vivo studies. Similarly, isolated platelets or anticoagulated whole blood are incubated with the compound and a platelet agonist (usually ADP). The evaluation of P-selectin and GIIb/IIIa is commonly performed in nonhuman primates and mice to detect activated platelets. These molecules are upregulated on the surface of activated platelets (see Chapter 79).

The PFA-100 instrument presents a different approach to platelet dysfunction: blood is aspirated at high shear through an aperture into a disposable cartridge coated with agonists and closure time is measured until a platelet plug is formed. In nonclinical studies the PFA is considered an interesting translational Nonclinical studies provide interesting translational markers from laboratory species to humans. from laboratory species to humans. It can be used in NHP and dogs, but does not function in rats because of the low reactivity of rat platelets in whole blood to the different agonists.

CONCLUSION

The evaluation of compound-related effects on hemostasis remains essential in nonclinical studies to determine their safety for administration to humans. In vitro assays of compound effects are gaining popularity because they limit the use of laboratory animals; however, evaluation of their predictive values can be challenging and comparison of newly developed compounds with already marketed compounds is sometimes necessary. The use of thrombosis or hemophilic models may be necessary to determine the doses for human administration. Due to the large species-specific variability in concentrations of coagulation factors, platelet numbers, and platelet function, the role of the veterinary clinical pathologist is essential to evaluate compound effects in regulatory nonclinical studies.

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CHAPTER 14

Preclinical Evaluation of Immunotoxicity

KRISTIN L. HENSON

Immunotoxicity and (Bio) pharmaceuticals
Immunosuppression
Immunostimulation
Hypersensitivity and Autoimmunity
  Hypersensitivity
  Autoimmunity
Regulatory Guidelines
Immunological Endpoints in Preclinical Toxicity Studies
  Tier 1 Assessments
    Hematology
    Clinical Chemistry
    Histopathology
    Stress and Immunotoxicity
  Tier 2 Assessments
    Flow Cytometry
    T-cell-Dependent Antibody Response
    Cell-Mediated Immunity
    Inflammatory Mediators
  Tier 3 Assessments
Immunogenicity
Developmental Immunotoxicity Evaluations

Acronyms and Abbreviations

ADA, anti-drug antibodies; ADC, antibody-dependent cellular cytotoxicity; ADR, adverse drug reaction; ATA, anti-therapeutic antibodies; CDC, complement-dependent cytotoxicity; CRA, cytokine release assay; CRS, cytokine release syndrome; CTL, cytotoxic T lymphocytes; DTH, delayed-type hypersensitivity; ELISA, enzyme-linked immunosorbent assay; EMA, European Medicines Agency; ICH, International Counsel for Harmonisation; KLH, keyhole limpet hemocyanin; LLNA, local lymph node assay; LMW, low molecular weight; NHP, nonhuman primate; NK, natural killer (cells); (e)PPND, (enhanced) prenatal and postnatal development; STS, standard toxicity studies; TDAR, T-cell-dependent antibody response assay; TIAR, T-cell-independent antibody response assay

IMMUNOTOXICITY AND (BIO) PHARMACEUTICALS

Preclinical immunotoxicity evaluations in drug development are used to identify test-article-related changes to the innate and adaptive immune system, the mechanism of immunotoxic effects, and the consequences of these changes for normal immune function and homeostasis.[12] All investigational new drugs should be evaluated for immunotoxic potential through assessment of immune-relevant endpoints in standard toxicity studies (STS) or specialized immunotoxicity studies. Potential mechanisms of immunotoxicity include exaggerated or prolonged pharmacology on an immune system target, modulation of an immune target with multiple immune functions or a target expressed on other immune cells, or off-target cross-reactivity. Results of preclinical immunosafety assessment for compounds with immunotoxic potential inform first-in-human dosing and dose escalation, clinical trial inclusion and exclusion criteria, the clinical safety monitoring plan, and, ultimately, drug labeling for any identified toxicological effects on the immune system.

IMMUNOSUPPRESSION

Test-article-related effects on the immune system potentially resulting in immune suppression include depletion of circulating leukocytes and lymphoid tissues, decreased hematopoiesis, or alterations in immune function resulting in increased incidence of infections or

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neoplasia. On-target or exaggerated immunopharmacology resulting in immunosuppression can be seen with (bio)pharmaceuticals intended for inflammatory and autoimmune diseases with effects mediated by binding to cell surface receptors resulting in cell depletion (rituximab), signaling pathway molecules (abatacept and belatacept), or inhibition of soluble mediators and cytokines (adalimumab).[29,31,36,42]

IMMUNOSTIMULATION

The safety implications of immune stimulation as a therapeutic target received much attention after administration of an anti-CD28 superagonist monoclonal antibody (TGN1412) to healthy volunteers resulting in life-threatening complications due to induction of cytokine storm.[39] Subsequently, intensive investigations and published reviews described potential mechanisms for the lack of predictivity for cytokine storm in nonhuman primate (NHP) preclinical toxicity testing, assays for improved assessment of potential for cytokine release syndrome (CRS), and strategies to mitigate the risk of immune stimulation in first-in-human studies and clinical trials. [15,19] Recently, immunotherapies, such as toll-like receptor agonists, immune cell checkpoint inhibitors, T-cell tumor antigen bispecific antibodies, and chimeric antigen receptor T-cell therapy, have demonstrated remarkable therapeutic benefit of targeted immune stimulation in cancer treatment. Safety considerations for cancer immunotherapies, including CRS and tissue inflammation, as well as the translatability of preclinical toxicity models to evaluate these safety findings have been reviewed.[11,22,23] Gene therapy is a rapidly progressing field with the preclinical safety assessment considerations for gene therapy products recently published.[1] Immunological considerations for gene therapy development include both the immune response to the therapeutic transgene and the viral vectors used to deliver the transgene, which may have pharmacokinetic, efficacy, and safety implications.[7]

HYPERSENSITIVITY AND AUTOIMMUNITY

Hypersensitivity

Adverse drug reactions (ADR) are unintended and undesired effects of drugs that occur during usual clinical use (see Chapters 12 and 15). Drug-induced hypersensitivity reactions are uncommon, unpredictable, dose-independent, and affect a small population (Type B ADR). Anaphylactoid or pseudoallergic drug reactions, due to complement or mast cell activation, may occur as an infusion reaction following the first dose.[43] Currently, no regulatory guidelines are in place identify the risk of drug-induced hypersensitivity in preclinical evaluations, and the complexity and unpredictability of the reactions make preclinical testing difficult. The in vivo local lymph node assay (LLNA), used for topical drugs, to screen for Type IV hypersensitivity risk has shown some promise for predictivity, especially if immunologically specific endpoints are included.[3] In vitro assays to assess drug-related T-cell and dendritic-cell activation also show promise for screening for hypersensitivity potential.[18] Currently, preclinical testing for drug-induced hypersensitivity should be considered on a case-by-case basis based upon pharmacology and results of preclinical testing and clinical findings.

Autoimmunity

Autoimmunity is the loss of immune tolerance to self-antigens with resulting immune-mediated destruction of cells and tissues. Potential drug-related causes for autoimmunity include molecular mimicry of the drug or metabolite to tissue antigens, binding of drug to cells or tissues resulting in a new antigenic target, and drug-related alterations in the immune system resulting in loss of tolerance, e.g., suppression of T regulatory cell function.[13] Due to the complexity of factors influencing autoimmune risk, preclinical assessment of the autoimmunity-inducing potential of a (bio)pharmaceutical is difficult. As immunoenhancement may increase risk of autoimmunity, methods to detect increased immune responses, including evaluation of tissue inflammation, T-cell populations involved in regulation of immune response, and immune function assessments (LLNA, delayed-type hypersensitivity (DTH), and antigen-specific immune challenge assays), may be considered for autoimmunity risk assessment. If there is clinical evidence of autoimmune risk or if the drug target or pathway raises concern for autoimmune potential, measurement of anti-DNA and anti-histone antibodies and screening for immune complex formation are additional screening methods with the recognition that these assays are not well validated in animal models for clinical translatability.[13]

REGULATORY GUIDELINES

The key guideline for (bio)pharmaceutical preclinical immunotoxicity assessment is the ICH (International Conference for Harmonisation) S8 guideline on immunotoxicity testing of human pharmaceuticals.[49] This guideline was the result of harmonization of immunotoxicity testing guidelines previously published by regulatory agencies (Table 14.1). For the purpose of the ICH S8 guideline, immunotoxicity is specified as unintended immunosuppression or enhancement with drug-induced hypersensitivity and autoimmunity excluded. The ICH S8 guidance recommends the evaluation of all new human pharmaceuticals for immunotoxic potential using a weight-of-evidence approach. Criteria for evaluating immunotoxicity include results of standard toxicity studies, pharmacological properties of the test compound, intended patient population, structural similarity to known immunomodulatory agents, disposition of the drug, and/or observations in clinical trials or clinical

TABLE 14.1 Regulatory guidelines for preclinical immunotoxicity testing of (bio)pharmaceutical.

Guideline Title	Issued by	Year	Reference
Note for guidance on repeated dose toxicity (Section 6.4 and Appendix B)	EMEA[a]	2000	44
Draft guidance on immunotoxicity testing	MHLW/JPMA[b]	2001	30
Immunotoxicology evaluation of investigational new drugs	FDA[c]	2002	47
ICH S8: Immunotoxicity studies for human pharmaceuticals	ICH[d]	2006	49
ICH S6(R1): Preclinical safety evaluation of biotechnology-derived pharmaceuticals	ICH	Parent—1997 Revision (R1): 2011	50
Guideline on similar biological medicinal products containing monoclonal antibodies—nonclinical and clinical issues	EMA[e]	2012	45
Immunogenicity assessment for therapeutic protein products	FDA	2014	48
Guideline on immunogenicity assessment of therapeutic proteins (Revision 1)	EMA	2017	46

[a]EMEA: European Agency for the Evaluation of Medicinal Products;
[b]MHLW/JPMA: Ministry of Health, Labour and Welfare of Japan/Japan Pharmaceutical Manufacturers Association;
[c]FDA: Food and Drug Administration;
[d]ICH: International Conference for Harmonisation; [e]EMA: European Medicines Agency.

use. If the potential for immunotoxicity is identified, then follow-up immunotoxicity testing using both morphological and functional assays should be conducted. The guideline provides recommendations on the duration, dosing, and types of immune testing to be included in the additional immunotoxicity studies.

Biotechnology-derived pharmaceuticals are considered out of scope for the ICH S8 guideline and reference is made to ICH S6 (R1) (preclinical safety evaluation of biotechnology-derived pharmaceuticals) published in 1997 and revised in 2011.[50] Regarding immunotoxic potential, ICH S6 (R1) notes that biotechnology-derived pharmaceuticals may induce either immunosuppression and immunoenhancement as well as immunogenicity, and testing strategies may require screening studies followed by mechanistic studies to clarify issues. However, routine tiered testing approaches or standard testing batteries are not recommended. For biosimilar therapeutic antibodies, the European Medicines Agency (EMA) guidance, while not specifically addressing immunotoxicity screening, recommends that Fc-receptor-mediated effects of the biosimilar monoclonal antibodies are similar to the reference product through use of in vitro assays evaluating antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and complement activation.[45]

IMMUNOLOGICAL ENDPOINTS IN PRECLINICAL TOXICITY STUDIES

Based upon the weight-of-evidence approach described in ICH S8, a tiered testing strategy comprising immune-relevant endpoints in STS (tier 1), specialized structural and/or functional immune parameters (tier 2), and host resistance studies (tier 3) are utilized for the preclinical immunosafety assessment of (bio)pharmaceuticals.

Tier 1 Assessments

The immune-relevant endpoints in STS (tier 1) include hematology, serum globulin concentrations, lymphoid organ weights, and histopathology of lymphoid tissues and bone marrow. The observation of increased infections or tumor incidence is also a tier 1 immunological assessment and may be the only indicator of an immunotoxic effect if the function of a single cell type is affected, such as neutrophil or macrophage phagocytosis.[37]

Hematology

The preclinical evaluation of hematology parameters is discussed in Chapters 11 and 12. From an immunotoxicity perspective, increases in peripheral leukocyte absolute counts may indicate test-article-related immunostimulation/enhancement or infection secondary to immunosuppression. Decreases in circulating leukocyte numbers occur with bone marrow or lymphocyte cytotoxicity, alterations in leukocyte trafficking, targeted depletion of immune cells, or increased tissue utilization due to infection or inflammation.

Clinical Chemistry

Decreases in lymphocyte numbers or suppression of lymphocyte function may result in decreased immunoglobulin production with concomitant decrease in globulin concentrations as measured in the standard chemistry panel. Changes in serum globulins are interpreted in conjunction with renal and/or liver parameters and histopathology to rule out non-immune-related causes for the globulin change. If a test-article-related impact on globulin production is anticipated, inclusion of serum immunoglobulin subclass evaluations (IgM, IgG, IgA) in STS may be considered for additional characterization. As increases in acute phase proteins related to immunostimulation and/or inflammation are generally

not detectable on serum globulin assessment alone, evaluation of acute phase proteins may be added to STS if immune stimulation is anticipated or if evidence of inflammation is detected.[13]

Histopathology

Organ weight of lymphoid organs and histopathological evaluation of lymphoid tissue and bone marrow are important tier 1 immunotoxicity assessments. Increases in lymphoid organ weights suggest immunostimulation related to either infiltration of immune cells (inflammation or altered trafficking) or immune cell hyperplasia. Decreases in lymphoid (spleen, thymus) organ weights are suggestive of immune suppression related to lymphoid depletion, which may be an on-target effect, related to cytotoxicity, or secondary to stress. Age-related decreases in thymic weights due to thymic involution, most apparent in dogs and NHP, can make interpretation of a direct test-article-related effect on thymic lymphopoiesis difficult. Selection of tissues for appropriate tier 1 assessment of immunotoxic potential and considerations for reporting of findings have been reviewed.[21]

Stress and Immunotoxicity

Animal handling and test article doses at or near the maximum tolerated dose can result in changes to the immune system related to stress due to cortisol, corticosterone, or other mediators.[16] Differentiation of direct test-article-related effects on the immune system from stress-related immune suppression is a key component of the weight-of-evidence review for immunotoxic potential. The impacts of stress on immune cell populations and immune organs should be interpreted in conjunction with the overall degree of changes in the study.

Tier 2 Assessments

If results of STS and/or other considerations in the weight-of-evidence review suggest immunotoxic potential, tier 2 assessments consisting of morphological and functional tests should be implemented in either a standalone immunotoxicity study or repeated dose toxicity.[41]

Flow Cytometry

Flow cytometry is a frequently applied methodology within immunotoxicity evaluations to assess changes in immune cell populations (see Chapter 142). For immunotoxicity evaluations, flow cytometry is most commonly used for immunophenotyping lymphoid subsets in blood and lymphoid tissue (spleen, thymus, and, less frequently, lymph node).[2] Flow cytometry also can be used to evaluate test-article-related changes in neutrophil and monocyte populations, T regulatory (suppressor) cells, memory subsets, immune cell activation, proliferation potential, phagocytic function, production of intracellular cytokines, and evaluation of pharmacodynamic markers, such as target receptor occupancy. Due to intra-animal variability and changes in lymphocyte subset numbers related to excitement and handling, two predose samples for immunophenotyping are typically collected in large animals (dogs, NHP, minipig) for baseline comparisons. Application of immunophenotyping and flow cytometry assessments in laboratory animals (rodents, dogs, NHP, minipig) has been reviewed.[25,28,34,35]

T-Cell-Dependent Antibody Response

The T-cell-dependent antigen response (TDAR) is the most commonly applied immune function test in preclinical immunotoxicity assessments. The TDAR involves challenge with an antigen followed by evaluation of the primary and secondary antibody assessment.[17] A neoantigen to which the animal has not been previously exposed may be used to evaluate both primary (IgM) and secondary (IgG) responses and/or a recall antigen can be administered to evaluate recall (memory) antibody (IgG) response. As the TDAR involves antigen presentation, T-cell activation and regulation, antibody production, and immunoglobulin class switching, the assay is considered a broad functional assessment of immune function. In the pharmaceutical industry, the most frequently applied neo-antigen is keyhole limpet hemocyanin (KLH) with tetanus toxoid often used as a recall antigen.[20] Methodologies and considerations for TDAR in toxicological species have been described.[26–28,35] With increased interest in biomarkers of immune stimulation for immunotherapies, modifications of the TDAR in cynomolgus monkeys using doses of KLH to elicit sub-maximal antibody responses allowing for detection of increases anti-KLH IgM and IgG have been reported.[33] Although not commonly used, a T-cell-independent antibody response (TIAR) assay using T-cell-independent antigens (e.g., pneumococcal vaccine) can be used to evaluate potential effects on T-independent antibody responses, such as a targeted effect on the lymphoid marginal zone.[10]

Cell-Mediated Immunity

The effect of (bio)pharmaceuticals on innate immune cell and T-cell responses can be evaluated using both ex vivo and in vivo assays. Ex vivo assays to evaluate phagocytosis/oxidative burst, cell killing mediated by natural killer (NK) cells or cytotoxic T lymphocytes (CTL), basophil function, and T- and B-cell antigen-specific responses (activation, intracellular cytokine production, cytokine release) are described.[24,28,33,35] The DTH assay is an in vivo assay that evaluates T-cell-mediated responses (Type IV hypersensitivity) following an intradermal antigen challenge and is used as a screen for unintended suppression of cell-mediated immunity or as a pharmacodynamic marker for enhanced T-cell responses. [4,9,35]

Inflammatory Mediators

Cytokines and chemokines can be measured using ex vivo cytokine release assays (CRA) or serum or plasma samples from in vivo toxicity studies to evaluate the potential for immunostimulation. Methods for cytokine

and chemokine evaluation include ELISA or bead-based flow cytometric (Luminex®) or electrochemiluminescent (Meso Scale Discovery®) multiplex technologies. Considerations for cytokine detection in preclinical laboratory species and cytokine release assays have been described.[19,28,33,35,40] Cross-species reactivity should be confirmed for the method used for cytokine detection. Single cytokine or chemokine measurements using ELISA can be considered if the analyte is a known target or key component of the modulated immune pathway or is a pharmacodynamic marker. If multiplex assessments are available, evaluation of a panel of both inflammatory cytokines (IL-1β, IL-2, IL-5, IL-6, IL-12, interferon-λ, and TNF-α) and anti-inflammatory cytokines (IL-10, IL-1RA) is a useful screen for immune enhancement. Additional analytes may be included based on pharmacology of the compound. As cytokine/chemokine responses are time-dependent and often transient, samples are collected at predose and multiple times postdosing (0.5 hour, 4–6 hours, and 24 hours postdose). Excitement and animal handling may increase circulating levels of cytokines, such as IL-6.[40] Thus, two predose samples for baseline comparisons should be considered (when allowed by blood volume collection limits) with results interpreted in conjunction with sampling schedule and other markers of inflammation.

Evaluation of complement activity can be used to assess the risk for complement activation for compound classes with known activating potential, for compounds specifically targeting the complement system, or when complement activation is suspected based upon results of STS. Similar to cytokine evaluations, samples to evaluate complement activation should be collected predosing and shortly after dosing (15–30 minutes).[28,33]

Tier 3 Assessments

Host resistance studies involve the administration of an infectious agent (bacteria, virus, fungus, parasite) or tumor challenge to rodents in presence of test compound to assess the immunotoxic (suppressive) effect of the test compound.[6] These studies may be considered on a case-by-case basis for compounds that are broadly immunosuppressive, to investigate a clinical infection risk signal, or for compounds against targets with a known clinical infection risk. As these studies are performed only in specialized biohazard laboratories and the clinical translatability is uncertain, host resistance studies are infrequently conducted in the pharmaceutical industry.

IMMUNOGENICITY

Immunogenicity is the ability of a drug to elicit an immune response and is a particular consideration for large molecule biopharmaceuticals. While not considered predictive for development of immunogenicity in humans, the evaluation of anti-drug or anti-therapeutic antibodies (ADA or ATA) in preclinical toxicity studies is important for the accurate assessment of the toxicity study. ADA may impact preclinical study pharmacokinetics, pharmacodynamics, and safety via alterations in drug clearance, neutralization of target binding, or hypersensitivity responses (type I and type II). Regulatory guidelines for the assessment of ADA are listed in Table 14.1. The assessment and predictivity of ADA evaluations have been reviewed. [5,33]

DEVELOPMENTAL IMMUNOTOXICITY EVALUATIONS

If the drug target, immunopharmacology, or observations of adolescent or adult animals in STS suggest a cause for concern for immunotoxicity on the developing immune system, morphological and functional assays can be incorporated into reproductive toxicity studies. For low-molecular-weight (LMW) compounds or biotherapeutics that are cross-reactive in rodents, these assays are included into rodent prenatal and postnatal studies (PPND). For biotherapeutics that are only cross-reactive in NHP, immunosafety assessment can be included in the enhanced prenatal and postnatal development (ePPND) study recommended in ICH S6 (R1). Study designs for incorporation of immune endpoints in rodent and NHP developmental toxicity studies are described. [8,14,38,50]

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CHAPTER 15

Blood and Bone Marrow Toxicity Induced by Drugs, Heavy Metals, Chemicals, and Toxic Plants

DOUGLAS J. WEISS

Drug-Induced Hematologic Disorders
- Evaluation of Suspected Adverse Drug Reactions
- Adverse Drug Reactions in Dogs
  - Antineoplastic Drugs
  - Estrogen
  - Anti-Inflammatory Drugs
  - Antibacterial Drugs
  - Anticonvulsant Drugs
  - Antiparasitic Drugs
  - Cardiovascular Drugs
- Adverse Drug Reactions in Cats
  - Antineoplastic Drugs
  - Azathioprine
  - Propylthiouracil/Methimazole
  - Griseofulvin
  - Albendazole
  - Antiviral Agents
- Adverse Drug Reactions in Horses
  - Drug-Induced Immune-Mediated Hemolysis
  - Drug-Induced Non-Immune-Mediated Hemolysis

Heavy-Metal-Induced Hematopoietic Injury
- Lead
- Mercury
- Arsenic

Chemical-Induced Hematopoietic Injury
- Benzene
- Chlorinated Hydrocarbons

Plant-Toxin-Induced Hematopoietic Injury
- Mycotoxins
- Bracken Fern

Acronyms and Abbreviations

ADR, adverse drug reaction; FDA, Food and Drug Administration; DDT, chlorophenothane; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; ppm, parts per million, IMHA, immune-mediated hemolytic anemia; RBC, red blood cell; RNA, ribonucleic acid.

DRUG-INDUCED HEMATOLOGIC DISORDERS

A growing number of therapeutic drugs have been incriminated in adverse drug reactions (ADRs) associated with the hematologic system.[74,79] These drug toxicities have been categorized as dose-dependent or idiosyncratic (see Chapter 12 for more detailed discussion).[74] In dose-dependent reactions, toxicity increases with the dose and most members of a species are affected at higher doses. Idiosyncratic drug toxicities occur at therapeutic doses and only a small percentage of susceptible individuals in a population is affected.[74] Idiosyncratic drug toxicities frequently result from individual variation in the metabolism of drugs producing reactive metabolites that cause tissue injury in susceptible individuals. These reactive metabolites may cause oxidative injury or bind to cellular proteins forming haptens that trigger humoral or cellular immune responses.[74]

The site and mechanism by which a drug acts are important in determining the clinical syndrome and prognosis; however, some drugs act at multiple sites or

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by several mechanisms making this determination difficult (Table 15.1).[75] Destruction of erythrocytes, leukocytes, or platelets in the peripheral blood results in rapid onset of cytopenias. The bone marrow can compensate by increasing production and cell numbers may return to normal despite continued administration of the drug. RBCs are particularly sensitive to oxidative injury. Oxidant drugs or those that are metabolized to oxidants tend to be dose-dependent because tissue injury is largely dependent on the degree of oxidative injury (see Chapter 32). Rapidly proliferating progenitor and proliferative cells in bone marrow are susceptible to chemotherapeutic agents. The development of cytopenias in the blood follows a predictable course.[17] Some drugs induce hematopoietic stem cell destruction or alter the hematopoietic microenvironment. The timing of development of cytopenias is more unpredictable and recovery is uncertain and delayed when it occurs. Endothelial cells of marrow capillaries and sinusoids also are vulnerable to toxic injury (see Chapter 16).

Evaluation of Suspected Adverse Drug Reactions

The mere temporal association of a drug treatment with a hematologic disorder does not in itself provide proof of an ADR. In each case, other potential causes of the hematologic condition should be eliminated by evaluation of the history, and clinicopathologic findings. When an ADR is suspected, the temporal relationship between onset of treatment and detection of the hematologic dyscrasia, previous exposure to the drug in question, and recovery from the hematologic dyscrasia after drug withdrawal should be documented.[42] This information not only documents the likelihood of a cause and effect relationship but may suggest a possible mechanism for the drug toxicity. When multiple drugs have been administered before detection of a hematologic dyscrasia, the Food and Drug Administration (FDA) database (http://www.fda.gov/cvm/adetoc.htm) can be reviewed or a literature search conducted to determine if other similar toxicities have been reported.[43] The FDA database can also be used to report suspected toxicities.

TABLE 15.1 Drugs suspected or confirmed to cause hematologic dyscrasias in animals.

Class and drug	Species	Suspected mechanism
Antineoplastics		
Class effect	All	Marrow suppression, dysmyelopoiesis
Endocrine		
Estrogen	Dog	Marrow suppression
Propylthiouracil	Cat	Immune-mediated
Methimazole	Cat	Immune-mediated
Anti-inflammatory		
Phenylbutazone	Dog, Cat	Marrow suppression, immune-mediated
Meclofenamic acid	Dog	Marrow suppression
Carprofen	Dog	Marrow suppression
Azathioprine	Dog, Cat	Marrow suppression
Naproxen	Dog	Blood loss
Antibacterial		
Chloramphenicol	Dog	Dysmyelopoiesis
Cephalosporin	Dog	Immune-mediated, marrow suppression, dysmyelopoiesis
Penicillin	Horse	Immune-mediated
Sulfonamides	Dog	Immune-mediated, marrow suppression
	Horse	Immune-mediated
Antifungal		
Griseofulvin	Cat	Marrow suppression
Antiviral		
Azidothymidine/Acyclovir	Cat	Marrow suppression
Anticonvulsants		
Phenobarbital/Primidone	Dog	Marrow suppression
Phenytoin	Dog	Marrow suppression
Antiparasitics		
Levamisole	Dog	Marrow suppression/immune-mediated
Albendazole	Dog, Cat	Marrow suppression

(*Continued*)

TABLE 15.1 (Continued)

Class and drug	Species	Suspected mechanism
Metronidazole	Dog	Marrow suppression
Fenbendazole	Dog	Marrow suppression
Amitraz	Dog	Unknown
Cardiovascular		
Amiodarone	Dog	Immune-mediated
Captopril	Dog	Marrow suppression
Quinidine gluconate	Dog	Unknown
Analgesics		
Acetaminophen	Dog, Cat	Oxidant
Aspirin	Dog, Cat	Oxidant
Phenacetin	Dog	Oxidant
Anesthetics		
Benzocaine	Dog, Cat	Oxidant
Cetacaine	Cat	Oxidant
Propofol	Cat	Oxidant
Urinary acidifiers/antiseptics		
Methylene blue	Dog, Cat	Oxidant
Diphenylhydrazine	Dog	Oxidant
DL-Methionine	Cat	Oxidant
Phenazopyridine	Cat	Oxidant
Other drugs		
Human erythropoietin/Darbepoetin	Dog	Immune-mediated
Mitotane	Dog	Marrow suppression
Colchicine	Dog	Marrow suppression
Azidothymidine (AZT)	Cat	Marrow suppression
Phenothiazine	Horse	Oxidant
Dimethyl sulfoxide	Horse	Oxidant
Heparin	Horse	Hemolysis

TABLE 15.2 Myelosuppressive potential of chemotherapeutic drugs used in dogs and cats.

High	Moderate	Low to none
Cyclophosphamide	Methotrexate	Corticosteroids
Cytosine arabinoside	Chlorambucil	L-Asparaginase
Doxorubicin	6-Mercaptopurine	Bleomycin
Vinblastine	Melphalan	
Hydroxyurea	5-Fluorouracil vincristine	

Usually, this information combined with serial complete blood counts, tests for immune-mediated hematologic diseases, and bone marrow aspiration and core biopsy specimens is the only data available to link a drug to a clinical ADR. Other tests that can be considered include evaluation of the hemolytic effects of the drug and evaluation of the effects of the drug, patient serum, or patient lymphocytes on blood cell or bone marrow cytotoxicity or on in vitro bone marrow cell culture.[25,39,60]

Adverse Drug Reactions in Dogs

Antineoplastic Drugs

Chemotherapeutic agents cause dose-dependent ADR. Because of their high mitotic rate, progenitor and proliferative cells in bone marrow are predisposed to injury by chemotherapeutic drugs. The myelosuppressive potential of chemotherapeutic agents varies (Table 15.2).[17]

Cytopenias are the most frequent chemotherapeutic toxicity identified and often necessitate temporary or permanent discontinuation of treatment. Bone marrow suppression follows a predictable course based primarily on the half-life of cells in the blood. Neutropenia occurs 5–7 days after myelosuppression followed by thrombocytopenia 9–14 days after myelosuppression.[16] Anemia as a direct result of chemotherapy is usually not seen because of the comparatively long half-life of RBCs.[17] Neutrophil and platelet counts usually return to baseline values within 72–96 hours after discontinuing therapy.[17]

Beyond destruction of hematopoietic cells, chemotherapeutic drugs have few adverse effects on the hematopoietic system. Multifocal areas of coagulation-type necrosis have been reported in association with administration of cyclophosphamide and vincristine.[78] An increase in dysplastic hematopoietic cells and atypical mitotic figures (i.e., secondary dysmyelopoiesis) is a frequent finding and should not be confused with a myelodysplastic syndrome (see Chapter 67).[1,81] Doxorubicin administration to dogs has been associated with poikilocytosis including the presence of ovalocytes, echinocytes, schistocytes, and keratocytes.[5] Doxorubicin is thought to bind to spectrin, a major cell membrane structural protein.[5]

Estrogen

Dog and ferret bone marrow is highly susceptible to estrogen-induced myelosuppression, and both dose-dependent and idiosyncratic reactions have been documented in dogs (aplastic anemia is discussed in Chapter 40).[44,67,82] In dogs, a single large dose of estradiol, therapeutic doses of estradiol, and therapeutic doses of diethylstilbestrol have been documented to result in hematologic dyscrasias. Therapeutic doses of estradiol or diethylstilbestrol, used in treatment of mammary tumors or prostatic hyperplasia, consistently cause a transient decrease in platelet count and neutrophilia 10–20 days after administration.[21] Administration of a single large dose of estradiol to dogs consistently results in leukocytosis, thrombocytopenia, and mild but progressive nonregenerative anemia in the first 3 weeks after injection of 1–3 mg/kg body mass of estradiol. Pancytopenia with bone marrow aplasia develops between 3 and 4 weeks after injection and most dogs begin to recover after 4 weeks. Although the mechanism is complex and poorly understood, serum inhibitors of hematopoiesis produced by T cells have been identified.[23] Myelofibrosis, bone marrow necrosis, and dysmyelopoiesis have been observed in dogs given therapeutic doses of estradiol propionate.[82]

Individual dogs appear to be uniquely sensitive to the effects of estrogen and develop severe aplastic anemia. Dogs given repeated therapeutic doses of estradiol or dogs having elevated endogenous estrogen levels resulting from cystic ovarian follicles, ovarian granulosa cell tumors, or Sertoli cell tumors may develop hematologic dyscrasias.[21] Dogs with endogenous estrogen toxicity typically are pancytopenic, but some are bicytopenic.[44,67,82] The bone marrow is typically aplastic.[23,82] This appears to be the result of hematopoietic stem cell destruction. The prognosis for these dogs is poor, but recovery after weeks to months of supportive care has been documented.[82] However, recent studies have reported recovery after removal of Sertoli cell tumors.[44,64]

Anti-Inflammatory Drugs

Phenylbutazone/Meclofenamic Acid Many cases of phenylbutazone-associated hematologic dyscrasias have been reported and one case of meclofenamic-acid-associated aplastic anemia has been reported.[38] Several types of idiosyncratic phenylbutazone-associated hematologic dyscrasias have been observed in dogs.[66] One type is a transient agranulocytosis that typically occurs within 2 weeks after starting treatment. Affected dogs frequently recover promptly when the drug is discontinued. This condition is most probably immune-mediated, but inhibition of bone marrow cell division or DNA synthesis is also a possible explanation. In another type of hematologic dyscrasia, dogs develop aplastic anemia months or years after initiation of treatment, presumably as a result of bone marrow toxicity.[77,82] In the experience of the author, the prognosis for recovery is poor. Additionally, phenylbutazone therapy has been associated with myelonecrosis and myelofibrosis.[77,82]

Carprofen Coagulation-type myelonecrosis has been reported in bone marrow from several dogs treated with therapeutic doses of carprofen.[78] Another dog was reported to have a neutrophilic dermatitis, vasculitis, immune-mediated hemolytic anemia (IMHA), and thrombocytopenia associated with carprofen adminstration.[45]

Azathioprine Neutropenia and thrombocytopenia or pancytopenia have been observed in dogs one to several months after initiation of treatment with immunosuppressive doses of azathioprine.[62] Bone marrow is aplastic, and some dogs have mild myelofibrosis. Peripheral blood cell numbers slowly return to normal once the drug is discontinued, but complete recovery may be delayed.

Naproxen Naproxen administration has been associated with blood loss anemia in dogs.[20,28] Gastrointestinal erosions or ulcerations are suspected to be the cause of the blood loss. This may result from local irritation or inhibition of prostaglandin synthesis.

Antibacterial Drugs

Sulfonamides Adverse drug events have been reported for sulfadiazine, sulfamethoxazole, and sulfadimethoxine.[27,82] These drugs induce an idiosyncratic syndrome that includes neutropenia, thrombocytopenia, hemolytic anemia, fever, polyarthropathy, or hepatocellular necrosis.[27,74] Doberman Pinschers may be at increased risk of sulfonamide hypersensitivity.[26] Others have reported sulfadiazine-associated aplastic anemia

(Figure 15.1).[69] The mechanism appears to be immune-mediated and does not appear to be related to folate deficiency.[27] Evidence of an immune-mediated etiology includes demonstration of drug-dependent antiplatelet antibodies and a positive direct Coombs test.[26] This immune response appears to be caused by an oxidized hydroxylamine metabolite that acts as a hapten when bound to proteins. Drug-specific T cells have been identified in humans and antidrug antibodies have been identified in dogs with sulfonamide hypersensitivity.[41,61] In dogs that are thrombocytopenic, antibodies recognize drug–platelet complexes.[42]

In dogs with sulfadiazine-induced aplastic anemia, pancytopenia usually occurs 10–14 days after initiation of treatment. The bone marrow is typically aplastic with the hematopoietic space replaced by adipose tissue.[80] The hematologic dyscrasia frequently resolves within 2 weeks after discontinuing treatment.

Chloramphenicol Severe aplastic anemia has been reported as an idiosyncratic reaction in humans treated with chloramphenicol, but has not been reported in dogs given therapeutic doses. One apparent idiosyncratic reaction was reported.[32] This dog had a moderate nonregenerative anemia with large numbers of siderocytes and ringed sideroblasts. This response suggests a reversible dysmyelopoiesis, perhaps secondary to drug-induced mitochondrial damage.

Cephalosporins Certain cephalosporins have been associated with several types of hematologic dyscrasias in dogs.[10,18] High dose or prolonged administration of cephalosporins can induce agranulocytosis, thrombocytosis, and a positive direct Coombs test.[15] Approximately half of dogs given high doses of cefonicid or cefazedone intravenously developed pancytopenia within 6–10 weeks after initiation of treatment.[10,18] The mechanism appears to be complex. In one study, the presence of anti-RBC, antineutrophil, and antiplatelet antibodies was consistent with immune-mediated destruction.[10] Cephalosporins also induce alteration in the bone marrow. Granulocytic and erythroid maturation arrest has been reported in dogs treated with cephalosporins.[18] The most prominent ultrastructural change in bone marrow was mitochondrial damage to hematopoietic and nonhematopoietic cells.[18] The hematologic dyscrasia resolved rapidly after discontinuing the drug. Aplastic anemia associated with persistent suppression of hematopoietic stem cell activity also has been reported when dogs were administered high doses of cefazedone.[15] Further, cefoperazone, cefamandole, and moxalactam inhibit synthesis of vitamin-K-dependent clotting factors and can lead to hypoprothrombinemia and a resultant coagulopathy.[15]

Anticonvulsant Drugs

Phenobarbital/Primidone Phenobarbital and primidone therapy has been associated with several types of hematologic dyscrasias in dogs. Because primidone is metabolized to phenobarbital, the mechanism of toxicity may be similar for both drugs. The mildest hematologic disorders are neutropenia and thrombocytopenia.[37,78] The presence of granulocyte hyperplasia in bone marrow indicates that the neutropenia is due to destruction of mature granulocytes. A more frequent ADR is myelonecrosis or myelofibrosis (Figure 15.2).[78,83] Dogs with myelonecrosis are frequently bicytopenic or pancytopenic. Bone marrow is characterized by multifocal areas of coagulation-type necrosis with variable degrees of myelofibrosis present.[83] Dogs with myelofibrosis, without concurrent necrosis, frequently have a severe nonregenerative anemia. The myelofibrosis appears to represent the chronic stage of myelonecrosis.

In a retrospective study of 37 dogs treated for 1 month or more with phenobarbital 8 (22%) had hematologic

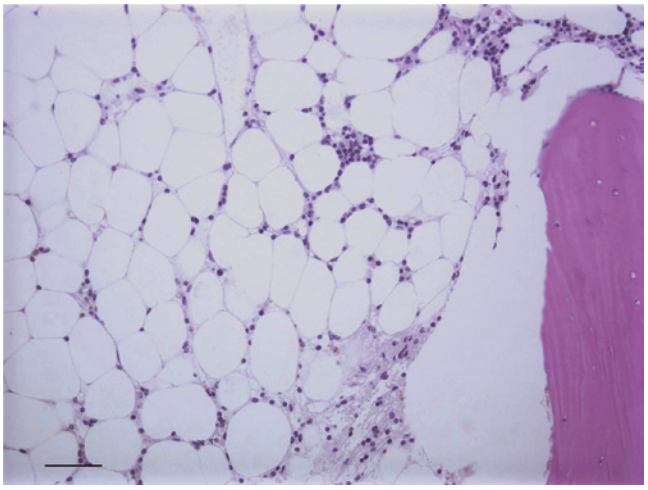


FIGURE 15.1 Bone marrow core biopsy from a dog treated with trimethoprim–sulfadiazine for 15 days. Note the presence of marrow aplasia. Cells present are mostly small lymphocytes. H&E stain; bar, 200 μm.

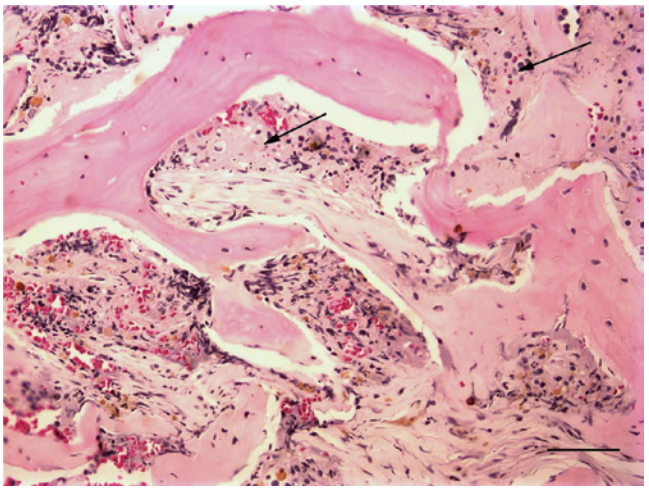


FIGURE 15.2 Bone marrow core biopsy from a dog treated with therapeutic doses of phenobarbital. Note the presence of coagulation-type necrosis (arrows) and myelofibrosis. H&E stain; bar, 200 μm.

changes.[29] Hematologic alterations varied from mild anemia to severe pancytopenia.

Both phenobarbital and primidone induce cytochrome P450 that may in part be responsible for their toxicity.[74] Alternatively, in human patients with anticonvulsant hypersensitivity syndrome, drug-specific T cells appear to elicit a secondary immune response.[74]

Phenytoin Long-term administration of phenytoin to dogs results in RBC macrocytosis, neutropenia, thrombocytopenia, and neutrophil hypersegmentation.[13] These changes were thought to be due to folate deficiency; however, folate deficiency does not appear to cause RBC macrocytosis in dogs.[13] Another dog undergoing long-term phenytoin therapy developed a severe normocytic nonregenerative anemia associated with severe myelofibrosis in bone marrow.[83]

Antiparasitic Drugs

Several antiparasitic drugs have been associated with idiosyncratic drug toxicities. Levamisole-induced thrombocytopenia and direct Coombs positive hemolytic anemia have been reported in several dogs.[3,4] All dogs had hyperplastic bone marrow and recovered after discontinuing levamisole treatment. Albendazole toxicity has been reported in one dog.[71] The dog had pancytopenia and an aplastic bone marrow, but recovered within 1 week after discontinuing treatment. Three dogs treated with metronidazole had coagulation-type myelonecrosis.[78] All dogs had pancytopenia but recovered after discontinuation of treatment. Fenbendazole administration has been associated with bone marrow coagulation-type necrosis.[78] One dog had pancytopenia after repeated weekly administration of the organic arsenical thiacetarsamide for treatment of demodectic mange.[76] The bone marrow was hypercellular and the dog recovered after discontinuation of treatment. However, the dog subsequently developed bone marrow RBC aplasia and died. Pancytopenia was reported in one dog given with amitraz for treatment for demodicosis.[52] The dog had a cellular bone marrow and peripheral blood cell counts recovered despite continued administration of the drug.

Cardiovascular Drugs

Several cardiovascular drugs have been associated with idiosyncratic drug toxicities. Amiodarone is an antiarrhythmic drug that has been associated with hemolytic anemias and hepatopathies in dogs.[14,35] Hematologic alterations were characterized by thrombocytopenia and positive direct Coombs test results with or without the presence of anemia. Affected dogs recover when treatment is discontinued. One dog developed pancytopenia and aplastic bone marrow after 18 months of continuous administration of captopril for treatment of a heart murmur.[35] Withdrawal of the drug and treatment of the dog with human granulocyte colony-stimulating factor and erythropoietin resulted in prompt hematologic recovery. In vitro studies using canine bone marrow cell cultures indicated that the toxic effects of captopril were dependent on a reactive thiol group.[30] This thiol group reacted with copper to generate hydrogen peroxide that was toxic to canine neutrophil progenitor cells. One dog developed neutropenia and anemia after prolonged administration of quinidine gluconate.[82] Three days after discontinuing the drug, the neutrophil count returned to normal and bone marrow cellularity was normal.

Adverse Drug Reactions in Cats

Antineoplastic Drugs

Neutropenia and thrombocytopenia are frequently dose-limiting factors in chemotherapy.[17] As in other species, rapidly proliferating progenitor and proliferative cells in bone marrow are susceptible to destruction by chemotherapeutic agents. Beyond cytopenias, dysplastic hematopoietic cells and atypical mitotic figures (i.e., secondary dysmyelopoiesis) are frequently found in bone marrow. Doxorubicin administration to cats has been associated with poikilocytosis including the presence of ovalocytes, echinocytes, schistocytes, and keratocytes.[51]

Azathioprine

Cats appear to be highly sensitive to azathioprine-induced bone marrow suppression. Of five cats given 2.2 mg/kg of azathioprine on alternate days, four developed multiple cytopenia.[8] Azathioprine-induced bone marrow suppression in humans has been associated with homozygosity for the gene for thiopurine methyltransferase.[8] This enzyme plays an important role in the catabolism of azathioprine.

Propylthiouracil/Methimazole

Severe idiosyncratic drug toxicities, consisting of agranulocytosis and severe thrombocytopenia, have been reported in 8.6% of hyperthyroid cats treated with propylthiouracil and 3.8% of cats treated with methimazole.[56,57] Similar types of reactions have been reported for carbimazole, which is a prodrug of methimazole.[74] In addition, mild hematologic alterations, including eosinophilia, lymphocytosis, and mild leukopenia, were reported in 4% of cats treated with methimazole.[57] Clinical signs develop within 1–2 months after initiation of treatment and include lethargy, weakness, anorexia, and platelet-related bleeding diathesis. Many affected cats had positive fluorescent antinuclear antibody and direct Coombs test results, indicating that the hematologic dyscrasia was immune-mediated.[74] These hematologic abnormalities resolved within 2 weeks after discontinuing methimazole treatment.

Griseofulvin

Griseofulvin is a fungistatic antibiotic used for treatment of mycotic diseases.[34] Neutropenia or panleukopenia

occurs as an idiosyncratic reaction to griseofulvin in cats.[34] In most cases, this occurs several weeks after initiation of griseofulvin treatment.[63] The bone marrow is typically hypoplastic.[63] The majority of cats recover after treatment is discontinued.

Albendazole

Albendazole toxicity has been reported in two cats.[71] Both cats were pancytopenic, but had hypercellular bone marrow. The cats had rapid hematologic recovery within 1 week after discontinuing treatment.

Antiviral Agents

Several antiviral agents have been shown to suppress feline bone marrow. Azidothymidine is a dideoxynucleoside derivative reverse transcriptase inhibitor used for treatment of feline immunodeficiency virus and feline leukemia virus infections. A transient or progressive nonregenerative anemia develops after several weeks of treatment.[33] The anemia is primarily the result of bone marrow erythroid hypoplasia, but Heinz body formation may also be a contributing factor. Acyclovir appears to be highly toxic to cats. Treatment of cats with 60 mg/kg body weight resulted in severe bone marrow suppression in addition to renal and hepatic necrosis.[49]

Adverse Drug Reactions in Horses

Mechanisms associated with ADRs in horses include immune-mediated hemolytic anemias (penicillin and trimethoprim–sulfamethoxazole), non-immune-mediated hemolysis (phenothiazine, L-tryptophan, indole, dimethyl sulfoxide), RBC agglutination (heparin), and bone marrow suppression (phenylbutazone).

Drug-Induced Immune-Mediated Hemolysis

Penicillin administration to horses has been associated with acute allergic reactions and with IMHA.[11,70] These allergic reactions consist of acute anaphylaxis, collapse, shaking, urticaria, fever, leukopenia, eosinophilia, thrombocytopenia, and anemia, and are thought to be an adverse response to procaine.[11] Penicillin and its metabolites bind firmly to RBC membranes and in most horses induce only low titers of IgM antipenicillin antibodies, which do not induce hemolysis.[11] Alternatively, some horses induce higher titers of IgG antipenicillin antibodies that induce hemolysis.[11]

One horse developed IMHA during treatment with trimethoprim–sulfamethoxazole.[73] Prompt recovery after withdrawal of drug treatment incriminated the drug in the causation of the anemia.

Drug-Induced Non-Immune-Mediated Hemolysis

Phenothiazine poisoning has been associated with hemolytic anemia in a horse.[6] Although this was thought to be an oxidant-induced hemolysis, few Heinz bodies were identified.

Oral or intravenous administration of L-tryptophan or indole to ponies results in acute hemolytic anemia and hemoglobinuria.[53,54] Indole is the major metabolite of L-tryptophan that induces hemolysis. Indole has lipophilic properties and interacts with RBC membranes, perhaps explaining its hemolytic properties. However, some ponies treated with indole had Heinz bodies in RBCs suggesting a possible Heinz body anemia.

Rapid intravenous administration of a 40% solution of dimethyl sulfoxide results in intravascular hemolysis in horses.[2] The hemolytic effects of dimethyl sulfoxide appear to be concentration-dependent and administration of a more dilute solution significantly reduced or eliminated the hemolysis.[2] The mechanism of dimethylsulfoxide-induced hemolysis is uncertain, but appears to involve potassium leakage from erythrocytes before hemolysis.

Heparin Unfractionated heparin has been reported to cause marked thrombocytopenia as an idiosyncratic reaction in human patients within 4–14 days after initiation of treatment.[31] Heparin appears to exert a direct effect on platelets causing platelet activation and aggregation, and also induces antiplatelet antibodies. In horses, the most frequent complication associated with heparin administration is anemia that is associated with RBC agglutination.[19,46] Heparin may bind to equine RBC and act as a hapten inducing agglutinating anti-RBC antibodies. Rapid increases in hematocrit occur after discontinuing heparin treatment. Because the increase in hematocrit exceeds the expected capacity of the bone marrow to replace erythrocytes, it is suspected that heparin causes in vivo agglutination of RBCs in the microvasculature.

HEAVY-METAL-INDUCED HEMATOPOIETIC INJURY

Several heavy metals, including lead, mercury, and arsenic, cause hematopoietic toxicity in addition to toxicity in other tissues. Zinc and copper toxicities cause oxidant-induced hemolysis and are discussed in Chapter 32.

Lead

Lead poisoning has been documented in dogs, cats, and cattle, as well as in waterfowl and some exotic species. The source of lead exposure varies by species. In dogs and cats, lead-containing paints; in cattle, discarded batteries and industrial contamination of pastures; and in waterfowl, lead shot are the major sources of exposure.[7,40,47] In dogs and cats, anemia can be associated with chronic lead poisoning. In dogs, basophilic stippling and metarubricytosis have been described as classical features. However, these changes appear to be infrequent findings. Collection of blood in EDTA may cause the stippling to disappear. The pathogenesis of the anemia in dogs involves a shortened RBC life span and

dyserythropoiesis. Lead may shorten RBC survival by inhibiting membrane ATPase. Dyserythropoiesis may result from lead interfering with several enzymes in the heme synthesis pathway and inhibiting pyrimidine 5′-nucleotidase that is involved in depolymerization of reticulocyte ribosomal RNA. Resultant retention of RNA in reticulocytes may be responsible for the basophilic stippling seen in some animals.

Mercury

Mercury has hematotoxic and immunotoxic effects.[69] Fish, waterfowl, and shore birds are at greatest risk of exposure to environmental mercury contaminants.[69] Exposure of egrets or ducks to low-dose methylmercury results in anemia with increased bone marrow cellularity.[69] This suggests a hemolytic anemia. Hemolysis may be due to the capacity of mercury to inhibit several RBC enzymes including glutathione reductase, glutathione-6-phosphate dehydrogenase, and acetylcholinesterase. Mercury also induces idiosyncratic immune-mediated hematologic disorders. Exposure of egrets to high-dose methylmercury results in bone marrow hypoplasia and depletion of lymphocytes in lymph nodes and thymus.[69] Mercury has been shown to be cytotoxic to hematopoietic cells and to cause dysmyelopoiesis in laboratory animals.[16]

Arsenic

Arsenic poisoning causes both hemolytic anemia and dyserythropoiesis, and has genotoxic effects. Arsenic in the form of arsine gas is a potent hemolytic agent; however, the main source of exposure is to humans in industrial settings. In the environment, arsenic exists in inorganic forms and the main source of exposure is through contaminated drinking water.[9] Severe poisoning in humans results in marked anemia, and in some cases neutropenia and thrombocytopenia.[24] Arsenic poisoning causes marked dyserythropoiesis characterized by karyorrhexis, megaloblastosis, and multinuclearity. This is thought to result from a direct toxic effect on DNA synthesis that results in altered nuclear division.[24] Results of several studies conclude that inorganic arsenic has genotoxic effects in a variety of laboratory animal species based on the presence of micronuclei and DNA strand breaks observed in bone marrow.[9]

CHEMICAL-INDUCED HEMATOPOIETIC INJURY

Benzene

Benzene and its metabolites are highly toxic to the hematopoietic system.[59] Benzene is widely used as a solvent in the manufacture of drugs, chemicals, dyes, and explosives. Benzene has both cytotoxic and genotoxic effects on the hematopoietic system. A variety of hematologic dyscrasias have been observed including hemolytic anemia, bone marrow hyperplasia, leukopenia, dysmyelopoiesis, aplastic anemia, myelodysplastic syndromes, leukemia, and lymphoma.[59] Concentrations as low as 100 parts per million (ppm) have been associated with leukopenias in approximately one-third of exposed workers.

Experimental inhalation or injection of benzene produces aplastic anemia in rabbits and mice.[59] The aplasia is preceded by dysmyelopoiesis. Despite the typical aplastic marrow and pancytopenia, stem cell numbers in marrow are normal suggesting that committed progenitor cells rather than stem cells are damaged by benzene. However, benzene-treated lethally irradiated marrow is not reconstituted by transplantation of normal marrow. This suggests that benzene damages the marrow microenvironment.

Chlorinated Hydrocarbons

A variety of chlorinated hydrocarbons are toxic to bone marrow and have the potential to cause clinical disease. These drugs include carbon tetrachloride, hexachlorophene, lindane, chlordane, chlorobenzene, chlorophenothane (DDT), chlorinated dioxins, pentachlorophenol, and trichloroethylene. Some of these compounds, including DDT, lindane (i.e., gamma-benzene hexachloride), and pentachlorophenol, are derivatives of benzene. Chlordane, DDT, lindane, and pentachlorophenol have been implicated as causes of aplastic anemia in humans and trichloroethylene has been implicated as a cause of aplastic anemia in cattle (see Chapter 40).[26,36,55] These compounds have multiple injurious effects on hematopoietic tissue. Chlorinated hydrocarbons appear to be toxic to primitive hematopoietic cells. Exposure of mice to various doses of lindane transiently reduced the number of pluripotent stem cells and progenitor cells.[36] In addition, residual long-term progenitor cell depletion has been demonstrated.[65] Dysmyelopoiesis is also a frequent finding associated with chlorinated hydrocarbon toxicity. Many chlorinated hydrocarbons also have genotoxic effects. The genotoxic potential of 1,1-dichloroethane has been evaluated in Swiss–Webster mice by evaluation of mitotic index, micronuclei, and chromosomal aberrations.[55] A dose-related increase in the number of chromosomal aberrations and micronuclei formation was observed.

Chlorinated dioxins cause anemia and progressive leukopenia in a variety of species including chickens, primates, and rats.[50] Chickens appear to be highly sensitive and develop clinical signs after ingestion of feeds containing fats industrially contaminated with dibenzo-*p*-dioxin. Administration of this material to chickens, primates, and rats resulted in hypoplasia of bone marrow and lymphoid tissue in all three species.[50]

Many fatal cases of aplastic anemia in cattle in Minnesota in the 1950s were traced to feeding of trichloroethylene-extracted soybean oil meal.[58] Although no longer used for this purpose, trichloroethylene is widely used as a solvent. In experimental studies, tetrachloroethylene-treated mice had a reduction in erythroid and granulocytic progenitor cells, but no decrease in pluripotent stem cells after inhalation exposure.[72]

Trichloroethylene also has been shown to inhibit delta-aminolevulinate dehydratase, an enzyme involved in heme synthesis, in rats and has genotoxic effects.[65]

PLANT-TOXIN-INDUCED HEMATOPOIETIC INJURY

Mycotoxins

A variety of mycotoxins produce aplastic anemia in animals. Mycotoxin exposure occurs with ingestion of food contaminated with fungal organisms. For animals, this is frequently the result of ingestion of grain that has been allowed to become damp during storage. Aplastic anemia has been documented in horses and cattle ingesting feed contaminated with *Stachybotrys alternans*. Many mycotoxins, including T-2 mycotoxin, trichothecene mycotoxin, trichothecene vomitoxin, ochratoxin, and zearalenone, produce toxicity in humans.[22] These toxins appear to be directly cytotoxic to a variety of cells including hematopoietic precursor cells and lymphocytes. As a result, many mycotoxins cause bone marrow hypoplasia/aplasia and lymphocyte depletion. Depletion of stem cells and granulocyte-macrophage progenitor cells was detected when mice were exposed to ochratoxin.[12] A marked increase in apoptotic hematopoietic cells was detected in bone marrow after injection of T-2 mycotoxin into mice.[68] The effect of mycotoxins on bone marrow and the immune system appears to be amplified by exposure to lipopolysaccharide. Genotoxic effects of some mycotoxins have also been documented in mouse studies.

Bracken Fern

Bracken fern (*Pteridium aquilinum*) is one of the most widely distributed plants on earth. Animals repeatedly ingesting bracken fern develop aplastic anemia (see Chapter 40). Cattle, horses, sheep, and pigs may be affected. The major toxin in bracken is ptaquiloside.[84] Ptaquiloside is a thiaminase.[84] After repeated exposure and thiamine depletion, cattle tend to develop aplastic anemia. This results in anemia, hemorrhage, and increased susceptibility to infection. Other species develop neurological signs due to polioencephalomalacia.

Chronic enzootic hematuria in cattle has also been linked to development of urinary bladder neoplasia after prolonged ingestion of bracken fern.[48] Metabolites of ptaquiloside are carcinogenic and induce bladder and intestinal carcinomas in cattle and other farm animals, as well as in laboratory animals.[48] Lymphocytic leukemia and pulmonary tumors have also been detected in mice exposed to ptaquiloside.

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CHAPTER 16

Acute Myelotoxicity and Myelitis in Domestic and Laboratory Animals

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Acute Myelonecrosis and Myelitis in Domestic Animals
Microvascular Injury
Myelonecrosis
Acute Myelitis
Acute Myelonecrosis and Myelitis in Laboratory Animals
Myelonecrosis
Acute Myelitis

Acronyms and Abbreviations

DNA, deoxyribonucleic acid; IMHA, immune-mediated hemolytic anemia; RBC, red blood cell.

ACUTE MYELONECROSIS AND MYELITIS IN DOMESTIC ANIMALS

As a rapidly proliferating tissue, bone marrow is sensitive to toxic injury. Components most vulnerable to injury include progenitor and proliferating hematopoietic cells, and the microvasculature. Microvascular injury results in altered vascular permeability and manifests as edema, hemorrhage, inflammation, or ischemia.[23] Myelonecrosis occurs when ischemia is severe, and in the chronic stage, necrotic areas may become fibrotic.[7,15,20] If the initiating agent is eliminated and blood flow is reestablished, myelofibrosis is reversible and regeneration of the hematopoietic tissue is possible.

Hematopoietic cell injury can result in hematopoietic cell death (necrosis or apoptosis) at various stages of maturation and may result in maturation arrest with or without dysplastic changes.[17] Hematopoietic cell injury usually results in reduced hematopoietic marrow cellularity. In the acute stages of hematopoietic injury, myelotoxic changes are frequently observed in the cytoplasm of metamyelocytes and band neutrophils. Common morphologic abnormalities include foamy basophilic cytoplasm (RNA clumping) and/or the presence of Döhle bodies.[20] Some myelotoxic compounds also can induce distinct cytoplasmic vacuolation (Figure 16.1).[20] Toxic changes to the nucleus may consist of hyper and/or bizarre segmentation of neutrophils. Nuclear blebbing may be observed among late-stage erythroid precursors. Other morphologic features of hematopoietic toxicity include cells that stain poorly and/or have indistinct nuclear or cytoplasmic borders. Donut-shaped nuclei are a normal finding in rodents, but would be considered abnormal in domestic animals and primates. Donut-shaped nuclei have been reported in dogs in association with an acute inflammatory response.[16,19] Arrested maturation frequently is characterized by reduced cell counts in the blood, a cellular bone marrow with maturation arrest, and/or dysplastic changes in one or more cell lines. Dysplastic changes can be seen as nuclear or cytoplasmic maturation defects. Nuclear maturation defects result from interference with DNA synthesis and may include megaloblastosis, atypical mitotic figures, fragmented nuclei, nuclear blebbing, and bi/multinucleation. Dysplastic cytoplasmic changes are most frequently seen in erythroid precursor cells where hemoglobin production is inhibited.

Microvascular Injury

The bone marrow microvasculature also is susceptible to toxic injury.[6,18] This is supported by experimental studies in which mouse bone marrow was evaluated after injection of endotoxin and rabbit bone marrow was

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evaluated after saponin injection.[8,26] Early ultrastructural lesions in bone marrow were characterized by degeneration of sinusoidal endothelial cells. Later lesions consisted of dilated sinusoids, interstitial edema, hemorrhage, and necrosis. Chronic lesions consisted of myelofibrosis.

Similar lesions have been described in humans and a variety of animal species.[1,4,9,14,18,25] Acute vascular injury is accompanied by dilated sinusoids, interstitial edema, and hemorrhage.[4,18] Acute inflammation is characterized by exudation of fibrin that may be accompanied by an infiltration of neutrophils.[4,25] Vascular injury also results in varying degrees of ischemia. When severe, ischemia induces ischemic necrosis.

Myelonecrosis

Myelonecrosis is usually associated with bone marrow ischemia, although it has also been associated with the administration of certain antineoplastic pharmaceutical agents (i.e., presumably direct cytotoxicity).[1,7,20] It is important to distinguish true degenerative changes from those associated with postmortem autolysis; prominent shrinkage and condensation of megakaryocytic nuclei are common features of the latter.[19]

In histopathologic sections, myelonecrosis can appear as multifocal areas of coagulation-type necrosis or as individual necrotic cells (Figure 16.1).[20] Coagulation-type necrosis appears as a central area of karyorrhexis and pyknotic cells enmeshed in an amorphous eosinophilic background material. Macrophages are frequently increased in number. Severe myelonecrosis may be accompanied by osteonecrosis (Figure 16.1). Osteonecrosis is characterized by loss of osteocytes, osteoblasts, and osteoclasts in bone as well as a homogeneous appearance of bone with loss of striations. In some cases, the surface of the bone may become irregular and moth-eaten in appearance (Figure 16.1). For dogs, severe myelonecrosis and osteonecrosis are frequently associated with leg pain and even reluctance to walk.

Coagulation-type necrosis occurs relatively frequently in dogs, but is rarely seen in cats.[21] Alternatively, in one study, individual cell necrosis was observed in 8% of cat clinical bone marrow specimens and is characterized by large numbers of degenerating, frequently anuclear, hematopoietic cells and increased pink amorphous background material (Figure 16.2).[21]

In the acute stage of either coagulation or individual myelonecrosis, necrosis is frequently accompanied by acute inflammation or hemorrhage, whereas in subacute myelonecrosis, necrosis is associated with phagocytic macrophages presumably removing the necrotic cells. In chronic myelonecrosis, reticulin or collagen myelofibrosis is frequently present.

Myelonecrosis is difficult to appreciate in bone marrow aspiration smears. The necrotic marrow smears unevenly, resulting in irregular elongated clumps of necrotic cells. Microscopically, slides often have a diffuse amorphous eosinophilic background and cells stain poorly. Many of the cells are lysed or have a morphologic appearance that makes definitive identification impossible. Differentiating these necrotic features from poor collection techniques is problematic.

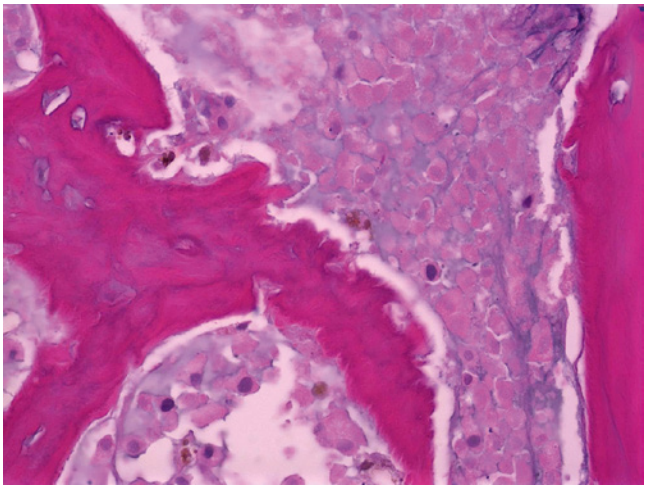


FIGURE 16.1 Diffuse coagulation necrosis with karyorrhexis and some pyknotic cells accompanied by osteonecrosis in a bone marrow biopsy section from a dog. Osteonecrosis is characterized by irregular border to trabeculae and loss of osteocytes, osteoblasts, and osteoclasts. H&E stain.

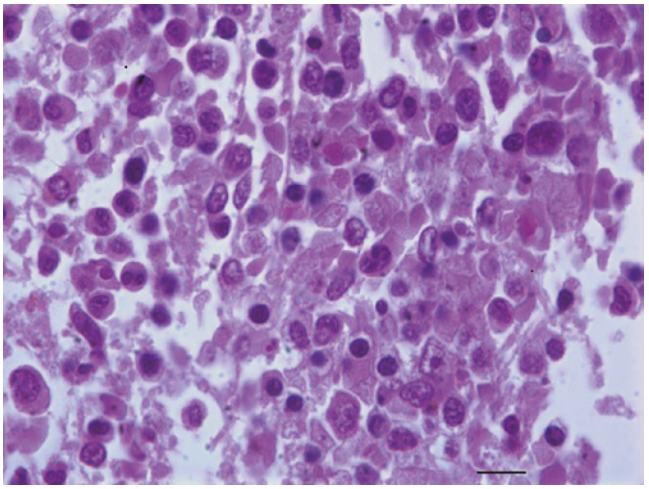


FIGURE 16.2 Individual cell necrosis in a core biopsy section from a cat with nonregenerative immune-mediated hemolytic anemia. Note the presence of many anuclear cells, cells with poorly staining nuclei, and cellular debris. H&E stain. (Douglas J. Weiss.)

Frequent causes of myelonecrosis in cats and dogs include immune-mediated hemolytic anemia (IMHA), sepsis, and leukemia/lymphoma. Less frequent causes identified in dogs include idiopathic causes, systemic lupus erythematosus, disseminated intravascular coagulopathy, and drug treatments.[20,22] Drugs associated with myelonecrosis include estradiol, phenobarbital, carprofen, mitotane, colchicine, metronidazole, fenbendazole, cephalosporins, and chemotherapeutic agents including cyclophosphamide and vincristine (see Chapter 15).

The mechanisms responsible for myelonecrosis have not been extensively investigated. In immune-mediated diseases and disseminated intravascular coagulopathy, thrombotic events may induce marrow ischemia. The necrosis associated with systemic lupus erythematosus in humans has been attributed to the presence of antiphospholipid antibodies. In sepsis, endotoxin or other bacterial toxins may induce sinusoidal injury as well as being toxic to hematopoietic precursor cells. Leukemic cells in bone marrow frequently outgrow their blood supply resulting in myelonecrosis.

Dogs and cats with myelonecrosis have various combinations of nonregenerative anemia, leukopenia, and thrombocytopenia.[20,22] In some instances, the cytopenias may be the result of myelonecrosis, whereas, in other animals, they are the result of a combination of the primary disease process and myelonecrosis. Treatment and prognosis for myelonecrosis should be based on the cause of the necrosis.[20,22] In general, if the primary condition can be successfully treated, the prognosis for recovery is good.[20]

Acute Myelitis

Acute myelitis, like myelonecrosis, is thought to be associated with acute injury to bone marrow sinusoids.[4] These changes are only visible in bone marrow histopathologic sections. In one study, acute myelitis was observed in approximately 3% of canine clinical bone marrow specimens.[23] Altered vascular permeability was the initial lesion identified in bone marrow. Histopathologic alterations include dilated or congested sinusoids, interstitial edema, and hemorrhage. Interstitial edema is seen as amorphous pink proteinaceous material between hematopoietic cells (Figure 16.3).[23,24] Pathologic hemorrhage must be differentiated from artifactual hemorrhage induced by the biopsy procedure. When the sample is obtained by needle biopsy, artifactual hemorrhage is frequently present on either end of the biopsy specimen and may be present on the edges of the specimen. Pathologic hemorrhage should be observed within the hematopoietic space away from the ends or edges of the specimen and should be accompanied by evidence of vascular alterations or interstitial edema. These alterations cannot be reliably evaluated in bone marrow specimens obtained post mortem because dilated sinusoids and altered permeability can occur as agonal changes or perhaps be induced by administration of euthanasia solution.

Acute microvascular injury also can result in exudation that is characterized by multifocal fibrin deposits or multifocal infiltrates of neutrophils (Figure 16.4).[23,24] Fibrin deposits are particularly prominent in cat bone marrow.[22,24] Fibrin appears as pink fibrillar material. The presence of fibrin can be confirmed by selective staining with Fraser–Lendrum stain.[25] Frequently, these fibrin deposits contain variable numbers of segmented neutrophils. In cases of sepsis, discrete microabscesses may be present in bone marrow.[23] These structures may represent septic thrombi.

Causes of acute myelitis in dog bone marrow include nonregenerative IMHA, systemic lupus erythematosus, and bacterial sepsis; in cat, they include IMHA and bacterial sepsis.[22–24]

In horses and cattle, acute myelitis has been associated with bacterial sepsis.[25] Acute myelitis has been identified in septicemic foals. Lesions are characterized by large multifocal infiltrates of neutrophils resembling microabscesses. Neutrophils in the lesions may appear degenerate and the centers of the microabscesses may be necrotic. Other lesions included dilated sinusoids, hemorrhage, and fibrin deposition. Bacteria are usually not seen in bone marrow sections, but can, in some cases, be cultured from bone marrow. Perivascular neutrophilic infiltrates in bone marrow were reported in a steer with chronic pneumonia caused by *Mannheimia haemolytica*.[24] Other cattle with a diagnosis of bacterial pneumonia and septicemia had fibrin deposits in bone marrow.

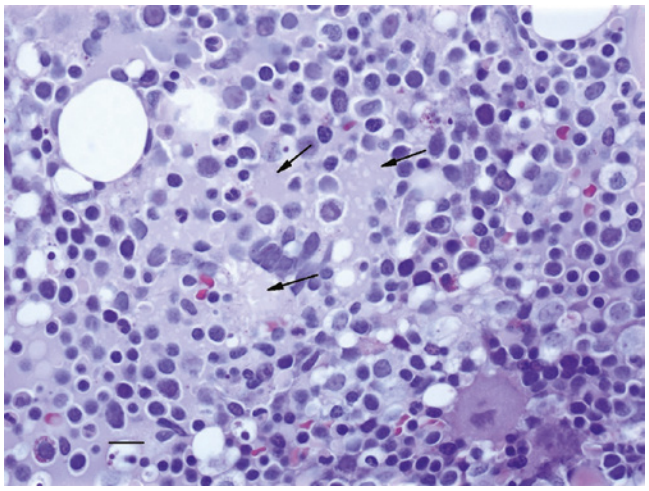


FIGURE 16.3 Interstitial edema in a bone marrow core biopsy section from a dog with immune-mediated hemolytic anemia. H&E stain. (Douglas J. Weiss.)

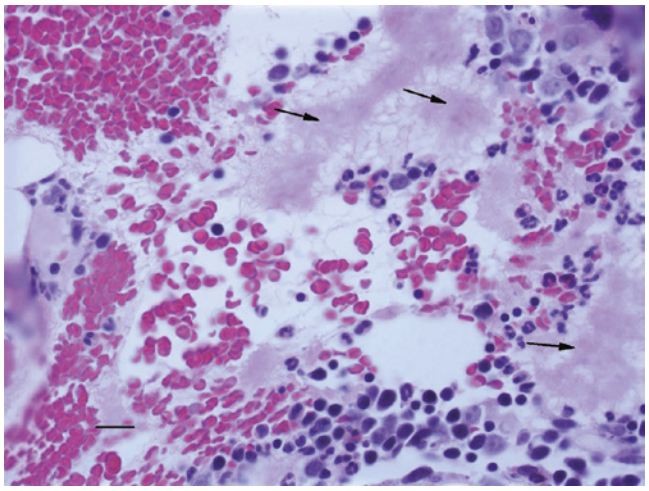


FIGURE 16.4 Fibrinous inflammation in a bone marrow core biopsy section from a dog with immune-mediated hemolytic anemia. Note the presence of fibrin and hemorrhage. H&E stain.

Eosinophilic inflammatory responses are less well-defined. A cow and a sheep with fibrinous inflammation and large numbers of eosinophils in bone marrow have been described.[24] The cow had bacterial enteritis and the sheep had a systemic vasculitis of unknown origin.

ACUTE MYELONECROSIS AND MYELITIS IN LABORATORY ANIMALS

Myelonecrosis

In laboratory animals, degeneration/necrosis of bone marrow tissues may be a direct consequence of test compound administration, but also can be a sequela of an infectious (e.g., bacterial sepsis) or neoplastic disease processes.[5,11,12,19] Similar to domestic animals, these lesions may be focal or multifocal and are characterized by karyorrhexis, karyolysis, cytoplasmic vacuolation, and/or nuclear pyknosis of hematopoietic cells. In early-stage lesions, findings may be limited to hematopoietic cells that poorly take up stain and/or have indistinct cell borders.[2] Varying degrees of associated inflammatory cells, hemorrhage, sinusoidal dilation, and edema may also be seen. Ischemic bone marrow injury resulting in necrosis does occur in laboratory animals and is most often the result of vascular pathology and/or infarction as related to disseminated intravascular coagulation, thrombosis, malignant neoplasia, or sepsis.[2] In almost all cases of bone marrow injury, associated decreases in circulating blood cells affecting one or more cell lines will be present. Peripheral cytopenias often precede or are a more sensitive indicator of marrow hypocellularity as observed histologically.

In rodents, prolonged and severe dietary restriction/anorexia (i.e., <25% of ad libitum food intake for 1–2 weeks) is associated with myelonecrosis involving hematopoietic and stromal bone marrow elements (Figure 16.5).[10,13] This phenomenon is relatively common in toxicology studies; however, it is often difficult to make a distinction between direct compound-related marrow injury and effects secondary to general moribundity/anorexia, hence, lesions solely related to decreased food intake are rarely encountered. In rats, peripheral blood reticulocyte counts are generally the most sensitive indicator of the myelodegenerative effects of severe anorexia/feed restriction (i.e., ≥25% reductions in food consumption), and often become evident before the appearance of histologic alterations.[10,13] Given sufficient time, reduced reticulocyte counts can result in overt decreases in circulating red cell mass; however, shorter fasting periods (48 hours or less) may actually cause an initial increase in red cell mass as a reflection of hemoconcentration.[13] Bone marrow degeneration/necrosis also has been described in cachectic animals, and as sequela of general moribundity in severely debilitated rats.[12]

Bone marrow necrosis, hypocellularity, and/or stromal proliferation have been described in several laboratory animal species in response to a variety of anticancer compounds.[3,11] Bone marrow lesions most often include decreases in hematopoietic cellularity with varying degrees of marrow necrosis, fibrin deposits, stromal or myelostromal proliferation, and/or new woven bone formation. Inflammation was not noted as a significant component of these lesions.

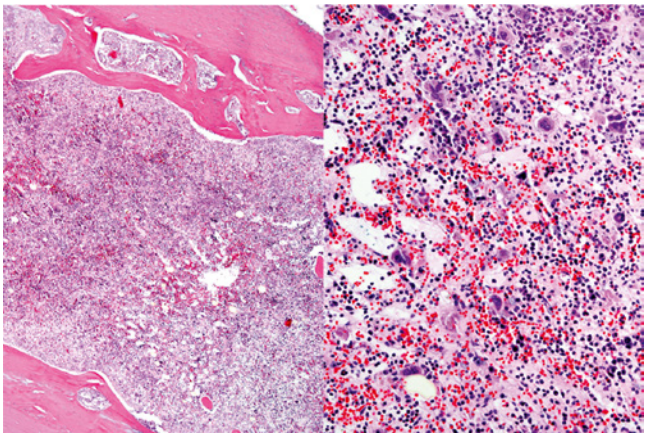


FIGURE 16.5 Myelonecrosis and hemorrhage in a rat. Note the paucity of myeloid and erythroid cells and the presence of karyorrhexis, karyolysis, and nuclear pyknosis of hematopoietic cells. Extravasation of erythrocytes and edema are also present. H&E stain (original magnification: Left 40× Right 200×) (Images courtesy of Michelle Cora, National Toxicology Program).

Acute Myelitis

Although it is widely recognized that bone marrow is exquisitely sensitive to a variety of drugs, test compounds, and toxins, acute myelitis is an uncommon finding in laboratory animals involved in nonclinical toxicology studies. The timing of tissue collections in toxicology studies likely contributes to this, as study designs and necropsy practices are not usually aimed at capturing the early stages of bone marrow effects. Acute inflammation of bone marrow in laboratory animals most often occurs secondary to systemic bacterial sepsis, which may be incidental, or secondary to experimentally induced immunosuppression.[2,12,19] Lesions are characterized by the presence of neutrophils (degenerate and/or nondegenerate), amorphous cell debris, edema, hemorrhage, and fibrin, with fewer numbers of macrophages, plasma cells, and/or small lymphocytes. Hematology findings are typically consistent with an acute inflammatory response as related to sepsis. Acute myelitis is generally not a significant component of the marrow response to myelotoxic effects of anticancer compounds.[3,11]

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CHAPTER 17

Chronic Inflammation and Secondary Myelofibrosis in Domestic and Laboratory Animals

ADAM D. AULBACH and DOUGLAS J. WEISS

Chronic Inflammation and Myelofibrosis in Domestic Animals
- Chronic Inflammation
  - Lymphocytosis
  - Lymphoid Aggregates
  - Plasmacytosis
  - Macrophage Proliferation
- Myelofibrosis
  - Osteosclerosis
  - Serous Atrophy of Fat/Gelatinous Transformation

Chronic Inflammation and Myelofibrosis in Laboratory Animals
- Chronic Inflammation
- Myelofibrosis
- Myelostromal Proliferation

Acronyms and Abbreviations

FeLV, feline leukemia virus; IMHA, immune-mediated hemolytic anemia; PRCA, pure red cell aplasia.

Chronic bone marrow injury can occur as a sequela of myelonecrosis or acute myelitis (see Chapter 16), be associated with chronic conditions, or result from prolonged or recurrent exposure to a toxic agent.[17,22,30] These chronic responses in bone marrow include chronic inflammatory responses (e.g., lymphocytosis, plasmacytosis, histiocytic proliferation, and granulomatous inflammation), secondary myelofibrosis, osteosclerosis, osteomyelitis, and serous atrophy of fat/gelatinous transformation.[2,10,32,42] As with acute bone marrow injury, many of these lesions are difficult or impossible to detect in bone marrow aspirates. Therefore, it is essential to evaluate both bone marrow aspirates and histopathologic sections to detect chronic bone marrow injury.

CHRONIC INFLAMMATION AND MYELOFIBROSIS IN DOMESTIC ANIMALS

Chronic Inflammation

Lymphocytosis

In general, domestic animals and avian species typically have less than 10% lymphocytes in cellular bone marrow specimens.[31] However, reference intervals reported for the percentage of lymphocytes in cat bone marrow vary with most indicating that up to 15% should be considered normal.[16,35] In healthy animals, most lymphocytes in bone marrow are small lymphocytes. Benign

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lymphocytosis is the most frequent type of chronic inflammation in cats, but occurs infrequently in dogs.[36,37] In a retrospective study of 203 consecutive feline bone marrow specimens, lymphocytosis (defined as lymphocytes >15% of all nucleated cells) was observed in 15.6% of the specimens.[36] In some cats, lymphocyte numbers exceeded 50% of all nucleated cells in bone marrow.[35] Greater than 80% of cats with lymphocytosis had a diagnosis of nonregenerative immune-mediated hemolytic anemia (IMHA) or pure red cell aplasia (PRCA).[35,36] Differentiating benign lymphocytosis from chronic lymphocytic leukemia can be problematic, but lymphocyte distribution and phenotype may be helpful. Benign lymphocytes are frequently arranged in small multifocal aggregates in bone marrow, whereas, in chronic lymphocytic leukemia, they are diffusely distributed throughout the marrow.[35,43] Immunophenotyping of a few cases has revealed that most lymphocytes in reactive lymphocytosis are B cells, whereas malignant lymphocytes in chronic lymphocytic leukemia were T cells.[35,43]

Unlike cats, only 1% of canine clinical bone marrow specimens had lymphocytosis.[37] These cases were associated with immune-mediated diseases including IMHA and PRCA. Therefore, at least in the Northern USA, bone marrow lymphocytosis in both cats and dogs correlated with immune-mediated hematologic diseases.

Lymphoid Aggregates

In a review of 17 species of mammals, lymphoid aggregates were identified only in humans and cats.[11] Lymphoid aggregates are small clusters of mature small lymphocytes with no evidence of organized structure (Figure 17.1). However, results of large retrospective studies indicate that lymphoid aggregates occur in both cat and dog bone marrow core biopsy specimens.[37,38] When present, lymphoid aggregates correlate with the presence of immune-mediated hematologic diseases or other chronic systemic immunologic stimulation.[35,41]

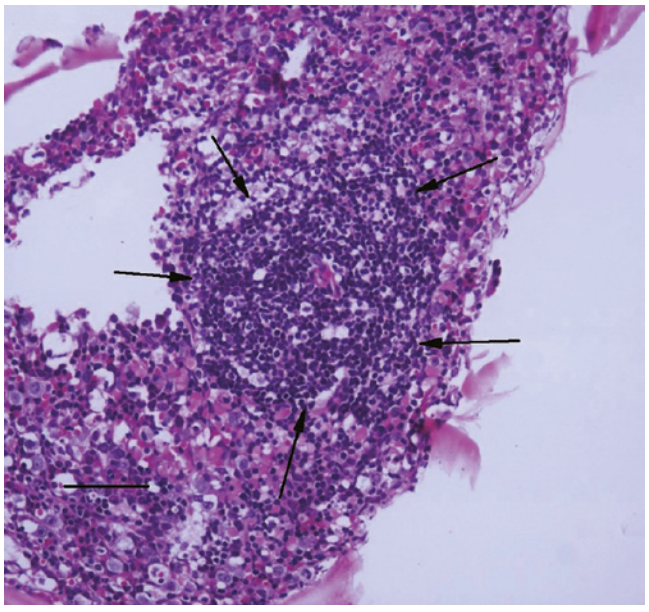


FIGURE 17.1 Lymphoid aggregate (arrows) in a bone marrow core biopsy section from a cat with nonregenerative immune-mediated hemolytic anemia. H&E stain; bar, 200 µm.

Plasmacytosis

Plasmacytosis is usually evaluated subjectively in bone marrow aspirates or core biopsy specimens because of their low numbers and uneven distribution. Plasmacytosis has been observed in approximately 10% of canine clinical bone marrow specimens and 6% of cat bone marrow specimens.[37,38] Plasmacytosis in cats has been associated with IMHA, PRCA, immune-mediated thrombocytopenia, and feline infectious peritonitis. Plasmacytosis is a consistent finding in bone marrow of dogs with immune-mediated diseases including IMHA, PRCA, and immune-mediated thrombocytopenia. Other causes of bone marrow plasmacytosis that should be considered include *Ehrlichia* spp. and *Leishmania* spp. infections.

Macrophage Proliferation

The presence of increased numbers of macrophages in bone marrow is associated with a variety of disorders. Macrophages can range from relatively immature cells with minimal phagocytosis to mature cells that have a foamy cytoplasm or contain phagocytic vacuoles. These phagocytic vacuoles may contain cells, cytoplasmic debris, hemosiderin, or organisms. Four general types of mononuclear phagocyte proliferations can be observed in bone marrow. Differentiating these types can be problematic and may require the use of flow cytometry or immunophenotyping.[34] The four types include reactive macrophage proliferation, reactive hemophagocytic syndrome[39] (see Chapter 49), lipid storage diseases (see Chapter 49), and malignant proliferations of monocytes (acute monocytic or myelomonocytic leukemia, chronic myelomonocytic leukemia; see Chapters 66 and 68) and dendritic cells (malignant histiocytosis, see Chapter 74). Reactive macrophage proliferations are macrophages responding to a need to remove necrotic debris or infectious agents. Increased necrotic debris is present in the subacute stages of myelonecrosis and in conditions of ineffective hematopoiesis.[36] Ineffective hematopoiesis is present in myelodysplastic syndromes, immune-mediated hematologic diseases, and in some leukemias.

A variety of infectious agents cause reactive macrophage proliferations in bone marrow. These vary with the species and geographic distribution of infectious organisms. In dogs, leishmaniasis, parvovirus infection, and deep fungal infections have been associated with macrophage proliferations (see Chapter 18).[4] In leishmaniasis and deep fungal infections, macrophages appear to be part of an inflammatory response in that organisms are found in the marrow in these conditions. Additionally, the presence of increased numbers of neutrophils is consistent with a diagnosis of a pyogranulomatous inflammation.

In parvovirus infection, macrophages may be reacting to the need to remove necrotic cells.

Granulomatous inflammation typically consists of a mixed infiltrate of macrophages, giant cells, small lymphocytes, and plasma cells, and may form distinct granulomas. Granulomas are distinct compact aggregates of inflammatory cells (Figure 17.2). These may contain epithelioid macrophages that have a large amount of pale cytoplasm and elongated nuclei with a dispersed chromatin pattern. Epithelioid cells may fuse to form giant cells. Other cells associated with the granuloma may include lymphocytes, plasma cells, and neutrophils. Discrete granulomas are infrequently seen in bone marrow of domestic animals.[36,37] Granulomatous inflammation has been associated with disseminated histoplasmosis in dogs and cats and *Mycobacterium avium* subsp. *avium* infection in dogs (see Chapter 18). A horse with an idiopathic systemic granulomatous disease was reported.[42] Lipid granulomas were observed in a cow with serous atrophy of fat.[42] Lipid granulomas consist of aggregates of lipid-laden macrophages. Other cells that may be present in lipid granulomas include lymphocytes, plasma cells, epithelioid cells, and giant cells. In humans, bone marrow granulomas have been associated with a variety of drug hypersensitivities. These drugs include phenytoin, procainamide, phenylbutazone, chlorpropamide, sulfasalazine, ibuprofen, indometacin, allopurinol, and carbamazepine.[2]

Myelofibrosis

Myelofibrosis is a bone marrow disorder characterized by proliferation of fibroblasts or collagen or reticulin fibers in the hematopoietic space (Figure 17.3).[1,2] Secondary myelofibrosis must be differentiated from primary idiopathic myelofibrosis. Primary idiopathic myelofibrosis is a relatively rare chronic myeloproliferative disease characterized by trilineage extramedullary hematopoiesis throughout the body and myelofibrosis.[13,14,21] In human primary idiopathic myelofibrosis, the myelofibrosis appears to occur secondary to the myeloproliferative disease and may be induced by secretion of platelet-derived growth factor, transforming growth factor-beta, and/or platelet factor-4 by megakaryocytes.

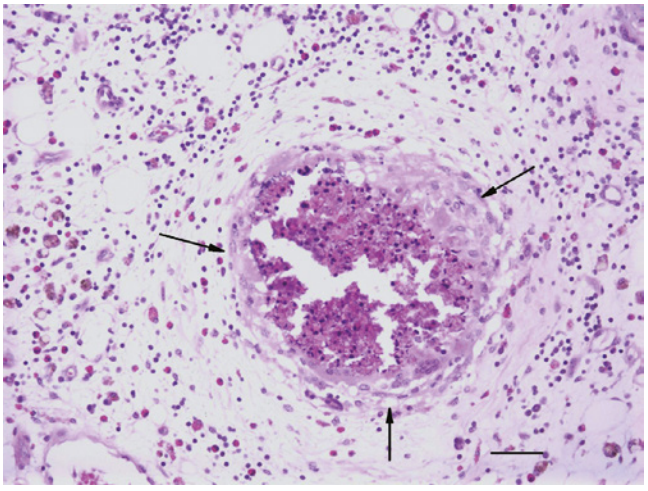


FIGURE 17.2 Granuloma in a bone marrow core biopsy section from a dog with a disseminated fungal infection. H&E stain; bar, 200 µm.

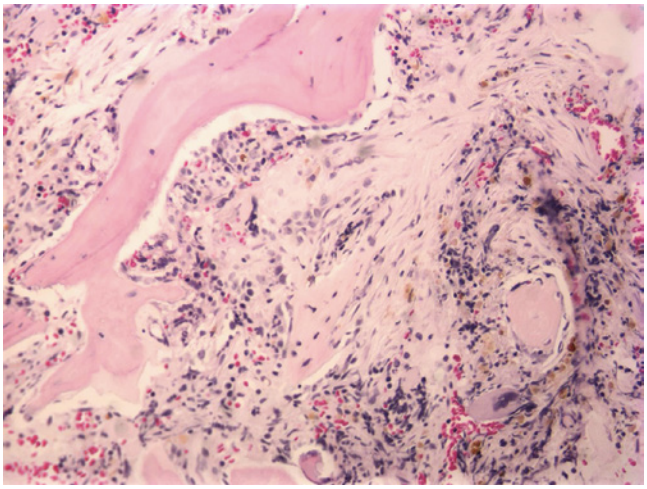


FIGURE 17.3 Secondary myelofibrosis in a bone marrow core biopsy section from a dog. H&E stain.

The etiopathogenesis of secondary myelofibrosis is uncertain. The coexistence of myelofibrosis and myelonecrosis in many dog and cat bone marrow specimens strongly suggests that fibrosis may be equivalent to scar tissue in other damaged tissues. Several fibrogenic cytokines have been identified. These include transforming growth factor-β, platelet-derived growth factor, platelet factor-4, and epidermal growth factor. In bone marrow, fibrogenic cytokines are produced by megakaryocytes and macrophages. Constant high levels of thrombopoietin in mice induce megakaryocyte hyperplasia and myelofibrosis.[7,44] Myelofibrosis has been induced in rodents by injection of a variety of cytokines, including thrombopoietin, erythropoietin, and megakaryocyte growth factor.[44,45] Increased megakaryocytes or dysmegakaryopoiesis has been seen infrequently in dogs with secondary myelofibrosis. Hemosiderosis has also been incriminated as a cause of secondary myelofibrosis.

Secondary myelofibrosis occurs relatively frequently and is associated with moderate to severe nonregenerative anemia and less frequently with thrombocytopenia or leukopenia.[33] If the causative agent is removed or effectively treated, the fibrosis is typically reversible with the tissue reverting to normal hematopoietic tissue.[28,33] Collagen and fibroblastic myelofibrosis has been observed in 4.2% of canine clinical bone marrow specimens and in 9% of feline clinical bone marrow specimens evaluated at a veterinary teaching hospital.[36,37] The distribution of fibrosis in marrow sections varies. Fibrosis can be multifocal or diffuse and randomly distributed or paratrabecular. Reticulin fibers are most frequently diffusely distributed in the marrow, but in some early lesions can be focally distributed around blood vessels.

In dogs, secondary myelofibrosis has been associated with IMHA, acute leukemias/lymphomas, idiopathic myelonecrosis, congenital pyruvate kinase deficiency, congenital nonspherocytic anemia, nonhemic malignant tumors, whole body irradiation, and certain drug treatments (see Chapters 15 and 16).[18,26,33,38,42] Most affected dogs have a moderate to severe nonregenerative anemia, some are thrombocytopenic, but leukopenia is a rare finding. Ovalocytes are infrequently seen in the peripheral blood. In one study of 19 dogs with secondary myelofibrosis, half of the dogs recovered from their anemia with supportive therapy. Treatments with immunosuppressive doses of prednisone or with erythropoietin may be useful.

A periparturient myelofibrosis, associated with severe nonregenerative anemia, has been reported in young female beagle dogs.[20] Myelonecrosis was identified in one of three dogs reported; therefore, the fibrosis may have occurred secondary to acute bone marrow injury.

Secondary myelofibrosis is a frequent finding in cat bone marrow specimens.[3,38] Both collagen and reticulin fibrosis are frequent causes of failure to obtain a sample when aspirating cat bone marrow. Major causes of secondary myelofibrosis in cats include myelodysplastic syndromes, acute myeloid leukemia, IMHA, feline infectious peritonitis, and chronic renal failure.[3,33]

Myelofibrosis has been reported as an inherited disorder in pygmy goats.[5] At birth, these goats have a nonregenerative anemia, neutropenia, and thrombocytopenia. All animals die at 6–12 weeks of age. Severe myelofibrosis was identified at necropsy. Other necropsy findings included extramedullary hematopoiesis and megakaryocytic hyperplasia and dysplasia.

Osteosclerosis

Osteosclerosis (also termed myelosclerosis) is a condition of excessive and unorganized production of trabecular bone. Alternatively, osteopetrosis is a congenital condition of dogs and humans characterized by severe nonregenerative anemia or pancytopenia and generalized increased bone density.[15] Osteosclerosis appears as irregular fingers or spurs of bone extending from the trabeculae into the hematopoietic space (Figure 17.4). These sites usually contain increased numbers of osteoblasts and osteoclasts.[18] When severe, the associated marked thickening of bone can be visualized radiographically.[9,12,18] Osteosclerosis is almost always associated with concurrent severe myelofibrosis. Severe osteosclerosis can obliterate or markedly reduce the size of the hematopoietic space. Osteosclerosis along with severe myelofibrosis occurs terminally in dogs with pyruvate kinase deficiency and has been associated with feline leukemia virus (FeLV) infection.[9,12,22]

Serous Atrophy of Fat/Gelatinous Transformation

The paratrabecular space within normal marrow is occupied almost totally by hematopoietic cells or adipocytes. In processing paraffin-embedded tissues, the lipid is dissolved out and the space within adipocytes appears clear and homogeneous. Serous atrophy of fat/gelatinous transformation is a condition in which there is loss of fat cells as well as depletion of hematopoietic cells from bone marrow and replacement by increased amounts of ground substance (Figure 17.5).[25] This is usually associated with starvation. Remaining adipocytes, depleted of fat, tend to take on a pink to blue hue. Acid mucopolysaccharides in the background may stain pink or blue with H&E stain and stain with Alcian blue and periodic acid shift stains.[25] This material may appear granular or fibrillar.

Serous atrophy of fat/gelatinous transformation has been documented in sheep, goats, primates, rodents, and cats. In sheep and goats, it has been associated with neglect or loss of teeth. In cats, it has been associated with prolonged anorexia due to chronic disease.[40,41] Affected cats had pancytopenia in the blood and marked hypoplasia of bone marrow. Associated conditions included chronic renal failure and oral and gastric ulcers.

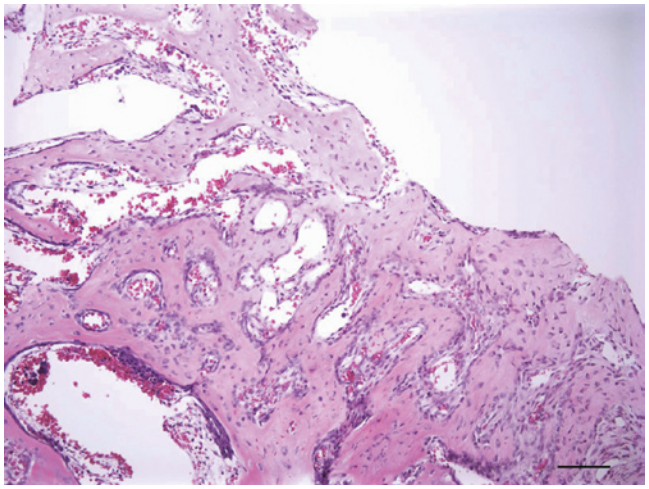


FIGURE 17.4 Osteosclerosis in a bone marrow core biopsy section from a feline-leukemia-virus-infected cat. H&E stain.

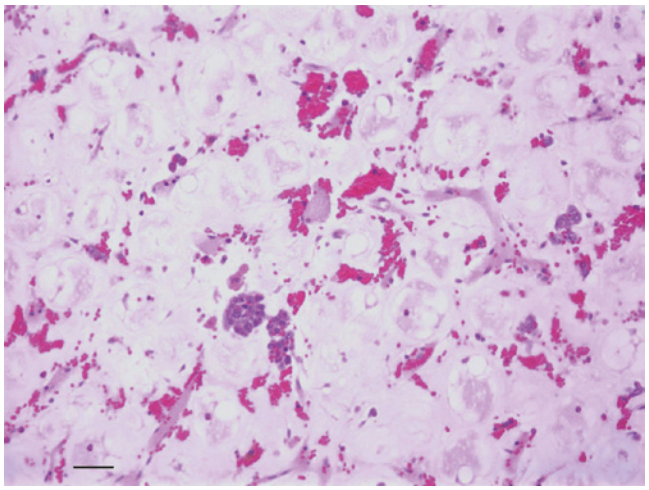


FIGURE 17.5 Gelatinous transformation in a bone marrow core biopsy section from a cat with chronic renal failure. H&E stain.

CHRONIC INFLAMMATION AND MYELOFIBROSIS IN LABORATORY ANIMALS

Chronic Inflammation

Increased numbers of macrophages can be seen in the bone marrow of laboratory animals in response to test compound-related cytotoxicity, or as a background incidental finding.[8] Granulomatous inflammation has been reported in rodents as a background finding, but also as a direct compound-related effect. Lesions usually consist of focal or multifocal aggregates of epithelioid macrophages that are sometimes vacuolated, with or without the presence of multinucleated giant cells and phagocytized cell debris (Figure 17.6). As lesions become more chronic, hemosiderin-laden macrophages or cholesterol clefts may be seen.

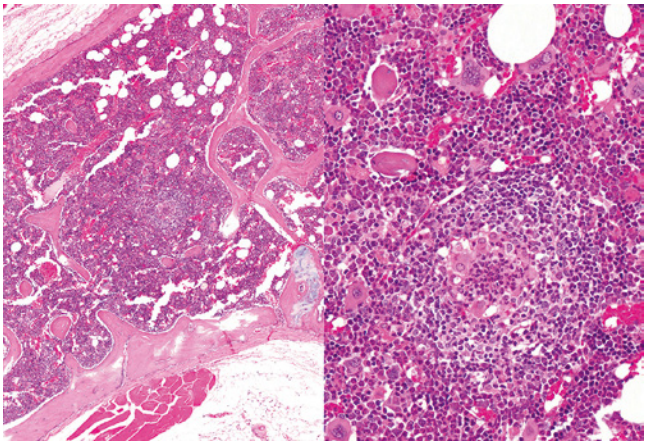


FIGURE 17.6 Granulomatous inflammation in a mouse. Note the presence of degenerate neutrophils and cellular debris surrounded by epithelioid macrophages. H&E stain (left 8× right 40×). (**Source:** Images courtesy of Michelle Cora, National Toxicology Program.)

Myelofibrosis

Fibrosis of the bone marrow has been reported in various forms in laboratory animals. Myelofibrosis can be seen as a background finding in aging rats, but also a consequence of compound administration, whole body irradiation, and certain viral infections.[19,27,29] As opposed to the human condition, primary idiopathic myelofibrosis as a specific condition has not been documented in rodents. Lesions may be focal or multifocal and are characterized by an increase in fibroblasts, stromal cells, and eosinophilic reticulin/collagen fibers (Figure 17.7).[8] In aged mice, myelofibrosis was observed in 43% of female mice, but in <1% of male mice.[24] In another study of aged NMRI mice, >90% of females and ovariectomized females had evidence of myelofibrosis as well as some castrated males.[23] This study indicates that estrogen may not be a major factor in development of myelofibrosis in mice.

In toxicology studies, fibrosis is occasionally seen in association with bone marrow cytotoxicity, but is frequently associated with test compounds that stimulate megakaryocyte or platelet production.[19] Examples include thrombopoietic agents (e.g., romiplostim) and polyethylene glycolylated peptide MPL (myeloproliferative leukemia virus oncogene) agonists. Similar to other species, cytokine products of megakaryocytes, platelets,

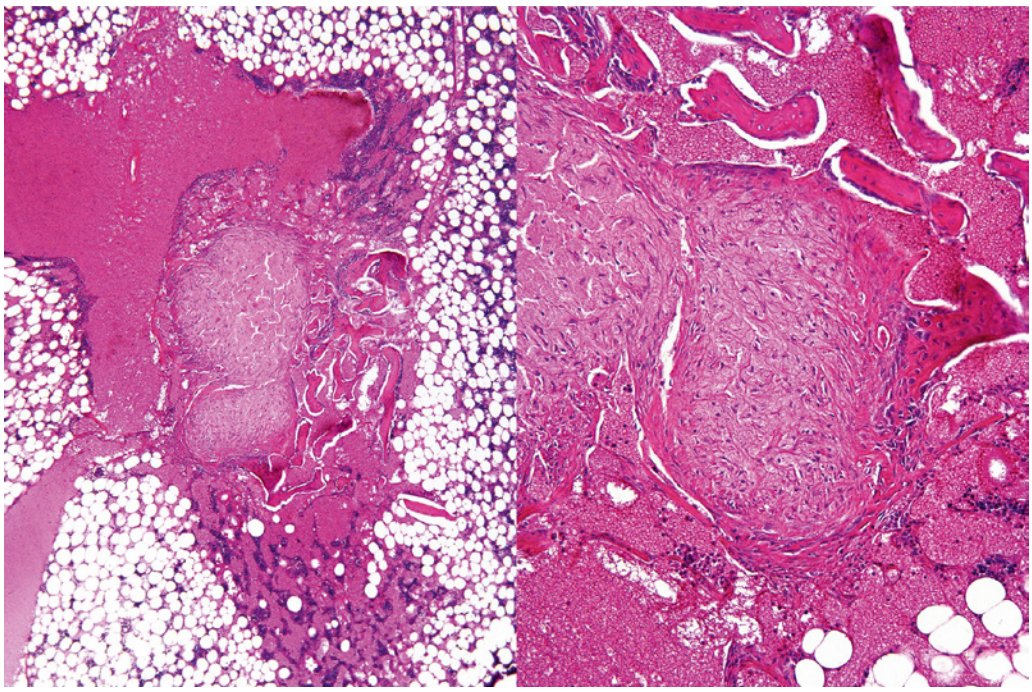


FIGURE 17.7 Fibrosis and hemorrhage in the bone marrow of a rat. Note the presence of increased trabecular bone around and adjacent to the area of fibrosis. H&E stain (original objective magnification: left 6× right 16×). (**Source:** Images courtesy of Michelle Cora, National Toxicology Program.)

and monocytes (e.g., transforming growth factor-β and platelet-derived growth factor) have been implicated as contributing to the development of myelofibrosis in rodents.[14] A myelofibrosis-like condition was described in genetically modified mice that have a deletion in megakaryocytic regulatory genes (GATA-1).[6]

Myelostromal Proliferation

Myelostromal proliferation has been described as a rare condition of bone marrow in the rat.[29] Lesions are characterized by a relatively nondescript uniform population of stromal cells or histiocytes with finely vacuolated cytoplasm that sometimes contains iron-positive inclusions. Extracellular stroma that is trichrome-negative often surrounds individual cells; however, overt atypia is not usually seen. Notably, the appearance of these lesions has been compared to that of histiocytic sarcoma.[29]

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CHAPTER 18

Infectious Injury to Bone Marrow

K. JANE WARDROP

Bone Marrow Pathology
Rickettsial Infection
Protozoal Infection—Leishmania
Viral Infection
  Parvovirus
  Retrovirus
  Equine Infectious Anemia virus
  Bovine Viral Diarrhea Virus and Classical Swine Fever Virus
Fungal Infection

Acronyms and Abbreviations

BLV, bovine leukemia virus; BMSC, bone marrow stromal cell; BVDV, bovine viral diarrhea virus; CFU-GM, colony-forming units-granulocyte–monocyte; CPV, canine parvovirus; CSFV, classical swine fever virus; EIAV, equine infectious anemia virus; FeLV, feline leukemia virus; FIV, feline immunodeficiency virus; FPV, feline panleukopenia virus; PCR, polymerase chain reaction.

BONE MARROW PATHOLOGY

The response of the bone marrow to infectious agents is variable, depending on the organism involved, the nature and chronicity of infection, and the presence of other diseases. Responses are generally nonspecific, and can occur with other, noninfectious disease. Visualization of the organism on marrow cytology or histology can occur in some infections, facilitating the diagnosis. The marrow response to common companion or domestic animal infectious diseases known for their marrow involvement is described in the following text.

RICKETTSIAL INFECTION

Bacteria within the families *Rickettsiaceae* and *Anaplasmataceae* of the order *Rickettsiales* cause a variety of diseases in dogs, cats, horses, and cattle; however, marrow changes are best documented for infections caused by *Ehrlichia canis*. *Ehrlichia canis* is the etiologic agent of canine monocytic ehrlichiosis, and is transmitted most commonly by the vector *Rhipicephalus sanguineus*. The disease has acute, chronic, and subclinical syndromes. Bone marrow in the acute phase of the disease is typically hypercellular. An increase in the bone marrow myeloid to erythroid ratio was described three to four weeks post infection in experimental canine ehrlichiosis.[22,31] Megakaryocytes were increased one week after infection, reflecting the hematopoietic response to the persistent thrombocytopenia seen with this disease (see Chapter 80). Morulae can be present in bone marrow but are difficult to find, with one to four morulae per 1000 oil immersion fields seen in one study of acute ehrlichiosis.[15] Chronic ehrlichiosis produces a pancytopenia, secondary to severe bone marrow hypocellularity (see Chapters 33 and 40).[3] Marrow aspirates are poorly cellular, with cells consisting of low numbers of stromal cells, fibroblasts, and macrophages. Hematopoietic precursors are few or absent. Core biopsies show a severely hypocellular marrow filled with adipose tissue.[3] Mastocytosis also has been reported in the chronic phase of ehrlichiosis.[17] Bone marrow plasmacytosis has been reported with ehrlichiosis, and is independent of the phase.[16,33] Myelofibrosis does not appear to play a significant role in the development of the myelosuppression seen with chronic ehrlichiosis.[14]

Anaplasma platys is another rickettsial pathogen that can affect the bone marrow. Although previously thought to only affect circulating platelets, thus producing a thrombocytopenia, the organisms have also been

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documented to infect megakaryocytes and promegakaryocytes within bone marrow.[25]

PROTOZOAL INFECTION—LEISHMANIA

Leishmaniasis is a disease caused by protozoal organisms of the genus *Leishmania*, and is transmitted in Mediterranean regions by the bite of an infected female sand fly. Several forms of the disease, including cutaneous, mucocutaneous, and visceral forms, have been identified in dogs. A mild anemia and thrombocytopenia are the most frequent hematologic findings. Microscopic examination of fine-needle aspirates of lymph nodes or bone marrow and serology and PCR are frequently used diagnostic methods (Figure 18.1). In one study, *Leishmania* spp. amastigotes were observed in the marrow of 92.6% of clinically affected dogs.[26] Another study in dogs naturally infected with *Leishmania infantum* showed that the most common pathological features of the bone marrow were megakaryocytic dysplasia, erythrophagocytosis, erythroid dysplasia, and emperipolesis.[5] Severe histiocytosis and a moderate to marked plasmacytosis of the marrow have also been reported.[18,19]

VIRAL INFECTION

Parvovirus

Feline panleukopenia virus (FPV) and canine parvovirus (CPV) are both parvoviruses that cause bone marrow injury and leukopenia, neutropenia, and lymphopenia. These viruses have a broad tropism for mitotically active cells. It is unknown whether the reduction in marrow cells is due primarily to virus replicating in and killing cells, or due to more indirect effects. FPV can kill both erythroid and myeloid colony progenitor cells in bone marrow cultures, although at low viral doses there is greater suppression of the formation of myeloid colony-forming units-granulocyte–monocyte (CFU-GM).[10] Viral replication of FPV in myeloid cells may lead to the neutropenia seen after FPV infection. In a study of 76 cats with clinical FPV, 84% showed complete bone marrow depopulation and dilation of the vascular sinuses, 5% had no significant marrow change, and 6% had marrow hypercellularity with granulocytic hyperplasia.[4]

In 75 dogs with clinical CPV, 92% had marrow depopulation, one dog showed no changes, and five dogs had marrow hypoplasia.[4] In another study using experimentally infected puppies, leukopenia and neutropenia only occurred in dogs with severe enteric disease. Viral replication in the bone marrow was sparse.[12] In both FPV and CPV, the severe neutropenia observed is probably the result of both neutrophil loss or consumption in the infected gut and bone marrow suppression.[20]

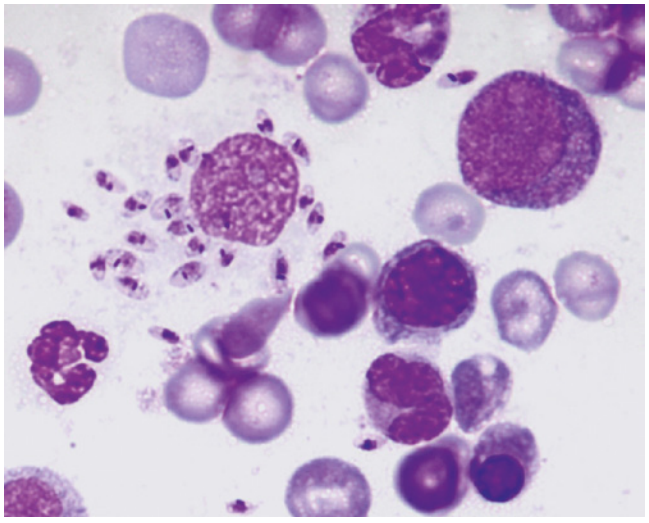


FIGURE 18.1 *Leishmania* organisms in bone marrow from a dog; Wright–Giemsa stain. (**Source:** Courtesy of Dr. Rick Cowell)

Retrovirus

Feline leukemia virus (FeLV, subfamily *Oncornavirinae*) and feline immunodeficiency virus (FIV, subfamily *Lentivirinae*) are retroviruses that are major infectious pathogens in domestic cats (see Chapters 53 and 60). FeLV infects hematopoietic cells, lymphoid cells, and accessory cells in the hematopoietic microenvironment. Marrow nucleated cells become infected with the virus two to six weeks after exposure, and virus can be detected in circulating leukocytes at that time.[8] After regression, one third to one half of cats transiently harbor latent virus in marrow myelomonocytic cells and stromal fibroblasts.[11] The types of marrow change seen in FeLV are highly variable. Early viremia can be accompanied by pancytopenia and marrow hypocellularity.[21] Marrow necrosis, with nuclear swelling and karyolysis, has rarely been described.[28] FeLV-C-induced pure red cell aplasia shows almost a total lack of marrow erythroid precursor cells, with normal myeloid and megakaryocytic precursors (see Chapters 38 and 60).[27] FeLV-induced myelodysplastic syndromes can show marrow hypercellularity, megaloblastic maturation abnormalities, reticulin fibrosis, and increased numbers of immature cells and blast cells (see Chapter 67).[2,13] Marrow myelophthisic disease, where the marrow is filled with blast cells, can be observed in FeLV-induced leukemias or lymphoma. Active viral replication is thought to be required for the majority of pathogenic mechanisms in which FeLV causes marrow suppression; however, regressive FeLV infection without viremia may also be responsible.[9,29]

FIV produces progressive CD4 T-cell depletion. Marrow morphologic abnormalities, marrow suppression, and peripheral blood cytopenias can be seen. Cytopenias (neutropenia, anemia) have been attributable to direct infection of the bone marrow stromal cell (BMSC) population.[24] BMSC types include fibroblastic/adventitial/reticular cells, macrophages, endothelial cells, adipocytes, and myofibroblasts, of which macrophages are productively infected with FIV.[1]

Bovine leukemia virus (BLV) is an oncogenic retrovirus, which produces lymphoma in adult cattle. Bone marrow involvement is not a prominent feature.

Equine Infectious Anemia Virus

The equine infectious anemia virus (EIAV) is a lentivirus of the family *Retroviridae* (see Chapters 34 and 37). Infection of horses with EIAV produces a persistent viremia and recurrent episodes of anemia, thrombocytopenia, and fever. In one study using experimentally infected foals with severe combined immunodeficiency, bone marrow changes were described after the onset of thrombocytopenia.[32] The postthrombocytopenia samples had a moderate to marked decrease in bone marrow cellularity, with an increase in intercellular homogeneous eosinophilic material. Individual cell necrosis or apoptosis was not observed. A moderate increase in adipocytes and reticulin fibers was noted. Denuded megakaryocyte nuclei were also observed. In another study, the total megakaryocyte area and megakaryocyte nuclear area were increased. Numbers of megakaryocytes were slightly increased.[23]

Bovine Viral Diarrhea Virus and Classical Swine Fever Virus

Bovine viral diarrhea virus (BVDV) and classical swine fever virus (CSFV) are related viruses (family *Flaviviridae*, genus Pestivirus), both known to infect and damage megakaryocytes of the marrow and produce a subsequent thrombocytopenia, but other marrow lesions are not present. In a study using calves infected with type II BVDV, histologic evaluation of the bone marrow showed an increased number of megakaryocytes. Approximately one half of the megakaryocytes were immature or had undergone nuclear loss or pyknosis.[30] Infection of calves with type II BVDV also reduced the number of CFU-GM cultured from bone marrow. This suggests that suppression of granulopoiesis may contribute to the prolonged neutropenia associated with BVDV infection. Experimental infection of pigs with a virulent strain of CSFV also produced changes in megakaryocytes, with abnormal nuclei and micromegakaryocytes seen.[7] Myeloid and erythroid cell depletion and lymphocyte apoptosis have also been described in experimentally infected pigs.[8]

FUNGAL INFECTION

Blastomycosis, histoplasmosis, coccidioidomycosis, and cryptococcosis are common systemic fungal diseases of the dog and cat. Although all of these diseases can involve the bone marrow, marrow involvement is most frequently described in histoplasmosis.

Histoplasma capsulatum is a soil-borne, dimorphic fungus that infects dogs and cats through inhalation of conidia from the mycelial phase, which subsequently converts to yeast in the body. The disseminated form of the disease predominantly affects the liver, spleen, gastrointestinal tract, bone, bone marrow, integument, and eyes. Primary gastrointestinal and pulmonary histoplasmosis can also occur.

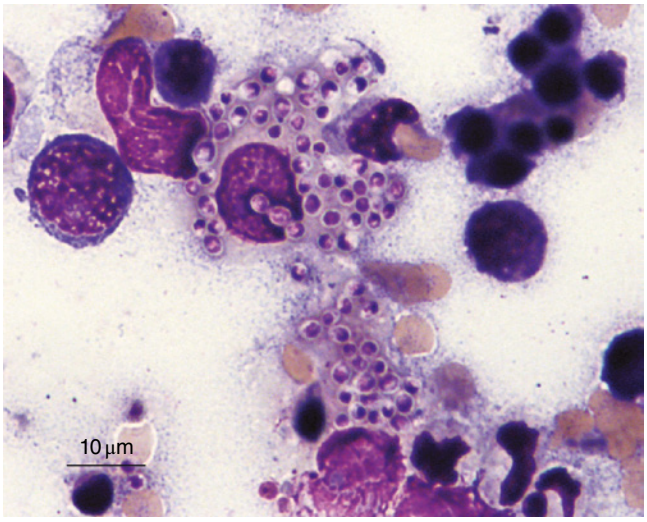


FIGURE 18.2 Histoplasma organisms in bone marrow from a cat; Wright–Giemsa stain. (**Source:** Courtesy of Dr. James Meinkoth, Oklahoma State University)

In the bone marrow, large numbers of organisms can be found within macrophages (Figure 18.2). Anemia is a frequent finding, but pancytopenia accompanied by granulomatous inflammation of the bone marrow has also been reported.[6]

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SECTION III
Erythrocytes

CHAPTER 19

Erythropoiesis

CHRISTINE SWARDSON OLVER

Definitions
Stem Cell to Erythrocyte
  General
  Development and Maturation of Reticulocytes
  Reticulocytes in Veterinary Species
Molecular Control of Erythropoiesis
  Cytokines and Growth Factors Regulating Erythropoiesis
  Transcription Factors
  Pleiotropic Transcription Factors
  Erythroid-Specific Transcription Factors
  Other Molecules and miRNAs
  Negative Regulators of Erythropoiesis
Microenvironment
  Molecules Involved in Adhesion
Erythrokinetics

Acronyms and Abbreviations

BFU-E, burst-forming unit-erythroid; CD, cluster of differentiation; CFU-E, colony-forming unit-erythroid; CMP, common myeloid progenitor; DNA, deoxyribonucleic acid; E2A, Transcription Factor 3; EKLF, erythroid Krüppel-like transcription factor; EMP, erythrocyte macrophage protein; EPO, erythropoietin; EPOR, erythropoietin receptor; FasL, Fas ligand; FOG-1, friend of GATA; GATA1 and GATA2, GATA-binding protein 1 and 2; GM-CSF, granulocyte/macrophage colony-stimulating factor; HIF-1, hypoxia-inducing factor-1; HSC, hematopoietic stem cell; ICAM-4, intercellular adhesion molecule-4; IGF-I, insulin-like growth factor-I; IL, interleukin; IFN-γ, interferon gamma; JAK2, Janus kinase 2; kDa, kilodalton; LDB1, LIM Domain Binding 1; LMO2, LIM Domain Only 2; LRF, Leukemia/Lymphoma Related Factor; mRNA, messenger ribonucleic acid; MEP, megakaryocyte/erythroid progenitor; RBC, red blood cell; RNA, ribonucleic acid; RT-PCR, reverse transcriptase polymerase chain reaction; TNF, tumor necrosis factor; SCF, stem cell factor; SCL, stem cell leukemia; SOCS, suppressor of cytokine signaling; STAT, signal transducers and activators of transcription; TNF, tumor necrosis factor; TPO, thrombopoietin; TRAIL, TNF-related apoptosis-inducing ligand; VCAM, vascular cell adhesion molecule; VLA-4, very late antigen-4.

Erythropoiesis is the proliferation and progressive differentiation of hematopoietic stem cells (HSCs) (see Chapters 2 and 4) into hemoglobinized RBCs. In normal animals, erythropoiesis and RBC mass are regulated by cellular oxygen levels. RBCs originate from HSCs by first committing to the erythroid lineage to form erythroid progenitor cells, which morphologically resemble lymphocytes, then differentiating into precursors cells that can be identified morphologically, and finally undergoing a series of division and differentiation steps until the nucleus is extruded (in mammals) to form reticulocytes. Each of these stages is initiated and regulated by specific humoral, microenvironmental, cell surface and transcription factors. This chapter describes erythropoiesis using information gained primarily from studies in mice, rats, and humans. Species-specific information is provided when available.

DEFINITIONS

Basal erythropoiesis is erythropoiesis that occurs to replace naturally senescent RBCs and therefore maintain normal RBC mass. Stress erythropoiesis refers to erythropoiesis that occurs in response to an anemic stimulus, such as blood loss, hemolysis, or pregnancy.

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STEM CELL TO ERYTHROCYTE

General

The commitment of multipotent HSCs into cells committed to specific blood cell lineages is depicted in Figure 19.1. Differentiation from the common myeloid progenitor (CMP) to megakaryocytic/erythroid progenitor (MEP) is associated with expression of various gene-specific transcription factors, growth factor receptors, functional proteins, and cell surface receptors, and is influenced by a variety of growth factors.[2] Gene-specific transcription factors are predominantly activators of transcription and usually function as a complex of proteins that binds to specific DNA sequences. MEPs differentiate into burst-forming unit-erythroids (BFU-Es), which are programed to differentiate only into erythroid cells, but resemble lymphocytes microscopically. These cells are identified by their growth from a single cell to several thousand cells in culture. The colony-forming unit-erythroid (CFU-E) differentiates from BFU-E. In culture, each CFU-E divides into 8–32 cells. These progenitor cells differentiate into microscopically recognizable precursor cells. Figure 19.2 depicts the various stages of erythroid precursor differentiation. The earliest precursor is a rubriblast (Figure 19.2), followed by the prorubricyte, basophilic rubricyte, polychromatophilic rubricyte, and metarubricyte. The erythroid precursor differentiation process is a series of differentiation steps that results in a progressive decrease in cell size, a gradual increase in cytoplasmic hemoglobin concentration, and gradual condensation of nuclear chromatin. In general, this results in a gradual decrease in cytoplasmic basophilia along with decreasing size of the nucleus. The metarubricyte nuclei are then extruded to produce polychromatophilic RBCs, which present as reticulocytes with vital stains such as new methylene blue. All of the precursors except

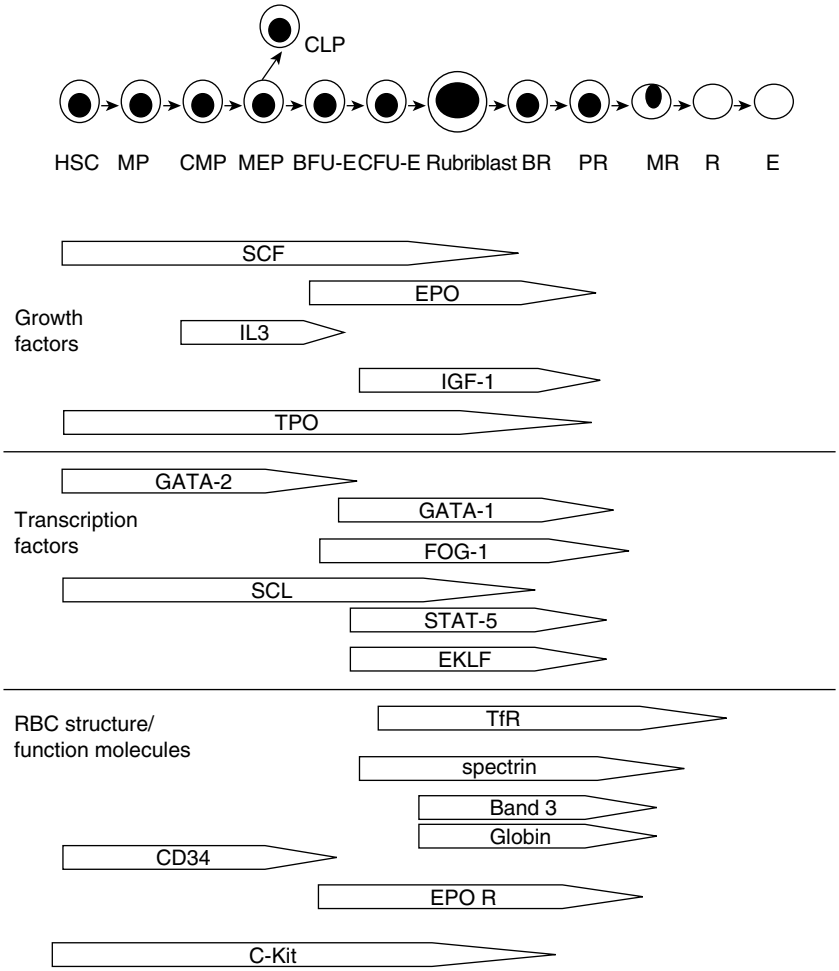


FIGURE 19.1 Approximate timing of the response to certain growth factors, activation of transcription factors, and expression of erythroid-specific molecules in erythropoiesis. Abbreviations: BFU-E, burst-forming unit-erythroid; BR, prorubricyte/basophilic rubricyte; CFU-E, colony-forming unit-erythroid; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; EKLF, erythroid Krüppel-like factor; EPO, erythropoietin; EPOR, erythropoietin receptor; FOG-1, friend of GATA1; HSC, hematopoietic stem cell; IGF-1, insulin-like growth factor-1; IL-3, interleukin-3; MEP, megakaryocytic/erythroid progenitor; MP, multipotential progenitor; MR, metarubricyte; PR, polychromatophilic rubricyte; R, reticulocyte; Rub, rubriblast; SCF, stem cell factor; SCL, stem cell leukemia gene; STAT, signal transducer and activator of transcription; TfR, transferrin receptor; TPO, thrombopoietin.

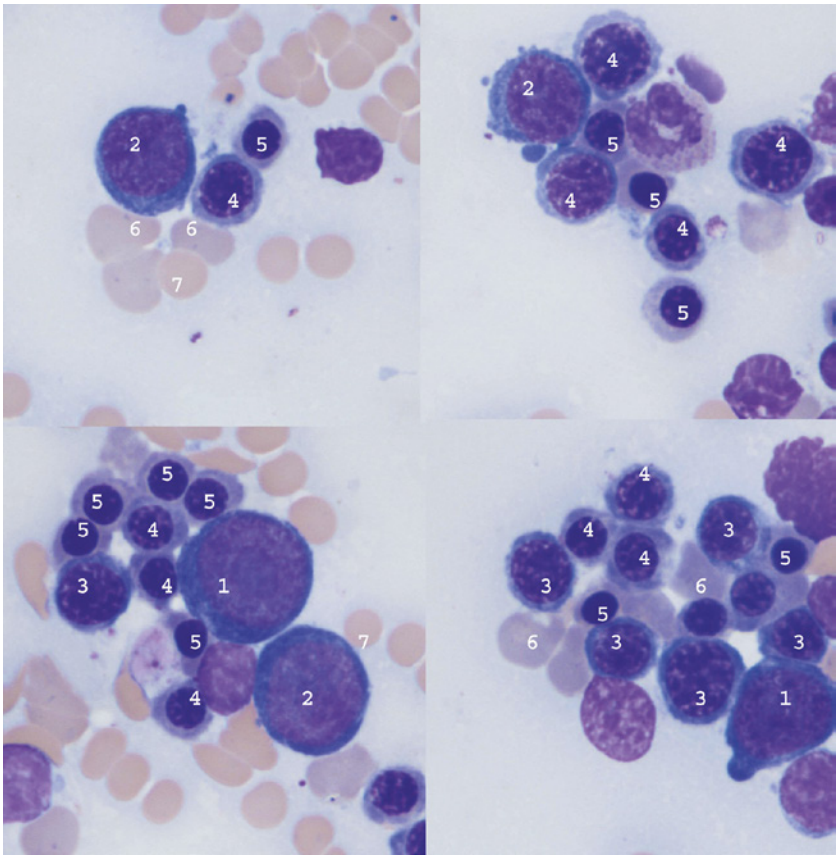


FIGURE 19.2 The various stages of erythroid precursors are shown. (1) Rubriblast; (2) prorubricyte; (3) basophilic rubricyte; (4) polychromatophilic rubricyte; (5) metarubricyte; (6) reticulocyte; (7) RBC.

metarubricytes are capable of division. In theory, 16 metarubricytes will be produced from each rubriblast; however, the actual percentages of each precursor type in a marrow evaluation may be slightly different from this ratio.

This is likely because each stage matures for a variable number of days, and thus a sample of marrow at a snapshot in time will not reflect the true "pyramid."

The nomenclature for erythroid precursors in humans and mice differs from that used in veterinary medicine. The earliest erythroid precursor is called a proerythroblast and subsequent cells are called basophilic erythroblasts, polychromatophilic erythroblasts, and orthochromatic erythroblasts (Table 19.1).

TABLE 19.1 Nomenclature of erythroid precursors.

Veterinary terminology	Human/mouse/research terminology
Rubriblast	Proerythroblast
Prorubricyte	Basophilic erythroblast
Basophilic rubricyte	Basophilic erythroblast
Polychromatophilic rubricyte	Polychromatophilic erythroblast
Metarubricyte	Orthochromatic erythroblast
Reticulocyte	Reticulocyte
Erythrocyte	Erythrocyte

Development and Maturation of Reticulocytes

Nuclear expulsion from metarubricytes is associated with changes in intermediate filaments and microtubules that result in rearrangement of the membrane cytoskeleton.[21] Extruded metarubricyte nuclei express phosphatidylserine and are rapidly ingested by bone marrow macrophages. Reticulocytes then exit the bone marrow, although the exact mechanism is unknown. Reticulocytes contain residual mitochondria, Golgi membranes, ribosomes, and microtubular components, and can have approximately 35% more volume than mature RBCs.[36] During maturation in the circulation, reticulocytes gain hemoglobin and lose organelles, membrane surface area, volume, and numerous cell-surface proteins. Cell-surface proteins are lost in the form of exosomes, or membrane blebs, that contain proteins specifically selected for removal (see Chapter 22).[19]

Stress reticulocytes are released from the bone marrow during times of erythropoietic stress.[25] Stress reticulocytes are larger and less mature than normal reticulocytes, and contain more mitochondria, ribosomes, and other organelles. Their membranes are more mechanically rigid and unstable, and they possess adhesive proteins that are normally lost before release.

Reticulocytes in Veterinary Species

The number of reticulocytes circulating during health varies among species (see Chapter 23).[17] Some species have circulating reticulocytes in health (dogs, cats, rodents, rabbits, pigs, guinea pigs), whereas others (cows, goats, sheep) have circulating reticulocytes only during a regenerative response. Horses, with rare exceptions,[40] do not have circulating reticulocytes even during a regenerative response.[6,30]

MOLECULAR CONTROL OF ERYTHROPOIESIS

Proliferation and differentiation of erythroid cells include cell divisions accompanied by a serial reduction of proliferative ability and increased erythroid-specific gene expression. The general sequence of molecular events for erythroid proliferation and differentiation is: (1) growth factor binding to its receptor; (2) activation of membrane-associated kinases; (3) phosphorylation of the cytoplasmic portion of the receptor and of other intermediate molecules; (4) activation of transcription factors; and (5) transcription-factor-dependent synthesis of either additional transcription factors or erythroid-specific functional proteins, depending upon the stage of erythropoiesis. Examples of erythroid-specific functional proteins include RBC-specific spectrins, globins, ankyrin, band 4.1, and glycophorins (cell-surface molecules).[21]

Cytokines and Growth Factors Regulating Erythropoiesis

Erythropoietin (EPO) is the principal hormonal regulator of erythropoiesis. EPO is a 34.4 kilodalton (kDa) glycoprotein (165 amino acids) that is produced primarily by peritubular interstitial fibroblasts of the kidney in adult animals. A small amount of EPO is produced in the liver during anemia.[20] Low tissue oxygen tension induces *EPO* gene expression through transcriptional activation and also induces *EPO* messenger ribonucleic acid (mRNA) stabilization.[5,7,41] Hypoxia upregulates EPO by allowing accumulation of hypoxia-inducible factors-1 and -2 (HIF-1 and HIF-2). These transcription factors bind a responsive element in the EPO gene. In severe hypoxia, production of EPO is increased up to 1000-fold.[7] EPO is highly conserved among species, and recombinant human EPO can support proliferation and differentiation of erythroid progenitors from a variety of species including horses and cats.

The EPO receptor (EPOR) is an integral membrane protein with seven tyrosine phosphorylation sites in its cytoplasmic domain. Each of these domains appears to regulate different aspects of erythropoiesis. Some phosphorylation sites may be dispensable for steady-state erythropoiesis, but required for stress erythropoiesis.[22] Signaling through the EPOR is mediated by prebound Janus kinase 2 (JAK2), a phosphokinase. JAK2 phosphorylates several tyrosine residues in the cytoplasmic domain of the EPOR, which results in recruitment of a number of cytoplasmic signaling proteins. Several pathways critical to survival and differentiation of erythroid progenitors and precursors are then activated. The signal transducers and activators of transcription (STAT)5 pathway, which results in upregulation of the Bcl-x_L anti-apoptotic protein, is an important example of one of these pathways. The EPOR is expressed on early cluster of differentiation of 34-positive (CD34+) hematopoietic progenitors, increases in number to its highest in CFU-E and rubriblasts, and then decreases gradually during precursor differentiation (Figure 19.1). Neither EPO nor EPOR is required for generation of BFU-E or CFU-E, but both are required for proliferation and development of later stages of erythroid precursors and for production of mature RBCs.[42]

Interleukin-3 (IL-3) is a multilineage cytokine produced by macrophages, activated T lymphocytes, mast cells, eosinophils, and bone marrow stromal cells. IL-3 acts on HSCs and progenitor cells to induce proliferation and self-renewal. It also primes progenitor cells to respond more vigorously to EPO.[1]

Stem cell factor (SCF), also known as steel factor, mast cell growth factor, and c-kit ligand, is expressed by bone marrow stromal cells and acts through its receptor, c-kit (CD117).[4] Its mRNA is also expressed in BFU-E, suggesting a potential autocrine or paracrine function. SCF markedly enhances the activity of EPO, allowing for proliferation and differentiation of progenitors and precursors.

Granulocyte–macrophage colony-stimulating factor (GM-CSF), produced by T cells, stimulates production of primarily neutrophils, monocytes, and eosinophils. GM-CSF may enhance erythroid progenitor development, although GM-CSF knockout mice are hematologically normal.

Thrombopoietin (TPO) is a 45–70 kDa (depending on glycosylation) glycoprotein produced in the liver, kidney, skeletal muscle, and bone marrow stroma. It appears to be required for maintenance of HSCs in G_0 and therefore their self-renewal capacity.[28] TPO also acts as a costimulatory agent with EPO to promote BFU-E growth in vitro and to support proliferation and maintenance of erythroid progenitor cells in vivo.[3]

Insulin-like growth factor-1 (IGF-1) and insulin both enhance the growth and differentiation of erythroid cells. IGF-1 decreases apoptosis of progenitors and enhances heme synthesis and nuclear condensation and enucleation.[24]

Glucocorticoids enhance erythroid colony formation in vitro and in vivo by stimulating erythroid progenitor proliferation and inhibiting differentiation.[39] Glucocorticoids act through a nuclear glucocorticoid receptor that subsequently directly affects transcription by binding to glucocorticoid response elements in DNA.

Humoral inhibitors of erythropoiesis include tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand (TRAIL), IL-1, and interferon gamma (IFN-γ).[31] TNF and IL-1 not only inhibit erythroid progenitors, but also suppress EPO secretion. Immature erythroid cells are susceptible to cell death due to TNF and IFN-γ upregulation of "death" receptors.[9]

Transcription Factors

Several transcription factors are activated during RBC development as a result of binding of hematopoietic cytokines to cell-surface receptors. The activated transcription factor then translocates to the nucleus to enhance transcription of specific genes. The DNA that is transcribed is either an erythroid-specific structural or functional gene (e.g., spectrin and globin) or a gene encoding a protein that is involved in initiation of the next step in differentiation (e.g., another transcription factor).

Pleiotropic Transcription Factors

The stem cell leukemia (*SCL*) gene is a pleiotropic (not erythroid-specific) transcription factor that is crucial for hematopoiesis; *SCL* null mice die in utero at day 8 without any blood cell formation.[33] *SCL* also enhances erythroid proliferation and differentiation while repressing myeloid differentiation.

GATA-binding protein 2 (GATA2) is expressed in early HSCs. It appears to be necessary for proliferation and survival of stem cells, but dispensable for terminal erythroid differentiation. Erythroid progenitor cells of GATA2 null mice stop differentiating at the rubriblast stage.[37]

Erythroid-Specific Transcription Factors

STAT5 is required for erythropoiesis and is enhanced by EPO and SCF. STAT molecules are mobile cytoplasmic transcription factors that are activated by the JAK family of protein tyrosine kinases. STAT molecules are latent, but become activated and translocate to the nucleus upon tyrosine phosphorylation. STAT5 binds to specific DNA sequences to upregulate genes involved in tissue-specific functions and regulation of cell growth.[34] STAT5 knockout mice are severely anemic in utero and their fetal liver cells show increased apoptosis. Some adult STAT5 knockout mice have normal hematocrits, but have ineffective hematopoiesis during erythropoietic stress.[34] These mice generate increased numbers of proerythroblasts during erythropoietic stress, but have increased apoptosis in the more mature stages.

GATA-binding protein 1 (GATA1) is essential for both primitive and definitive erythropoiesis, and plays a key role in the differentiation of rubriblasts to mature RBCs.[42] GATA1 is responsible for expression of many erythroid-specific or erythroid-predominant genes, including cytoskeletal proteins, the transferrin receptor, EPOR, and the friend of GATA1 transcription factor (FOG-1). GATA1 also maintains expression of Bcl-x_L and EPOR, which promote late erythroid precursor survival and differentiation, while repressing GATA2, a molecule that induces proliferation of earlier stages.

Erythroid Krüppel-like transcription factor (EKLF) is essential for primitive and definitive erythropoiesis and for adult β-hemoglobin production. Its disruption in mice results in accumulation of fetal γ-globin and inability to produce adult β-globin.[27] EKLF appears also to regulate expression of other erythroid-specific genes including dematin, cytoskeletal proteins, transcription factors, and heme synthesis proteins.[15]

LMO2 (LIM domain only 2), LDB1 (LIM domain binding 1), E2A (transcription factor 3), and LRF (leukemia/lymphoma related factor) are all transcription factors that have been defined as important for erythropoiesis using murine knockout models, and, in general, act as multiprotein complexes to stimulate the production of RNAs essential for erythropoiesis.[14]

Other Molecules and miRNAs

Bcl-x_L is an anti-apoptotic protein that is associated with the mitochondrial membrane. Bcl-x_L prevents apoptosis of hemoglobinizing rubricytes.[29] Mice that have a conditional knockout of the Bcl-x_L gene have severe anemia and splenomegaly. The bone marrow and spleens of these mice accumulate early rubricytes, but are deficient in maturing cells. Bcl-x_L expression is EPO-dependent via STAT5, and is increased during terminal differentiation stages when hemoglobin synthesis is greatest.[12]

JAK2 is associated with the EPOR and is responsible for downstream signaling. EPO binding to the EPOR changes conformation of the receptor and thereby activates JAK2, which causes tyrosine phosphorylation of the cytoplasmic domain, and subsequent recruitment of signaling proteins including STAT5. JAK2 knockout mice die during embryogenesis with severe anemia. The most important function of JAK2 appears to be activation of STAT5.[11]

FOG-1 is a nuclear protein that binds to GATA1 and acts as its cofactor in transcription.[18] FOG-1 mediates both transcriptional activation as well as repression (e.g., of GATA2)and FOG-1 knockout mice die of anemia in utero.[38] GATA2 repression is dependent on FOG-1, and thus FOG-1 may facilitate GATA switching, leading to erythroid differentiation.

miRNAs are small RNAs that regulate the expression of their specific genes. Typically, they downregulate mRNA by either degradation or translational inhibition (or sometimes both), and therefore decrease the expression of their target gene. miRNAs regulate several aspects of erythropoiesis including erythroid lineage determination, erythroid progenitor proliferation, terminal erythroid differentiation, and enucleation. miR-24, -150, -221, -222, and -223 appear to inhibit erythroid differentiation and proliferation. For example, miR-223 downregulates LMO2, which is a critical transcription factor that is upregulated during erythroid differentiation of CD34+ progenitor cells. By contrast, miR-144 and -451 are induced during erythroid differentiation, and appear important for late erythroid cell maturation.[14]

Negative Regulators of Erythropoiesis

Fas and Fas ligand (FasL) are members of the TNF receptor and TNF superfamilies, respectively, and are expressed as integral membrane proteins. When FasL from one cell interacts with Fas from another cell, clustering of Fas initiates an intracellular caspase cascade

and apoptosis. These molecules are coexpressed on a small percentage of immature erythroid cells in murine bone marrow and are responsible for erythroid precursor apoptosis during basal erythropoiesis.[22] Both of these molecules are downregulated in response to EPO, allowing erythroid precursor survival and differentiation.

Suppressor of cytokine signaling (SOCS) competes for the binding site of STAT5 on the cytoplasmic tail of the EPOR. STAT5 can then no longer bind or be activated to produce its downstream effects. SOCS also participates in the degradation of JAK2. SOCS is induced by a number of cytokines, including EPO.[32]

MICROENVIRONMENT

Erythroblastic islands are one component of the microenvironment (see Chapters 3 and 4) that is essential for erythropoiesis. Erythroblastic islands comprise a central macrophage surrounded by 10–30 erythroid cells from CFU-Es to reticulocytes.[23] The macrophage projects cytoplasmic processes to which erythroid cells adhere via erythroblast macrophage protein (EMP), very late antigen-4 (VLA-4), and intercellular adhesion molecule-4 (ICAM-4). These proteins bind to the macrophage receptor proteins EMP, vascular cell adhesion molecule-1 (VCAM-1), and integrins αv and α4, respectively. Interestingly, the transcription factor EKLF upregulates ICAM-4 on erythroid precursor cells.[16] The macrophage cell surface molecule CD163 also may be important in binding to erythroid cells and in promoting erythropoiesis.[8]

The central macrophage has a unique phenotype, including a high level of expression of F4/80 and the Forssman glycosphingolipid (a 7-transmembrane spanning receptor, best described in the mouse), marked adhesiveness, a pronounced ability to endocytose, and inability to generate a respiratory burst. Although erythroblastic islands are critical for erythroblast proliferation and differentiation, macrophage function related to erythropoiesis is incompletely understood. Central macrophages may produce positive and negative regulators of erythropoiesis, supply iron for hemoglobin production, and play a role in nuclear extrusion and phagocytosis of the extruded nucleus.

Molecules Involved in Adhesion

ICAM-4 is a transmembrane protein belonging to the immunoglobulin superfamily of adhesion receptors, and is predominantly expressed on erythroid cells.[35] ICAM-4 mediates binding of rubricytes to rubricytes and rubricytes to macrophages, and is upregulated by the transcription factor EKLF. It is expressed during terminal differentiation of erythroid cells, beginning with the rubriblast. EMP is a 36 kDa transmembrane protein expressed on the surface of erythroid cells and macrophages, and is involved with interactions between erythroid cells and between erythroid cells and macrophages.[23] Absence of EMP severely limits the number of erythroblastic islands and results in marked apoptosis of erythroid cells. VLA-4, also known as $\alpha 4\beta_1$ integrin, is expressed on immature erythroid cells and binds to VCAM-1 on macrophages. Integrin αV is expressed on macrophages and binds to ICAM-4 on immature erythroid cells. Fibronectin is an extracellular matrix glycoprotein that binds to all stages of immature erythroid cells via the VLA-4 and VLA-5 integrins.[26]

ERYTHROKINETICS

Massive amplification from HSC to mature RBCs is required to create 4×10^{11} mature RBCs from 2×10^4 stem cells.[4] Erythrokinetics encompasses the rates of proliferation and maturation from HSC to mature RBCs. Long-term and short-term repopulating cells have been identified in transplantation experiments in mice, and probably represent hematopoietic cells that are less mature than BFU-Es. These cells take months to differentiate into later stages of erythroid cells.[13] BFU-Es take about 10–14 days to develop into rubriblasts in culture, whereas CFU-E maturation takes about 3–5 days. In health, there are approximately 50 rubricytes and 113 reticulocytes for every rubriblast in the bone marrow.[10] Approximately five mitotic divisions occur over 5 days before a rubriblast loses its nucleus and becomes a reticulocyte. In most species, reticulocytes spend 2–3 days maturing in bone marrow before being released to the circulation.

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CHAPTER 20

Erythrocyte Structure and Function

CHRISTINE SWARDSON OLVER

Erythrocyte Structure
- Overall Structure
- Membrane Structure
  - Membrane Lipids
  - Lipid Rafts
  - Erythrocyte Deformability
  - Cytoskeletal Proteins
  - Integral Membrane Proteins

Hemoglobin Structure, Synthesis, and Metabolism
- Hemoglobin Structure
- Hemoglobin Synthesis
- Control of Hemoglobin Synthesis
- Hemoglobin Turnover
- Heme Catabolism and Bilirubin Formation
- Fetal Hemoglobin
- Hemoglobin Types in Animals

Iron Metabolism
- Absorption in Intestine
  - Intestinal Absorption of Nonheme and Heme Iron
- Cellular Iron Uptake
- Macrophage Handling of Iron
- Selected Molecules Involved in Iron Metabolism
  - Divalent Metal Iron Transporter-1
  - Ferrireductase Duodenal Cytochrome b
  - Hephaestin
  - Ferroportin
  - Hepcidin
  - Transferrin Receptor 1
- Transport and Storage of Iron
  - Transport of Iron
  - Storage of Iron
- Iron Regulatory Proteins

Acronyms and Abbreviations

ADP, adenosine diphosphate; dCytb, duodenal cytochrome B; DMT-1, divalent metal iron transporter-1; Hgb, hemoglobin; ID, iron deficiency; IRP, iron regulatory protein; kD, kilodalton; MPS, mononuclear phagocyte system; NADH, nicotinamide adenine dinucleotide (reduced form); NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; RBC, red blood cell; SM, sphingomyelin; SMAD, small mothers against decapentaplegic; TfR, transferrin receptor.

The main function of RBCs is to carry oxygen to tissues. All energy in RBCs that is devoted to maintaining cell shape, membrane structure, enzymatic functions, reduced iron in hemoglobin, and other functions does so to optimize oxygen delivery to tissues. Recently, mammalian erythrocytes have also been shown to participate in the innate immune response by binding inflammatory molecules and producing antimicrobial reactive oxygen species.[2] It is well known that the erythrocytes of birds, amphibians, and fishes can produce cytokine-like factors, upregulate viral response genes, and sequester pathogens through surface binding or phagocytosis.[2] Erythrocytes also play numerous roles in hemostasis in both procoagulant and anticoagulant ways.[59] For example, erythrocytes release ADP and thromboxane A_2, both of which increase platelet adhesion and aggregation (procoagulant). Another procoagulant mechanism is the exposure of phosphatidylserine (PS) on the outer membrane leaflet. Alternatively, RBCs make the fibrin network more porous, which is anticoagulant.

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ERYTHROCYTE STRUCTURE

Overall Structure

Mammalian RBCs have no nuclei and no organelles, and thereby no ability to synthesize proteins. The full complement of functional proteins must be present by the time the reticulocyte matures. Human RBCs are composed (by mass) of 61% water, 32% protein (mostly hemoglobin), 7% carbohydrates, and 0.4% lipids. Isolated RBC membranes are composed of approximately 20% water, 40% protein, 35% lipid, and 6% carbohydrate.[43]

Membrane Structure

Membrane Lipids

The RBC membrane phospholipid bilayer is composed of phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), and PS, as well as other less abundant phospholipids.[56] This composition varies with species. For instance, sheep, cows, and goats have little to no PC, whereas horses, dog, and rats have abundant PC.[51] Sheep have approximately twice the SM content as other species.[19] The bilayer is vertically asymmetrical, with PS and PE located almost exclusively on an inner PL layer. The asymmetry is maintained and/or altered by the activities of enzymes termed flippases[14] and floppases, Type-IV P-type ATPases, and ABC transporters.[34,50] Other lipids in the bilayer include glycolipids and cholesterol, and these are embedded in the phospholipids.

Lipid Rafts

Lipids rafts are specialized membrane microdomains that are rich in cholesterol and sphingolipids and also contain specific membrane proteins. They are isolated as detergent-resistant membranes because of their insolubility in nonionic detergents.[30] Examples of proteins localized in these microdomains include glycophosphatidylinositol-anchored proteins, flotillins, and stomatin.[39,45]

Erythrocyte Deformability

The erythrocyte membrane must be flexible and resilient in order to repeatedly pass through small capillaries for oxygen delivery to the tissues, all the while maintaining a life span of at least 6 weeks (depending on the species). The deformability and resistance to fragmentation are dependent on the biconcave shape, the viscoelastic properties dependent on the spectrin cytoskeleton, and the red cell viscosity, which is dependent on hemoglobin content. The first two properties are related to the structure of the membrane cytoskeleton. The exact structure of the RBC membrane and its associated structural and integral membrane proteins is still being elucidated. A general explanation of what is currently known is given in the following text.

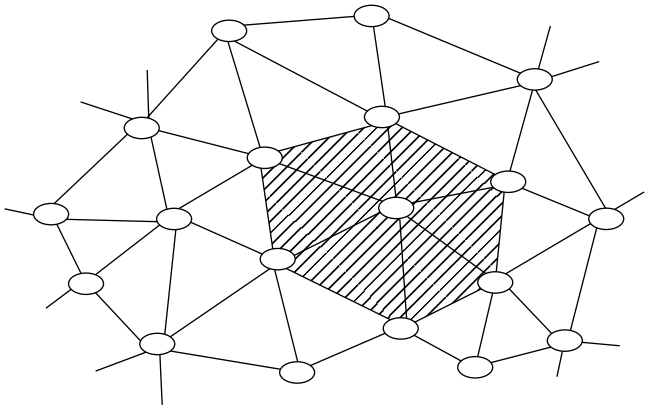


FIGURE 20.1 Lattice structure provided by spectrin tetrameters in the cytoskeleton of erythrocytes.

Cytoskeletal Proteins

Cytoskeletal proteins are located on the cytoplasmic side of the plasma membrane and function to help maintain cell shape, membrane deformability, membrane stability, and the lateral mobility of some integral membrane proteins.[60] They are considered "peripheral proteins" because they are not embedded within the membrane but rather are anchored to the cytoplasmic side of the bilayer.

Alpha and beta spectrin subunits are the largest and most abundant proteins in the cytoskeleton. They are elongated and self-associate to form $\alpha_2\beta_2$ tetramers, which then associate end to end into a pseudohexagonal network of molecules that lies just underneath the plasma membrane. The spectrin lattice is anchored to other proteins and to the bilayer at select areas of the hexagon. The most current model of the erythrocyte membrane shows that spectrin molecules are secured to the lipid bilayer at "nodes" called junctional complexes (Figure 20.1). One of these complexes, the ankyrin complex, is made up of ankryin, band 3, band 4.2, CD47, and glycophorin A. The spectrin molecule is directly associated with the ankyrin molecule. The other complex, the junctional complex, is made up of glut 1, band 3, stomatin, glycophorin C, band 4.1, p55, adducins, and is associated with the membrane via actin, tropomyosin, and tropomodulin. Bands 4.1 and 4.2 proteins are named for their migration during one-dimensional electrophoresis.[3]

Integral Membrane Proteins

Band 3 or the anion exchange protein is an integral membrane protein composed of dimers, tetramers, and other large oligomers. It is responsible for anchoring the cytoskeleton to the membrane, anion exchange, and binding of glycolytic enzymes, hemoglobin, and hemichromes. The anion exchange function is primarily for delivering CO_2 to the lungs in exchange for Cl^-.

Glycophorins are heavily glycosylated integral membrane proteins. They carry most of the sialic acid residues and, therefore, negative charge on the surface of the RBC. Other cell surface molecules that RBCs carry are shown in Table 20.1.

TABLE 20.1 Selected Erythrocyte Surface Molecules in the Human and/or Mouse.

Molecule	Synonyms	Function
CD35	Complement receptor 1	Binds complement fragments
CD36	Platelet glycoprotein IV	Unknown
CD38	N/A	NAD glycohydrolase
CD44	H-CAM, gp85	Hyaluronic acid binding
CD47	Integrin-associated protein	May be a senescence marker
CD49d/ CD29[a]	VLA-4, α4β1 integrin	Adhesion to fibronectin, V-CAM
CD55	Decay-accelerating factor	Neutralizes complement activation
CD59	Complement-regulatory protein	Inhibits complement membrane
CD71[a]	Transferrin receptor	Iron acquisition
CD147	N/A	Recirculation of RBC from spleen
CD239	Basal cell adhesion molecule/Lutheran	Laminin binding

[a]Reticulocytes only.

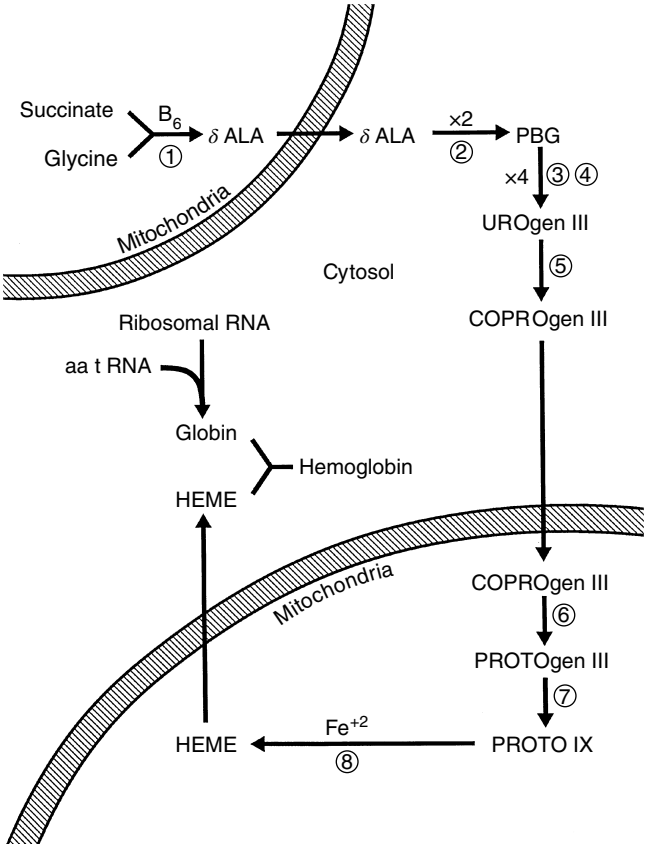


FIGURE 20.2 Pathway for protoporphyrin, heme, and hemoglobin synthesis emphasizing the partitioning of the pathway between the mitochondrial and the cytosolic compartments of the cell. The circled numbers represent the following enzymes of heme synthesis: 1, ALA-Syn; 2, ALA-D; 3, UROgenI-Syn; 4, UROgenIII-Cosyn; 5, UROgen-D; 6, COPROgenIII-Ox; 7, PROTOgenIII-Ox; 8, FER-Ch. (**Source:** Courtesy of Christine S. Olver.)

HEMOGLOBIN STRUCTURE, SYNTHESIS, AND METABOLISM

Hemoglobin is an iron–porphyrin–protein complex. Other iron–porphyrin–protein complexes include myoglobin and heme-containing enzymes such as catalase, peroxidase, and cytochromes. The porphyrin–iron–protein hemoglobin molecule occupies a central role in physiology by binding, transporting, and delivering oxygen to tissues. Hemoglobin is synthesized within developing RBCs and its synthesis is coordinated with the developmental stages of the erythroid precursors.

Hemoglobin Structure

Hemoglobin is composed of two α-polypeptide and two β-polypeptide chains and is approximately 64 kDa in molecular weight. Each chain contains a heme prosthetic group held firmly within a hydrophobic cleft (otherwise known as the "heme pocket"). The entire molecule is, therefore, a globular tetramer. This globular structure permits a cooperative interaction of oxygen binding that gives the sigmoid oxygen–Hgb saturation curve.

Hemoglobin Synthesis

Heme is a planar molecule composed of two elements, a tetrapyrrole protoporphyrin IX and a central iron molecule. The iron is supplied by ferritin, the cytosolic storage form of iron. Heme biosynthesis is an enzymatic process requiring both mitochondrial and cytosolic enzymes. Figure 20.2 shows the sequence of reactions leading to the synthesis of PROTO IX, heme, and Hgb. The initial step of heme synthesis occurs in mitochondria. It requires vitamin B_6 (pyridoxine) and is catalyzed by δ-aminolevulinic acid (ALA) synthase. ALA is then translocated to the cytosol. In the cytosol, two moles of ALA are condensed to form porphobilinogen (PBG), a reaction catalyzed by ALA-dehydratase (ALA-D). ALA-D is strongly inhibited by lead, leading to the anemia associated with lead poisoning. The remainder of the process is depicted in Figure 20.2.

Synthesis of α- and β-globin chains occurs in the ribosomes and polyribosomes in the cytoplasm. Each globin chain contains a cleft or heme pocket that is lined by largely nonpolar amino acid side chains that impart a hydrophobic nature to the pocket. The nonpolar (vinyl and methyl groups) side of heme is positioned in the hydrophobic pocket of each globin chain. The addition of heme to the pockets of globin is followed by a dimerization of the α- and β-globin chains, which is then followed by spontaneous formation of hemoglobin tetramers. Hemoglobin synthesis is ongoing through the reticulocyte stage.

Control of Hemoglobin Synthesis

Synthesis of heme and globin chains is finely coordinated so that there is little or no free heme or globin in the cytoplasm of developing erythroid cells. Heme governs ribosomal translation of globin chain synthesis.[55] Moreover, α- and β-chain synthesis is coordinated. Excess α-chains inhibit their own synthesis while stimulating β-chain synthesis, while excess β-chains inhibit their own synthesis.[28,52]

Hemoglobin Turnover

Hemoglobin is turned over after the extravascular or intravascular destruction of RBCs. In health, extravascular hemolysis occurs as RBCs age and are subsequently trapped in the spleen and phagocytosed by splenic macrophages. Hemoglobin is catabolized within a cell. When RBCs undergo intravascular hemolysis, the hemoglobin is released into the circulation in its free form. Free plasma Hgb is quickly cleared by several mechanisms. The most important is binding of free Hgb to haptoglobin followed by clearance of the complex by macrophages.[27] The half-time for free Hgb clearance is 20–30 minutes. Minor pathways for Hgb clearance are (1) oxidation to methemoglobin (MetHgb) that can be excreted, (2) hydrolysis of the MetHgb to release its ferriheme (ferric heme, hematin, hemin) that is complexed to hemopexin for transport to the mononuclear phagocyte system (MPS), and (3) binding of ferriheme to albumin (methemalbumin) for transport to the MPS. When there is excess intravascular hemolysis, such that the binding capacity of plasma haptoglobin is exceeded, Hgb may be cleared via glomerular filtration. In this event, hemoglobinuria may occur, or the proximal renal tubular cells may catabolize Hgb.[17] When Hgb is internalized into macrophages, it is degraded by hydrolysis into its globin and heme moieties. The globin chains are systematically degraded by proteolytic enzymes to release the constituent amino acids.

Heme Catabolism and Bilirubin Formation

The initial step in the heme catabolic pathway is enzymatic cleavage of the heme ring at the α-methene bridge to release linear tetrapyrrole biliverdin, iron, and carbon monoxide. This reaction is catalyzed by microsomal heme oxygenase in the presence of cytochrome P-450, oxygen, and reduced NADPH. Heme oxygenase activity is highest in the spleen, and there is some activity in the liver, bone marrow, and renal tubular cells. Iron is oxidized to the ferric form, released, and transported as transferrin for storage as hemosiderin or ferritin in the liver and as ferritin in the bone marrow for subsequent reuse.[53] Biliverdin, a green pigment, is reduced in macrophages to bilirubin, a yellow pigment, by the action of the enzyme biliverdin reductase in the presence of NADPH. The virtual absence of biliverdin reductase in birds accounts for the green color of avian bile. Bilirubin is a nonpolar compound and must be bound to albumin to remain soluble in aqueous plasma. The bilirubin–albumin complex is transported to the liver, and at the hepatocyte surface, albumin is released. The first step involving the hepatocyte surface is the uptake or transport of bilirubin across the hepatocyte membrane by a transporter system. The second step in the hepatocyte is conjugation of bilirubin to glucuronic acid, primarily as the diglucuronide and with some monoglucuronide. This reaction is catalyzed by the enzyme glucuronyl transferase. The bilirubin glucuronides are highly polar and are therefore soluble compounds. The third step in the hepatocyte is the transport of bilirubin glucuronide into the bile canaliculi and thence the biliary system. This canalicular transport is the rate-limiting step in hepatic bilirubin metabolism.[29] A small amount of the glucuronides may also be transported back into the circulation, so that, normally, both unconjugated and conjugated bilirubin are present in the circulation.

Fetal Hemoglobin

The synthesis of α-globin chains remains constant during stages of gestational development and throughout adult life. Different types of non-α-globin chains are synthesized and account for the different types of Hgb appearing during development and in adult life. At the embryonic stage, most animals synthesize only embryonic chains designated as ε chains (i.e., embryonal Hgbs). Fetal Hgb (HgbF) is composed of two γ-chains and two α-chains, although not all animals produce HgbF. Ruminants possess a mixture of adult and fetal Hgb at birth, and HgbF is replaced by adult Hgb in the first few months of life.[4] Although HgbF rapidly declines after birth, the capacity to synthesize it remains, and HgbF may appear in responsive anemias. Dogs, cats, horses, and pigs lack HgbF and are born with adult Hgb that has replaced the embryonal Hgb during gestation.[10,32]

Hemoglobin Types in Animals

With the possible exception of pigs, two or more types of Hgb normally occur in several domestic animal species.[9,31,32] Most polymorphisms are determined genetically and are usually associated with multiple amino acid substitutions.[32] The Hgb of cats has a unique structure containing 8–10 reactive sulfhydryl groups per molecule compared to other animals that have only two to four per Hgb molecule.[37] The presence of the readily oxidizable groups is regarded as the basis for the ease of Heinz body formation in the cat (see Chapter 32). Sheep and goats synthesize HgbC in response to severe anemia.[26] Sheep normally have HgbA and goats HgbA and HgbB, and the switch to HgbC during anemia is mediated by erythropoietin.[5] Carbon dioxide decreases oxygen affinity for HgbC more than it does for normal adult Hgbs.[26,61]

IRON METABOLISM

Iron is an essential component not only of hemoglobin and myoglobin, but also of many enzymes. Iron-containing enzymes include NADH dehydrogenase,

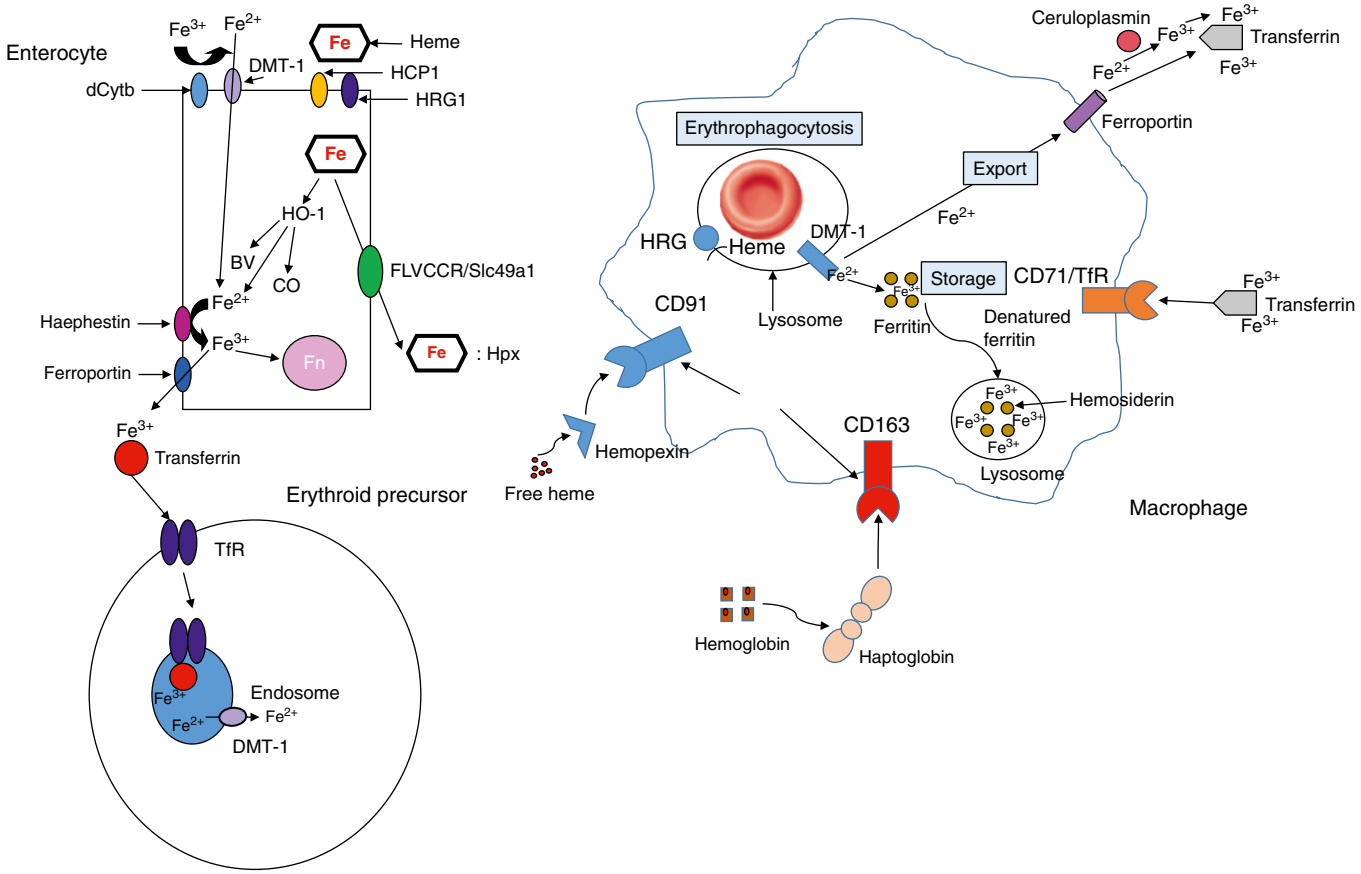


FIGURE 20.3 A schematic of iron metabolism including absorption from enterocytes, transport in circulation, utilization by the erythroid precursors, and recycling by macrophages.

lipoxygenases, superoxide dismutase, ribonucleotide reductase, fatty acid desaturases, and phosphatases.[38] These enzymes function in energy generation, prostaglandin synthesis, free radical detoxification, synthesis of DNA, synthesis of fatty acids, and signal transduction. Iron is provided to cells of the body by three sources: (1) absorption from the gut, (2) recycling from senescent RBCs, and (3) liver and macrophage storage iron (Figure 20.3).

Absorption in Intestine

Absorption of iron is regulated; excretion is not (iron is lost through epithelial sloughing of gut, skin, urothelium). Iron absorption takes place in the proximal duodenum, and it is absorbed either as heme or nonheme iron. Although most iron utilized in physiologic mechanisms is that recycled from hemoglobin iron, total body iron stores are regulated by the duodenum to prevent whole-body iron accumulation or deficit.

Intestinal Absorption of Nonheme and Heme Iron

Iron is stabilized in the ferric form by the acidity of the gastric fluid. Apical duodenal cytochrome B (dCytb) reduces ferric to ferrous iron. Ferrous iron is transported to the basolateral membrane by intracellular mechanisms that are unclear. At the basolateral surface, hephaestin, a copper-containing enzyme, reoxidizes iron to its ferric state. Ferroportin then exports iron out of the cells into the portal circulation, where it is carried bound to transferrin. Once in the circulation, iron is carried to the liver to be stored, or to the erythroid precursors in the bone marrow to be incorporated into hemoglobin. Heme iron is absorbed via heme by the apical heme transporter (apical heme carrier protein 1).[46] Iron is then released from heme via the action of heme oxygenase. It can then be exported from the cell via ferroportin. Heme can also be exported directly using the FLVCR/SLC49A1 transport protein.[16]

Cellular Iron Uptake

Iron is taken up by most cells (i.e., not enterocytes) via the cell surface transferrin receptor (TfR). The expression level of this receptor is directly related to the iron requirements of the cell.[6,15] The cell surface transferrin receptor functions as a dimer, with each 90 kDa monomer embedded in the membrane with one single transmembrane-spanning region. Iron-binding transferrin (holotransferrin) binds to the TfR at physiologic pH and the transferrin–iron–TfR complex is internalized into an endosome. The low pH of the endosome

causes the release of Fe^{3+} from the transferrin and the iron is then reduced to Fe^{2+}. Ferrous iron is then exported to the cytoplasm.[41,47] A newly discovered protein, the product of the *HFE* gene, forms a complex with the TfR and appears to regulate its ability to import iron.[7]

Macrophage Handling of Iron

Intracellular iron is derived from many sources. These include erythrophagocytosis of senescent red cells, scavenging hemopexin-bound heme and haptoglobin-bound hemoglobin, and endocytosis of transferrin bound iron via transferrin receptor 1. Once senescent RBCs, heme, and hemoglobin are endocytosed, heme is degraded by heme oxygenase-1 (HO-1) to release iron (and biliverdin), which is then exported from the endosome via divalent metal iron transporter-1 (DMT-1). Alternatively, heme can be exported from the phagolysosome via HRG1 (slc48a1). Once iron is present in macrophages, it can be exported through ferroportin, where it is oxidized by ceruloplasmin so it can bind transferrin, or it can be stored in the macrophage cytoplasm in ferritin. Should ferritin stores become maximally filled, then hemosiderin begins to accumulate in the lysosome (Figure 20.2).

Selected Molecules Involved in Iron Metabolism

The uptake of iron by the duodenum and by individual cells in the body is regulated by total body iron levels and intracellular iron levels, respectively.[1]

Many molecules involved in iron regulation have been discovered in recent years. Although there are still gaps in our knowledge of the physiologic processes, several important molecules have been identified in research involving humans and rodents, and are described in the following text.

Divalent Metal Iron Transporter-1

DMT-1 is expressed in the enterocytes of the villi and not in the crypts,[11] and is responsible for transporting iron from the enterocyte to the circulation, and from endosomes of macrophages to the cytoplasm. It is associated with phagosomal membranes in macrophages and plasma membranes and endosomes of cells that use the TfR complex for iron acquisition.[23,24]

Ferrireductase Duodenal Cytochrome b

DMT-1 transports Fe^{2+} into the enterocyte from the duodenal lumen. Since Fe^{3+} is the dietary form that exists in the lumen, it must be reduced. The duodenal cytochrome b, located on the apical membrane of the enterocyte, acts as such a ferric reductase.[11]

Hephaestin

Hephaestin, a copper-dependent transmembrane ferroxidase, is an approximately 150–160 kDa protein with 50% homology to ceruloplasmin. It is highly expressed in the small intestine, primarily in the basolateral membrane of enterocytes, and is colocalized with ferroportin.[44] Hephaestin is responsible for oxidizing Fe^{2+} to Fe^{3+} so that it can be transported out of the enterocyte into the plasma.

Ferroportin

Ferroportin is expressed on the membrane of duodenal enterocytes (basolateral), placental trophoblasts, and macrophages distributed in various tissues.[1,13,18] It is the only mammalian cellular iron exporter. It exports iron from enterocytes into the circulation (from dietary iron) and from macrophages to the circulation (from recycled erythrocyte iron). Its expression levels are regulated by hepcidin binding and internalization of the protein, and also by iron levels.

Hepcidin

Hepcidin should be considered a molecule that regulates iron systemically. Hepcidin regulates iron export from enterocytes and macrophages by binding to and causing degradation of ferroportin.[22] It is synthesized as a propeptide in the liver and circulates as a 25 amino acid disulfide-rich peptide. Synthesis is increased by iron loading to decrease iron absorption, and decreased by anemia and hypoxia to increase iron absorption.[42,58] It is also increased in inflammation, secondary to interleukin-6 production, and causes the iron trapping in macrophages during anemia of inflammatory disease.[22] Iron may control hepcidin synthesis through a signaling pathway dependent on bone morphogenic protein 6 (BMP) which acts as a paracrine factor through the BMP receptor (BMPR) in hepatocytes, causing the small mothers against decapentaplegic (SMAD) pathway to induce the hepcidin promoter.[57] BMPR has a coreceptor, hemojuvelin, the loss of which can cause significant iron overload because of impaired hepcidin production.[36] Kupffer cells also appear to regulate hepcidin expression; when Kupffer cells are eliminated, hepcidin expression is increased and serum iron is decreased.[54]

Anemia and hypoxia decrease hepcidin synthesis by increasing erythropoietic activity.[33] Erythroid precursors produce erythroferrone (ERFE) in response to erythropoietin, and ERFE downregulates hepcidin expression.[57]

Transferrin Receptor 1

The transferrin receptor is described under the heading "Cellular Iron Uptake."

Transport and Storage of Iron

Transport of Iron

Transport of iron from one compartment or tissue to another through the circulation (e.g., enterocytes to bone marrow cells) is accomplished by the carrier

protein transferrin. Serum transferrin is a soluble 78 kDa protein that binds one or two iron atoms in their ferric (3^+) form.

Storage of Iron

Iron is stored in developing RBCs, and other cells, as ferritin, an iron–protein complex.[48,49] The protein moiety, apoferritin, consists of at least 24 monomers of either H or L subunits. Each subunit is shaped like a short rod and these rods are assembled as a hollow sphere. Apoferritin has a molecular weight of 441 kDa and can store as much as 4500 molecules (31%) of iron.[35] The maximal weight of ferritin can be approximately 800 kDa, but ferritin more commonly is less than that (620 kDa) with roughly 18% iron. The method of iron movement in and out of the molecule is through "channels," and it enters as the ferrous form and is oxidized in the catalytic ferroxidase center to be stored as the ferric form.[8,12] Iron is released either by a reversal of the process or by digestion of ferritin by lysosomes and reduction to the ferrous form.[25] Hemosiderin is found in cells of the MPS, predominantly in macrophages of the liver (Kupffer cells), spleen, and bone marrow. Hemosiderin can be seen as golden granules in hematoxylin-and-eosin-stained histologic sections, and may appear green to black in Wright–Giemsa-stained cytologic preparations. It is a "stripped down" version of ferritin, whereby the apoferritin protein shell has been removed, leaving approximately 25–30% of iron by weight.[20]

Iron Regulatory Proteins

Proteins involved in iron metabolism are predominantly regulated at the post-translational level by the interplay of iron-responsive elements (IREs) and cytosolic iron-binding proteins referred to as iron regulatory proteins (IRPs). The IREs are stem-loop or hairpin structures located in the 3′ or 5′ untranslated regions of the messenger RNAs (mRNAs) that act as nucleic-acid-binding sites for the IRPs. The binding affinity of IRPs is reversibly regulated by the intracellular concentration of iron. When the intracellular concentration of iron is low, IRPs have a high affinity for IREs, and when cells are iron replete, the affinity is low.

Ferritin and aminolevulinate synthase (rate-limiting enzyme in heme biosynthesis) mRNAs have a single IRE in the 5′ end at the beginning of the coding region. Binding of the IRP inhibits translation; thus, when iron is low, the IRP binds to the IRE and translation is inhibited, whereas when iron is high, IRPs lose affinity and translation of those proteins occurs.[40] Thus, when ferritin is required for storage of increased intracellular iron, translation is upregulated. On the other hand, the transferrin receptor mRNA has an IRE on the 3′ end. When iron is low, the binding of the IRP inhibits the breakdown of the mRNA and thereby stimulates the translation of the protein. Thus, low iron upregulates TfR so that cells become more iron avid.[21] In summary, IRPs regulate mRNA translation by binding to IREs, and binding is stimulated by low iron. However, sometimes binding stimulates translation (TfR) and sometimes binding inhibits translation (ferritin).[40]

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CHAPTER 21

Erythrocyte Biochemistry

JOHN W. HARVEY

Membrane Transport
Carbohydrate Metabolism
Embden–Meyerhof Pathway and ATP Production
Diphosphoglycerate Pathway and Oxygen Affinity of Hemoglobin
Oxidants and Oxidative Injury
Pentose Phosphate Pathway and Protection Against Oxidants
Methemoglobin Formation and Reduction

Acronyms and Abbreviations

Cb_5R, cytochrome-b_5 reductase; DPGM, diphosphoglycerate mutase; DPGP, diphosphoglycerate phosphatase; 1,3DPG, 1,3-diphosphoglycerate; 2,3DPG, 2,3-diphosphoglycerate; EMP, Embden–Meyerhof pathway; FAD, flavin adenine dinucleotide; GAPD, glyceraldehyde phosphate dehydrogenase; G6P, glucose 6-phosphate; G6PD, glucose-6-phosphate dehydrogenase; GPx, glutathione peroxidase; GR, glutathione reduction; GSH, reduced glutathione; GSSG, oxidized glutathione; HK, hexokinase; HK^+, high potassium; LK^+, low potassium; LMB, leucomethylene blue; MB, methylene blue; O_2^-, superoxide; PFK, phosphofructokinase; 3PG, 3-phosphoglycerate; P_i, inorganic phosphate; PGK, phosphoglycerate kinase; PK, pyruvate kinase; PPP, pentose phosphate pathway; SOD, superoxide dismutase.

Erythrocytes provide vital functions of oxygen transport, carbon dioxide transport, and buffering of hydrogen ions. These functions do not require energy per se, but energy in the form of ATP, NADH, and NADPH is needed to keep the cells circulating for months in a functional state despite repeated exposures to mechanical and metabolic insults.

Mature mammalian erythrocytes do not have nuclei; consequently, they cannot synthesize nucleic acids or proteins. The loss of mitochondria during the maturation of reticulocytes results in a loss of Krebs cycle and oxidative phosphorylation capabilities, and prevents the synthesis of heme or lipids de novo in erythrocytes. Energy needs of mature erythrocytes are met solely by anaerobic glycolysis in the Embden–Meyerhof pathway (EMP). Although metabolic demands are lower than in other blood cell types, erythrocytes still require energy in the form of ATP for maintenance of shape, deformability, phosphorylation of membrane phospholipids and proteins, active membrane transport of various molecules, partial synthesis of purine and pyrimidine nucleotides, and synthesis of glutathione. A minor shunt for carbohydrate metabolism, the pentose phosphate pathway (PPP), provides the erythrocyte with additional critical protection against oxidative damage.[10]

MEMBRANE TRANSPORT

The erythrocyte lipid bilayer is impermeable to most molecules. Consequently, various membrane protein transport systems are utilized for movement of molecules into and out of erythrocytes. Water and carbon dioxide are transported across erythrocyte membranes using water channels called aquaporins.[10] Band 3 appears to function as a channel for the movement of anions, especially bicarbonate and chloride, and various compounds including phosphoenolpyruvate and pyridoxal-5-phosphate.[7] Defective anion transport and marked spherocytosis with membrane instability occur in anemic cattle with an inherited deficiency of band 3 protein.[13]

Major interspecies, and in some cases intraspecies, differences occur in cation transport and subsequently

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in intracellular Na^+ and K^+ concentrations.[10] Animal species with high intracellular K^+ concentrations (horse, pig, and some ruminants) have an active Na^+/K^+-pump that exchanges intracellular Na^+ for extracellular K^+ with the hydrolysis of ATP. In addition to individuals with high K^+ erythrocytes, some sheep, goats, buffalo, and most cattle erythrocytes have relatively low K^+ and, consequently, high Na^+ erythrocytes. These low K^+ erythrocytes have low Na^+/K^+-pump activity and high passive K^+ permeability.

Nucleated erythroid precursors and reticulocytes in cats and dogs are high K^+ cells. However, mature erythrocytes from cats and most dogs are low K^+ erythrocytes. With reticulocyte maturation, the Na^+/K^+-pump activity disappears, resulting Na^+ and K^+ concentrations near those predicted for the Donnan equilibrium with plasma.[15] However, many clinically normal Akitas, Shibas, Jindos, and other East Asian dog breeds have high K^+ erythrocytes.[3,10] Erythrocytes from these dogs have substantial Na^+/K^+-ATPase activity, and some of these dogs also have increased glutamate transport, which results in high reduced glutathione (GSH) concentrations. These high K^+/high GSH erythrocytes promote *Babesia gibsoni* replication compared to low K^+/normal GSH erythrocytes.[26] Other pathways of sodium and potassium transport occur to variable degrees in certain species.[10]

Excessive intracellular Ca^{2+} promotes the suicidal death of erythrocytes (eryptosis);[23] consequently, erythrocytes actively extrude Ca^{2+} using a calcium pump having Ca^{2+}-activated, Mg^{2+}-dependent ATPase activity and operating as Ca^{2+}/H^+ exchanger. The calcium pump is activated by a calcium-binding protein called calmodulin.[10]

Amino acid transport in erythrocytes provides amino acids for synthesis of glutathione.[10] In addition, amino acid transporters may be responsible for efflux of amino acids during reticulocyte maturation.[10]

Species vary in their permeability to glucose, with human erythrocytes being very permeable and pig erythrocytes being poorly permeable. Erythrocytes of other domestic animals appear to be intermediate between these extremes.[10] Glucose movement into erythrocytes requires glucose transporters. Glucose transporter 1 is present in mature erythrocytes of mammals that cannot synthesize ascorbic acid (humans, higher primates, guinea pigs, and fruit bats). Glucose transporter 1 is also present in neonatal erythrocytes of other mammals, but it is replaced by glucose transporter 4 in adult erythrocytes of these species.[18] An exception is that adult erythrocytes from all Japanese Akita dogs and some Shiba dogs contain both glucose transporters.[20,21] Erythrocytes from adult pigs lack a functional glucose transporter and, therefore, have limited ability to utilize glucose for energy.[10]

Erythrocyte cell membranes from most animal species have a nucleoside transporter. Rabbit, pig, and human erythrocytes exhibit substantially more adenosine uptake than those of other species studied. Erythrocytes from dogs exhibit more adenosine uptake than those of cats, goats, or cattle, and erythrocytes from horses and most sheep appear to be nearly impermeable to adenosine. While dog erythrocytes are permeable to adenosine, they are impermeable to inosine. Dog and cat erythrocytes exhibit adenine uptake and incorporation into nucleotides, but values are much lower than those of human, rabbit, or rodent erythrocytes.[10]

Other erythrocyte transporters include urea transporter-1 and ferroportin-1. The lipid-raft, integral membrane stomatin appears to play a role as a membrane-bound scaffolding protein that can modulate some transport proteins.[24] Because membrane proteins interact with one another, a deficiency in one protein can result in secondary deficiencies in other proteins. Decreased amounts of several membrane proteins were reported in a dog with hemolytic anemia believed to have a defect in band 3.[1]

CARBOHYDRATE METABOLISM

Although substrates such as ribose, fructose, mannose, galactose, dihydroxyacetone, glyceraldehyde, adenosine, and inosine may be metabolized to some extent, depending on the species, glucose is the primary substrate for energy needs of erythrocytes from all species except the pig. Inosine appears to be the major substrate for pig erythrocytes. Its production by the liver is sufficient to meet erythrocyte energy requirements.[27]

Once glucose enters the cell, it is phosphorylated to glucose 6-phosphate utilizing the hexokinase enzyme. The glucose 6-phosphate is then metabolized through either the Embden–Meyerhof pathway or the pentose phosphate pathway as shown in Figure 21.1.

EMBDEN–MEYERHOF PATHWAY AND ATP PRODUCTION

A net of two molecules of ATP is produced for each molecule of glucose metabolized to two molecules of lactate in the Embden–Meyerhof pathway. Because mature erythrocytes lack mitochondria, the Embden–Meyerhof pathway is the only source of ATP production in these cells. Reactions catalyzed by hexokinase, phosphofructokinase (PFK), and pyruvate kinase (PK) appear to be rate-limiting steps in glycolysis, with the PFK reaction being most important under physiologic steady-state conditions.[10]

At physiologic pH values, high concentrations of inorganic phosphate (P_i) stimulate glycolysis through the Embden–Meyerhof pathway by reducing the ATP inhibition of PFK. Conversely, glycolysis is inhibited by short-term phosphate deficiency, primarily by decreasing intracellular inorganic phosphate for glyceraldehyde phosphate dehydrogenase. Decreased glycolytic rates result in decreased erythrocyte ATP concentrations and hemolytic anemia in experimental dogs made severely hypophosphatemic by hyperalimentation. Hemolytic anemia associated with hypophosphatemia has also been reported in diabetic cats and a diabetic

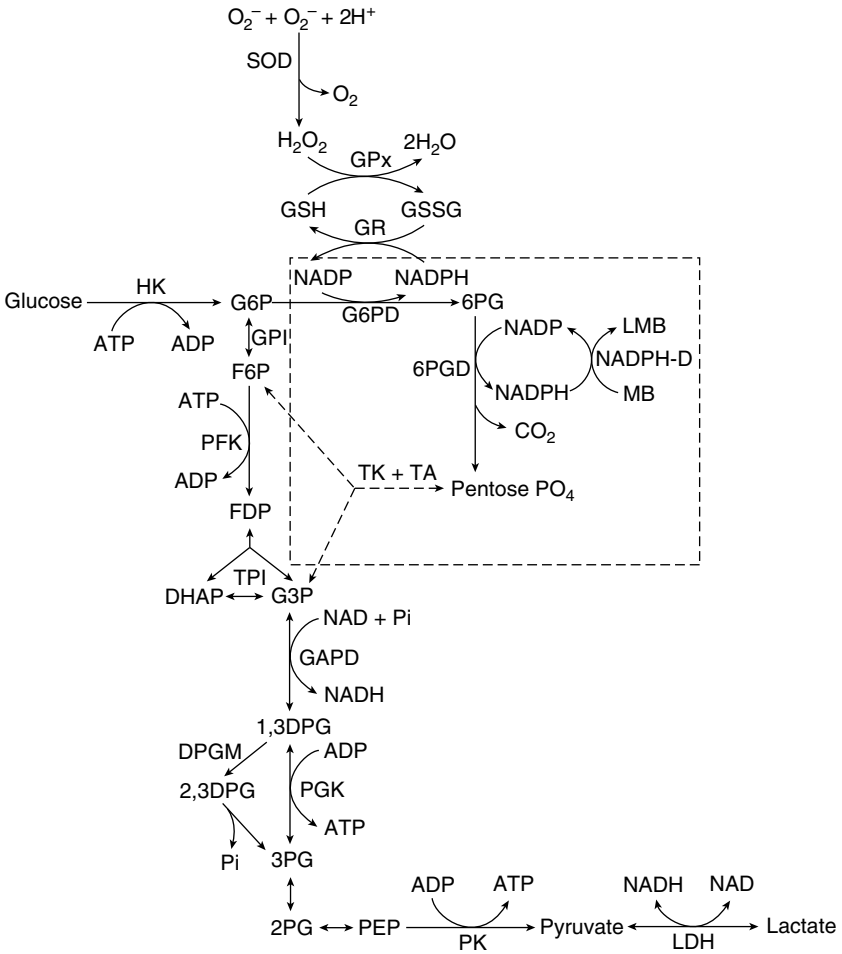


FIGURE 21.1 Metabolic pathways of the mature erythrocytes. HK, hexokinase; GPI, glucose phosphate isomerase; PFK, phosphofructokinase; TPI, triosephosphate isomerase; GAPD, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; MPGM, monophosphoglycerate mutase; DPGM, diphosphoglycerate mutase; PK, pyruvate kinase; G6PD, glucose-6-phosphate dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase; LDH, lactate dehydrogenase; LMB, leucomethylene blue; MB, methylene blue; GR, glutathione reductase; GPx, glutathione peroxidase; TK, transketolase; TA, transaldolase; GSSG, oxidized glutathione; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; FDP, fructose 1,6-diphosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; 1,3-DPG, 1,3-diphosphoglycerate; 2,3-DPG, 2,3-diphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; NAD, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NADPH-D, reduced nicotinamide adenine dinucleotide phosphate diaphorase; GSH, reduced glutathione; P_i, inorganic phosphate; SOD, superoxide dismutase. (**Source:** Courtesy of John Harvey. This figure was published in Harvey.[10] ©Elsevier 2008)

dog following insulin therapy, in a cat with hepatic lipidosis, and in postparturient cattle in which decreased erythrocyte ATP concentrations have been measured.[10]

Because mature erythrocytes depend solely on anaerobic glycolysis for ATP generation, deficiencies of enzymes involved in glycolysis result in shortened erythrocyte survival. Insufficient ATP generation in deficient erythrocytes can result in echinocyte formation. PK-deficient dogs and cats have mild to severe regenerative hemolytic anemia. PK-deficient dogs exhibit marked iron accumulation in the liver and die from liver failure or myelofibrosis.[9] PFK-deficient dogs have compensated hemolytic anemia with sporadic episodes of intravascular hemolysis and hemoglobinuria.[9] PFK-deficient dog erythrocytes are alkaline fragile, because 2,3-diphosphoglycerate (2,3DPG) concentration is decreased in these cells. A decrease in 2,3DPG, the major impermeant anion in dog erythrocytes, results in higher intracellular pH. Episodes of intravascular hemolysis occur when PFK-deficient dogs develop alkalemia secondary to hyperventilation.[9]

DIPHOSPHOGLYCERATE PATHWAY AND OXYGEN AFFINITY OF HEMOGLOBIN

Molecules of 1,3-diphosphoglycerate produced by the glyceraldehyde phosphate dehydrogenase reaction may be utilized by the phosphoglycerate kinase reaction in

the Embden–Meyerhof pathway or may be converted to 2,3DPG by the diphosphoglycerate mutase reaction (Figure 21.1). 2,3DPG degradation to 3-phosphoglycerate is catalyzed by diphosphoglycerate phosphatase activity. The diphosphoglycerate pathway or shunt bypasses the ATP-generating phosphoglycerate kinase step in glycolysis; consequently, no net ATP is generated when glucose is metabolized through this pathway.

Erythrocytes of dogs, horses, pigs, and man normally contain high concentrations of 2,3DPG, whereas those of cats and domestic ruminants have low concentrations. In erythrocytes from most mammalian species, 2,3DPG decreases the oxygen affinity of hemoglobin. When the oxygen affinity of hemoglobin is studied in hemolysates, removal of 2,3DPG and ATP (termed "stripping") from species with low 2,3DPG erythrocytes results in considerably lower oxygen affinities than removal from hemoglobin of species with high 2,3DPG erythrocytes. Because stripped hemoglobins from species with high 2,3DPG erythrocytes have high oxygen affinities, it appears that 2,3DPG is needed within erythrocytes of these species to maintain hemoglobin oxygen affinity within a physiologically useful range.[10]

The flow through the diphosphoglycerate pathway is regulated by the overall glycolytic rate. The formation of 2,3DPG is stimulated by increased phosphate concentration and increased pH. Hypoxic conditions stimulate 2,3DPG synthesis primarily by inducing hyperventilation with resulting alkalosis. Conversely, acidosis and hypophosphatemia result in decreased 2,3DPG concentrations. PFK deficiency inhibits glycolysis above the diphosphoglycerate shunt and results in decreased 2,3DPG concentration. By contrast, the concentration of 2,3DPG is increased in dogs with PK deficiency because the metabolic block occurs below the diphosphoglycerate shunt.[9]

Erythrocyte 2,3DPG increases in anemic dogs and cats. The resultant decrease in hemoglobin oxygen affinity would seem to be beneficial in response to anemia in the dog. 2,3DPG concentration is much lower in cat erythrocytes than in dog erythrocytes, and cat hemoglobins are generally less responsive to 2,3DPG than dog hemoglobin; consequently, the physiologic significance of this increase seen in anemic cats is unclear.[10] Increased 2,3DPG has also been reported in erythrocytes from horses with hypoxic conditions.[4] In the case of severe hypoxic hypoxemia, the response might be detrimental, because hemoglobin cannot be fully saturated.[10] Erythrocyte 2,3DPG, as well as ATP, decreases in blood stored for transfusions.[2,14]

OXIDANTS AND OXIDATIVE INJURY

Animals are exposed to low levels of oxidants in their environment and from normal metabolic processes in the body. Reactive oxygen species and reactive nitrogen species are formed as products of normal cellular metabolism. At low to moderate concentrations, nitric oxide and superoxide free radicals are involved in signal transductions between cells. When generated at higher concentrations in disease states, these free radicals (and even more potent oxidative metabolites that they produce) can overwhelm protective systems within the body, producing cellular injury and/or destruction.[10]

Cumulative injury from these exposures may account for the normal aging and removal of circulating erythrocytes. Some metabolic disorders, including diabetes, inflammation, hyperthyroidism, neoplasia, erythrocyte parasites, intense exercise, and ischemia/reperfusion, can generate oxidants in sufficient amounts to result in increased oxidant injury and shortened erythrocyte life spans, but the anemia (when present) is generally mild. A wide variety of drugs and environmental agents either exist as free radicals or can be converted to free radicals by cellular metabolic processes. These free radicals can be more damaging than reactive oxygen species and reactive nitrogen species.[10]

Oxidants may damage erythrocyte hemoglobin, enzymes (especially sulfhydryl groups), and membranes (especially polyunsaturated lipids). Methemoglobin forms when hemoglobin iron is oxidized from the ferrous to the ferric state. Methemoglobin is unable to bind oxygen, but its presence alone does not result in shortened erythrocyte life span.[10]

Heinz bodies are inclusions that form within erythrocytes following the oxidative denaturation of the globin portion of hemoglobin. They bind to the inner surface of erythrocyte membranes, and their presence can result in premature erythrocyte phagocytosis. Heinz bodies are frequently recognized in erythrocytes from cats because of the susceptibility of cat hemoglobin to form Heinz bodies, combined with a poor ability of the cat spleen to remove Heinz bodies from erythrocytes. Even normal cats may have low numbers of Heinz bodies (<5%).[10]

Oxidative membrane damage results in premature phagocytosis of injured erythrocytes. Oxidative injury to erythrocyte membranes may result in the adhesion of opposing areas of the cytoplasmic face of the erythrocyte membrane and the formation of eccentrocytes and pyknocytes. Eccentrocytes have primarily been recognized in dog and horse erythrocytes.[11] Intravascular hemolysis may occur when severe membrane injury is present.

PENTOSE PHOSPHATE PATHWAY AND PROTECTION AGAINST OXIDANTS

The pentose phosphate pathway generates NADPH, the major source of reducing equivalents in the protection of erythrocytes against oxidative injury. Normally, only about 5–13% of glucose metabolized by erythrocytes flows through the pentose phosphate pathway (Figure 21.1), but this flow can be accelerated markedly by oxidants.[10] NADPH is needed to maintain glutathione and thioredoxin in their reduced states, and it is important in maintaining catalase in a functional form. Defects in the pentose phosphate pathway can render erythrocytes susceptible to endogenous and

exogenous oxidant injury. Glucose-6-phosphate dehydrogenase is the rate-controlling enzyme in the pentose phosphate pathway. A persistent hemolytic anemia with eccentrocytosis has been described in an American saddlebred colt with <1% of normal glucose-6-phosphate dehydrogenase activity.[25]

Glutathione is of central importance in the protection against oxidant injury. It is a tripeptide of glutamic acid, cysteine, and glycine that is synthesized de novo in erythrocytes of animals from constituent amino acids via two ATP-requiring reactions. Glutathione deficiency occurs in some sheep because of a deficiency of γ-glutamylcysteine synthetase, the first enzyme involved in glutathione synthesis, and in other sheep deficient in the amino acid transporter responsible for cysteine transport into erythrocytes, thereby limiting cysteine uptake and restricting glutathione synthesis.[10]

Reduced glutathione (GSH) has a highly reactive sulfhydryl group that may act nonenzymatically as a free radical acceptor to counteract oxidant damage. GSH also functions as an electron donor in various reductive enzyme reactions including glutathione peroxidase, phospholipid hydroperoxide glutathione peroxidase, glutathione S-transferase, and glutaredoxin.[10]

GSH binds to various oxidized thiols, including those present in hemoglobin and peroxiredoxins,[10,22] which contributes to the reduced GSH concentrations measured during oxidative injury to erythrocytes. These glutathionylation reactions can be reversed by glutaredoxin and GSH.[22]

Oxidized glutathione produced by these various reactions is reduced back to GSH by using the flavin adenine dinucleotide (FAD)-dependent glutathione reductase reaction that requires NADPH. Horses with erythrocyte FAD deficiency have markedly reduced glutathione reductase activity, decreased GSH concentration, and prominent eccentrocytosis.[12] If not reduced, oxidized glutathione can be transported out of erythrocytes.[10]

Superoxide dismutase within erythrocytes is a copper- and zinc-containing enzyme that promotes the dismutation of two superoxide (O_2^-) molecules to H_2O_2 and oxygen (Figure 21.1). The importance of superoxide dismutase as an oxidant defense in erythrocytes is unclear.[6] Although generally considered protective, superoxide dismutase may actually increase oxidant injury in conditions where H_2O_2 catabolism is compromised.[10]

Glutathione peroxidase catalyzes the conversion of H_2O_2 to water. It also catalyzes the reduction of fatty acid hydroperoxides and 1-monoacylglycerol hydroperoxides. Another glutathione peroxidase in erythrocytes termed phospholipid hydroperoxide glutathione peroxidase participates in the reduction of more complex phospholipid hydroperoxides using GSH.

Catalase is a heme-containing enzyme that also destroys H_2O_2 by conversion to water and oxygen. Except for dogs, mammalian erythrocytes generally have high catalase activities. Dogs and humans with acatalasemia do not develop hemolytic anemia.[10]

Recent studies suggest that peroxiredoxins may be more important in protecting against H_2O_2 than glutathione peroxidase or catalase.[16] Peroxiredoxins are a ubiquitous family of enzymes that reduce hydrogen peroxide, organic hydroperoxides, lipid hydroperoxides, protein hydroperoxides, and peroxynitrite.[19] Peroxiredoxin-2 is the third most abundant protein in the cytoplasm of erythrocytes after hemoglobin (98% of the total protein) and carbonic anhydrase. Peroxiredoxin-2 functions primarily as a noncatalytic scavenger of hydrogen peroxide; however, oxidized peroxiredoxin-2 is slowly regenerated using reduced thioredoxin, and oxidized thioredoxin is reduced by NADPH using thioredoxin reductase.[16] Peroxiredoxin-2 maintains hemoglobin stability and is essential in protecting erythrocytes from oxidative stress.[8]

Thioredoxin may also be important in the direct scavenging of free radicals. Thioredoxin and glutaredoxin are small proteins with two closely associated cysteines in their active sites. The reduced forms of these proteins interact with and reduce intramolecular protein disulfides that form in oxidatively damaged proteins. Oxidized glutaredoxin is reduced by GSH.[10]

Selenium is incorporated as selenocysteine at the active site of a wide range of selenoproteins, including glutathione peroxidase, phospholipid hydroperoxide glutathione peroxidase, and thioredoxin reductase in erythrocytes.[10] Heinz body hemolytic anemia has been reported in selenium-deficient cattle grazing on St. Augustine grass.[10]

Vitamin E (α-tocopherol) is lipid-soluble and the most important antioxidant in the cell membrane. It donates reducing equivalents to lipid peroxyl radicals, converting them to less toxic lipid hydroperoxides.[10] Oxidized vitamin E can be reduced by ascorbate, presumably at the aqueous–lipid interface of the lipid bilayer.[10] Hemolytic anemia secondary to vitamin E deficiency has been reported in monkeys and premature human infants, but Heinz body formation has not been recognized.[5]

Dehydroascorbate can be reduced to ascorbate nonenzymatically by GSH and enzymatically via the glutaredoxin reaction, by a separate GSH-dependent dehydroascorbate reductase enzyme, and by the thioredoxin reductase reaction. Reduced ascorbate serves as an antioxidant by donating one or two electrons to a variety of oxidants, including oxygen-free radicals and peroxides.[10]

METHEMOGLOBIN FORMATION AND REDUCTION

About 3% of hemoglobin within erythrocytes is oxidized to methemoglobin each day in normal animals. However, methemoglobin usually accounts for less than 1% of total hemoglobin, because it is constantly reduced back to hemoglobin by a methemoglobin reductase, also called cytochrome-b_5 reductase, enzyme reaction present in erythrocytes.[10] In this reaction, electrons are transferred from NADH generated in glycolysis to

ferricytochrome-b_5 using FAD as the enzyme-bound prosthetic group; then the resulting ferrocytochrome-b_5 reduces methemoglobin nonenzymatically to hemoglobin. Methemoglobin reduction is more corrective than protective. Methemoglobin forms at much higher levels in the presence of oxidative compounds. An inherited deficiency in cytochrome-b_5 reductase in dogs and cats results in persistent methemoglobinemia with minimal or no clinical signs.[9] Methemoglobinemia also occurs in horses that have decreased cytochrome-b_5-reductase activity secondary to erythrocyte FAD deficiency.[12] One dog with congenital methemoglobinemia has been reported with a cytochrome-b_5 defect rather than a cytochrome-b_5-reductase defect.[17]

Methylene blue is used to treat toxic methemoglobinemia. It causes methemoglobin to be reduced faster than occurs by the relatively slow NADH-dependent methemoglobin reductase reaction. Methylene blue is reduced to leucomethylene blue by NADPH-dependent diaphorase activity (Figure 21.1), and leucomethylene blue reacts spontaneously with methemoglobin, reducing it to hemoglobin and regenerating methylene blue.[10]

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CHAPTER 22

Erythrokinetics and Erythrocyte Destruction

ANDREA PIRES DOS SANTOS, and JOHN A. CHRISTIAN

Reticulocyte Maturation
  Formation of Mature RBCs
  Reticulocyte Release
  Reticulocyte Identification and Counting
RBC Life Span
  Species Variation
  Rheological Compromise
    Decreased Metabolic Rate
Mechanisms of RBC Clearance
  Morphological Changes and Membrane Modulation
  Accumulation of Oxidative Damage
  IgG Binding
  Complement
  RBC Programmed Cell Death
RBC Destruction

Acronyms and Abbreviations

AChE, acetylcholinesterase; AST, aspartate amino transferase; Bc, conjugated bilirubin; BRBC, biotinylated RBCs; Bu, unconjugated bilirubin; CAT, catalase; CD47, thrombospondin-1 receptor; ECM, extracellular matrix; Fas, first apoptosis signal receptor; FADD, Fas-associated death domain FSC, forward scatter; GLUT1, glucose transporter 1; GPx, glutathione peroxidase; GPA, glycophorin A; GSH, glutathione; Hct, hematocrit; Hgb, hemoglobin; HO-1, heme oxygenase-1; Hsp 70, heat shock protein 70; KCC, potassium chloride cotransporter; LC3, microtubule-associated protein light chain 3; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; NMB, new methylene blue; NO, nitric oxide; PAF, platelet -activating factor; PRDX2, peroxiredoxin-2; PS, phosphatidylserine; RNA, ribonucleic acid; ROS, reactive oxygen species; RPI, reticulocyte production index; SIRPα, signal-regulatory protein alpha; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; FLS, fluorescent light scatter; SOD, superoxide dismutase; TfR, transferrin receptor; TrxR, thioredoxin reductase; Trx, thioredoxin.

The anuclear RBC life span is characterized by three phases. A brief reticulocyte maturation period featuring rapid changes is supported by a high rate of metabolism and translation activity. The mature cell phase involves months of precise functioning under rigorous circulatory conditions. In the final, relatively brief senescence phase, RBCs incur age-associated functional and structural lesions culminating in removal from the circulation, predominantly by cells of the mononuclear phagocyte system, and are recycled. The synthetic activities of reticulocytes, their maturation and release, and the processes of erythrocyte senescence and destruction are covered in this chapter.

RETICULOCYTE MATURATION

Formation of Mature RBCs

A reticulocyte is produced when a late-stage nucleated erythroid precursor (metarubricyte) expels its nucleus. The reticulocyte then develops into a mature circulating RBC in 3–4 days, but at inception it is quite distinctive from the mature cell.[23] In contrast to the relatively uniform biconcave shape of mature cells in many species, reticulocytes are larger, round to lobulated cells with considerably more surface area and less cytoplasm.

Late-stage metarubricytes and maturing reticulocytes are found primarily in erythroblastic islands in the bone

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marrow where they can be sheltered until better prepared for circulatory stresses.[2] The maturation process from reticulocyte to mature erythrocyte involves complex mechanisms that include: (1) hemoglobinization for optimal oxygen transport; (2) plasma membrane remodeling; (3) cytoskeleton rearrangement; (4) removal of obsolete organelles; and (5) release into circulation.

Hemoglobinization is achieved by endocytic activities mediated through internalized membrane vesicles and clathrin-coated pits.[29] Clathrin proteins form a hexamer in the plasma membrane to facilitate the uptake of macromolecules. Together with clathrin-coated pits, transmembrane receptors, transporter proteins, and other transport/endocytic functions actively provide essential "building blocks" (amino acids, iron, etc.) for terminal protein translation and synthesis of other cellular components. At this stage, the remaining 20–30% of total hemoglobin must still be produced.[74] These production activities require an actively synthesizing cytoplasm containing ribosomes, polyribosomes, RNA, and mitochondria, all of which will be removed within the next 3–4 days.

In vitro studies indicate that the youngest reticulocytes have significantly decreased membrane stability and deformability compared to later stage reticulocytes and mature RBCs.[85] The junctional complex interactions between spectrin–actin–protein 4.1 are considered frail in reticulocytes, in part explaining the superior mechanical stability in mature RBCs.[46] Exosomes play a major role in the cell membrane remodeling process by reducing membrane surface area.[5,21] Briefly, exosomes are formed by endocytic invaginations of membrane that are assembled into multivesicular structures. These structures then fuse with the internal cell surface where small (~60 nm) membrane-bound vesicles (exosomes) are released. Some estimates suggest that exosomes account for most of the membrane loss associated with maturation, about 30% in rats. The membranes of released exosomes have features such as phosphatidylserine (PS) exposure that allow recognition and removal by macrophages as apoptotic bodies, avoiding the incitation of inflammation or possibly an autoimmune response.[5] Exosome release coupled with cytoskeleton maturation and stability leads to smaller size and improved RBC deformability.

The remodeling and stability of the cell are also required to form the characteristic biconcave shape and to increase shear resistance. Studies in mice and humans show that reticulocyte tubulin and cytosolic actin are tagged by ubiquitin molecules and are degraded via the ubiquitin–proteasome pathway, whereas the major RBC cytoskeleton proteins such as spectrin, membrane actin, ankyrin, protein 4.1, protein 4.2, p55, and tropomodulin are stable during maturation.[26,46]

Late reticulocyte maturation requires degradation of cellular components that are not needed in mature RBCs; this process allows for a more efficient transport of hemoglobin. Several mechanisms are involved in the clean-up of organelles. Elimination of mitochondria occurs via programmed cell death (mitoptosis) and autophagy (mitophagy), and starts at the time of enucleation. These two mechanisms overlap and mitoptosis leads to autophagosome formation (mitophagy).[33,57] Simultaneously, a specific 15-lipoxygenase initiates degeneration of mitochondria by producing reactive oxygen species (ROS), which will target the mitochondria for proteasomal degradation. Damaged mitochondria can form small vesicles that are subsequently released by membrane blebbing as mitoptotic bodies.[21,50]

Exocytosis via the endosome–exosome pathway promotes the clearance of organelles such as smooth and rough endoplasmic reticulum (ER), ribosomes, and the Golgi apparatus.[57] Other cell components not needed in the mature RBC are selectively enriched in exosomes, including transferrin receptor (TfR or CD71), fibronectin, insulin, glucose transporters, nucleosides, and amino acids.[21] TfR removal is important to prevent oxidative damage caused by iron overload; thus, after the extrusion of the nucleus and loss of mitochondria, the process of heme formation stops and there is enough intracellular iron for heme formation. Heat shock protein 70 (Hsp 70), a chaperone protein, has been strongly implicated in the enrichment of TfR and probably other membrane proteins into exosomes.[21,22] Membrane Na+/K+-ATPase is also removed from reticulocytes of carnivores that switch to low K^+:high Na^+ cytoplasm in mature RBCs.

Autophagy also plays a major role in organelle clearance. Studies in vitro with human reticulocytes have shown that autophagosomes and endosomes will form large autophagic compartments that will fuse with the cell membrane to release cell contents by exocytosis.[27] The autophagosomes are formed from the double membrane originated in the ER, and contain autophagic proteins such as the microtubule-associated protein light chain 3 (LC3), which is a marker of autophagy. The autophagosome will then fuse with endosomes and lysosomes for further degradation. The formation of a hybrid autophagosome/endosome is demonstrated by the colocalization of LC3 and plasma-membrane-derived glycophorin A (GPA), band 3, and glucose transporter 1 (GLUT1).[26] These endosome vesicles, however, do not contain proteins such as actin, ankyrin, and spectrin, suggesting the membranes forming vesicles released by blebbing are unlinked to the cytoskeleton. This fine adjustment of the plasma membrane provides stability and deformability to the mature erythrocyte.[26]

While degradation of most organelles happens in the bone marrow, messenger RNAs (mRNAs) are degraded in the blood stream.[57] This process is mediated by RNAases such as the pyrimidine 5′ nucleotidase. The deficiency or inhibition (e.g., lead toxicity) of pyrimidine 5′ nucleotidase causes accumulation of RNA resulting in basophilic stippling.[77]

Reticulocyte Release

As cells mature, several changes prepare the cell for release from the marrow into the circulating pool. The membrane receptors that tether reticulocytes to fibronectin and thrombospondin in the marrow

extracellular matrix (ECM) are gradually lost, apparently through transfer to exosomes. In addition, surface sialic acids are also decreased, which reduces negative charge repulsion between reticulocytes and endothelial cells.

Subsequent contact of reticulocytes with adventitial and endothelial cells initiates thinning of the adventitia and separation between endothelial cell junctions that allows diapedesis of RBCs through pores into circulation.[4] Erythropoietin (EPO) stimulates the early release of reticulocytes.[30] In species where some reticulocytes are allowed to circulate in health, they appear to be preferentially sequestered for a period in the spleen where reticulocyte "ripening" allows for further hemoglobinization, membrane remodeling, and cytoskeletal stabilization. Splenic retention is apparently mediated through residual fibronectin receptors on reticulocytes.[65]

Reticulocyte Identification and Counting

Reticulocytes are identified based on their appearance when exposed to supravital stains (see Chapter 23). The maturation time for aggregate and punctate cells and the maturation stage at which reticulocytes are normally released from the marrow vary with species. Dogs normally release aggregate reticulocytes from the bone marrow (usually ≤1.0%/day), which then complete maturation in roughly 24 hours. Punctate forms are short-lived and are seen in insignificant numbers. Cats normally release low numbers of aggregate reticulocytes in health (<0.4%). These have a short circulating maturation time of about 12 hours to become punctate cells, which then circulate for 10–12 days and may represent 10% of RBCs in health.[81] Horses do not release reticulocytes from bone marrow in health and only rarely in regenerative anemia. However, RNA-containing equine RBCs can be recognized by automated technology, which might improve sensitivity in assessing equine anemia.[13] Reticulocytes are not typically seen in the peripheral blood of cattle, sheep, or goats except in response to anemia. Healthy laboratory animal species that have shorter RBC life spans and higher rates of erythropoiesis typically release reticulocytes at rates equal to or greater than are seen in dogs. Reticulocyte reference intervals have been published for some species (see section IX).

Cat aggregate reticulocyte counts and polychromatophilic cell proportions are lower than seen in healthy dogs or dogs with similar degrees of anemia, suggesting inherently inferior responsiveness to anemia. This species comparison may be misleading because bone marrow regenerative robustness is reportedly greatest in the cat among common domestic species.[81] Accordingly, the feline punctate reticulocyte counts may be quite high, exceeding $4 \times 10^6/\mu L$. It appears that the rapid aggregate cell maturation in circulation masks the overall intensity of regeneration in cats. Historically, punctate reticulocyte counts in cats have been omitted in many laboratories. Because aggregate cells are so short-lived and elevated punctate counts in cats do reflect a recent regenerative response, reporting of both types is advisable. Omission of punctate counts may lead to misclassification of anemia.

The implementation of flow-cytometry-based techniques of reticulocyte counting using nucleic acid stains such as thiazole orange has removed the subjectivity of manual methods and demonstrated that reticulocytes are a heterogeneous population of cells with different stages of maturation from enucleation to mature RBCs.[64]

However, RBC inclusions such as Howell–Jolly and Heinz bodies[71], presence of RBC-associated pathogens,[67,68] and other cellular elements such as leukocyte cytoplasmic fragments,[62] giant platelets,[71] and spurious artifacts may interfere with the counts. Concurrent evaluation of blood smears should minimize these possible interferences.

When assessing marrow responsiveness, the reticulocyte percent can be easily misinterpreted because the percentage depends not only on marrow production, but also on the number of mature cells in circulation. The same number of reticulocytes per microliter of blood will represent a very different percentage when accompanied by normal numbers of mature cells (Hct 45) versus greatly reduced mature RBC numbers (Hct 15). To correct for this, the reticulocyte percent is multiplied by the total RBC count to obtain the "absolute reticulocyte count" (reticulocytes/μL). Healthy dogs and cats, by manual methods, generally have ≤80 000/μL and ≤60 000/μL absolute reticulocyte counts, respectively. If the RBC count is not available, the Hct can be used to calculate a "corrected reticulocyte percent," which makes a similar adjustment for the effect of relative RBC mass on reticulocyte percent. This value is obtained by multiplying the patient reticulocyte percent times the patient Hct/normal Hct. The normal Hct value is typically 45 for dogs and 37 for cats, values that roughly represent the middle of their respective species reference intervals.

As severity of anemia increases, reticulocytes tend to be released earlier in the marrow maturation process. Because more immature reticulocytes circulate longer, reticulocyte counts will be higher, but this reflects prolonged circulation time rather than increased production. This overestimation of marrow responsiveness can be corrected by calculating the "reticulocyte production index" (RPI). In humans and presumably in dogs, it is estimated that the reticulocyte circulating maturation time increases by 0.5 days for each 10-unit decrease in Hct. At Hct 45, the normal maturation time is 1 day. Therefore, the maturation time at Hct 35 is 1.5 days, at Hct 25 it is 2 days, and at Hct 15 it is 2.5 days. The RPI is calculated by dividing the corrected reticulocyte percentage (or absolute reticulocyte count) by the above-mentioned maturation factor. For example, if a patient has a Hct of 25 (maturation time = 2 days) and a corrected reticulocyte percentage of 3, the RPI is 3.0/2 = 1.5. This substantially reduces the estimation of marrow responsiveness. The RPI adjustment is not recommended in other species due to lack of information on the impact of various Hct levels on circulating maturation times.

RBC LIFE SPAN

Species Variation

After release from marrow, RBCs circulate throughout the vascular system thousands of times before being removed, disassembled, and recycled by macrophages. The RBC life span varies characteristically between species and the differences are, at least partially, explained by different metabolic rates. Comparative studies reveal that RBC life span correlates positively with species longevity (years) and with body mass. That is, larger mammals tend to live longer and have longer RBC life spans when compared to smaller species. Smaller species have higher rates of oxidative metabolism and presumably accrue oxidative injury at higher rates, reducing both longevity and RBC life span. This relationship is illustrated by comparison among common mammalian species where the RBC life span is approximately 140–145 days for horses, 130 days for cattle, 115 days for goats, 100–115 days for dogs, 73 days for cats, and 43 days for mice.[78]

Other complementary experimental evidence suggests that RBC life span in health may be influenced by RBC antioxidant status, where the RBC potential life span in several mammalian species (n = 10) correlated significantly with the RBC levels of superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione (GSH).[41] Additionally, RBC viability may be negatively influenced by corporal aging, at least in small laboratory animals. RBCs from old rats are reported to have lower quantities of several antioxidant enzymes compared to young rat RBCs.[24] Reticulocytes from old rats have lower SOD activity, and increased quantities of catalytically inactive SOD, suggesting an age-associated defect in erythropoiesis.[24] These differences are associated with altered RBC survival as evidenced by a reported 40–60% shorter RBC life span in old mice and rats versus young animals.[1,25] Although limited information is available for larger animals, a similar pattern of shorter RBC life span with age has been suggested for greyhounds.[63]

Rheological Compromise

Based on evidence that cell density and cell age were positively correlated, early RBC aging studies concentrated heavily on identifying "worn out" cells with severely compromised rheological abilities. Since then, some well-designed comparative studies using in vivo aged rabbit biotinylated RBCs, hypertransfused mouse RBCs, and human dense RBCs have confirmed that some rheological compromise accompanies cell aging.[49,79] However, the magnitude and significance of these changes in explaining RBC clearance remain equivocal. Although mild decreases in deformability were observed, fairly constant surface area to volume ratios (sphericity) and a slight decrease in diameter offset that change; thus convincing support that the RBCs would be compromised in traversing sinusoids was lacking. More recently, Franco and collaborators,[20] using a combination of density gradient and flow cytometry techniques, reported that normal human RBCs fail to show increasing density past the early stages of circulation. Interestingly, they observed that biotin-labeled human RBCs retrieved at >126 days of age (end of life span) generally display an irregular morphology with uneven distribution of band 3 and increased presence of IgG; the latter potentially indicating a clearance mechanism. The authors suggest that the irregular morphology would likely be considered artifact or unspecific poikilocytosis in blood smear evaluations when they are in fact senescent RBCs.[20]

Decreased Metabolic Rate

Senescence of RBCs reportedly involves the decrease of metabolic rate evidenced by the decrease of multiple glycolytic enzymes such as pyruvate kinase (PK), phosphofructokinase (PFK), glucose-6-phosphate dehydrogenase (G6PD), and hexokinase in humans.[28,34] In addition, the transport of glucose across the RBC membrane is decreased.[7] Thus, the capacity to metabolize glucose is decreased by approximately 2.5 times in older RBCs.[48]

MECHANISMS OF RBC CLEARANCE

The mechanisms of RBC clearance may be multiple and the importance of each may vary with species. It appears that the best supported clearance mechanisms are designed to remove old RBCs well before significant decrement in individual cells might compromise overall RBC functionality. Thus, redundancy in mechanisms to ensure timely clearance is not surprising. RBC aging is reported to involve decreased metabolic rate, accumulation of oxidative damage, alterations in and loss of cell membrane, decreased cell volume, increased density, and increased immunogenicity.[48] Given the inability of RBCs to synthesize new proteins, aging changes depend mostly on modifications of pre-existing molecules that activate complex molecular pathways. These pathways will ultimately result in a nonlinear, nonrandom, rapid terminal stage process culminating in RBC clearance by the reticuloendothelial system.[3]

Morphological Changes and Membrane Modulation

The RBCs undergo multiple morphologic changes, such as loss of cell membrane with membrane-bound hemoglobin by vesiculation and loss of cations, which result in decreased cell volume and increased deformability.[48] Approximately 15–20% of hemoglobin is gradually lost by vesiculation over the RBC's life span with greater loss in the second half of the life span. These vesicles are removed, at least in part, by a process similar to the "pitting" of Heinz bodies by the spleen.[84] Vesiculation is also associated with the removal of senescent cell antigens, perhaps preventing premature removal of RBCs. These vesicles are characterized by the presence of glycophorin

A, PS, and IgG on their outer membrane and, thus, are rapidly removed from circulation by phagocytosis.[83]

Decreased cell volume and modulation of the cell membrane result in decreased membrane fluidity and decreased levels of sialic acid and thrombospodin-1 receptor (CD47). CD47 is an integrin member of the Ig superfamily that binds to the signal-regulatory protein alpha (SIRP α), a transmembrane protein on macrophages forming a "don't eat me" signal.[54] Conversely, there is an increase exposure of "eat me" signs, such as PS.[6,48]

Accumulation of Oxidative Damage

ROS are a family of unstable reactive molecules derived from molecular oxygen generated continuously as a by-product of metabolism.[87] The ROS family includes free radicals such as superoxide anion (O_2^-), nitric acid (NO^-), hydroxyl radical (HO^-), and reactive compounds such as hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl), and lipid radicals.[8] When in excess, ROS are implicated in the oxidation of biological molecules such as nucleic acids, proteins, carbohydrates, and lipids.[11]

RBCs are particularly susceptible to oxidative injury because of continuous exposure to endogenous and exogenous sources of ROS. Erythrocytes accumulate large amounts of hemoglobin during development. Hemoglobin is an iron–porphyrin–protein complex, whereas iron is a pro-oxidant element and transforms H_2O_2 into the most reactive free radical OH^-.[69] Auto-oxidation of hemoglobin to methemoglobin and O_2^- also represents an endogenous source of ROS production.[56] Exogenous sources include ROS released from neutrophils, macrophages, and endothelial cells, primarily in the microvasculature,[56] and exposure to oxidants.

Given their high susceptibility, RBCs also have intricate antioxidant mechanisms that prevent ROS generation, neutralize ROS, or limit deleterious effects of ROS. The primary oxidant defense mechanism consists of components that will directly intercept ROS intracellularly, including SOD, catalase (CAT), GPx,[56] thioredoxin reductase (TrxR)/thioredoxin (Trx),[58] and the peroxiredoxin-2 system (PRDX2).[60] Other mechanisms include plasma membrane oxidoreductases, which are electron transport systems that enable erythrocytes to respond to changes in redox environments.[40] The absence of nuclei in mature erythrocytes makes them even more susceptible to oxidative damage given their inability to synthesize new proteins in response to oxidative insults.

RBCs' aspartate amino transferase (AST) activity declines significantly with aging.[33] AST is one of the enzymes responsible for glutamate biosynthesis. The RBC membrane is impermeable for this amino acid. AST uses serum aspartate and α-ketoglutarate (permeable) to reversibly form oxaloacetate and glutamate.[18] Glutamate is necessary for the synthesis of GSH, which plays a central role protecting RBCs from oxidative damage.

RBC oxidative stress has been implicated in shortening of the RBC's life span and in impairing cellular deformability needed for effective oxygen transport and delivery to tissues.[56] Furthermore, defects in enzymes critical to antioxidant responses are implicated in the mechanism of mild chronic to severe acute hemolysis.[55]

The most common consequence of oxidant injury to RBCs is the formation of methemoglobin, eccentrocytes, and Heinz bodies. Cat erythrocytes are remarkably sensitive to systemic oxidative injury attributed to the presence of eight reactive sulfhydryl groups per hemoglobin molecule. When the number of free radicals exceeds antioxidant defenses, oxidation of the sulfhydryl groups on the globin chains will form Heinz bodies.[10] This can result in acute life-threatening hemolytic anemia (Heinz body anemia)[10] or can be responsible for decreased erythrocyte life span without anemia due to increased RBC clearance via oxidative stress-induced eryptosis (discussed in the following text).[42]

Several indications of compromised antioxidant capacity have been observed in dense and in vivo aged RBCs from various species. A few examples include decreases in total free thiol and reduced GSH, glutathione reductase activity, and increases in methemoglobin,[31] membrane-bound hemichrome (denatured hemoglobin),[35] and lipid peroxidation by-products.[32] Although there has been controversy over the significance of reported compromises in antioxidant defenses,[12] the presence of irreducible denatured hemoglobin bound to membranes is difficult to dismiss as clear evidence of antioxidant decline. At least two different pathways have been proposed whereby interaction of oxidized Hgb with the membrane might lead to RBC removal. One is hemoglobin–spectrin cross-linking leading to increased membrane rigidity, decreased deformability, and/or stimulation of membrane proteolysis with subsequent breakdown in membrane integrity.[75] In the other pathway, oxidized Hgb binds to and clusters band 3 protein leading to opsonization and clearance.[80,86]

A number of other lesions in senescent RBCs have been proposed as playing a role in clearance. With evidence for some decline in antioxidant capabilities, there is also a risk of lipid peroxidation that could facilitate RBC removal by either compromising deformability[66] or triggering recognition by macrophages.[72] The latter is suggested to occur through exposure of PS on the outer leaflet of the lipid bilayer. Whether secondary to oxidative injury, depletion of energy, or other mechanisms, there is ample evidence that the presentation of negatively charged PS on the outer layer of RBCs leads to phagocytosis by macrophages.[6,72]

IgG Binding

Autologous immunoglobulin binding to the most dense (presumably aged) human RBCs and increased phagocytosis of these RBCs by autologous macrophages were first described by Kay.[39] Fab-dependent IgG binding and phagocytosis suggested the involvement of specific receptor recognition by the IgG.[38] Supporting Kay's findings, several other density-gradient-centrifugation-based studies have also reported increased immunoglobulin binding in the most dense RBCs.[35] Furthermore,

using the mouse hypertransfusion model of in vivo aging, autologous IgG was identified only in the oldest RBCs and was associated with in vitro phagocytosis.[73] Increased IgG binding to dog biotinylated RBCs ≥104 days old has been demonstrated, although the ability of the IgG binding to mediate phagocytosis was not tested.[9] By contrast, in vivo aged rabbit biotinylated RBCs failed to show terminal increases in IgG binding,[14] which further highlights the need for caution in extrapolating information between species. In the rabbit biotinylation model, older RBCs were found to expose more PS than younger RBCs.[6] This may well be an important mechanism for clearance of senescent RBCs in the rabbit where IgG binding does not appear to be a significant factor.[14] Recognition of PS exposure on RBCs by macrophages can be at least partially mediated by lactadherin, a PS-binding glycoprotein produced by macrophages.[15]

The identity of the epitope responsible for antibody binding to senescent RBCs has been controversial, although it is not unreasonable to consider that more than one epitope may develop and that this could vary between species. The anion transport protein (band 3 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)) has received the most support as an important site, although other sites such as glycophorin A and aminophospholipids have also been proposed. Band 3 reportedly becomes antigenic through either proteolytic cleavage revealing a 62 kDa immunoreactive fragment, or by horizontal redistribution of the transmembrane protein into immunoreactive clusters. Exposure of the cryptic 62 kDa antigen by proteolysis is proposed to occur as a consequence of cumulative oxidative injury.[37] Interestingly, cat RBCs exposed to activated neutrophils show evidence of proteolysis and increased IgG binding.[82] These changes contribute to the accelerated clearance of RBCs in the anemia of inflammatory disease due to a combination of oxidation and serine protease activity. Lower level, cumulative injury could conceivably play a role in RBC injury and clearance under normal homeostasis, as described by Kay et al.[37]

The role of band 3 clustering in initiating autologous antibody binding has been well documented in senescent and diseased RBCs.[35] It is theorized that unclustered band 3 does not sustain antibody binding because the senescent cell IgG binds weakly and requires a two-point attachment to form a stable association. Normally, the distance between band 3 molecules is too great for the binding of both Fab portions of IgG. Clustering of band 3 can be accomplished by several methods, including binding of denatured Hgb at the cytoplasmic domain of band 3 or by chemical agents such as zinc, melittin, and acridine orange.[76] These chemical agents also cause autologous IgG binding apart from hemichrome formation, supporting the premise that it is the clustering of band 3 and not the oxidation process per se that creates the signal for IgG binding.[76] Removal of the clustering agents leads to reversal of the clustered distribution and elution of antibodies.

Denatured Hgb has a specific high-affinity binding site on the cytoplasmic domain of band 3. This binding leads to a horizontal redistribution of band 3 into a clustered arrangement that is then recognized on the external surface by autologous antibodies. This pathway illustrates how an early intracellular compromise in maintaining reduced Hgb could initiate a morphologic change on the external cell surface leading to opsonization and phagocytosis. Membrane aggregates showing enrichment and colocalization for hemichromes, band 3, and IgG have been identified in a variety of conditions, including dense human RBCs,[35] sickle RBCs,[36] and thalassemic RBCs.[86] The pathway of Hgb denaturation leading to band 3 clustering and opsonization is at least partially supported by in vivo RBC aging studies. Dog biotinylated RBCs show increased membrane-bound Hgb and increased IgG binding late in the RBC life span, although these changes have not yet been specifically colocalized to band 3 clusters,[70] as has been documented in the human studies.

Opsonization of RBCs secondary to band 3 clustering may play a notable role in the pathogenesis of several hemolytic anemias of significant importance to veterinary medicine. Heinz body hemolytic anemia occurs in several species as a result of exogenous intoxicants and, in the cat, associated with certain metabolic diseases. Because Heinz bodies bind to the cytoplasmic domain of band 3, subsequent clustering and opsonization may lead to an immune-mediated component in clearance of these cells.

Zinc intoxication in dogs is frequently associated with severe hemolytic anemia. Strong regenerative responses with spherocytosis and inflammatory leukograms have led clinicians to initially diagnose this as immune-mediated hemolytic anemia. Given the clear role of zinc in clustering of band 3 with subsequent opsonization, this toxicity is likely an immune-mediated process. Although initially counterintuitive, this condition is consistently reported as Coombs-test-negative. However, this is very consistent with the reversible nature of band 3 clustering by zinc. Washing of the RBCs removes zinc, and presumably leads to dispersion of the band 3 clusters and elution of the antibodies.

Complement

Another factor likely to be important in the efficient removal of IgG-opsonized RBCs is amplification of the signal by complement. Turrini and coworkers showed that when band 3 in human RBCs was clustered using $ZnCl_2$, a sequence of IgG binding, complement binding, and in vitro phagocytosis followed.[76] Phagocytosis was maximal in the presence of clustered band 3, IgG, and complement. Inactivation of complement reduced phagocytosis by 80%, which demonstrated IgG's weak capacity to initiate phagocytosis alone and emphasized complement's importance in amplification. However, phagocytosis was virtually eliminated if IgG or band 3 clustering was removed, showing the

absolute requirement for these steps. This work complemented previous reports describing the presence of C3b–IgG complexes on oxidatively stressed and density-gradient-derived "senescent" human RBCs.[47] Interestingly, only low numbers of IgG (an increase of <20 IgG/RBC) were needed to stimulate significant complement deposition.[47] Quantitative studies on antibody binding to dense RBCs have generally reported an increase of only a few hundred IgG per RBC.[35,38] With amplification, this degree of antibody binding should be a more than adequate signal to initiate phagocytosis.

RBC Programmed Cell Death

Programmed cell death of erythrocytes (eryptosis) is a coordinated mechanism to eliminate injured cells before senescence occurs. In this process, RBCs undergo shrinkage, blebbing, and surface exposure of PS with subsequent phagocytosis of eryptotic cells and particles.[42] Shrinkage happens, at least in part, by an increase in cytosolic Ca^{2+}, which stimulates calcium-sensitive K^+ channels and parallel efflux of Cl^-; thus, driving water out of the cell, which results in shrinkage. Increased intracellular Ca^{2+} is also involved in membrane blebbing by stimulation of the Ca^{2+}-dependent cysteine protease calpain, which degrades the cytoskeleton. In addition, Ca^{2+} stimulates the calcium-dependent enzyme scramblase, which flips the PS-rich cytosolic leaflet of the plasma membrane, exposing PS.[19]

Many substances trigger eryptosis by increasing cytosolic Ca^{2+} including arsenic, aluminum, lead, lithium, selenium, and zinc, as well as amphotericin B, leukotriene C4, retinoic acid, and hemin.[42] When cell shrinkage occurs, RBCs release platelet-activating factor (PAF), which activates an erythrocyte sphingomyelinase promoting the breakdown of sphingomyelin, resulting in ceramide formation. Ceramide further stimulates eryptosis by enhancing the effects of Ca^{2+} in eryptotic RBCs.[43] Given that PAF activity does not require increased cytosolic Ca^{2+}, this mechanism is Ca^{2+}-independent.[45] Triggers of eryptosis through the effect of ceramide include amyloid, copper, and vitamin K3.[42] Eryptosis may also be elicited by ligation to specific surface antigens such as CD47, CD95 (first apoptosis signal receptor, FasR), and glycophorin C.[19]

Oxidative stress may also trigger eryptosis by activation of Ca^{2+} and Cl^- channels promoting Ca^{2+} influx and Cl^- efflux, contributing to cell shrinkage.[17] RBCs also express oxidant-sensitive caspases; pro-caspase-3 present in mature RBCs is cleaved to active caspase-3 when oxidative stress is present.[53] Caspase-3 targets band 3 in RBC membranes weakening the band 3 and protein 4.2 interactions and increasing PS exposure.[51,53] Mature RBCs also contain Fas, FasL (Fas ligand), Fas-associated death domain (FADD), and caspase 8. Their presence is associated with loss of aminophospholipid translocase activity and with PS externalization, suggesting that Fas-dependent signaling processes play a role in regulating RBC clearance through PS exposure.[52]

Excessive eryptosis may cause anemia; although with adequate regeneration there may be a compensated hemolytic process (normal Hct and reticulocytosis). In addition, excessive eryptosis may hamper microcirculation due to the binding of eryptotic bodies to endothelial cells.[43]

Several disorders are associated with excessive eryptosis including iron-deficiency anemia, diabetes, renal insufficiency, hemolytic anemia, sepsis, and myelodysplastic syndrome.[42] Interestingly, acceleration of eryptosis tends to limit the life span of intraerythrocytic pathogens limiting their growth, as noted in *Plasmodium falciparum* infection in human RBCs.[44]

EPO, the most potent stimulator of erythropoiesis, can inhibit eryptosis by its actions on Ca^{2+}-permeable channels.[59] In the same fashion, catecholamines, endothelin 1, and NO also counteract eryptosis.[42]

Most of the studies of eryptosis are based on human or rodent animal model RBCs. Thus, it is likely that variation might occur between other veterinary species. Future studies to determine additional or species-specific triggers and inhibitors of eryptosis, additional pathways and interactions, as well as the role of eryptosis in the pathogenesis of diseases are needed for animal species.

RBC DESTRUCTION

The primary organs of RBC clearance are the spleen, liver, and bone marrow. Phagocytized RBCs are digested within macrophage phagolysosomes, and hemoglobin is dissociated into heme and globin. Globin is further digested and resulting amino acids are reutilized. Heme breakdown produces iron, biliverdin, and small amounts of carbon monoxide. Heme oxygenase-1 (HO-1) is the rate-limiting enzyme in heme degradation and is responsible for release of iron. Heme iron may be stored in macrophages or exported to the circulation by ferroportin.[16,61] Biliverdin is converted within the macrophage to bilirubin (unconjugated, Bu) that is relatively water-insoluble. As bilirubin leaves the cell, it attaches noncovalently to albumin to improve solubility and to be transported to the liver. Bu uptake by hepatocytes and conjugation with glucuronides (most species) produce the water-soluble conjugated bilirubin (Bc) that is excreted by active transport into biliary canaliculi for excretion into the intestines. There Bc is broken down to urobilinogen which is either reabsorbed or is converted to stercobilinogen, which contributes to the dark brown color of feces, and excreted. Reabsorbed urobilinogen is either taken up by hepatocytes and excreted in the bile or enters general circulation and is excreted in the urine.

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CHAPTER 23

Reticulocyte and Heinz Body Staining and Enumeration

HAROLD TVEDTEN and ANDREAS MORITZ

Reticulocyte Staining and Counting
Manual Methods
Automated Methods
Reporting Methods
Reticulocytosis without Anemia
Other Indicators of Reticulocyte Status
Heinz Body Staining and Counting
Manual Methods
Species Variations

Acronyms and Abbreviations

Advia, Advia 2120®, 120, or 2120I; AO, acridine orange; BCB, brilliant cresyl blue; CH retic, hemoglobin concentration reticulocyte; CHr, cell hemoglobin of reticulocytes; EDTA, ethylenediaminetetraacetic acid; LaserCyte® hematology analyzer; ProCyte dx, IDEXX Laboratories ProCyte dx® hematology analyzer; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCVr, mean corpuscular volume reticulocyte; mRNA, messenger ribonucleic acid; NMB, new methylene blue; RBC, red blood cell; Sysmex, Sysmex XT 2000iV® TO, thiazole orange.

This chapter describes technical aspects of enumeration of reticulocytes and Heinz bodies. Related material is described in Chapters 25, 32, 142, and other chapters. Evaluation of reticulocytes is a critical step in the evaluation of anemia (see Chapter 25). One major type of anemia is Heinz body anemia and that diagnosis requires detection and enumeration of the number and approximate mass of Heinz bodies in erythrocytes.

RETICULOCYTE STAINING AND COUNTING

Reticulocytes are the stage in the maturation of erythroid cells between the metarubricytes and mature RBCs. Reticulocytes lack a nucleus but retain some ribosomal mRNA material. The basic principle of different methods identifying reticulocytes is to stain mRNA in the cells so that they can be differentiated from mature RBCs and nucleated RBCs. Both manual and automated methods can be used. The preferred specimen for reticulocyte quantification is anticoagulated blood, usually as EDTA. Specimens are suitable for clinical diagnosis if stored at room temperature for up to 48 hours and likely up to 4 days at 4 °C. Specimens should be free of clots, hemolysis, or other gross defects. Sample-induced errors (usually false increases) in reticulocyte counting include Howell–Jolly bodies, Heinz bodies, basophilic stippling, blood parasites, and large platelets with abundant RNA.[19] Factors that affect determination of the total RBC count affect the absolute reticulocyte count.

Manual Methods

RNA is a polyanion and can be stained with many cationic dyes including brilliant cresyl blue (BCB), new methylene blue (NMB), purified azure B, acridine orange (AO), thiazole orange (TO), auramine O, oxazine 750, polymethine, and pyronin Y. The two stains used most frequently for manual counting of reticulocytes in blood smears are new methylene blue (1% NMB in saline plus 1.6% potassium oxalate) and brilliant cresyl

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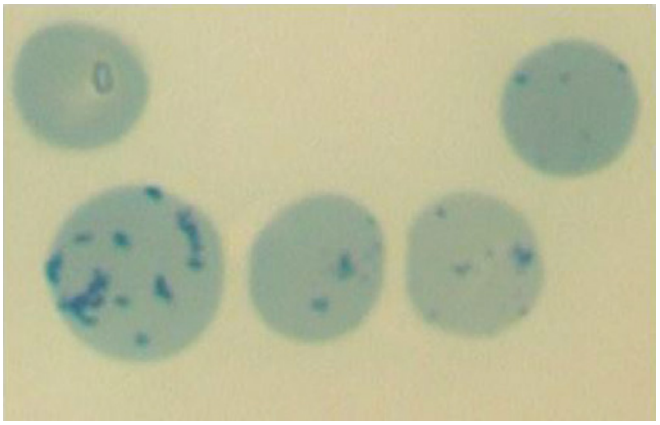


FIGURE 23.1 Feline reticulocytes. The photo shows three punctate reticulocytes on the right, an aggregate reticulocyte on the lower left, and an erythrocyte (with an artifact) on the upper left. The upper right punctate reticulocyte has three dots (ribosomal material) and it therefore just makes the definition of a reticulocyte.

blue (1% in saline). Equal amounts of blood and stain are mixed and allowed to stand at room temperature: 10 minutes for NMB and 15–30 minutes for BCB. One may increase the proportion of blood if the animal is very anemic. The mixture is remixed, and blood smears are made. Purified azure B has been recommended to replace new methylene blue or brilliant cresyl blue.[18] Counterstaining with a Romanowsky stain (e.g., Wright–Giemsa) gives a darker reticulum with BCB. For birds, mix three drops NMB to one drop EDTA blood and allow the mixture to incubate at room temperature for 10 minutes before making the smear.

Microscopic evaluation of 1000 stained non-nucleated erythroid cells is used to determine the percentage of reticulocytes. That percentage multiplied by the absolute erythrocyte count gives the absolute reticulocyte count per given volume (microliter or liter). Other granular material may be present and should be recognized. Heinz bodies stain a lighter blue with BCB and NMB than the more intensively colored purple reticulocyte granules.

Reticulocytes are defined as RBCs that contain more than two dots of ribosomal material. This is not to indicate that cells with one to two dots have too little mRNA to be reticulocytes, but that reticulocyte stains often have granular precipitate and cells with more than two dots are more certainly reticulocytes. Identification of the division between feline aggregate and punctate reticulocytes is more difficult and subjective (Figure 23.1). Tvedten defines aggregate reticulocytes as having more than 15 dots or as RBC having one or more aggregates of dots. Reticulocytes counted in birds should be the ring form reticulocyte, in which aggregates of ribosomes are arranged in a ring around at least half of the nucleus. Estimation of polychromasia is easier than identifying reticulocytes in birds.[12]

Automated Methods

Automated systems have better precision and accuracy than manual methods because much larger numbers of cells are enumerated, interobserver variation is avoided, and variation in staining quality such as stain precipitates and staining intensity is minimized. Time needed to perform the test is greatly decreased; therefore, reticulocyte evaluation has become routinely available. The coefficient of variation for manual counting of reticulocytes may be 8–23%, while for flow cytometric counting it may be less than 3%. However, stains and detection methods vary in sensitivity and specificity.[13] Automated reticulocyte counts may be erroneously increased due to the presence of Heinz bodies, Howell–Jolly bodies, blood parasites, and leukemic cells.[3,14]

Automated veterinary instruments, designed for reference laboratories and validated to enumerate canine, feline, and equine reticulocytes, include the Sysmex® and the Advia®.[13] The Sysmex XT 2000iV® uses fluorescence flow cytometry with a semiconductor laser for enumerating reticulocytes. Cells are stained with polymethine, a fluorescent stain, which is optimized to stain RNA. The IDEXX ProCyte dx® and the new Sysmex XN-V® also use that method. In one study, Sysmex® canine reticulocyte counts had high correlation ($r = 0.90$) with manual counts.[13] Sysmex® reticulocyte counts in cats (Figure 23.2) correlated well with manual aggregate reticulocyte counts ($r = 0.86$), but not with manual punctate reticulocyte counts ($r = 0.50$). A mean positive bias of the Sysmex® reticulocyte count over the manual aggregate count was about 6% (range −2% to +19%). This suggested that some punctate reticulocyte were included with the Sysmex® automated reticulocyte count. Equine reticulocytes were detected by the Sysmex®, but too few were seen in manual reticulocyte counts to validate any accuracy. The highest absolute reticulocyte counts were seen in horses with highest RBC counts and in foals.[13]

The automated reticulocyte analysis of the Advia uses a reagent solution formulated to maintain pH 7.4 as a preferred value, with an osmolality preferably about 292 ± 5 mOsm. At these pH and osmolality ranges, the concentration of the cationic dye, oxazine 750, required for RNA staining is preferably about 9.0 µg/mL to about 10.5 µg/mL. The buffer enhanced penetration results in the dye staining RNA in the reticulocytes in about 20–30 seconds or less. The concentration of dye in the reagent of the method minimizes nonreticulocyte staining of mature RBCs, which leads to a good signal separation from the background noise.[5,27]

The Advia 2120® automated reticulocyte counts (Figure 23.2) had excellent correlation to manual reticulocyte counts in 32 dogs ($r = 94$, slope 0.98) and to manual feline aggregate reticulocyte counts in 33 cats ($r = 94$, slope 1.16) (unpublished data). However, there was poor correlation with feline punctate reticulocytes. Equine reticulocytes are reported by the Advia.[25]

Two in-clinic hematology instruments (IDEXX LaserCyte® and Oxford Science ForCyte®) have been evaluated for reticulocyte counting in dogs and had fair correlation coefficients compared with the Advia, and moderate random error for canine reticulocyte counting.[1] The LaserCyte® compared well with the Advia for feline reticulocyte counts. The LaserCyte uses NMB to

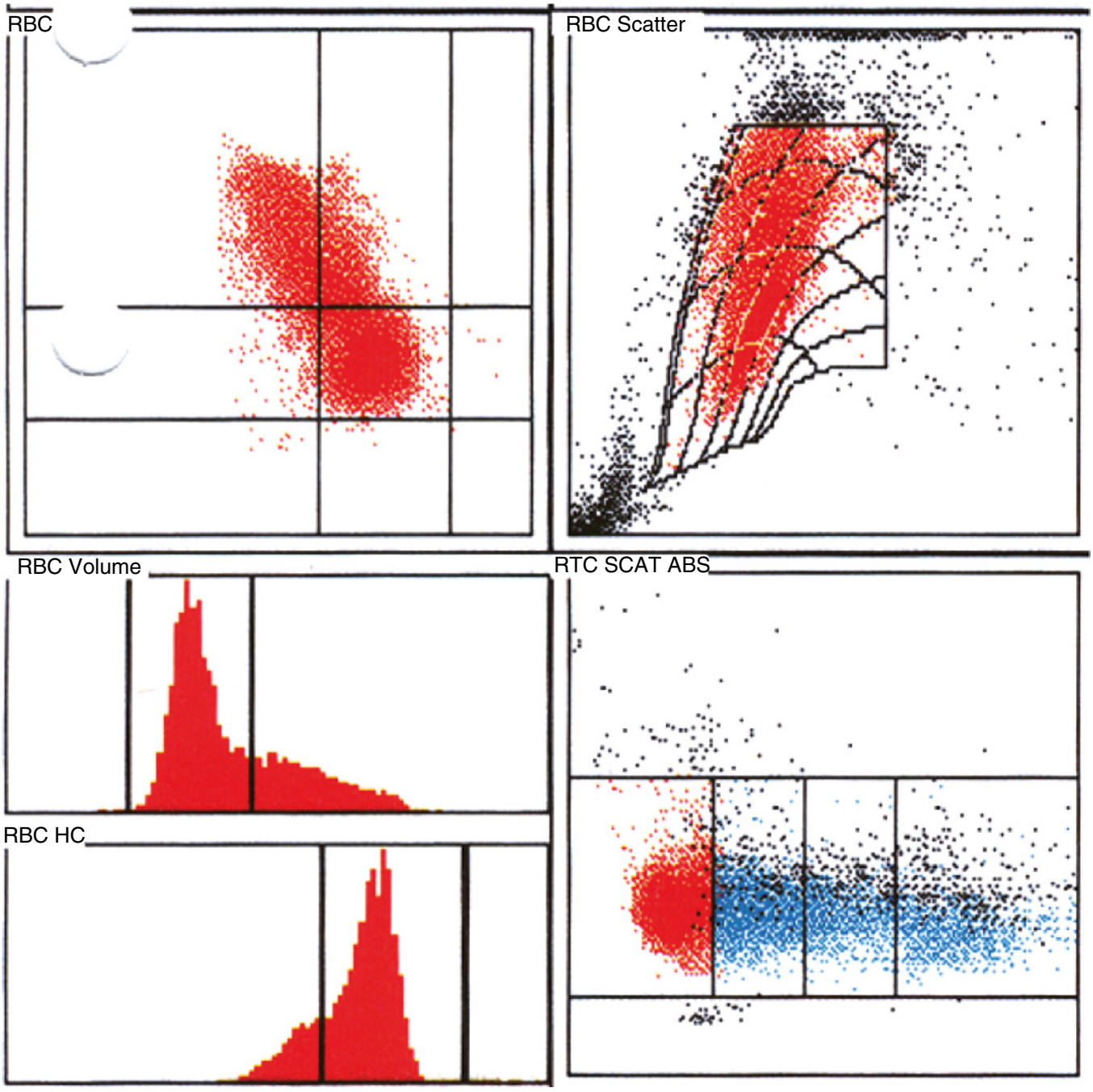


FIGURE 23.2 Advia 2120 graphics of a dog with a regenerative anemia and 21% reticulocytes. The RTC SCAT ABS cytogram (lower right) shows reticulocytes as blue dots extending to the right of mature RBCs (red). The reticulocytes are divided into three groups based on RNA content. Those to the far right have the most RNA. The other four graphics show RBCs analyzed by size and hemoglobin concentration. These illustrate well-distinct populations of mature normocytic normochromic RBCs and immature macrocytic hypochromic RBCs. The RBC Scatter cytogram colors cells red that were considered to be mature erythrocytes. Black dots were cells not considered to be erythrocytes or reticulocytes.

stain reticulocytes, which are then differentiated from RBCs by different extinction and laser light scatter. The LaserCyte was compared to Advia and manual reticulocyte counting in 250 anemic dogs in which two-thirds had a regenerative anemia defined by more than $60 \times 10^3/\mu L$ reticulocytes on manual counts.[26] Weissert et al. found fair correlation ($r = 0.66–0.72$), but a negative bias combined with a proportional error for the LaserCyte® absolute reticulocyte count compared to the manual and Advia methods. Thus, they recommended instrument specific cut-off values for the LaserCyte to separate nonregenerative from regenerative anemia which have reticulocytes of $<35 \times 10^3/\mu L$ or $>75 \times 10^3/\mu L$, respectively.[26] Evaluation of the IDEXX ProCyte

dx® showed moderate imprecision for reticulocyte counts in the dog and cat with coefficients of variation of 16% and 22%, respectively, compared to the Advia and fair correlation for cat reticulocytes (r_s = 0.77).[10]

Other stains, such as TO, AO, and auramine O, have been used for flow cytometry evaluation of reticulocytes.[5] Perkins and Grindem[18] showed that of these three dyes, TO best identified distinct peaks corresponding to aggregate and punctate reticulocytes from cats, while using a dedicated reticulocyte cytometer, the Sysmex R-1000, and a software package, Retic-count® (Becton Dickinson Immunocytometry Systems, San Jose, CA). Thiazole orange was brighter than auramine O or AO, but required longer incubation times, and the commercially prepared reagent was expensive. Punctate reticulocytes appeared as a widened RBC peak or a shoulder off the mature peak. Aggregate reticulocytes formed a separate peak. Indistinct separation of the punctate reticulocytes required a standardized protocol and use of template histogram regions. Percentages of automated counts correlated fairly well manual count results.[18]

Reporting Methods

Reticulocytes are reported both as a percentage of nonnucleated RBCs and absolute number per liter or microliter. The absolute number of reticulocytes is recommended for interpretation. However, the total RBC count is needed to calculate the absolute count (RBC × percent reticulocytes; see Chapter 22).

Reticulocytosis without Anemia

In a multicenter study, Fuchs et al. evaluated 3956 cats and 11 087 dogs admitted to seven German veterinary clinics.[7] Reticulocytosis without anemia (RWA) was observed in a low proportion of blood samples: 3.1% (124/3956) in cats and 4.4% (492/11 087) in dogs.[9] Scanning electron microscope studies on the red pulp in the spleen of cats and dogs indicate that immature erythroid cells attach to splenic macrophages.[2,11,22] Therefore, one explanation previously given for reticulocytosis in nonanemic dogs was that it could be a physiologic reaction to excitement, in which splenic contraction results in increased reticulocyte numbers in blood.[4] But only 1.8% of cats and 1.5% of dogs with reticulocytosis without anemia were considered healthy in the study by Fuchs.[9] Diseases associated with RWA included blood loss/anemia, cardiac/respiratory disorders, gastrointestinal disorders, and inflammatory disorders, as well as cancer. Increased RBC turnover may have resulted in a reticulocytosis without anemia. The severity of underlying causes/conditions is reflected in a mortality rate of approximately one-third of patients: 38% in cats and 30% in dogs with reticulocytosis without anemia.[9]

Other Indicators of Reticulocyte Status

The Advia hematology analyzer identifies the volume and hemoglobin concentration of each individual RBC analyzed and keeps track of which erythroid cells are reticulocytes or erythrocytes (Figures 23.3 and 23.4). The Advia can report reticulocyte size (MCVr) and hemoglobin concentration (CHr) and several other indices. CHr and MCVr have been shown to be sensitive indicators of iron deficiency and response to therapy in dogs (Figure 23.3).[6] Reticulocytes in iron deficiency are relatively smaller (more microcytic) than normal reticulocytes. But reticulocytes in iron deficiency are still relatively macrocytic and hypochromic compared to the patient's own erythrocytes (Figure 23.3). Reticulocyte indices may be monitored for diagnosis and response to treatment. For example, when assessing values after experimental iron replacement in iron-deficient dogs, the MCVr increased from 65 fL before iron treatment to 82 fL after 11–12 days of iron supplementation, and CHr increased from about 16 pg up to 22 pg.[6] Note that CHr may also be reduced in portosystemic shunting, anemia of inflammation, and breed-associated microcytosis.[21]

The hematology analyzer ProCyte dx reports a reticulocyte hemoglobin equivalent (RET-He, new: RETIC-HGB), which is used similarly to CH retic in the diagnosis of iron deficiency.[8]

HEINZ BODY STAINING AND COUNTING

Heinz bodies are formed from denatured, precipitated hemoglobin resulting from oxidative damage. See Chapter 32 for different causes. Oxidation of sulfhydryl groups causes denaturation of hemoglobin. Oxidation of the exposed β-93 cysteine residues present in hemoglobin results in sulfhemoglobin formation.[20] Sulfhemoglobin and denatured hemoglobin are less soluble than normal hemoglobin, so they precipitate to form Heinz bodies that bind to the cell membrane. Oxidation damage to the membrane causes eccentrocyte formation.

Manual Methods

Heinz bodies may be seen on blood smears routinely stained with Romanowsky-type stains if they are large enough to bulge from the surface of the RBC or if they appear lighter and somewhat refractive compared to the rest of the hemoglobin in the RBC. Heinz bodies are more clearly seen in ghost cells as hemoglobin colored bodies attached to the RBC membrane (Figure 23.4). Heinz bodies are more easily evaluated on blood smears stained for enumeration of reticulocytes (e.g., NMB- or BCB-stained smears) (Figure 23.4 and Chapter 32). For birds, two drops of NMB can be mixed with one drop fresh EDTA blood and incubated at room temperature for 30 minutes before making the smear.[15]

Heinz bodies may be small, granular, and numerous, and may be mistaken for reticulocytes (Figures 23.4 and 23.5) in blood smears with reticulocyte staining. This error may falsely elevate the reticulocyte count. Small granular Heinz bodies may not be

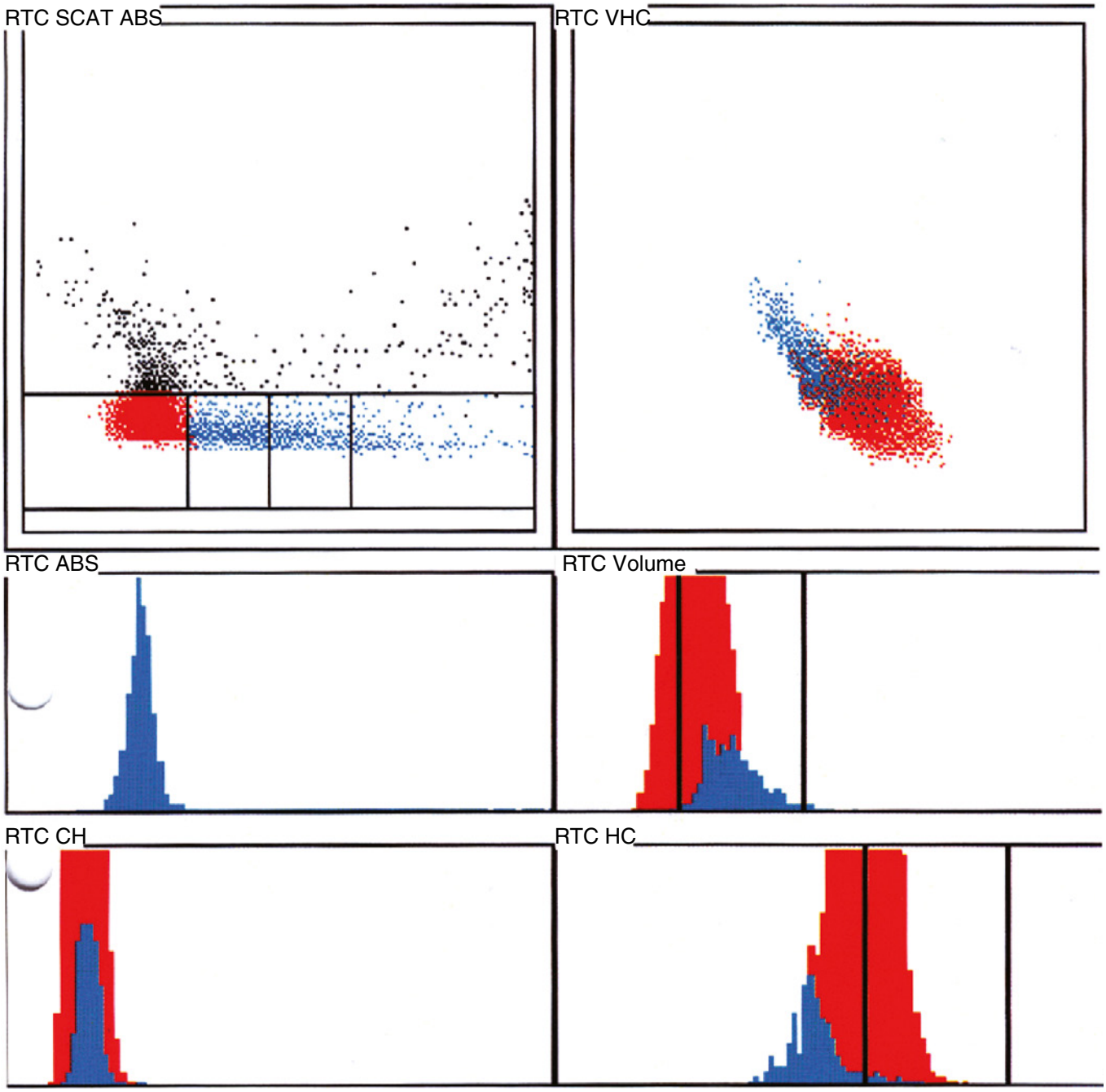


FIGURE 23.3 Advia 2120 reticulocyte graphics from a dog with iron deficiency. Note that the reticulocyte (RTC) cluster in RTC SCAT ABS cytogram (upper left) is lower along the y-axis than the cluster in Figure 23.2, reflecting that reticulocytes in iron deficiency anemia are smaller than normal reticulocytes. But the reticulocytes (colored blue) were macrocytic and hypochromic when compared to the patient's mature RBCs colored red RTC VHC cytogram, RTC volume histogram, and RTC HC histogram.

recognized as such to those expecting a single large round Heinz body seen typically in cats. Heinz bodies stain a lighter blue than ribosomal material in reticulocytes on NMB- or BCB-stained smears, so should not cause confusion with reticulocyte identification. Howell–Jolly bodies are nuclear remnants, stain a darker purple, and have a more distinct border than Heinz bodies.

The total mass of denatured hemoglobin (Heinz body material) is reflective of the magnitude of toxic injury; therefore, reports should indicate whether Heinz bodies are small or large, how much granular Heinz body material also is present in the RBC, and how much other damage to RBC is seen. Blood turbidity may be measured to estimate the amount of Heinz body material.[16]

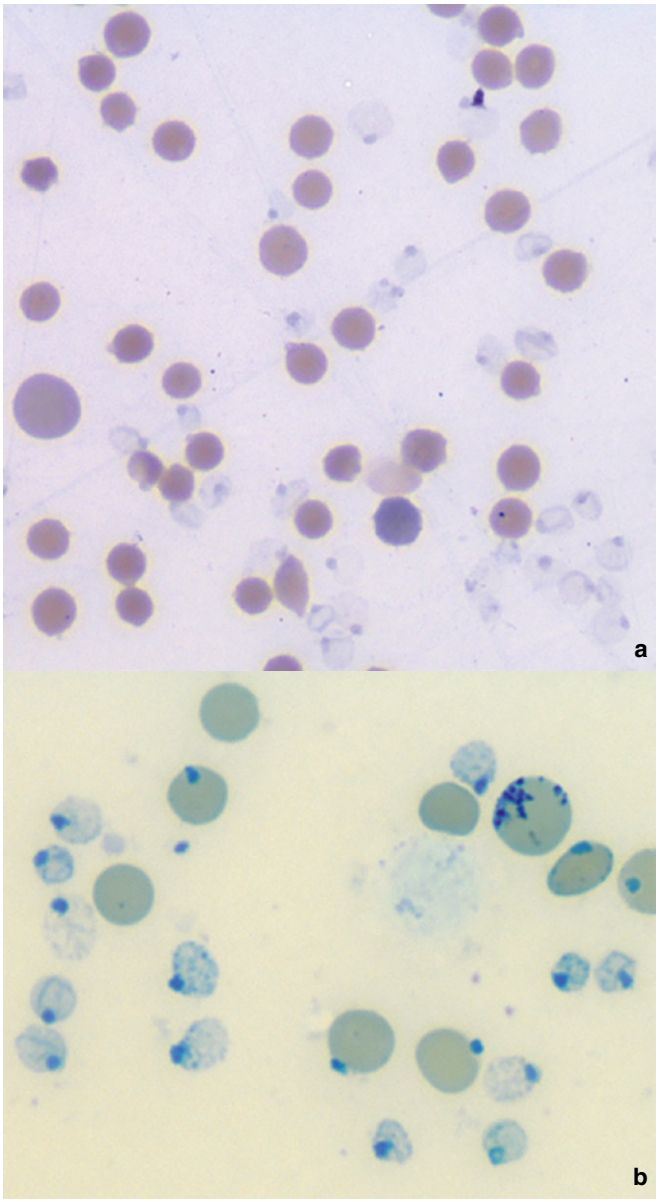


FIGURE 23.4 Blood smear of a lamb with copper poisoning. (a) May–Grünwald–Giemsa stain. Larger Heinz bodies are seen in some of the intact erythrocytes, but even many small Heinz bodies are seen in ghost cells; (b) A brilliant-cresyl-blue-stained smear in which Heinz bodies are more apparent. There is only one reticulocyte (upper right). The small Heinz bodies can easily be mistaken for reticulocytes.

Oxidative toxins that form Heinz bodies also cause other changes such as methemoglobin formation, eccentrocytes, ghost cells, and pyknocytes. These other disease changes (and the presence of Heinz-body-induced artifacts, see in the following text) should be evaluated before a final hematology report is released from the laboratory. The presence and severity of those additional changes should be included in the report. The pattern and amount of change in Heinz bodies,

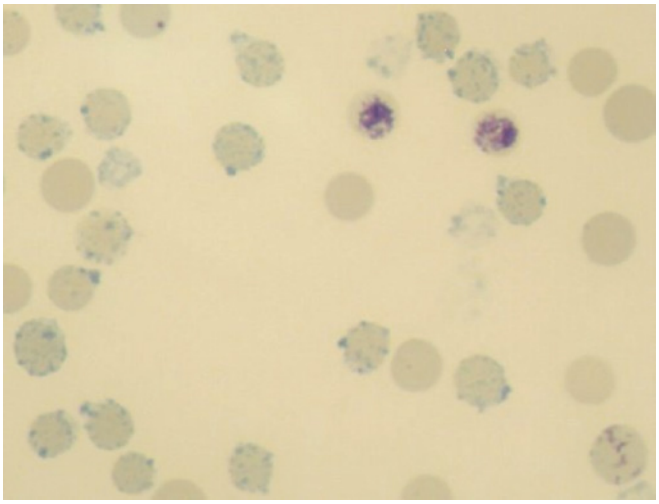


FIGURE 23.5 Brilliant-cresyl-blue-stained blood smear of a dog with onion toxicity. There are only two reticulocytes with dark blue–purple granules in the upper right. Most of the RBCs have many small Heinz bodies which are lighter-blue-stained granular bodies.

eccentrocytes, and methemoglobin vary with different oxidative toxins and species.[17]

Heinz bodies in the blood sample may cause artifacts such as false increases in the mean corpuscular hemoglobin (MCH) and the mean corpuscular hemoglobin concentration (MCHC). The errors cause an overestimation of hemoglobin concentration. These errors are due to suspended Heinz bodies causing increased optical density of lysates of blood for hemoglobin concentration determined by spectrophotometric methods and also by increased high-angle scatter of erythrocytes containing Heinz bodies evaluated by laser hematology instruments.[23]

Species Variations

The size of Heinz bodies varies greatly. Heinz described them as 1–2 μm in diameter in dogs, rabbits, and guinea pigs. They are largest in cats, in which they may be up to one-third the diameter of the RBC.[24] Heinz bodies in cats are predominately distinct, large round structures. However, in dogs and other species, they may be multiple, small granular bodies that can be mistaken for reticulocytes (Figures 23.4 and 23.5).

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CHAPTER 24

Erythrocyte Morphology

ANNE M. BARGER

Normal Erythrocyte Morphology and Distribution
Variation in Erythrocyte Morphology
  Hemoglobin Content
  Cell Size
  Poikilocytosis
    Acanthocytes
    Codocytes
    Dacryocytes
    Eccentrocytes
    Echinocytes
    Elliptocytes
    Keratocytes
    Leptocytes
    Schistocytes
    Spherocytes
    Stomatocytes
    Hemoglobin Crystals
Erythrocyte Inclusions

Acronyms and Abbreviations

DIC, disseminated intravascular coagulation; FAD, Flavin adenine dinucleotide; GN, glomerulonephritis; HJ, Howell–Jolly body; IMHA, immune-mediated hemolytic anemia; MCV, mean corpuscular volume; NMB, new methylene blue; nRBC, nucleated red blood cell.

Evaluation of RBC morphology is a critical step in thorough blood smear evaluation and can aid in identifying many different metabolic disorders, indicate oxidative damage, or help localize a disease process. There are four features of red blood cell morphology that should be evaluated: color, size, shape, and inclusions.

NORMAL ERYTHROCYTE MORPHOLOGY AND DISTRIBUTION

The typical RBC shape for multiple veterinary species is the disc or biconcave disc (i.e., discocyte) resulting in a high surface area to volume ratio making the RBCs deformable. Central pallor can be observed to variable degrees in these species on examination of a peripheral blood smear. Central pallor is most prominent in the dog and less so in cats and horses. Poikilocytosis is a frequent finding in young ruminants and is considered to be normal, especially in goats (Figure 24.1). In certain deer breeds, antelope, and some breeds of sheep and goats, drepanocytes (also termed *sickle cells*) can be observed in vitro. This phenomenon occurs when the oxygen tension of blood is high (e.g., exposure to atmospheric oxygen during mixing of samples or smear preparation) or the pH is between 7.6 and 7.8. The sickling is due to variants of hemoglobin that form insoluble, elongated polymers under these conditions.[25]

The monolayer of the peripheral blood smear allows for evaluation of red blood cell arrangement. In most species, RBCs are individualized; however, some species are prone to rouleaux formation in which the RBCs line up in a column, resembling a stack of coins (Figure 24.2). Although it may be a normal finding in cats and horses, it can be accentuated by elevated plasma protein concentrations.[25] Rouleaux must be differentiated from agglutination. Agglutination is disorderly aggregation of RBCs (Figure 24.3). Agglutination can be observed in immune-mediated hemolytic anemia (IMHA) and as an in vitro change in horses given heparin.[33] Rouleaux is disrupted by diluting the blood with saline, but agglutination is not (see Chapter 107).

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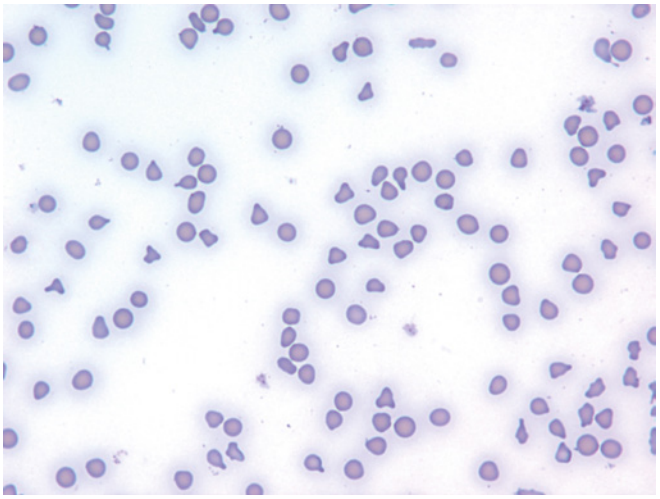


FIGURE 24.1 Blood smear from a goat showing typical poikilocytosis. Wright–Giemsa stain.

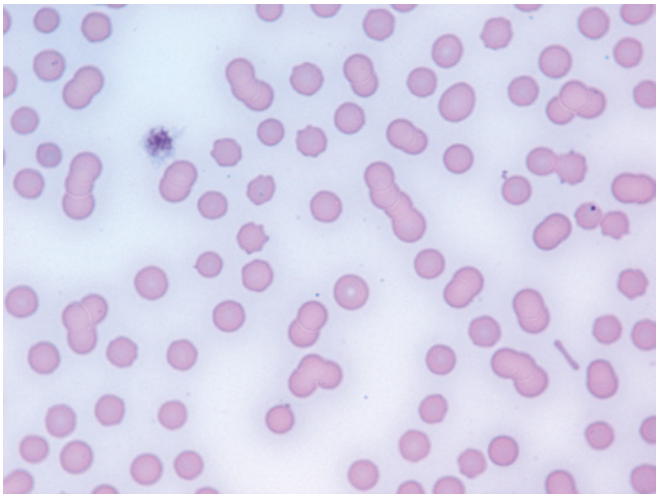


FIGURE 24.2 Blood smear from a cat showing rouleaux formation. Wright–Giemsa stain.

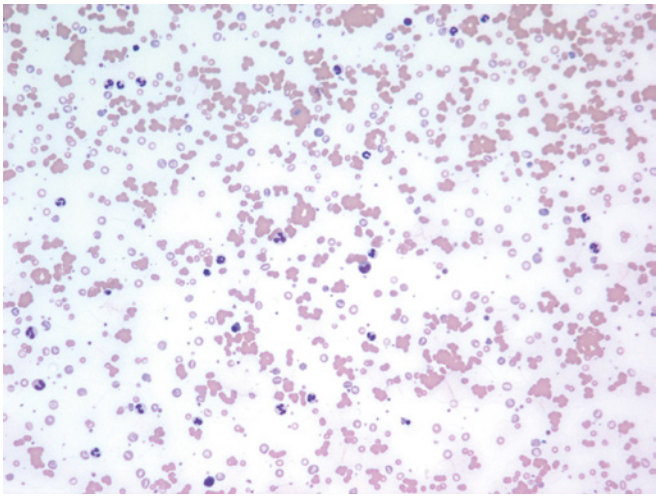


FIGURE 24.3 Blood smear from a dog with marked agglutination associated with immune-mediated hemolytic anemia. Wright–Giemsa stain.

VARIATION IN ERYTHROCYTE MORPHOLOGY

Hemoglobin Content

Many species will have low numbers of circulating reticulocytes. This can result in noticeable polychromasia and anisocytosis. A polychromatophilic RBC contains organelles such as ribosomal RNA and mitochondria. When stained with a Romanowsky stain, the presence of these structures will cause the cytoplasm to stain grayish-blue (Figure 24.4). The RNA network and organelles precipitate, becoming more obvious if the blood is stained with new methylene blue (NMB). Increased polychromasia can indicate a regenerative response to anemia, but is not as sensitive as a reticulocyte count because not all reticulocytes are polychromatophilic. Horses do not have circulating reticulocytes and thus do not have polychromasia in their peripheral blood.

Patients with iron deficiency anemia can develop hypochromasia (see Chapter 27). Since iron is necessary for heme synthesis, erythrocytes from patients with iron deficiency can have decreased hemoglobin content. These cells have a thin rim of hemoglobin with increased area of central pallor (Figure 24.5). True hypochromasia needs to be differentiated from torocytes. A torocyte is usually considered an artifact.[25] These cells have an increase in central pallor, but it is abrupt rather than a gradual decrease in the rim of hemoglobin. Other causes of hypochromasia include prolonged copper deficiency or deficiency of vitamin B6.[20]

Cell Size

Anisocytosis is a general term that describes variation in cell size. Healthy animals will have mild anisocytosis, likely from the presence of low numbers of circulating reticulocytes. Reticulocytes are often larger than mature erythrocytes, so they are described as macrocytes. Other causes of macrocytosis include feline leukemia virus,

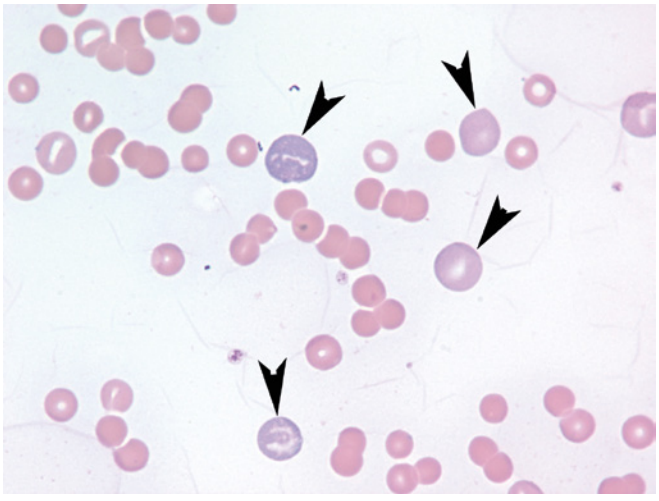


FIGURE 24.4 Blood smear from a dog showing polychromatophilic RBCs (arrows). These cells are larger than mature RBCs with grayish-blue cytoplasm. Wright–Giemsa stain.

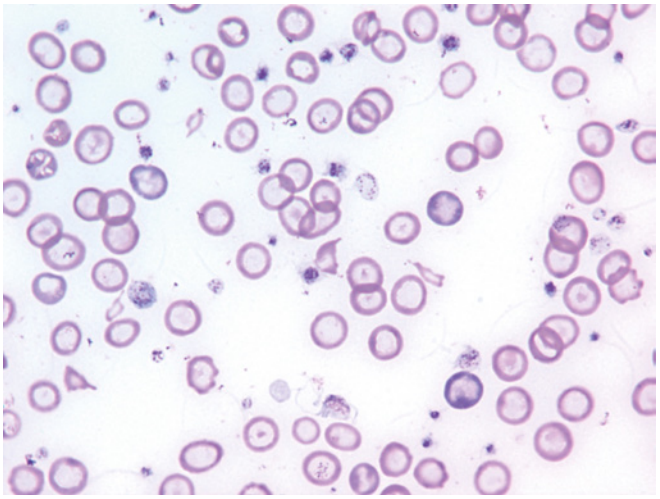


FIGURE 24.5 Blood smear from a dog with iron deficiency anemia. Notice marked hypochromasia. Wright–Giemsa stain.

myelodysplastic syndromes, and some congenital and hereditary conditions (see Chapters 30, 31, and 67). Generally, animals with these conditions will have an overall increase in their mean corpuscular volume (MCV). Microcytosis or small red blood cells can be observed with several different disease processes, the most common of which is iron deficiency (see Chapter 27). Due to the impaired heme synthesis, the red blood cells undergo an additional division. Other causes of microcytes include hereditary microcytosis (Akita and Shiba Inu) and familial dyserythropoiesis of English Springer Spaniels (see Chapter 31).[18,25]

Poikilocytosis

Poikilocytosis is a general term for variation in RBC shape; it can occur in a variety of conditions, so poikilocytosis is nonspecific. Further, some species are more prone to poikilocytosis than others. For instance, echinocytes are a common finding in pigs. Goat kids less than 3 months of age and some adults, particularly adult angora goats, are prone to marked poikilocytosis. Their RBC can vary in shape, and triangular, spindle, fusiform, matchstick, oblong, and pear-shaped erythrocytes can be observed. Anemic goats can form hemoglobin C, which makes them more prone to poikilocytosis.[25,27] Camelids, avians, and reptiles have oval RBCs as their normal shape. Types of poikilocytes and the common disease and associated species can be found in Table 24.1.

Acanthocytes

Specific changes occurring in the membrane structure, hemoglobin condition, and bone marrow architecture can result in specific changes in RBC morphology. Acanthocytes have irregularly spaced spicules generally 2–20/cell. The name is derived from the Greek for "thorn" (Figure 24.6). The changes in the RBC membrane of these cells are associated with increased cholesterol content of the membrane, which is thought to

TABLE 24.1 Erythrocyte shape changes associated with selected diseases or conditions.

Spherocyte	
Immune-mediated hemolytic anemias	Dog
Transfused blood	Dog
Blood parasite infections	Several species
Anaplasmosis	Cattle
Hereditary spherocytosis	Goat, cattle, dog
Zinc toxicity	Dog
Snake venom (coral snake, European viper)	Dog
Hypophosphatemia	Dog
Keratocyte/Schistocyte	
DIC	Dog, cat, cattle, horse
Congestive heart failure	Dog
Glomerulonephritis	Dog
Myelofibrosis	Dog
Hemangiosarcoma	Dog
Chronic doxorubicin toxicosis	Dog
Acanthocyte/Budding fragmentation	
DIC	Dog
Hemangiosarcoma	Dog
Portosystemic shunts	Dog
Chronic liver disease	Dog, cat
Lymphosarcoma	Dog
Glomerulonephritis	Dog
Echinocyte	
Crenation artifact	Dog, cat, cattle, horse
Lymphosarcoma	Dog
Glomerulonephritis	Dog
Doxorubicin toxicity	Dog
Electrolyte depletion	Horse, dog
Heinz bodies	
Onion, leek, garlic ingestion	Dog, cat, cow, horse, sheep
Vitamin K (mainly K_3)	Dog
Acetaminophen treatment	Cat
Phenothiazine treatment	Horse
Red maple leaf ingestion	Horse
Methylene blue treatment	Dog, cat
Phenazopyridine treatment	Cat
Propylene glycol in food	Dog, cat
Brassica spp. ingestion	Cattle
Selenium deficiency	Cattle
Copper toxicity	Sheep
Zinc toxicity	Dog

TABLE 24.1 (Continued)

Eccentrocytes/pyknocyte	
Vitamin K treatment	Dog
Onion ingestion	Dog
Acetylphenylhydrazine	Dog
Hydrogen peroxide intravenously	Cattle
Glucose-6-phosphate dehydrogenase enzyme deficiency	Horse
Hypochromasia	
Iron deficiency	Dog, cat, cattle, horse, pig, llama, goats, birds
Inflammation	Birds
Lead toxicity	Birds
Macrocytosis	
Regenerative anemia	Many species
Poodle macrocytosis	Dog
Stomatocytosis	Dog
Feline leukemia virus infection	Cat
Myelodysplasia	Cat
Anticonvulsant (antifolate) drugs	Dog
Prolonged storage of blood	Many species
Hypernatremia	Cat, dog
Microcytosis	
Iron deficiency	Dog, cat, cow, horse, pig
Hepatic portosystemic shunt	Dog
Hyponatremia	Dog
Normal breed characteristic	Dog (Akita, Shibas)

expand the outer layer of the lipid bilayer; however, other studies suggest acanthocytes can also result from RBC fragmentation.[47] In dogs, liver disease, portosystemic shunts, hemangiosarcoma, disseminated intravascular coagulation (DIC), lymphosarcoma, gastrointestinal disease, glomerulonephritis (GN), osteosarcoma, and high cholesterol diets have been associated with acanthocytosis.[22,43,47,48] In cats with liver disease, acanthocytes are reported as the most common poikilocyte.[10]

Codocytes

Codocytes (also termed target cells) resemble a bull's eye (Figure 24.7). They have a central area of hemoglobin surrounded by a pale rim with hemoglobin at the periphery of the cell. Similar to acanthocytes, codocytes have an increased amount of cholesterol resulting in an overall increase in the surface area of the erythrocyte membrane. Codocytes are often observed in patients with iron deficiency, cholestatic liver disease, and postsplenectomy; however, codocytes are also frequent findings in regenerative anemias.

Dacryocytes

Dacryocytes are teardrop-shaped cells that may occur when the RBC membrane is damaged and distorted as cells pass through narrow marrow or splenic sinusoids (Figure 24.8).[25] In human medicine, these cells are commonly associated with myelofibrosis or myelophthisis. In dogs, they have been reported with myeloproliferative disorders and hypersplenism.

Eccentrocytes

Eccentrocytes (also termed erythrocyte hemighosts) are RBCs with eccentrically shifted hemoglobin within the cell, leaving a pale, hemoglobin-free area.[21] These cells may progress to pyknocytes, which are eccentrocytes in

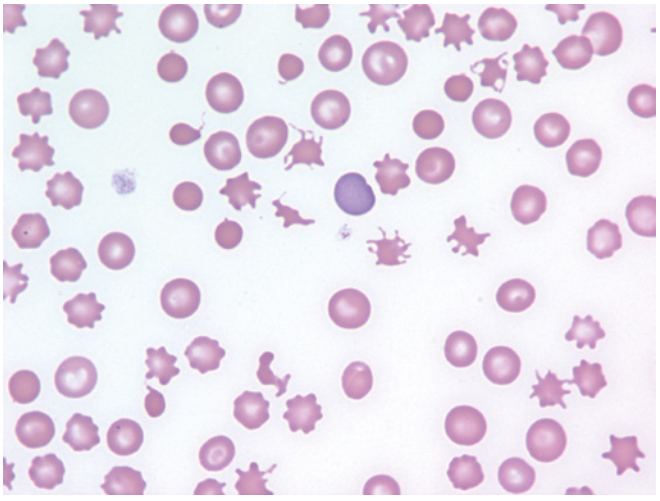


FIGURE 24.6 Blood smear from a dog showing acanthocytes with varying numbers of irregularly spaced projections. Wright–Giemsa stain.

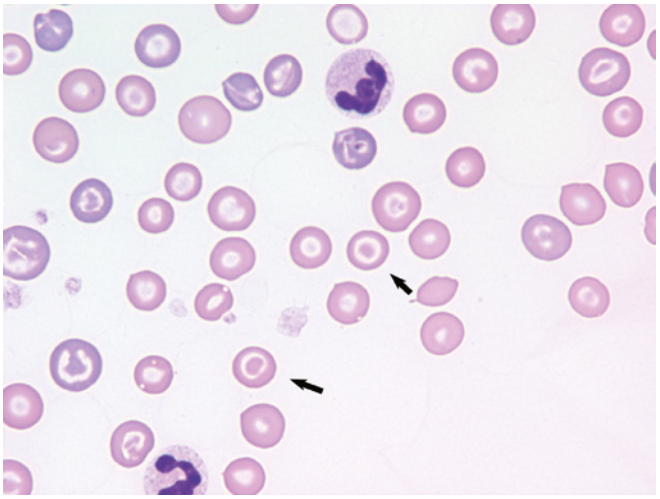


FIGURE 24.7 Blood smear from a dog with iron deficiency anemia. Codocytes are indicated with arrows. Wright–Giemsa stain.

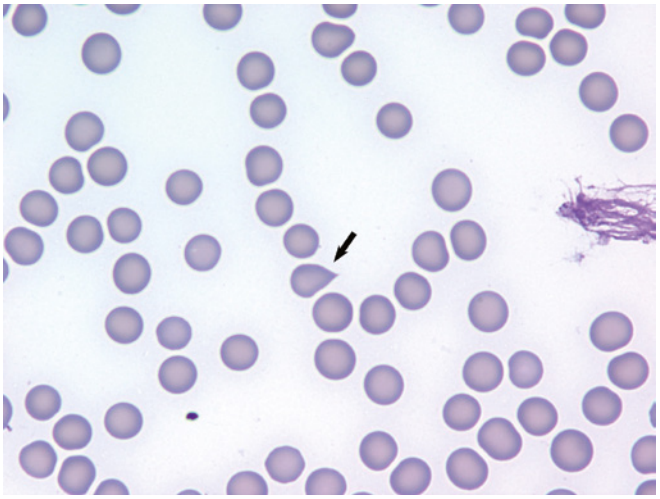


FIGURE 24.8 Blood smear from a dog. Dacryocyte is indicated with an arrow. Wright–Giemsa stain.

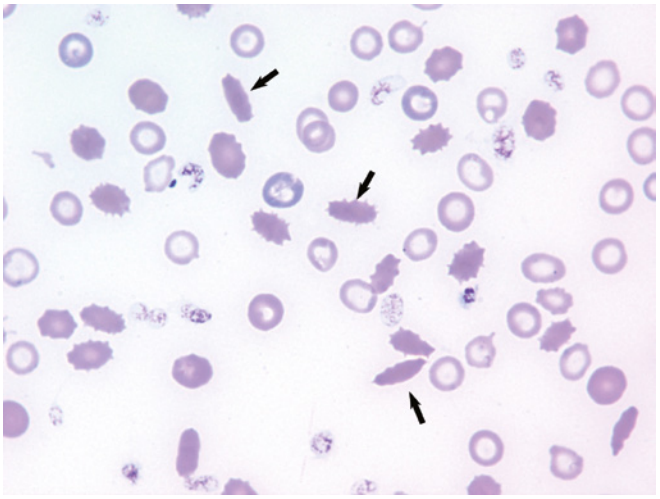


FIGURE 24.9 Blood smear from a dog. Ovaloechinocytes are indicated with arrows. Wright–Giemsa stain.

which the cell membrane has collapsed. Eccentrocytes have been reported to be easier to visualize on NMB-stained preparations.[36] These cells are associated with oxidative damage to the erythrocyte membrane and cytoskeleton.[6] In horses, eccentrocytes have been observed with red maple toxicity (*Acer rubrum*), erythrocyte glucose-6-phosphate dehydrogenase deficiency, and flavin adenine dinucleotide (FAD) deficiency (see Chapter 32).[21,41] In dogs, eccentrocytes have been observed with ingestion of substances known to cause oxidative damage, such as onions, vitamin K, garlic, and acetaminophen. Additionally, eccentrocytes have recently been described in dogs with diabetes mellitus, T cell lymphoma, and severe infections.[6] Cattle receiving intravenous hydrogen peroxidase may have circulating eccentrocytes.[42]

Echinocytes

Echinocytes are spiculated red blood cells with evenly dispersed, short projections. Their shape can vary from discoechinocytes to highly spiculated spheroechinocytes or even ovaloechinocytes (Figure 24.9). Echinocytes can be subclassified based on particular morphologic features. Type I echinocytes have irregular or angular edges, but do not have distinct spicules. Type II echinocytes have multiple blunt spicules. Type III echinocytes are ovoid or spherical with 10–30 regularly spaced spicules. Echinocytes (also termed crenation) can occur as an artifact from sample handling, prolonged sample storage, or during slide preparation. These cells occur commonly in pigs, in vitro.[15] Echinocytes form when the outer leaflet of the lipid bilayer of the RBC membrane is expanded relative to the inner layer. Mechanisms of echinocyte formation include depletion of ATP, administration of amphipathic drugs, calcium loading, or when RBCs are dehydrated. In dogs, echinocytes have been reported with glomerulonephritis, lymphoma, hemangiosarcoma, and other neoplasms, immune-mediated hemolytic anemia, pyruvate kinase deficiency, rattlesnake envenomation, and doxorubicin

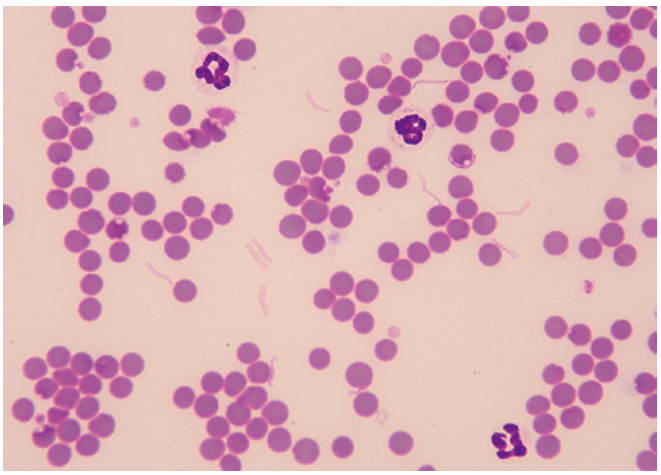


FIGURE 24.10 Blood smear from a dog with viper envenomation. Many erythroid loops are noted. Wright–Giemsa stain. (Image courtesy: Dr. Carlo Masserdotti.)

toxicosis among others.[3,5] In one study, dogs with greater than 90% echinocytes were used to confirm a clinical suspicion of rattlesnake envenomation[8] (Figure 24.10). In addition to echinocytes, small structures, termed "erythroid loops," have been identified as a result of viper venom.[31] Cats likely have echinocytes with many of these diseases as well, but echinocytes have been specifically reported with chronic doxorubicin administration.[35] In horses, echinocytes have been observed with diseases causing hyponatremia and hypochloremia, such as colitis or exercise.[17,25]

Elliptocytes

Elliptocytes are oval-shaped, non-nucleated RBCs that are normal in camelid species. In people, hereditary ovalocytosis has been associated with a protein band 3 abnormality as well as mutated spectrin.[12,37] In veterinary species, ovalocytes are occasionally described

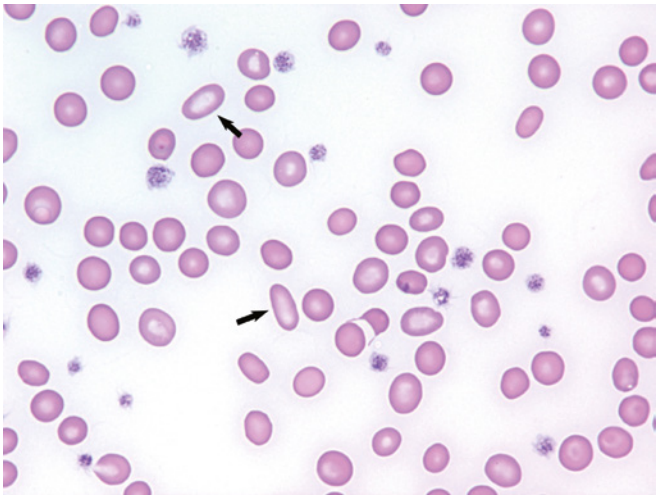


FIGURE 24.11 Blood smear from a dog. Ovalocytes are indicated with arrows. Wright–Giemsa stain.

(Figures 24.9 and 24.11). Hereditary ovalocytosis has been reported in a dog with protein band 4.1 deficiency,[40] but also in myelofibrosis, glomerulonephritis, and myelodysplastic disease.[23] In cats, elliptocytes have been reported with myeloproliferative disorders, hepatic disease, and with chronic doxorubicin administration.[10,35]

Keratocytes

Keratocytes are spiculated RBCs that frequently have only one to two spicules and indicate RBC fragmentation (Figure 24.12); thus, the RBC appears to have "horns" (*kerat* is the Latin term for *horn*). The formation of keratocytes has been associated with microvascular injury, especially when there is deposition of fibrin strands. The opposing sides of the damaged RBC may fuse leading to formation of a blister. Bursting of the blister is thought to result in progression to a keratocyte.[25] Keratocytes have been reported in cats with liver disease[10] and in dogs and cats with chronic doxorubicin administration and hemangiosarcoma.

Leptocytes

Leptocytes are thin RBCs that can fold and take on the shape of other poikilocytes such as codocytes, knizocytes (central fold giving the appearance of a central bar of hemoglobin), or stomatocytes. Leptocytes can be observed in iron deficiency and cholestatic liver disease.[25]

Schistocytes

Schistocytes are red blood cell fragments usually resulting from direct physical damage to RBCs secondary to vascular abnormalities or turbulent blood flow. The shape of the fragments may vary from pointed or triangular to spiculated (Figure 24.13). Microangiopathic fragmentation has been described in dogs in a several different disorders including DIC, GN, hemangiosarcoma, myelofibrosis, dyserythropoiesis, and chronic doxorubicin toxicosis.[3,13,47] Schistocytes have also been identified as a common feature of DIC in calves.[24] However, they are uncommon in the horse and cat,[14] though schistocytes have been reported in cats with hepatic disease.[10]

Spherocytes

Another result of RBC fragmentation is spherocytes. Spherocytes are RBCs that have lost their biconcave shape and have become round like a sphere. On a blood smear, these cells have lost their central pallor and appear slightly darker and often a little smaller than healthy RBCs (Figure 24.14). IMHA is a common cause of this poikilocyte. They occur secondary to antibody binding on the membrane or complement fixation. Part of the membrane may be phagocytized or just punctured resulting in the change in shape. These cells have

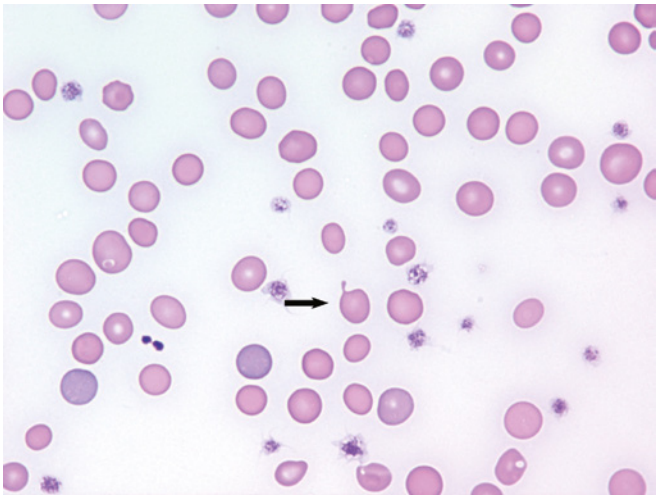


FIGURE 24.12 Blood smear from a dog. Occasional keratocytes are indicated by arrow. Wright–Giemsa stain.

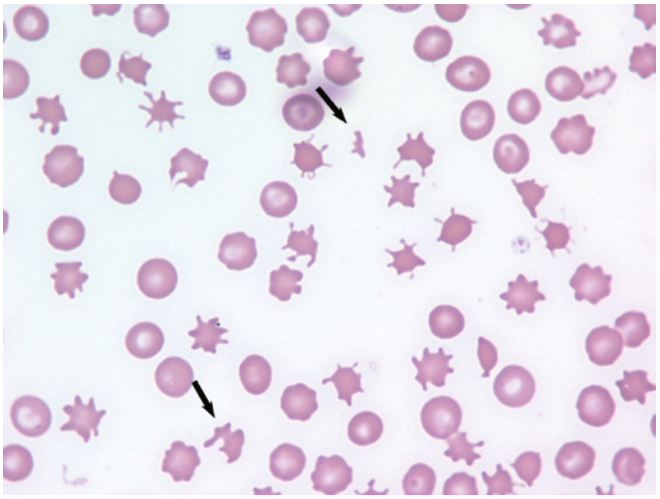


FIGURE 24.13 Blood smear from a dog. Severe poikilocytosis with schistocytes indicated with arrows. Wright–Giemsa stain.

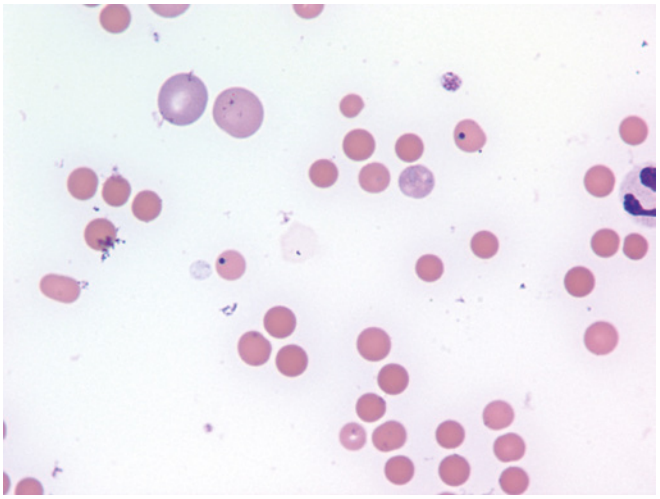


FIGURE 24.14 Blood smear from a dog showing many spherocytes. Wright–Giemsa stain.

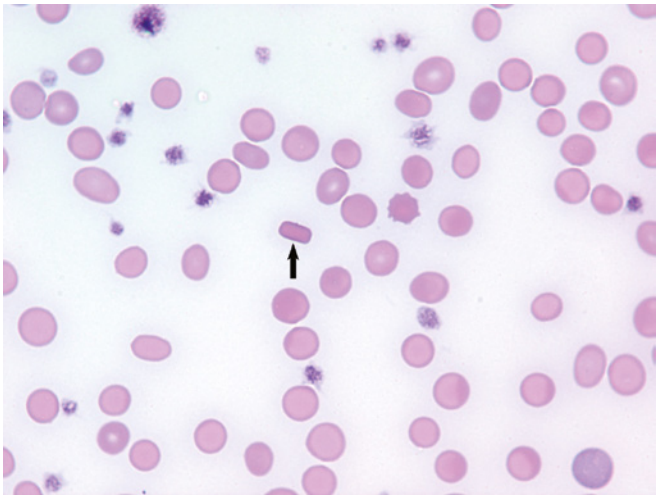


FIGURE 24.15 Blood smear from a dog showing hemoglobin crystals (arrow). Wright–Giemsa stain.

been associated with other diseases as well, including coral snake, rattlesnake and bee sting envenomation, and anaplasmosis infection in cattle.[34] Spheroechinocytes have been reported in Basenjis with pyruvate kinase deficiency and in goat erythrocytes treated with lysolecithin.[5,25,26] Spherocytes are most obvious in dogs, likely because they have prominent central pallor. In other species such as the cat and horse, spherocytes are difficult to identify.

Not all spherocytes are formed secondary to fragmentation. Spherocytes can be caused by a molecular defect in one or more of the proteins of the RBC cytoskeletal network. Double knockout mice with defects in actin-binding proteins affect erythrocyte shape and membrane integrity leading to pronounced spherocyte formation.[9] Spectrin deficiency has been identified in a group of Dutch Golden Retrievers (see Chapter 30).[39]

Stomatocytes

Stomatocytes are RBCs that have a narrow elongated band of central pallor resembling a mouth on dried blood smears. In actuality, these cells are more bowl-shaped in wet-mount preparations. Several breeds of dogs have been identified with hereditary stomatocytosis, including Alaskan Malamutes with concurrent chondrodysplastic disease, Drentse Patrijshond with stomatocytosis-hypertrophic gastritis, and Miniature and Standard Schnauzers (see Chapter 30).[4,38] The Drentse Patrijshonds often have a normal MCV, whereas the Schnauzers and Malamutes have an increased MCV.[4,38]

Hemoglobin Crystals

Hemoglobin crystals are unstable hemoglobin that precipitates in the RBC. These dense staining, rhomboid or rod-shaped crystals lead to decreased deformability of the erythrocyte (Figure 24.15). Crystals may be found in human patients with Hemoglobin C disease and sickle cell anemia, particularly after splenectomy. In veterinary medicine, hemoglobin crystals are infrequently reported, and their cause is uncertain. Nonetheless, they have been described in dogs, llamas, and horses with FAD deficiency.[7,41]

ERYTHROCYTE INCLUSIONS

Basophilic stippling represents the spontaneous aggregation of ribosomes and polyribosomes in RBC. In Romanowsky-stained samples, affected RBCs contain uniformly distributed punctate, basophilic structures (Figure 24.16). Basophilic stippling may be seen in cattle and other ruminants as a part of the regenerative response to anemia. It may be associated with exuberant regeneration in other species including the dog and cat. However, if basophilic stippling and increased numbers

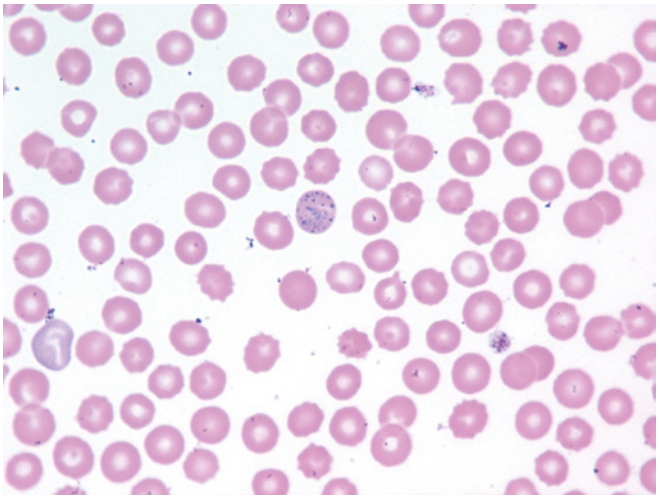


FIGURE 24.16 Blood smear from a dog showing basophilic stippling. Wright–Giemsa stain.

of nucleated RBC (nRBC) are present in the absence of a severe anemia, lead toxicity should be considered.

Cabot rings are ring or figure-eight-shaped structures identified as a normal finding in camelids and are thought to originate from the mitotic spindle.[29] These structures are not considered clinically significant in camelids; however, they have been reported in dogs with dyserythropoiesis.[29]

Nucleated RBCs in circulation can also be part of a regenerative response to anemia. These cells, depending on their level of maturity, usually have polychromatophilic or hemoglobinized cytoplasm and a round nucleus (Figure 24.17). The metarubricytes have a small, pyknotic nucleus, but less mature cells have a stippled chromatin pattern. When nRBCs accompany a regenerative response, polychromasia and anemia should also be observed. However, nRBCs may occur in the absence of anemia in diseases affecting the spleen and bone marrow. Myeloproliferative disorders and infiltrative marrow diseases such as solid tumor metastases and myelofibrosis can result in high numbers of nRBCs, occasionally with less mature erythrocyte precursors, rubriblasts, and prorubricytes in circulation. Low numbers of circulating nRBCs can be observed in splenectomized animals or in dogs or cats with hemangiosarcoma, hemophagocytic histiocytic sarcoma, and pure red cell aplasia.[19,30,46] Cats with myelodysplastic syndromes, hepatic lipidosis, upper respiratory infections, and hemangiosarcoma can have normoblastemia.[19] Lead toxicity is commonly associated with metarubricytosis; however, these patients are usually not anemic. Polled Hereford calves with congenital dyserythropoietic anemia and dyskeratosis are reported to have increased nucleated red blood cells in circulation and these cells often exhibit an atypical morphology.[28]

Nuclear remnants, not expelled as RBCs leave the bone marrow, are termed Howell–Jolly (HJ) bodies. They are round, deeply basophilic structures that may vary in size (Figure 24.18). Howell–Jolly bodies are normally removed by the spleen. However, in cats and horses, species having a nonsinusoidal spleen, low numbers are normally seen in circulation. Increased numbers of HJ bodies can be seen as part of a regenerative response to anemia, in animals with hypofunctioning spleens, or in splenectomized patients. There are also reports of increased numbers of HJ bodies in nonanemic miniature and toy poodles with hereditary macrocytosis (see Chapter 31).

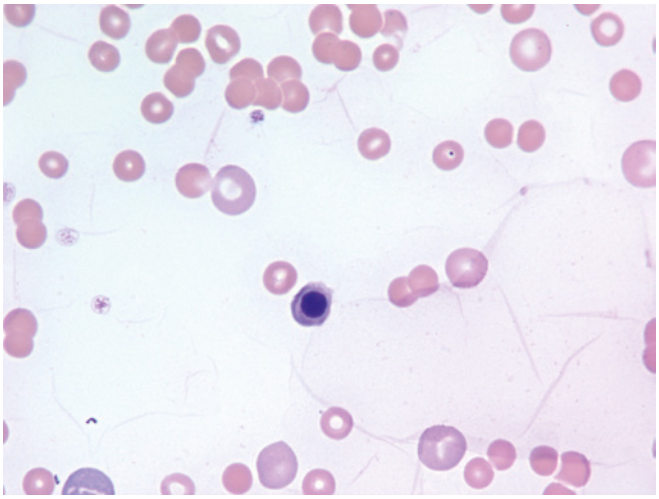


FIGURE 24.17 Blood smear from a dog showing occasional metarubricytes as part of a regenerative response. Wright–Giemsa stain.

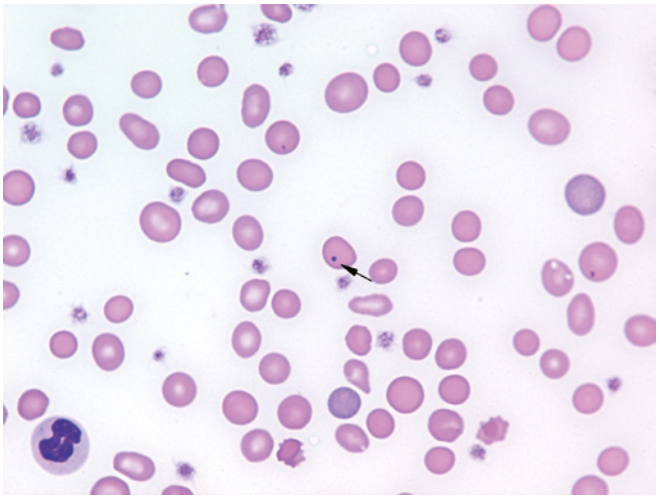


FIGURE 24.18 Blood smear from a dog. Howell–Jolly bodies are nuclear remnants observed in the cytoplasm of red blood cells (arrow). Wright–Giemsa stain.

Oxidative damage can result in a number of morphologic changes to the RBCs including the formation of Heinz bodies.[1,2,11,16,32,49] Heinz bodies are small aggregates of oxidized hemoglobin that can occur as hemoglobinized projections extending from the cell or as pale areas within the cell (Figure 24.19a). With Romanowsky stains, the Heinz bodies are pale or hemoglobinized, but with NMB, they are bluish-green and much more obvious (Figure 24.19b). Many toxins and diseases have been reported to cause Heinz bodies (see Chapter 32).

Iron observed in mature RBCs is referred to as siderocytes or Pappenheimer bodies. With Romanowsky stain, the iron appears as irregularly shaped, pale basophilic material, whereas ferric iron forms a bright blue pigment when stained with Prussian blue. Siderocytes have been reported in the blood in association with myeloproliferative disorders, lead toxicity, and hemolytic anemia. Sideroblasts are nucleated erythroid precursors with iron in their cytoplasm. These cells have been reported with myelodysplastic syndromes and myeloproliferative disorders and in inflammatory diseases in dogs and cats.[44–46]

Artifactual changes can occur that mimic true pathologic fragmentation or inclusions. Stain precipitate or drying artifact can mimic red blood cell parasites or inclusions such as HJ bodies. A commonly observed artifactual change associated with prolonged drying of blood smear preparations produces refractile RBC inclusions. Additionally, crenation of RBCs can occur as an artifact of drying. Usually, artifactual crenation will occur in thick areas of blood smear.

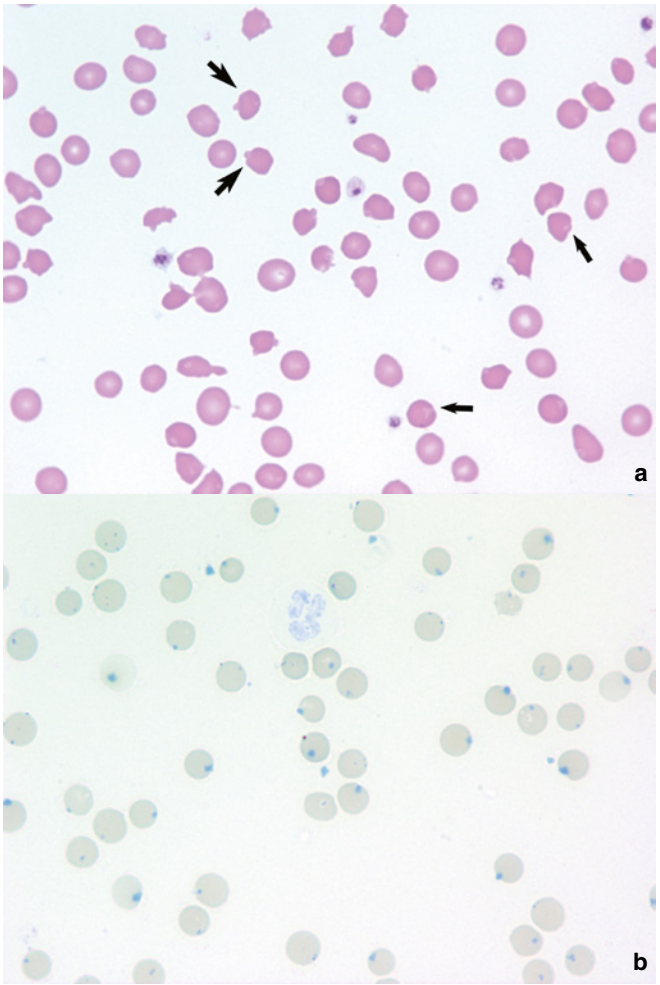


FIGURE 24.19 Blood smear from a cat. (a) Wright–Giemsa stain: Many Heinz bodies are observed and can appear as projections from the RBC membrane (large arrow) or as pale areas near the membrane (small arrow); (b) New methylene blue stain: Heinz bodies are more obvious with vital stains.

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CHAPTER 25

Classification and Laboratory Evaluation of Anemia

HAROLD TVEDTEN

Clinical Manifestations of Anemia
Presence and Severity of Anemia
  Appearance of Microhematocrit Tubes
Classification Systems
  Classification by Strength of Erythropoiesis
  Classification by Erythrocyte Volume and Hemoglobin Concentration
  Classification by Blood Smear Morphology
  Classification by Etiology
Diagnostic Approaches
Blood Loss Anemia
Nonregenerative Anemia
  Bone Marrow Evaluation

Acronyms and Abbreviations

Advia, Siemens Advia 2120® hematology system; CRP, corrected reticulocyte percentage; EIA, equine infectious anemia; FeLV, feline leukemia virus; fL, femtoliter; Hct, hematocrit; Hgb, hemoglobin; IMHA, immune-mediated hemolytic anemia; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; NRBC, nucleated red blood cell; PRCA, pure red cell aplasia; RDW, red cell distribution width; RPI, reticulocyte production index; RTC-VHC, reticulocyte volume hemoglobin concentration cytograms; Sysmex, Sysmex XT-2000iV® hematology system.

This chapter discusses initial and general diagnosis of anemia based primarily on laboratory findings. Erythrocyte morphology (see Chapter 24) and many specific causes of anemia are described elsewhere. Blood loss anemia diagnosis has expanded discussion in this chapter. Diagnosis of the cause of anemia uses certain characteristics of blood to place the anemia into pre-established classes or categories useful for diagnosis. Several classification systems are used and are based on various tests and observations. The systems overlap and classifications by one system (e.g., macrocytic hypochromic anemia) may be identifying the same change or process in RBCs as another classification (e.g., reticulocytosis, polychromasia, and regenerative anemia). Thus, different terms frequently lead to a similar diagnosis. Despite the classification system used, the goal of diagnosis should be to provide the most specific and correct diagnosis to allow appropriate treatment or other action by the attending clinician.

CLINICAL MANIFESTATIONS OF ANEMIA

The clinical signs that prompt an animal's owner to seek a clinician to diagnose and then treat an anemia include weakness, lack of stamina, pale mucous membranes, icterus, and hemoglobinuria or hematuria (red urine). Anemia which is too mild to cause these signs is more often detected by initial hematological examination of patients. The purpose of laboratory testing is to provide unbiased, objective evidence that supports or refutes a clinical or tentative diagnosis. Laboratory diagnosis of the cause of anemia should be based first on laboratory conclusions founded on numerical test results of hematology instruments, but also importantly on subjective interpretation of cell morphology in blood smears and graphical reports from hematology instruments. This avoids bias, which can come from submitting the clinician's tentative diagnosis, clinical signs, age, breed, and other clinical information. After making the basic

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laboratory conclusions, the other sources of information should be utilized to influence the final conclusions or diagnosis. This permits laboratory results to suggest new possibilities not considered by the veterinarian submitting the sample and avoid "premature closure" too early in the diagnostic process.

PRESENCE AND SEVERITY OF ANEMIA

The first step in evaluation is to determine if the animal has anemia and, if present, to evaluate the severity. Laboratory parameters routinely used are hematocrit (Hct), total erythrocyte count (RBC count), and hemoglobin (Hgb) concentration. PCV is essentially the same as Hct, though implies a centrifugal method of analysis. Hematocrit will be used in this chapter to describe severity of anemia, but Hgb concentration is used often in Europe and in human medicine. Table 25.1 gives useful approximate guidelines. Hct versus PCV will vary a few percentage units.

If Hct, RBC count, and Hgb concentration are not similarly decreased in anemia, then erythrocytes are not normal in size and Hgb content. Changes in erythrocyte indices (mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC)) should help explain any discordance in relative decreases (see section on classification by erythrocyte volume and Hgb concentration later in this chapter). For example, in iron deficiency anemia with severe microcytosis, RBC count will not be decreased as much as Hgb concentration or Hct, and therefore not reflect the severity of the anemia as well. Even normal numbers of microcytic erythrocytes may not contribute to a normal Hgb concentration per unit volume of blood and the smaller erythrocytes will be packed into a smaller volume of the blood (PCV).

Pre-analytical and analytical errors may also cause discordance among Hct, Hgb concentration, and RBC count. For example, lipemia may erroneously increase Hgb concentration (and MCHC). This error among others indicates that hematology samples should be taken from fasted animals just as is recommended for chemistry analysis. Erythrocytes swell in EDTA with time, so Hct (and MCV) will be falsely increased in older samples (mailed in), while Hgb should still reflect the severity of an anemia. Heinz bodies may make erythrocytes more fragile, so they lyse in the microhematocrit centrifuge, lowering the PCV. Heinz bodies remaining in suspension after erythrocytes are lysed increase the optical density and falsely increase Hgb concentration. Heinz bodies in erythrocytes make them more optically dense to laser hematology instruments, also causing a falsely increased cell Hgb concentration and MCHC.

Severity of an anemia is useful in diagnosis. Table 25.1 provides some guidelines for classifying severity. Moderate to severe anemias are more likely important or primary problems. For example, primary bone marrow disorders like chronic myelofibrosis can cause very severe anemia (e.g., Hct 6–8%), while secondary suppression of bone marrow (e.g., anemia of inflammation) is characteristically mild to moderate. Severe anemia or rapid decline in erythroid mass should stimulate more aggressive diagnosis and treatment, while mild to moderate anemia in animals with severe inflammatory or neoplastic disorders is expected as a secondary problem.

Clinical information such as breed and age affect interpretation (see Chapter 140). Sight hounds like greyhounds normally have a higher Hct (about 50–65%) compared to other dog breeds (37–55%), and therefore a mild anemia in a greyhound will have a Hct within the reference intervals for most other canine breeds. Puppies have lower Hct than adult dogs (as well as lower plasma protein and higher reticulocyte numbers). For example, a 6-week-old puppy can have a Hct of 26–30%, reticulocytes of 4.5%, and plasma protein of 5.0–5.6 g/dL. Thus, a normal puppy can appear to have a regenerative, blood-loss-type anemia when using adult reference intervals for interpretation.

The patient's hydration status must be considered. Hematocrit reflects severity of anemia only when the animal has regained normal hydration and blood volume. It may take 1–2 days after blood loss before blood volume is restored to normal and the Hct shows the severity of the anemia.[2] Acute hemolytic anemia may also not have a Hct reflecting the severity of anemia at the time of presentation to a clinic.

TABLE 25.1 Guidelines for classification of severity of anemia.

	Dog	Horse	Cat/ruminant
Mild	30–37[a]	30–33	20–26
Moderate	20–29	20–29	14–19
Severe	13–19	13–19	10–13
Very severe	<13	<13	<10

[a]Values are hematocrit or packed cell volume in percent. Guidelines were determined by D. J. Weiss.

Appearance of Microhematocrit Tubes

Use the appearance of the plasma and buffy coat of the microhematocrit tubes to aid in diagnosis (Figure 25.1). Hemolytic anemia has often icterus or hemolysis seen in the plasma. The severity of the icterus or hemolysis is reflected by the intensity of the color. RBC fragmentation can be detected by noting a red layer on top of the buffy coat. Methemoglobin can give a brown or at least a darker color to the blood. Lipemia can be detected. Lipemia predisposes erythrocytes to lysis as an artifact. Lipemia will increase osmotic fragility, Hgb, and MCHC as an artifact.

CLASSIFICATION SYSTEMS

Classification by Strength of Erythropoiesis

Determination of bone marrow's effective erythropoiesis (function) divides anemia into those with no or relatively weak evidence of erythropoiesis (nonregenerative) or those with good evidence of active and effective erythropoiesis (regenerative) (Table 25.2). The anemia is classified



FIGURE 25.1 Examples of different appearances of microhematocrit tubes. (a) Tube from a normal dog; (b) Tube showing severe anemia with an increased buffy coat, which indicates an increase in leukocytes or platelets; (c) Severe anemia with icteric plasma and probable extravascular hemolysis or perhaps hepatic disease; (d) Severe anemia with hemolytic plasma, indicating possible intravascular hemolysis. A red layer above the buffy coat indicates the presence of RBC fragments; (e) Severe hemolysis and a thick layer of RBC fragments over the buffy coat; (f) Dog with leukemia and a very thick buffy coat; (g) Lamb with copper poisoning and severe methemoglobinemia indicated by the brown color; (h) Lipemia with whitish turbid plasma and a faint layer of RBC fragments on top of the buffy coat.

TABLE 25.2 Canine and feline relative and absolute reticulocyte guidelines.

		Feline	
Strength of regeneration	**Canine reticulocytes (%)**	**Aggregate reticulocytes (%)**	**Punctate reticulocytes (%)**
Relative			
None	1[a]	0–0.4[a]	1–10[a]
Weak	1–4	0.5–2	10–20
Moderate	5–20	3–4	20–50
Strong	21–50	>5	>50
Absolute			
None	60[b]	<15[b]	<200[b]
Weak	150	50	500
Moderate	300	100	1000
Strong	>500	>200	>1500

[a]Values are percentage of non-nucleated RBCs that are reticulocytes. The percentage of canine reticulocytes or feline aggregate reticulocytes should approximately predict the percentage of polychromatophils on a blood smear.
[b]Reticulocytes × 10^9/L or reticulocytes × 10^3/μL.

as regenerative if signs of increased erythropoiesis seem adequate for the severity of anemia, duration, species characteristics, treatments, and likelihood of multiple etiologies. Regenerative or responsive anemia should have normal bone marrow function. Regenerative anemias are caused by loss of erythrocytes from the body (external blood loss) or lysis of the erythrocytes within the body (hemolytic anemia and internal blood loss). Regenerative anemia is usually easier to diagnose than nonregenerative anemia. Nonregenerative anemia should have significant primary or secondary bone marrow dysfunction, which is often without an apparent cause.

Bone marrow regeneration in species that display consistent reticulocyte responses (e.g., dog, cat, pig, and rat) is usually evaluated by the reticulocyte response (see chapters on individual species characteristics). The strength of increase in the absolute reticulocyte count per unit volume of blood best reflects how effective bone marrow erythropoiesis is in the patient (Table 25.2). The percentage of reticulocytes is a ratio of reticulocytes to total non-nucleated erythroid cells. With increasingly more severe anemia, there are increasingly fewer mature erythroid cells. This exaggerates the % reticulocytes. If 1% reticulocytes is normal for a dog, 3% reticulocytes may sound increased, but if there are 1/3 as many erythrocytes, there has been no increase in the absolute number of reticulocytes released from the bone marrow. Absolute counts (% reticulocytes × total erythrocyte count) are preferred for interpretation. The corrected reticulocyte percentage (CRP) and reticulocyte production index (RPI) are other methods for interpreting the reticulocyte response when the total erythrocyte count (RBC) is not available to calculate an absolute reticulocyte count. Guidelines for and the clinical usefulness of CRP or RPI are not well established or widely understood in veterinary species.

Use Table 25.2 for approximate guidelines to interpret approximately how great an increase in reticulocytes indicates mild, moderate, or marked bone marrow responses. The value of 60 reticulocytes × 10^9/L (or 60 000 reticulocytes × 10^3/μL) in Table 25.2 is a mean, reference value for canine reticulocytes in general and should not be interpreted to mean that less than 60 × 10^9/L is nonregenerative and greater than 60 × 10^9/L is a regenerative anemia. This is a common error in interpretation and diagnosis. Diagnosis is not mathematics with one correct number. Use reference values for reticulocytes established for the particular hematology instrument used and species. For example, a reference interval for canine reticulocytes with the Advia 2120® is 10–110 × 10^9/L not <60 × 10^9/L.

Reticulocyte numbers are best interpreted at the time of an expected peak of reticulocytes, which is 4–8 days

after onset of the anemia. Guidelines for interpretation of reticulocyte numbers in Table 25.2 are for the peak reticulocyte response and may be misleading early in an anemia (i.e., pre-regenerative) when reticulocyte numbers have not increased yet, or late in an anemia when reticulocyte numbers decline (10–14 days).[6] Late in an anemia, the number of macrocytic hypochromic erythrocytes (Figure 25.2) is more reflective of the strength of the initial regenerative response than the reticulocyte number, which may be declining or back to normal.

Feline punctate reticulocytes have a long half-life in blood and therefore may accumulate in large numbers (Table 25.2). The kinetics of feline aggregate reticulocyte responses are similar to those of other species, but maximal responses are weaker (Table 25.2) than those expected in dogs.[6] Aggregate reticulocytes may not be increased unless the anemia is severe.

Microscopic identification of aggregate and punctate reticulocytes is time consuming and subjective when many reticulocytes are present (see Chapter 23). The automated reticulocyte counts of the Advia 2120 and Sysmex XT-2000iV® detect primarily feline aggregate reticulocytes and not punctate reticulocytes.[3] Use a quick screen of a reticulocyte-stained blood smear for an estimate of punctate reticulocyte percentages.

It is important that laboratories indicate the type of feline reticulocyte reported. Sometimes, only "reticulocytes" are reported without specifying the type. In Table 25.2, note that the number of aggregate reticulocytes indicating a strongly regenerative anemia is the same absolute number as the number of punctate reticulocytes indicating no regeneration. Duration of the feline regenerative response may be interpreted by pattern of changes in the two types of reticulocytes. A strong increase in aggregate reticulocytes but little to no increase in punctate reticulocytes indicates an early response (3–6 days after onset of anemia), while late in a response (9–20 days) there may be very many punctate reticulocytes, but no increase in aggregate reticulocytes.[6] The Advia erythroid graphics illustrate well the increased numbers of macrocytic hypochromic cells, and in the author's opinion are a better indication of an erythroid response than absolute reticulocyte numbers, especially later in the response.

Other indicators of effective erythropoiesis in anemia are less specific for erythropoiesis than reticulocytosis; however, these may be useful in species without consistent reticulocyte responses. Nucleated RBCs (NRBCs) are useful indicators in ruminants (e.g., cattle, llamas) to reflect regeneration.[5] NRBC numbers are frequently reported as a relative ratio of NRBC/100WBC, so marked changes in WBC count can affect this ratio. Basophilic stippling, especially in ruminants, and Howell–Jolly bodies suggest a regenerative anemia (Figure 25.5f). RBCs produced during active and increased erythropoiesis are larger. Thus, macrocytosis and anisocytosis suggest active erythropoiesis and may be the only indicators in peripheral blood of horses. Horses release very few or no reticulocytes into circulation. Macrocytosis and anisocytosis can be detected by the MCV (e.g., >52 fL in horses), RBC cytograms and histograms (Figure 25.3), or blood smear evaluation. RBC distribution width (RDW) indicates the amount of anisocytosis. NRBC, macrocytosis, Howell–Jolly bodies, and anisocytosis are caused by disorders other than regenerative anemia, such as glucocorticoid treatment, heat stroke, dysmyelopoiesis, myeloproliferative disorders, prolonged storage of EDTA blood, etc., and are thus not specific indicators of regeneration.

Classification by Erythrocyte Volume and Hemoglobin Concentration

Disease changes in erythrocytes may cause sizes that are microcytic (smaller), normocytic, or macrocytic (larger), and erythrocyte hemoglobin concentration changes are hypochromic (reduced) and normochromic (normal concentration). Hyperchromic changes (increased MCHC or increased optical density detected in erythrocytes by hematology instruments) indicate an erroneous result and not a disease-related change. Pre-analytic and analytic errors, such as hemolysis, lipemia, or Heinz bodies, cause artifacts that imply the erythrocytes contain more hemoglobin per unit volume than a normal cell filled with hemoglobin. The error causing a hyperchromic result should be identified by the laboratory and those results not reported to the veterinarian without explanation.

The three important diagnostic patterns are macrocytic hypochromic anemias (regenerative anemias with large, young RBCs that are not fully hemoglobinized), normocytic normochromic anemias (nonregenerative anemias with residual normal erythrocytes), and microcytic hypochromic anemias, which are usually iron deficiency anemias.

The MCV and MCHC are reported by most hematology instruments and have been used for this type of classification. MCV and MCHC are mean values (one single number) and as such unfortunately are too insensitive to usually and correctly identify the three main patterns. The numbers of abnormal erythrocytes are often too small to pull mean values like MCV or MCHC outside of wide reference intervals. Blind use of MCV and MCHC results may give erroneous conclusions like macrocytic normochromic anemia, which can suggest a completely incorrect cause.

Computer graphics (cytograms and histograms) of hematology analyzers such as the Advia 2120 (see Figure 25.2) can detect diagnostic changes in erythrocyte volume or Hgb concentration and are exceptionally good tools in anemia diagnosis. For example, microcytic hypochromic cells can often be detected in iron deficiency while the MCV and MCHC are still normal.

The Advia determines hemoglobin concentration by a spectrophotometric method (like most instruments), but also based on a summation of individual cell hemoglobin concentrations from its analysis of laser light scatter (Mie analysis). The latter is called cell hemoglobin concentration mean (CHCM), which is more correct in the case of lipemia causing a false increase in the spectrophotometric (cyanmethemoglobin) method.

Regenerative anemia in horses can be diagnosed by a macrocytic normochromic pattern on Advia cytograms

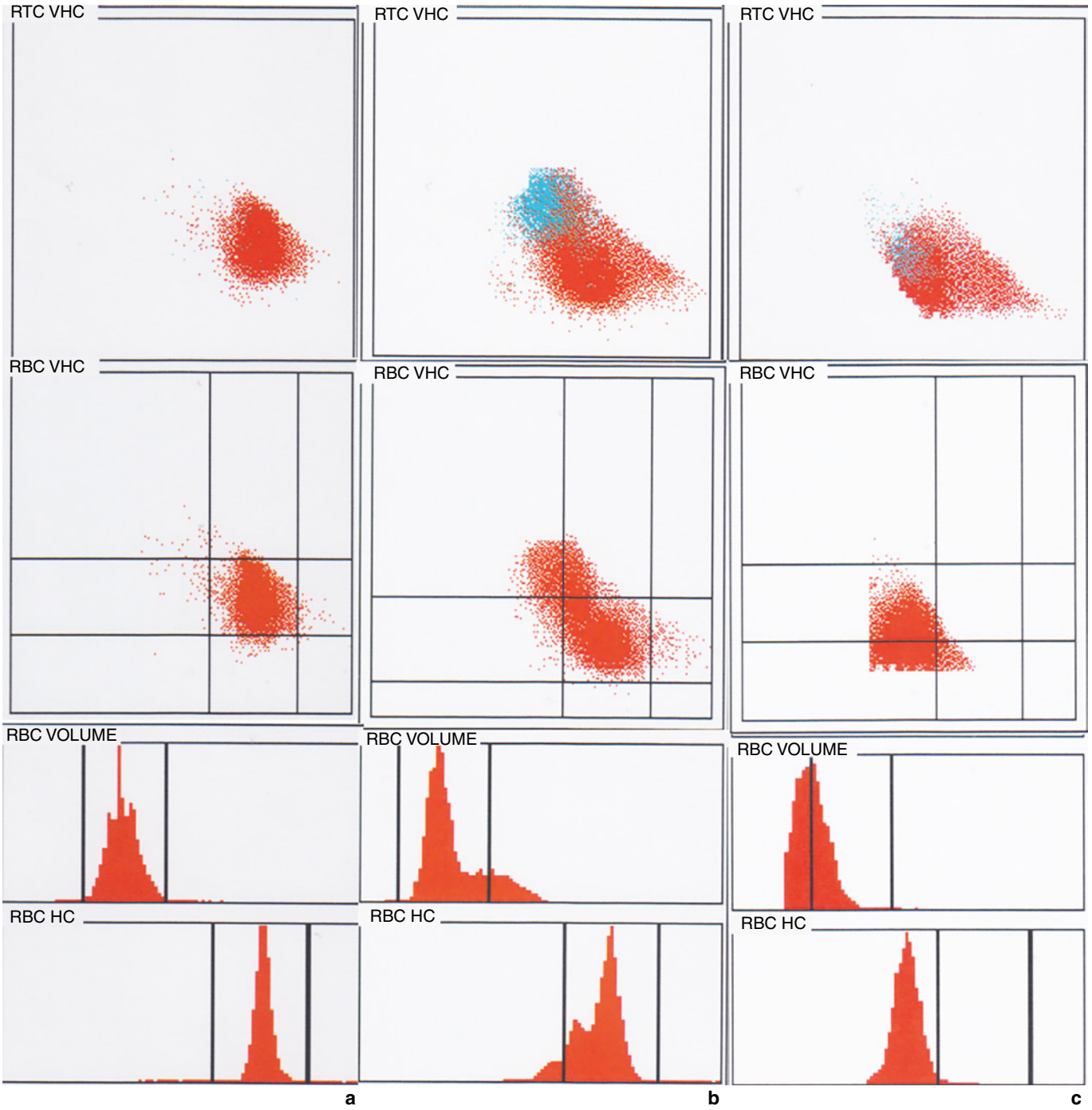


FIGURE 25.2 Composite of Advia 2120 reticulocyte volume hemoglobin concentration cytograms (RTC VHC), RBC cytograms (RBC VHC), RBC volume histograms, and Hgb concentration histograms (RBC HC) from two dogs and one cat that illustrate the three main types of anemia based on size and hemoglobin concentration of RBC. (a) The left column was from a dog with nonregenerative IMHA and had only occasional reticulocytes (blue dots) in the RTC VHC. It was a normocytic normochromic anemia with almost all RBCs in the central box of the 9-box RBC cytograms (RBC VHC); (b) The center column was from a cat with blood loss anemia. The RTC VHC and RBC VHC cytograms show two populations of erythrocytes. The macrocytic hypochromic (upper left cluster) erythrocytes in RBC VHC are blue reticulocytes in the RTC VHC cytogram. A second population of normocytic normochromic erythrocytes is in the central box of the nine-box RBC cytogram. These erythrocytes were present prior to blood loss. Part of the cluster of macrocytic hypochromic cells remains in the central box; (c) The right column was from a dog with iron deficiency and microcytic hypochromic anemia. Its reticulocytes were also microcytic and are lower in the right RTC VHC cytogram compared with the cat.

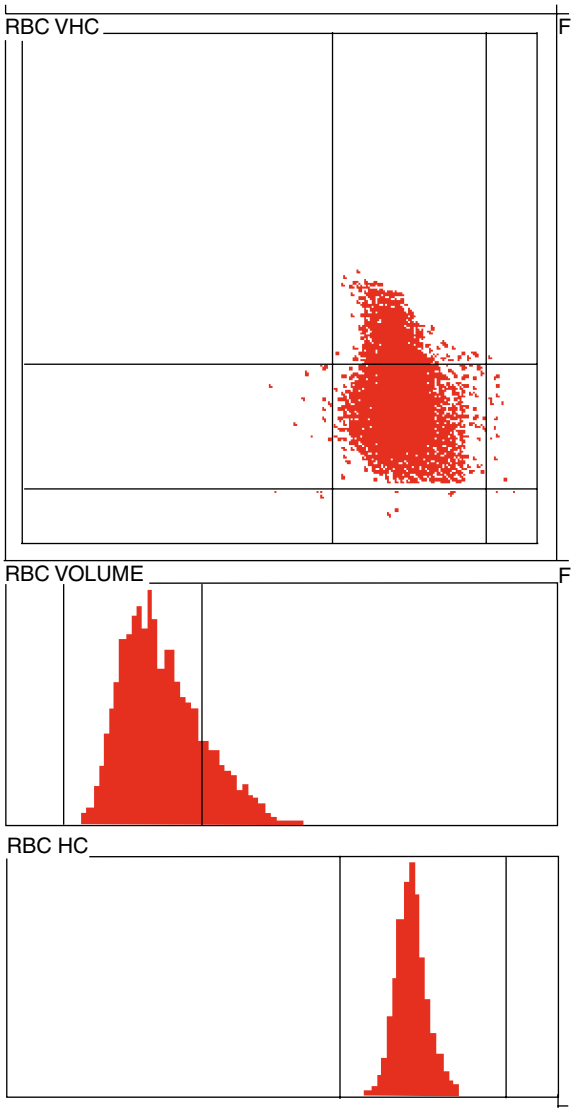


FIGURE 25.3 Regenerative response in a horse with immune-mediated hemolytic anemia. There is a macrocytic normochromic population of RBCs seen in the Advia 2120 RBC graphics. This horse did not have increased reticulocytes detected by the Advia 2120.

and histograms (Figure 25.3). Horses even with a strong regenerative anemia often have no reticulocyte response. Cats have relatively weak aggregate reticulocyte responses, which often fails to affect the MCHC and MCV, but may be clearly detected in Advia's RBC graphics (Figures 25.2 and 25.4). Macrocytic normochromic erythrocytes in the absence of polychromasia in cats can suggest feline leukemia virus (FeLV) infection or myelodysplastic syndromes. Some poodles have macrocytic erythrocytes as a familial defect. Swelling of erythrocytes is a common artifact in day-old samples. Macrocytic hypochromic RBCs occur (as in stomatocytosis) because the RBCs swell greatly and quickly in vitro. In contrast to pernicious anemia in people, macrocytic normochromic changes in MCV and MCHC should not indicate vitamin B12/folic acid deficiency in veterinary patients. Vitamin B12/folic acid deficiency is a rare condition in domestic animals. When it occurs, it is not a cause of macrocytic normochromic anemias. Cobalamin (vitamin B12) malabsorption has been reported in Giant Schnauzers, but causes a normocytic normochromic anemia (see Chapter 31).[1]

Classification by Blood Smear Morphology

Blood smear evaluation is an essential procedure in classification of anemia, especially hemolytic anemias. Many subjective observations critical to diagnosis can only be made by a trained microscopist. Some examples include identification of blood parasites, spherocytes, autoagglutination, ghost cells, Heinz bodies, eccentrocytes, pyknocytes, and hypochromasia (Figure 25.5). Many morphological changes may be seen and reported with erythrocytes, but reports should be restricted to findings likely to affect the final diagnosis. Changes that can suggest numerical errors include clumping of platelets or leukocytes (see Chapter 24).

Classification by Etiology

The ultimate goal of diagnosis is correct classification of anemia by etiology or etiologies in case of multiple causes (see chapters on specific causes). The path to the final diagnosis varies with each case. The likely etiology may be identified in the history. Substances known to cause Heinz body anemia, such as acetaminophen/paracetamol in cats or red maple tree branches and leaves in horses, can be identified by proper questioning of the owner.

DIAGNOSTIC APPROACHES

Anemia diagnosis should begin with a CBC including blood smear evaluation. Hematology instruments provide abundant automated enumerations such as reticulocyte counts, but many diagnoses will be missed without blood smear evaluation. Instrument graphics can illustrate changes in erythrocyte populations well. If the diagnosis is not obvious from the history, physical examination, and CBC, then additional testing is indicated. Additional tests are chosen based on the situation, species, and probability of different causes of anemia for a location. For example, anemia with hemoglobinuria in a young dog with a tendency to eat foreign bodies can indicate radiographic examination to look for zinc-containing metallic foreign bodies in the stomach. Gastric radiographs are not usually considered a diagnostic method for anemia, but may be the most important diagnostic test in that situation.

A simple, algorithmic approach to selected anemias reflects the previous discussion (Figure 25.6). One must add one's own interpretation and knowledge to any algorithm to consider additional information or factors in a given situation. After documenting the presence and severity of the anemia, the strength of the bone marrow's response should be determined. Hemolytic

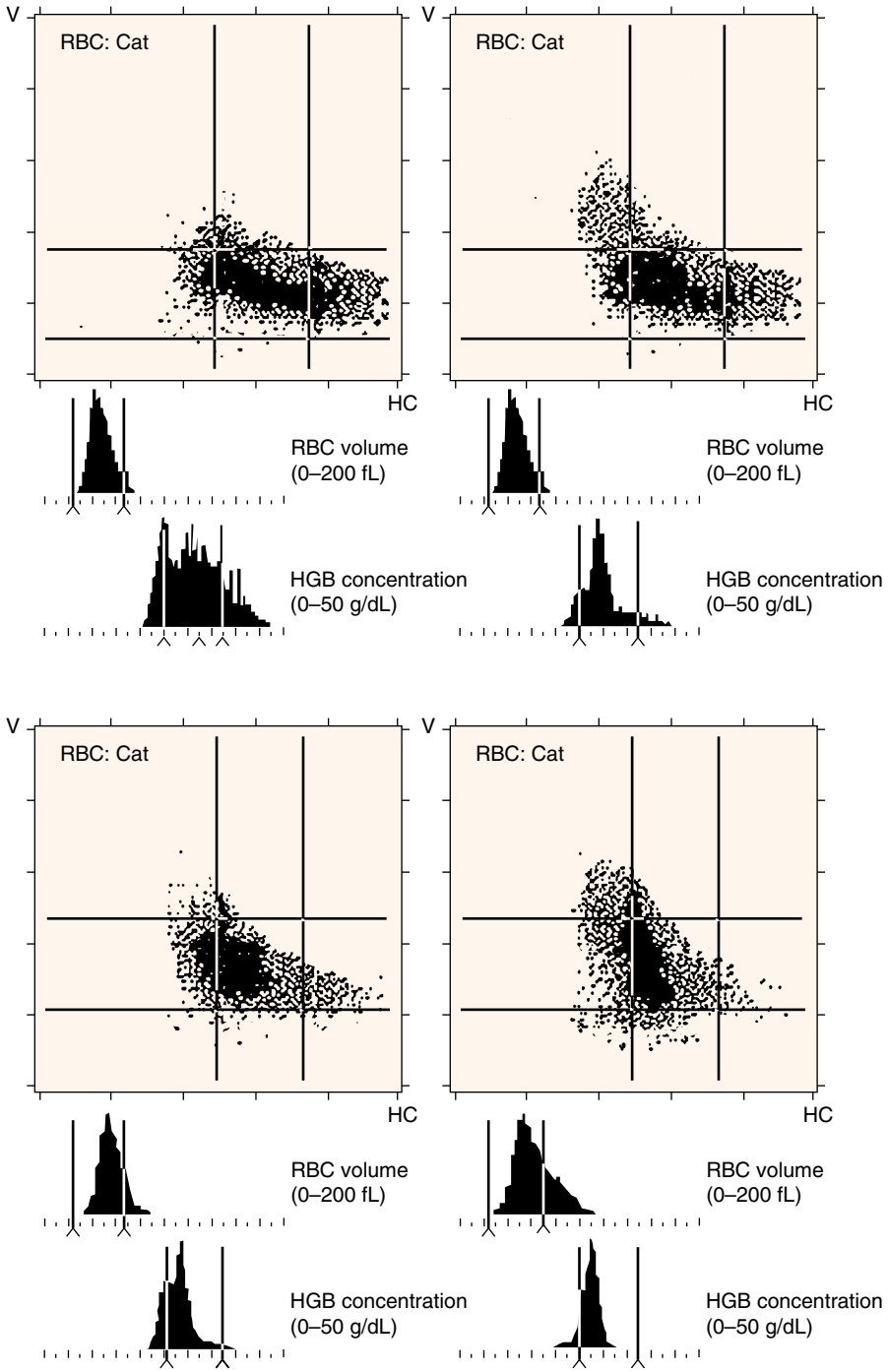


FIGURE 25.4 Composite of four sets of RBC cytograms and histograms over 1 week from a cat with Heinz body anemia from eating baby food containing onion powder. The sequence begins on the upper left, and progresses to upper right, lower left, and finally lower right. The third set (lower left) looks the most normal and may be used for comparison. That RBC cytogram has one dark, round cluster that is normocytic normochromic erythrocytes. The cells should be in the center box, but the grid was not aligned well. The lightly dispersed dots extending to the right are RBCs with Heinz bodies that appeared hyperchromic. Note that, on the first set (upper left), this extension is very dark, indicating many RBCs appeared hyperchromic because 84% contained large Heinz bodies. The MCHC was falsely high (44 g/dL) on that day. The Hgb concentration histogram was bimodal with a larger right peak of hyperchromic RBC. The smaller left peak was normochromic cells (grid was offset). The final set of graphics (lower right) shows a good regenerative response in the RBC cytogram with mainly macrocytic and slightly hypochromic RBC (i.e., reticulocytes) extending above and to the left of the normal cells. The RBC volume histogram shows many of the cells (about 1/3) were macrocytic. The HGB histogram shows very few hyperchromic cells, because RBCs with Heinz bodies dropped to 17% and Heinz bodies were smaller. The sequence shows well the loss of hyperchromic erythrocytes with Heinz bodies and the developing regenerative response with young, macrocytic cells.

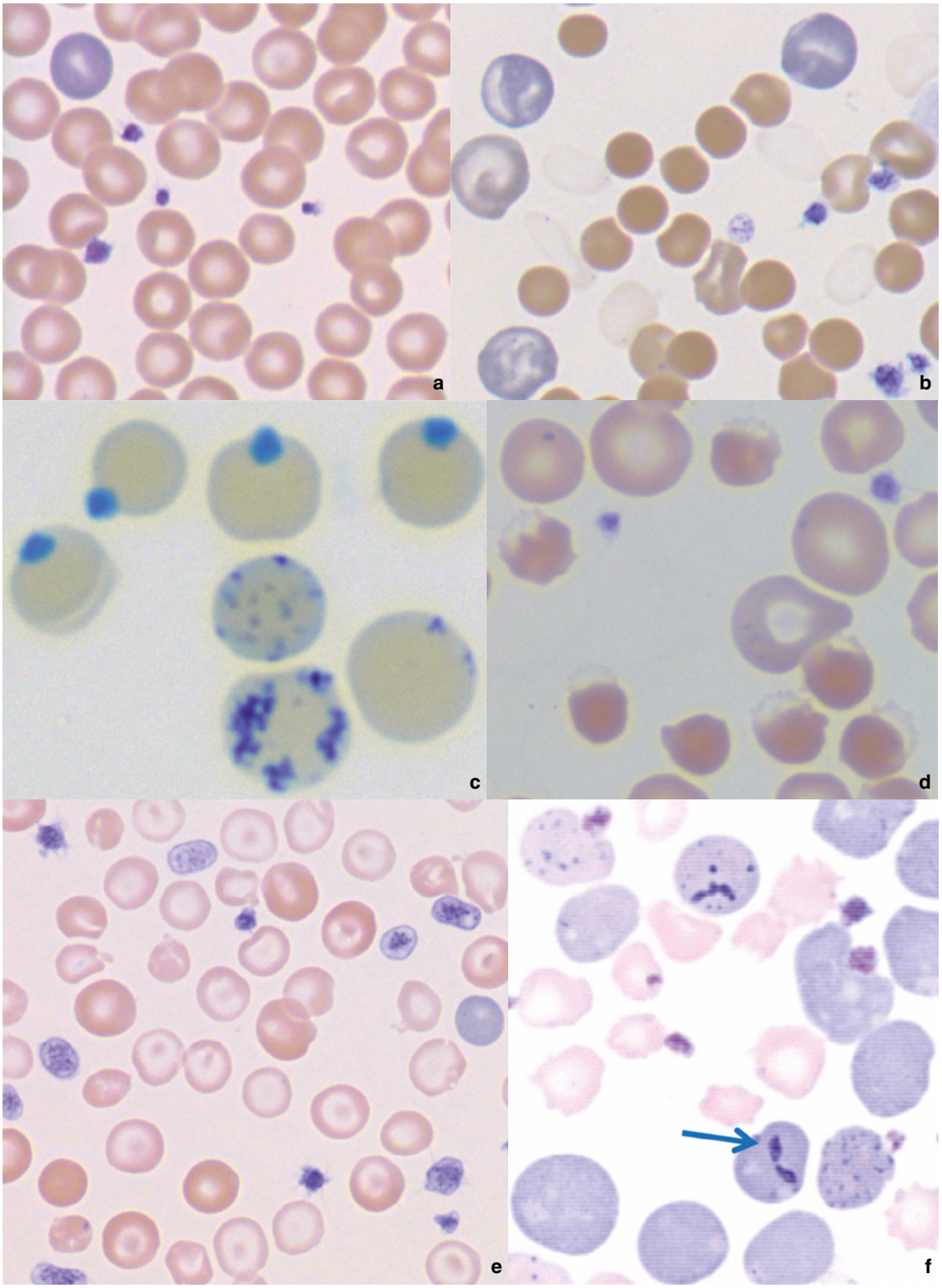


FIGURE 25.5 Composite of six photomicrographs of blood smears with diagnostic morphology. Use erythrocytes for size comparison (about 5–7 μm in diameter). Smears were stained with Giemsa except for brilliant cresyl blue stain in panel (c). (a) Normal dog; (b) Dog with IMHA. The photo has four polychromatophils (reticulocytes), more than 10 spherocytes, and six ghost red cells; (c) Four feline erythrocytes with Heinz bodies, one punctate reticulocyte with only three to four dots, one aggregate reticulocyte with 18 dots, and one aggregate reticulocyte with four aggregates of dots; (d) Eccentrocytes from a dog with onion poisoning. Indistinct Heinz bodies are also present. The spherical portion of eccentrocytes (pyknocytes) may be confused with spherocytes; (e) Smear from a dog with iron deficiency and severe hypochromasia; (f) *Babesia* (arrow), polychromatophils, and basophilic stippling in a roe deer. See Chapter 24 for more on erythrocyte morphology.

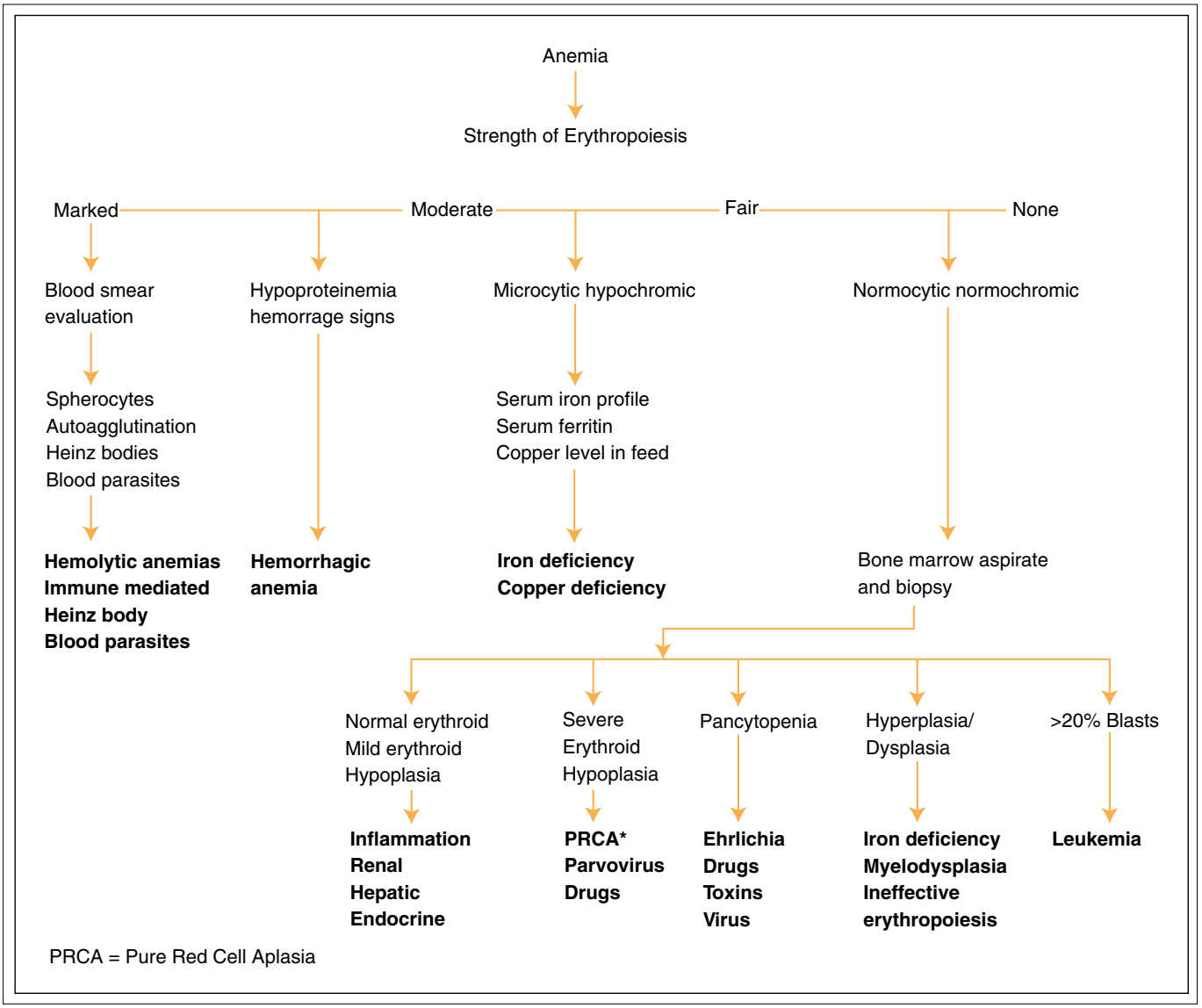


FIGURE 25.6 Algorithm for diagnosis of anemia. Anemia is documented and evaluated for severity. The strength of erythropoiesis is determined usually by reticulocyte evaluation. The marked to moderately regenerative anemias should be hemolytic or hemorrhagic. Blood smear evaluation frequently identifies the type of hemolytic anemia. Diagnoses are given in bold. Hypoproteinemia and clinical signs indicate hemorrhagic anemia. Microcytic hypochromic anemia most often indicates iron deficiency that varies from nonregenerative to moderately regenerative. Nonregenerative and inadequately regenerative anemias with normocytic and normochromic RBCs may be diagnosed by bone marrow evaluation.

anemias are usually the most regenerative (Table 25.3). Blood smear analysis frequently provides specific evidence of the cause of the hemolysis such as spherocytes, autoagglutination, ghost cells, Heinz bodies, eccentrocytes, or blood parasites (Figure 25.5). Icteric or hemolytic plasma, splenomegaly, or urinalysis evidence of hemoglobinuria suggests hemolytic anemia. If a regenerative anemia lacks indicators of hemolysis, then a blood loss anemia should be suspected. Hypoproteinemia or low normal plasma protein can support external blood loss as a diagnosis.

BLOOD LOSS ANEMIA

Blood loss can be external or internal (Table 25.4). Internal blood loss mimics hemolytic anemia in that erythrocytes are broken down within the body and plasma proteins are not lost from the body. External blood loss causes loss of plasma proteins as well as erythrocytes, so plasma protein concentration should be evaluated. Hypoproteinemia or low–normal plasma protein combined with regenerative anemia strongly suggests external blood loss. Plasma protein is replaced much quicker than erythrocytes, so hypoproteinemia is more associated with recent and

TABLE 25.3 Selective causes of hemolytic anemias.

Immune-mediated	Idiopathic, viral (equine infectious anemia, EIA), drugs, blood parasites, neonatal isoerythrolysis, transfusion reaction
Heinz body	Onions, vitamin K_3, phenothiazine, acetaminophen, phenacetin, acetanilide, phenylhydrazine-HCl, benzocaine, red maple, methylene blue, phenazopyridine, copper toxicity, selenium deficiency, *Brassica* sp. ingestion
Toxic hemolysis	Zinc, copper, water in calves, l-sorbose
Hypophosphatemia	Postparturient hemoglobinuria, refeeding syndrome, diabetes mellitus
Blood parasites	*Anaplasma, Babesia, Haemobartonella, Eperythrozoon, Theileria, Trypanosoma, Sarcocystis, Cytauxzoon*
Bacteria	Leptospira, *Clostridium novyi*
Hereditary RBC enzyme deficiencies	Pyruvate kinase, phosphofructokinase, glucose-6-phosphate dehydrogenase

TABLE 25.4 Selected examples of hemorrhagic anemia.

Trauma	Hit by car, penetrating wounds, surgical trauma
Bleeding lesions	Intestinal neoplasms, gastrointestinal tract ulceration, burns, infections
	Parasites
	Hookworms, coccidia, *Haemonchus*
	Fleas, lice
	Hemostatic disorders
	Thrombocytopenia, thrombopathy
	Acquired coagulopathy (e.g., warfarin toxicity, hepatic disease)
	Hereditary coagulopathy, von Willebrand disease, disseminated intravascular coagulation

large volume blood loss. Evidence of bleeding comes from history, diagnostic imaging, fluid cytology, etc. Chronic blood loss causes iron deficiency anemia. Iron deficiency anemia due to bleeding occurs more quickly in young, growing animals that are rapidly producing erythrocytes and using iron stores.

Time after onset of blood loss greatly affects the pattern of laboratory results. Indicators of the erythroid mass in the body (Hct, Hgb concentration, RBC count) may even increase during the first few hours after blood loss. This is most prominent in horses with very contractile spleens, but can be seen in dogs during controlled experimental situations.[4] The spleen holds about 20% of the erythrocyte mass, and splenic contraction can mask an anemia for the first 1–2 hours after blood loss, especially in horses.

The rapidity of fluid replacement and restoration of normal vascular volume after bleeding affect how rapidly remaining erythrocytes and plasma protein in the vascular system are diluted. It may take 1–2 days for the Hct to reflect the true severity of the anemia (Figure 25.7). Withholding access to water can delay decreases in the Hct. Aggressive fluid therapy can reduce the Hct rapidly and even cause hemodilution.

Time affects the expected number of reticulocytes. Blood loss anemia is regenerative, but no increase in reticulocytes is expected for the first 1–3 days during which it is termed a preregenerative anemia. Peak reticulocytosis is expected 4–8 days after onset (Figure 25.7).[6] Reticulocyte numbers decrease by 2 weeks after onset, but macrocytic hypochromic erythrocytes remain in circulation to give evidence of prior regeneration. This late stage macrocytic hypochromic response may be shown by Advia graphics.

Blood loss anemia (Table 25.4) becomes less regenerative with time due to loss of nutrients like iron and protein. Chronic, severe blood loss causes iron deficiency anemia, which becomes progressively more nonregenerative (see Chapter 27). About half of iron deficiency anemia cases still have enough reticulocytosis to be called regenerative.The author believes that confusion has occurred with interpretation of platelet counts related to blood loss. A common misconception is that blood loss by itself causes thrombocytopenia. This belief may cause other potential sources of thrombocytopenia to be overlooked. Actually, blood loss is an experimental method to cause thrombocytosis in dogs (Figure 25.7b).[2] Severe thrombocytopenia causes blood loss, but not vice versa. Blood loss together with aggressive fluid therapy can cause thrombocytopenia.

NONREGENERATIVE ANEMIA

Mild to moderate nonregenerative anemia is often secondary to conditions such as inflammation, renal or hepatic disease, endocrine disease, neoplasia, etc. The primary problems are diagnosed by tests more specific for them (e.g., leukogram, clinical chemistry, endocrinology, cytology, etc.). The cause of the anemia is usually clinically inferred from the type of disease present (e.g., if renal failure is present, then the anemia is likely anemia of renal disease).

Bone Marrow Evaluation

Bone marrow evaluation is indicated in nonregenerative anemias that have had adequate time (e.g., 3–6 days) to respond. Bone marrow is especially indicated if there is a bicytopenia or pancytopenia present. The anemia should be a nonregenerative type. Pancytopenia is a deficiency of cells in all three cell lineages. Likely causes include *Ehrlichia canis*, other bacteria, virus of that species, and exposure to various drugs and toxins.

Bone marrow evaluation is most complete when based on diagnostically useful smears of an aspirational biopsy, histologic sections of a cortex-to-cortex biopsy, and a CBC on blood taken the same day. However, small core cortex-to-cortex biopsies may have artifacts that inhibit adequate diagnosis. Erythroid hypoplasia, pure red cell aplasia (PRCA), myelofibrosis, aplastic

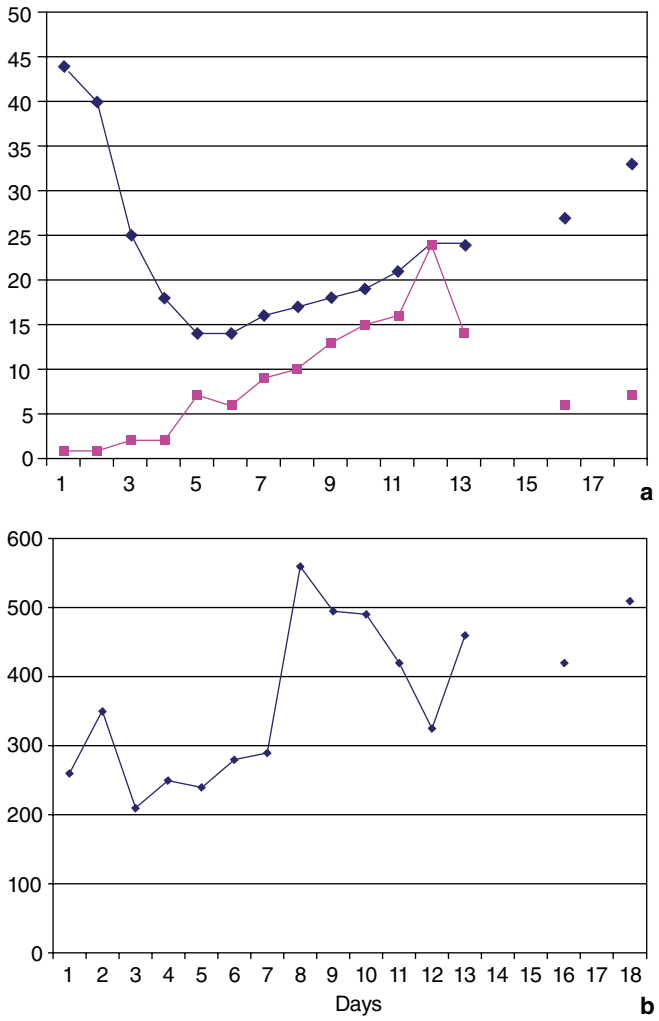


FIGURE 25.7 (a) Hematologic changes associated with canine blood loss anemia. A mature, healthy, research Beagle dog had 200–250 mL of blood removed each day for 3 consecutive days.[2] Equal volumes of saline and serum were infused intravenously to replace lost blood volume. Blood removal occurred on days 2–4 in the figure.[2] Hematocrit (blue line with diamonds) and reticulocyte values (red line with squares) are given in percent on *y*-axis. Days are on the *x*-axis in panel (a). Day 1 was the control value before bleeding. Note that hematocrit does not reach its lowest values until days 5 and 6. The hematocrit was still low (33%) on day 18, which was 14 days after the last bleeding. Reticulocytes peaked at 24% on day 12, which was 8 days after the last bleeding and fell to 6–7% on days 16 and 18; (b) Thrombocytosis occurring after canine blood loss. Platelet counts ($\times 10^9$/L) on the *y*-axis are from the same dog in Figure 24.5a. The dog had normal platelet counts before, during bleeding on days 2–4, and 3 days after the last bleeding. It had thrombocytosis on day 8 and higher than baseline platelet counts on days 9–18, which was 5–14 days after the last bleeding.

pancytopenia (fatty marrow), and leukemia clearly cause nonregenerative anemia.[7] A severe decrease in only the erythroid line is called pure red cell aplasia and may be caused by immune-mediated damage or viruses such as parvovirus in dogs and FeLV in cats (see Chapter 38). Drugs and toxins cause a wide variety of effects on the bone marrow (see Chapters 15 and 16).

Ineffective erythropoiesis, with an adequate to increased number of erythroid cells seen in the marrow, but a nonregenerative anemia seen in the CBC, can also occur. Causes of ineffective erythropoiesis include infections, drug reactions, myelodysplastic syndromes, and idiopathic causes. Erythroid hyperplasia and nonregenerative anemia occur in iron deficiency and will be accompanied by a lack of hemosiderin in macrophages. Precursor-directed immune-mediated anemia may be suggested by finding active phagocytosis of rubricytes by macrophages in the marrow (personal communication, Mike Scott).

Myelodysplastic syndromes are indicated by the presence of a hypercellular marrow, cytopenias, and abnormal cell morphology such as megaloblastic rubricytes, dwarf megakaryocytes, binucleated cells, nuclear fragments, and increased blast cells (see Chapter 67).

ACKNOWLEDGEMENTS

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CHAPTER 26

Erythrocytosis

JOHN F. RANDOLPH, MARK E. PETERSON, and ERICA BEHLING-KELLY

Relative Erythrocytosis
Absolute Erythrocytosis
  Primary Erythrocytosis (Polycythemia Vera)
  Secondary Erythrocytosis
    Appropriate Secondary Erythrocytosis
    Inappropriate Secondary Erythrocytosis
  Endocrinopathy-Associated Erythrocytosis
Clinical Signs of Erythrocytosis
Diagnostic Approach
Treatment

Acronyms and Abbreviations

EPO, erythropoietin; JAK, Janus kinase; P_{50}, the partial pressure of oxygen at which hemoglobin is half-saturated with oxygen; P_aO_2, arterial partial pressure of oxygen; PCV, packed cell volume; RBC, red blood cell; rPDA, reversed patent ductus arteriosus; S_aO_2, arterial hemoglobin oxygen saturation.

RBC production is predominantly regulated by the hormone erythropoietin (EPO; see Chapter 19). When oxygen sensors located in the renal cortex become hypoxic, hypoxia-inducible factors are generated and incite EPO gene transcription.[26,54] The resultant EPO then stimulates erythropoiesis in the bone marrow, increasing RBC numbers and subsequently the oxygen-carrying capacity of the blood.

Erythrocytosis is defined as an increase in RBC numbers, hemoglobin concentration, and calculated hematocrit or PCV above established reference intervals. Erythrocytosis and the term polycythemia are often used interchangeably. However, the concurrent expansion in platelet and neutrophil numbers that typically accompanies the clonal form of polycythemia (polycythemia vera) in humans has led to a lack of consensus on the appropriateness of this exchange.[48] Based on pathogenesis, erythrocytosis can be classified into relative or absolute categories. Absolute erythrocytosis can be further characterized as primary (polycythemia vera) or secondary (Figure 26.1).

RELATIVE ERYTHROCYTOSIS

Relative erythrocytosis is characterized by an increase in PCV due to a decrease in plasma volume rather than an increase in RBC production and mass. In dogs and cats, this occurs secondary to fluid losses often coupled with insufficient water intake and is the most common form of erythrocytosis. In animals with a muscular splenic capsule, such as the horse and dog, catecholamine-induced splenic contraction can cause the release of stored RBCs from the red pulp resulting in a transient mild erythrocytosis unassociated with clinical signs; the increase in PCV occurs in the absence of any increase in RBC production.

ABSOLUTE ERYTHROCYTOSIS

Absolute erythrocytosis, defined as a true increase in RBC mass, can develop from primary or secondary causes.

Primary Erythrocytosis (Polycythemia Vera)

Primary erythrocytosis results from autonomous proliferation of erythroid precursors that require little to no EPO for differentiation. The prototypical primary erythrocytosis, also called polycythemia vera, is a chronic myeloproliferative disorder resulting from the clonal expansion of hematopoietic progenitor cells

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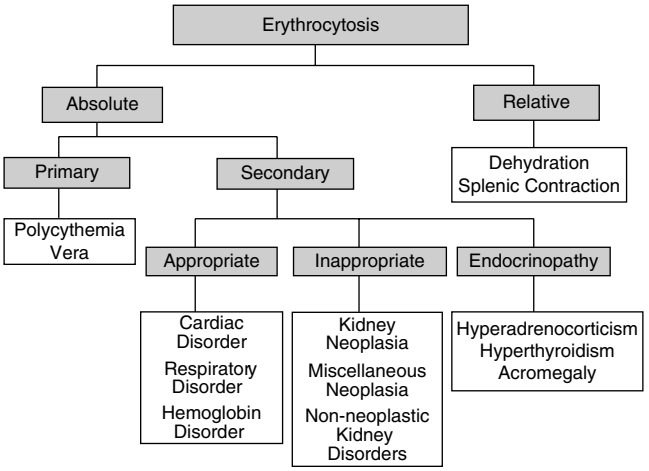


FIGURE 26.1 Flow chart for classification of erythrocytosis.

often driven by a Janus kinase 2 (JAK2) genetic mutation in humans.[31,59] An identical JAK2 mutation also has been identified in a dog with primary erythrocytosis.[1]

In humans, polycythemia vera is associated with splenomegaly, leukocytosis, and thrombocytosis, and may progress to myelofibrosis or leukemia.[26,48] However, many of those features are absent in dogs and cats with primary erythrocytosis. It remains unclear whether primary erythrocytosis in companion animals is the same disease as polycythemia vera in humans, or whether it represents a variant with isolated erythrocytosis. Regardless, primary erythrocytosis is a disease with variable age of onset in cats (median, 5–7 years) and dogs (median, 7 years).[10,62] Dogs with primary erythrocytosis are more frequently female, whereas affected cats are commonly male.[62] However, a recent report concluded no sex predilection for cats with primary erythrocytosis.[10] Primary erythrocytosis also has been diagnosed in the horse and ferret.[32,34]

Secondary Erythrocytosis

Secondary erythrocytosis develops from excessive production of EPO. If EPO is secreted in response to systemic hypoxia, then the resultant erythrocytosis represents an *appropriate* compensatory response. If, on the other hand, the increased EPO secretion is not associated with systemic hypoxia, then the response is *inappropriate* (Figure 26.1). Endocrinopathy-associated erythrocytosis is another type of secondary erythrocytosis resulting from hormonal (other than solely EPO-mediated) stimulation of erythropoiesis.

Appropriate Secondary Erythrocytosis

In physiologically appropriate erythrocytosis, the EPO-mediated increased RBC mass increases the oxygen-carrying capacity of the blood in an attempt to improve inadequate tissue oxygenation. For example, congenital heart anomalies resulting in right-to-left shunting with blood bypassing the lungs (e.g., tetralogy of Fallot, reversed patent ductus arteriosus [rPDA]) cause decreased oxygen saturation in the blood, which appropriately stimulates EPO production and resultant erythrocytosis.[5,6,19,20,28,37,40] Similarly, chronic severe pulmonary disease can cause decreased oxygenation of blood and may lead to erythrocytosis.[12,47] Hereditary high-oxygen affinity hemoglobinopathies in humans cause decreased release of oxygen to tissue and consequent erythrocytosis.[18,48] Although this type of hemoglobin disorder has not been described as a cause of pathologic erythrocytosis in companion animals, impaired oxygen transport from chronic methemoglobinemia in dogs and cats with methemoglobin reductase deficiency may cause mild erythrocytosis.[22] High-altitude dwelling also can result in an appropriate secondary erythrocytosis.[48]

Inappropriate Secondary Erythrocytosis

Excess production of EPO in the absence of systemic hypoxia, termed physiologically inappropriate erythrocytosis, is caused by: (1) EPO-secreting tumors of the kidneys or other organs, or (2) kidney disorders that result in regional hypoxia leading to increased EPO production. In humans, kidney neoplasms, as well as tumors of the liver, adrenal gland, uterus, and cerebellum, have been associated with inappropriate production of EPO and erythrocytosis.[48,54] Tumor-related erythrocytosis has been described with renal carcinoma,[9,46,60,61] renal lymphosarcoma,[5,16,38] renal fibrosarcoma,[21] nasal fibrosarcoma,[7] cecal leiomyosarcoma,[51] and schwannoma[65] in the dog, with renal carcinoma[5,25,29] and splenic hemangiosarcoma[52] in the cat, and with lymphoma in the horse.[30] Non-neoplastic kidney abnormalities in humans (e.g., renal cysts, hydronephrosis, glomerulonephritis)[54] and rarely in dogs (e.g., renal cryptococcosis, pyelonephritis)[27,61] have been reported as potential causes of erythrocytosis. For two dogs with glomerulonephropathy and erythrocytosis attributed to polycythemia vera, the kidney disorder was speculated to be a consequence, rather than a cause, of the erythrocytosis.[41,49] Genetic defects in the oxygen-sensing pathways responsible for some forms of congenital erythrocytosis in people have not yet been identified in companion animals.[48]

Endocrinopathy-Associated Erythrocytosis

Hormones other than EPO, such as cortisol, testosterone, thyroxine, and growth hormone, also may stimulate erythropoiesis either directly or indirectly through increased production of EPO or alternate pathophysiologic mechanisms.[13,48] In dogs, increased cortisol or testosterone concentrations associated with adrenal hyperactivity may produce mild erythrocytosis.[13] Similarly, cats with increased thyroxine concentrations from hyperthyroidism may have mildly increased RBC count.[45] Acromegalic cats with excess growth hormone may develop mild erythrocytosis,[45] but similar hematologic findings are usually absent in dogs with acromegaly.[50] The mild erythrocytosis in these endocrine

disorders is insufficient to result in clinical signs, and is usually discovered incidentally during routine diagnostic evaluation for the endocrinopathy.

CLINICAL SIGNS OF ERYTHROCYTOSIS

The clinical signs of both primary and secondary erythrocytosis include erythema (brick-red or ruddy color) of mucous membranes, neurologic disturbances (lethargy, ataxia, weakness, seizures, blindness, behavioral change), bleeding episodes (epistaxis, hematemesis, hematochezia, melena, hematuria), or polyuria and polydipsia. Most of these clinical manifestations are attributed to increased blood viscosity from the expanded RBC mass. In fact, the blood viscosity rises steeply as PCV increases.[4,55] The hyperviscosity slows blood flow, distends capillaries and small vessels, may increase the likelihood of thrombosis and rupture of these vessels, and may impair tissue oxygen delivery.[4,18,54] Ischemic myopathy may be responsible for the increased creatinine kinase activity reported in many cats with primary erythrocytosis.[10] Vascular changes characteristic of hyperviscosity frequently can be visualized on ophthalmologic examination (i.e., dilated and tortuous retinal vessels, sometimes accompanied by retinal hemorrhage). The polyuria and polydipsia have been attributed to hyperviscosity and increased blood volume leading to impaired vasopressin release.[60]

Laboratory test results may be skewed by extreme erythrocytosis. Hypoglycemia may be noted, and explained by enhanced glucose utilization by the increased number of RBCs in vivo or in vitro. Whole blood samples collected into sodium citrate anticoagulant tubes may result in prolonged prothrombin and activated partial thromboplastin times because of excess citrate concentration for the relatively smaller volume of plasma in patients with profound erythrocytosis.[26]

DIAGNOSTIC APPROACH

Before an extensive diagnostic workup is started, remember that many sighthound breeds, such as the Greyhound, normally have mild erythrocytosis when their PCV is compared to standard canine reference intervals.

In most animals, the first step in determining the reason for erythrocytosis is to exclude relative causes. Relative erythrocytosis usually results in a mild increase in PCV, rarely exceeding 60–65%.[23,62] However, the PCV may be as high as 70–80% in some dogs with the acute hemorrhagic diarrhea syndrome due to rapid fluid loss into the gut. This observation emphasizes that the magnitude of increase in PCV does not necessarily differentiate between relative and absolute erythrocytosis. The dehydration and hemoconcentration of relative polycythemia usually are identified by clinical findings (e.g., dry mucous membranes, reduced skin turgor), laboratory variables (e.g., hyperproteinemia, prerenal azotemia), and response to rehydration with intravenous fluid therapy. Excitable or anxious dogs with mild erythrocytosis suspected to be caused by splenic contraction should have another blood sample collected under less-stressful circumstances. Rarely, animals with repeatable mild increases in PCV and no obvious evidence of fluid loss may require direct measurement of RBC mass with radioisotope-labeled autologous RBCs to differentiate relative from absolute causes. However, these studies seldom are performed in clinical practice.

Once relative erythrocytosis is excluded, the next step is to differentiate primary and secondary causes of absolute erythrocytosis. Serum EPO measurement has been used in a number of published reports to differentiate between these two types of erythrocytosis. In secondary erythrocytosis, an increased EPO concentration would be expected, whereas EPO should be low in primary erythrocytosis. Although most dogs and cats with primary absolute erythrocytosis have low to low-normal EPO concentration and many animals with secondary absolute erythrocytosis have higher EPO values, results discordant with the expected findings and overlap with normal animals limit the diagnostic utility of EPO determinations.[3,5,17,24] Fluctuations in EPO secretion, production of hematopoietic growth factors not detected by the EPO assays, or renal hypoxia secondary to hyperviscosity may explain these findings.[5,33] Repeat EPO determinations, even following phlebotomy, have been suggested in problematic cases.[3,17,18,26,54] Because of these limitations, EPO measurements should complement but not supplant a thorough diagnostic evaluation of animals with erythrocytosis. Furthermore, current availability of validated EPO assays for dogs and cats is limited. Additional efforts to match diagnostic features of polycythemia vera in humans with primary erythrocytosis in companion animals have been unrewarding. Routine examination of bone marrow in affected dogs and cats is not useful because both primary and secondary erythrocytosis are characterized by erythroid hyperplasia. Therefore, a diagnosis of primary erythrocytosis typically is made by excluding common causes of secondary erythrocytosis.

Arterial blood gas analysis including determination of partial pressure of oxygen (P_aO_2) and hemoglobin oxygen saturation (S_aO_2) is useful in assessing tissue oxygenation. A P_aO_2 less than 80 mmHg indicates hypoxemia and S_aO_2 less than 95% reflects tissue hypoxia, both values warranting consideration of appropriate secondary erythrocytosis. If initial collection of arterial blood is thwarted by hyperviscosity, sampling may be repeated following cautious phlebotomy.[23] Alternatively, capillary S_aO_2 may be measured noninvasively with a pulse oximeter. Examination of cardiac and pulmonary systems by thoracic auscultation, thoracic radiographs, electrocardiography, and echocardiography may help determine the cause of appropriate erythrocytosis. Studies to confirm a right-to-left cardiac shunt include selective angiography and contrast echoaortography.[14,20] In dogs with rPDA, cyanosis of the vulvar or preputial (but not the oral) mucous membranes may be detected at rest or following exercise; similarly, lower P_aO_2 values

may be identified at the tail or hindlimbs compared to head or forelimbs.[14] This differential hypoxia in rPDA develops because deoxygenated blood shunts from the pulmonary artery into the aorta distal to the left subclavian artery and brachycephalic trunk, allowing more oxygenated blood to travel to the head and forelimbs. Abnormal hemoglobin variants with increased oxygen affinity in humans can be recognized by demonstrating normal P_aO_2 and S_aO_2 values, but reduced partial pressure of oxygen when hemoglobin is half-saturated with oxygen (P_{50}).[23,54] These hemoglobinopathies have yet to be confirmed as a cause of pathologic erythrocytosis in companion animals. However, some investigators have proposed that the higher PCV (and lower hemoglobin P_{50}) in Greyhounds may indicate that the hemoglobin of this breed has a greater affinity for oxygen.[56]

Once systemic hypoxia is excluded as the cause of erythrocytosis, conditions causing inappropriate EPO production should be considered. To locate the source of EPO in these disorders, results of physical and neurologic examinations, abdominal ultrasonography, intravenous urography, and computed tomography or magnetic resonance imaging should be helpful.

TREATMENT

With relative erythrocytosis due to dehydration, fluid therapy should be administered, and the underlying disorder should be addressed. The PCV will usually normalize with successful management. For relative erythrocytosis resulting from splenic contraction, no treatment is necessary.

In primary erythrocytosis, the goal of therapy is to reduce and maintain the PCV within reference intervals, thereby alleviating clinical signs and preventing any potential thrombotic and hemorrhagic complications. The optimal treatment, however, is unknown. In dogs and cats, the disease has been managed with phlebotomy alone[15,35,36] or in combination with other modalities, leeching,[39] radioactive phosphorous,[35,53,64] or chemotherapeutic agents, such as doxorubicin[41] and busulfan.[35] Phlebotomy and leeching must be repeated on a regular basis to maintain the PCV within reference intervals and can lead to iron deficiency and hypoproteinemia. Radioactive phosphorus induces prolonged remission and reduces morbidity, but is not readily available and may increase the risk of leukemia in treated humans.[26,48] Some of the chemotherapeutic drugs used to treat humans with polycythemia vera also may be leukemogenic.[26,48] However, it remains unknown whether leukemic transformation in these instances represents complications of treatment or natural progression of this chronic myeloproliferative disease.[26,48] The infrequent development of leukemia in dogs and cats with primary erythrocytosis may be further proof that it is indeed distinct from polycythemia vera in humans, or may merely reflect the prolonged time (10–15 years) needed for leukemic transformation in humans.[26] Anecdotal reports of successful control of primary erythrocytosis in dogs with chlorambucil after phlebotomy also have been mentioned.

Hydroxyurea, an antineoplastic drug, is most commonly used to manage primary erythrocytosis in dogs and cats. Side effects of hydroxyurea include anorexia, vomiting, bone marrow hypoplasia, mild alopecia, and sloughing of the nails.[44] Cats also may develop methemoglobinemia and hemolytic anemia with Heinz bodies, especially with high doses.[63] The effects of hydroxyurea are enhanced by reducing the PCV to approximately 50–60% with phlebotomy (10–20 mL/kg) and simultaneous fluid replacement.[43,44] Then, hydroxyurea is given at a loading dose of 30 mg/kg PO daily for 7–10 days, followed by 15 mg/kg PO daily in single or divided doses.[43,44,57] Alternate dosing schedules for cats have been reported.[8,10,11,63] Complete blood and platelet counts should be monitored at 7–14-day intervals until the PCV has normalized, then every 1–3 months. If leukopenia, thrombocytopenia, or anemia develops, hydroxyurea should be discontinued until the blood count returns to normal, and then resumed at a lower-maintenance dosage or dose frequency.[43,44] In humans, the JAK2 inhibitor ruxolitinib, pegylated interferon-alpha, and busulfan are considered for patients with polycythemia vera resistant to, or intolerant of, hydroxyurea.[42,58,59] Further studies on the safety and efficacy of these drugs for companion animals with primary erythrocytosis are needed.

In inappropriate secondary erythrocytosis, treatment should be directed toward the underlying cause, when feasible. Erythropoietin-secreting tumors should be surgically removed if practical, or managed with chemotherapy and/or radiation therapy. Preoperative phlebotomy to normalize the PCV may help reduce morbidity and mortality.

In appropriate erythrocytosis secondary to systemic hypoxia, as in some cardiac diseases, treatment of the underlying problem is recommended, if possible. Unfortunately, the two most common types of congenital heart defects associated with polycythemia in pets are not easily amenable to surgical correction: rPDA cannot be corrected surgically because of the attendant pulmonary hypertension, and mortality is high for attempted surgical correction of tetralogy of Fallot.[19] If correction is not possible, then long-term maintenance of the desired PCV can be accomplished with cautious phlebotomy (5–10 mL/kg) and hydroxyurea therapy as outlined for primary erythrocytosis; alternate protocols using hydroxyurea also have been successful.[37] However, because the erythrocytosis in these cases represents a compensatory response to tissue hypoxia, the PCV should be maintained between 55% and 60% rather than attempting normalization.[3] This degree of erythrocytosis seemingly is sufficient to minimize clinical signs associated with the systemic hypoxia as well as the polycythemia-associated hyperviscosity. Other investigators have recommended maintaining the PCV at even higher values (62–68%), or simply relieving clinical signs without concern about attaining a specific PCV.[2,6,48] Regardless, phlebotomy and drug administration in these patients should be performed with careful monitoring.

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CHAPTER 27

Iron and Copper Deficiencies, and Disorders of Iron Metabolism

LAUREN B. RADAKOVICH and CHRISTINE SWARDSON OLVER

Iron Physiology
Tests for Evaluation of Iron Disorders
  Serum Iron Concentration
  Percent Saturation, Serum Total Iron-Binding Capacity, and Unbound Iron-Binding Capacity
  Serum Ferritin
  Bone Marrow Iron
Iron Deficiency
  Types of Iron Deficiency and Causes
  Laboratory Features
    CBC Findings
    RBC Morphological Findings
  Portosystemic Shunts
Other Microcytic or Hypochromic Anemias
  Sideroblastic Anemias
Copper Deficiency
Iron Overload
  Acute Iron Toxicity
  Hemosiderosis and Hemochromatosis
    Primary Hemochromatosis
    Secondary Hemochromatosis

Acronyms and Abbreviations

MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; RDW, red blood cell distribution width; TIBC, total iron-binding capacity; CHr, reticulocyte hemoglobin content; MCVr, reticulocyte mean cell volume; PSS, portosystemic shunt.

Iron is an essential component of all living organisms, playing a role in many metabolic processes, particularly those involving electron transfer. Copper is a component of several metalloproteins including hephaestin, ceruloplasmin, cytochrome c oxidase, superoxide dismutase, urate oxidase, dopamine β-hydroxylase, ascorbic acid oxidase, and tyrosine oxidase. Iron absorption, metabolism, and utilization in erythropoiesis are discussed in Chapter 20. In this chapter, disorders of iron and copper metabolism will be discussed.

IRON PHYSIOLOGY

Iron is an element that is crucial to many biological processes. While its deficiency can lead to anemia, iron excess can pose serious problems, as iron readily participates in the Fenton reaction, resulting in deleterious free radical formation.[37,49] Because of the potential to induce oxidant damage, iron is tightly regulated in normal, healthy animals. Please see Figure 27.1 for an overview of mammalian iron regulation. Since there is no direct excretion mechanism,[54] iron levels are controlled by regulating the amount absorbed by the gastrointestinal tract. Both heme and nonheme forms of iron are transported into enterocytes, where iron can either be stored in ferritin when iron stores are replete, or absorbed into the bloodstream when there is a need for iron. Ferritin stores that have become oversaturated with iron degrade to become hemosiderin (see Chapter 20). When there is a need for iron, it is exported from the enterocyte via ferroportin,[6] oxidized by hephaestin, and then bound to the serum protein transferrin.[4] Transferrin shuttles iron to cells throughout the body, but the majority is used by erythroid precursors in the bone marrow to produce heme.

In addition to iron absorbed from the gastrointestinal tract, a large proportion of iron utilized by the body is recycled from the breakdown of senescent red blood cells by splenic macrophages.[56] These macrophages

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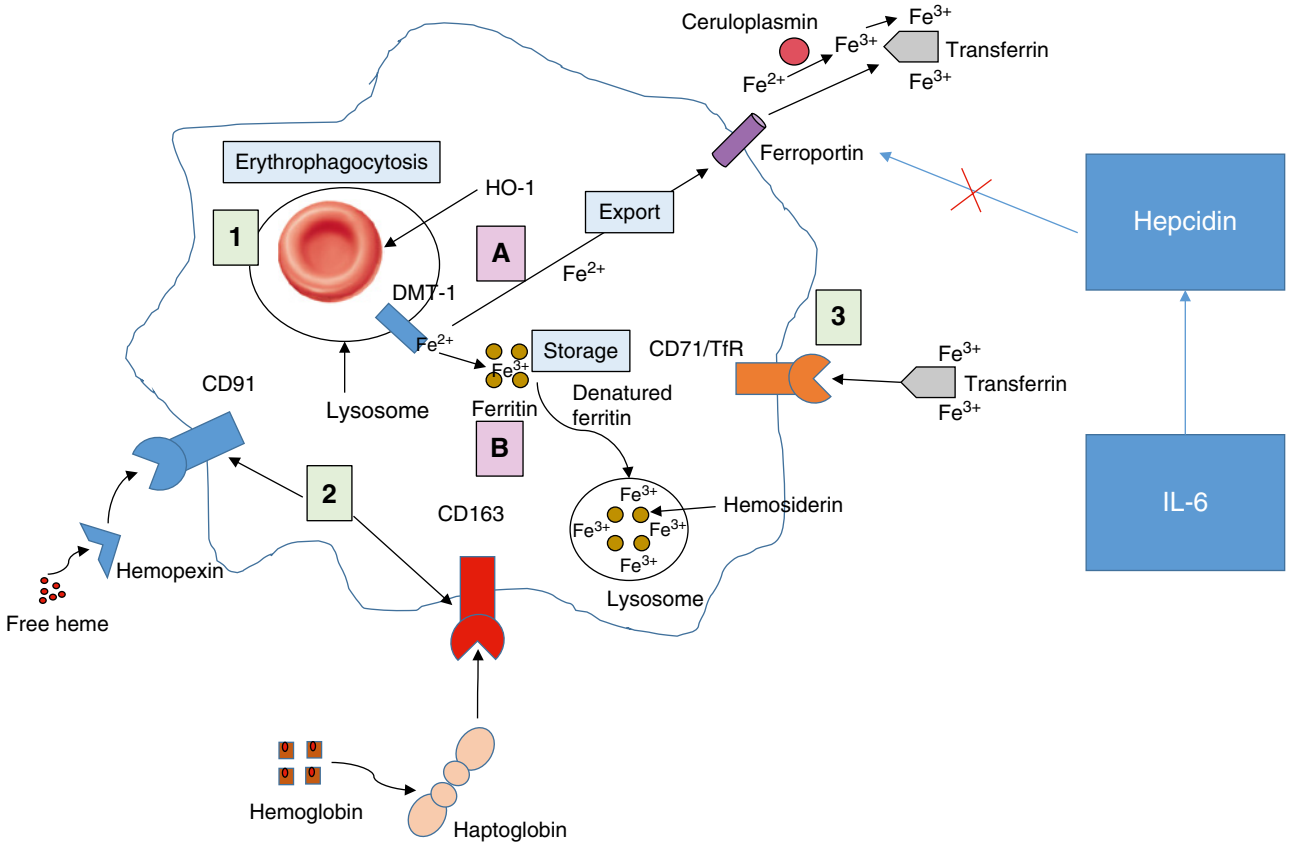


FIGURE 27.1 Iron regulation in mammals. This image demonstrates the mechanisms of iron entry and export from a macrophage. (1) Iron can enter the macrophage via phagocytosis of RBCs, (2) by receptor-mediated endocytosis of heme–hemopexin or hemoglobin–haptoglobin, (3) or by receptor-mediated endocytosis of transferrin-bound iron. After iron is broken down and released within the cell, it may be exported via (A) ferroportin, or (B) stored as ferritin. Once ferritin becomes saturated, it can become denatured over time, resulting in hemosiderin. In the presence of either iron overload or inflammation, hepcidin is produced by the liver, which results in the degradation of ferroportin, preventing iron export from cells.

phagocytize red blood cells and subsequently breakdown heme to release iron. This iron may be exported to the circulation via ferroportin, where it is then oxidized by ceruloplasmin, a copper-containing protein, to bind to transferrin. If not exported, it can be stored within the macrophages as ferritin.

The cellular export of iron is regulated by the peptide hormone hepcidin.[46] Hepcidin is produced by the liver in response to high tissue levels of iron and inflammation. Hepcidin binds to ferroportin, resulting in its degradation. This loss of ferroportin prevents iron from being absorbed from the gastrointestinal tract and also cuts off iron cycling from splenic macrophages. This causes a drop in serum iron levels and restricts iron availability to the bone marrow for heme production, which can eventually cause anemia,[32,24] as described in the following text.

TESTS FOR EVALUATION OF IRON DISORDERS

Serum Iron Concentration

Iron is transported in plasma by the iron transport protein apotransferrin, which carries up to two Fe^{3+} ions. The apotransferrin–Fe^{3+} complex is called *transferrin*. Under normal conditions, approximately one-third of the iron-binding sites of transferrin are occupied by Fe^{3+}. Serum iron concentration refers to the Fe^{3+} bound to transferrin and typically does not include hemoglobin-bound iron until hemolysis is moderately increased. Serum iron is quantified by releasing the iron bound to transferrin using strong acidification, followed by a series of reactions that culminate in a colored reaction product proportional to iron concentration. Serum iron is typically below the reference interval in iron deficiency, inflammatory diseases,[16,52] and after exogenous erythropoietin is administered without iron supplementation.[5] Serum iron concentration is also decreased by glucocorticoid administration to ruminants.[50] Serum iron is typically increased above the reference interval in hemolytic anemias, dyserythropoiesis, hypoplastic anemia, aplastic anemia, iron overload, and in dogs and horses given glucocorticoids.[17,42,55]

Percent Saturation, Serum Total Iron-Binding Capacity, and Unbound Iron-Binding Capacity

There are several tests that may be included as part of an iron panel. Percent saturation refers to the number of filled iron-binding sites present on the serum protein

transferrin. Each unit of transferrin can bind two iron molecules. Normally, transferrin is approximately at 30% saturation in domestic mammals. With iron deficiency, the percent saturation is typically decreased. The total iron-binding capacity (TIBC) is an indirect measure of the total serum transferrin concentration and can be measured either directly or indirectly. TIBC is directly measured in much the same way as serum iron, but preliminary steps allow full saturation of transferrin followed by release of transferrin-bound iron prior to measurement. Thus, the term "total iron-binding capacity." TIBC is increased in absolute iron deficiency in humans, horses, cattle, and pigs, but does not appear to be increased in clinical iron deficiency in dogs.[16] TIBC is decreased or in the low–normal range in inflammatory diseases.[7] Unbound iron-binding capacity, or UIBC, is an indirect measurement of TIBC. Since transferrin typically binds iron at only 30% of its capacity, the UIBC represents the remainder of binding sites for iron. Thus, the sum of the serum iron and the UIBC equals the TIBC. The UIBC is measured by adding a known quantity of iron, in excess, in order to fully saturate the transferrin. The remaining nonbound iron is then measured and subtracted from the known amount added.

Serum Ferritin

Serum ferritin is measured by immunologic methods. Assays to quantitate human serum ferritin are available in kit form and automated immunoassay instruments. In general, immunoassays to quantitate human serum ferritin cannot be used to quantify serum ferritin in other species. Quantitative immunoassays must be calibrated with a standard curve of known concentrations of the purified analyte from the species of interest in order to determine the concentration of the analyte in the unknown sample. Although there is serologic cross-reactivity between serum ferritins of some species,[28] in general, serum ferritins tend to be immunologically species-specific.[19] Therefore, it is likely that individual assays still must be developed and calibrated for each species.

ELISA tests have been developed for serum ferritin in the dog,[8] cat,[7] horse,[15,18,26] pig,[13,25] cow,[17] northern fur seals *(Callorhinus ursinus)*,[5] black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceroses,[23] and black and white ruffed lemur *(Varecia variegata)*.[3] In all cases, serum ferritin was purified from each species and quantitated by chemical methods. Polyclonal antisera or monoclonal antibodies were raised to the ferritin and used to develop tests for the dog, cat, horse, pig, and cattle. In some instances, there was sufficient species cross-reactivity to allow use of antisera or monoclonal antibodies to serum ferritin of one species to develop an ELISA for another species. Anti-equine ferritin polyclonal antibodies were used for the rhinoceros,[24] mouse monoclonal anti-dog ferritin antibody for the northern fur seal,[5] and anti-human ferritin polyclonal antibodies for the lemur ELISA test.[3] Each ELISA is calibrated with a standard curve of known concentrations of the ferritin purified from the species being measured. Non-human serum ferritin assays are offered by the Comparative Hematology Laboratory at Kansas State University.

Despite being an acute phase protein, serum ferritin correlates with tissue iron stores in normal domestic animals.[43] Increased serum ferritin occurs in animals with increased iron stores associated with chronic hemolytic anemias and repeated blood transfusions. Other causes of high serum ferritin concentrations include benign hemophagocytic disorders and malignant histiocytosis. Serum ferritin is also transiently increased in horses by intense exercise. Serum ferritin is decreased in animals with iron deficiency. Serum ferritin loses sensitivity when iron deficiency occurs in the face of inflammation, as the increase due to the acute phase response masks the decrease due to decreased storage.

Bone Marrow Iron

Bone marrow aspirates or core biopsies can be stained with Prussian blue to detect blue-staining hemosiderin, a tissue storage form of iron. Bone marrow aspirates used for Prussian blue staining should contain several unit particles to ensure adequate material is present for assessment of marrow iron. Animals with iron deficiency lack stainable iron in bone marrow; however, lack of stainable iron is not necessarily predictive of iron deficiency.[11] Healthy noniron-deficient cats and some cattle lack stainable iron in bone marrow.[14]

IRON DEFICIENCY

Types of Iron Deficiency and Causes

There are two broad classifications of iron deficiency: absolute and functional. Absolute iron deficiency is caused by insufficient gastrointestinal iron absorption, an iron-poor diet, or, most commonly in veterinary species, chronic hemorrhage.[31] Sources of hemorrhage include gastrointestinal ulcers, gastrointestinal neoplasia, hemorrhagic colitis, urinary bladder neoplasia, thrombocytopenia, inherited hemostatic disorders, and severe ectoparasite burden in small/young animals.[31] Deficiency due to an iron-poor diet is rare except in low-birth-weight neonatal animals that are nursing.[41] Milk contains low concentrations of iron; therefore, nursing animals can easily become iron-deficient as they grow.[23] Piglets reared without access to soil are highly susceptible to iron deficiency. This is prevented by administration of intramuscular iron dextran injections in piglets.[48] Inflammatory bowel disease and other disorders diffusely affecting the gastrointestinal tract may also cause iron deficiency due to decreased iron absorption by compromised bowel function in addition to mucosal blood loss.[33,38] Clinical features associated with iron deficiency vary somewhat depending on the cause of the iron deficiency and the presence of other concomitant disorders. Clinical signs that may be present include pale mucous membranes, lethargy, weakness, weight loss, stunted growth, hematuria, dermatitis, hematochezia, and melena. Iron-deficient animals have increased susceptibility to infection. This is because iron is required for normal T-cell function and bactericidal activity of macrophages.[20]

In contrast to absolute iron deficiency is functional iron deficiency, which is associated with inflammation.

In functional iron deficiency, iron stores within the body are adequate, but they are not accessible for hemoglobin production. When inflammation is present, the liver is stimulated to produce the peptide hormone hepcidin (Figure 27.1). Hepcidin binds to and degrades the only known cellular iron exporter, ferroportin. Without ferroportin, iron cannot enter the bloodstream from the gastrointestinal tract. Neither can it be exported from macrophages, which play a key role in iron cycling due to their primary function in breakdown of red blood cells. Clinical features of functional iron deficiency depend on the primary disease process, but these patients typically develop anemia, which is often referred to as anemia of chronic disease or anemia of inflammatory disease.

Laboratory Features

CBC Findings

Findings for absolute and functional iron deficiency are similar. Because iron is required for hemoglobin synthesis (see Chapter 20), deficiency impairs this process, altering erythrocyte and reticulocyte indices. Once iron deficiency has depleted iron stores, anemia usually develops, which is detected on CBCs by a decreased RBC count, hematocrit, and hemoglobin concentration. Mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) are two major erythrocyte indices that may also reflect iron deficiency. MCV usually decreases with absolute iron deficiency. One study theorized this may be due to iron-deficient cells undergoing additional cell divisions to achieve adequate hemoglobin concentration; however, this has not been proven.[47] MCHC also classically decreases with absolute iron deficiency, as less iron is available to produce hemoglobin. Functional iron deficiency is typically associated with a normocytic, normochromic anemia. However, microcytosis may develop if inflammation continues for an extended period of time.[22,32] The RBC distribution width (RDW) is frequently increased in absolute iron deficiency because of the coexistence of microcytes and normocytes in the blood. The greatest disadvantage of MCV and MCHC in evaluation of patient iron status is that their values do not move beyond the reference interval until late in the course of disease when there are enough affected erythrocytes to shift the mean value. Thus, these tests lack sensitivity in detection of iron deficiency. Visual inspection of cytograms generated by flow-cytometer-based hematology analyzers may detect low numbers of microcytic and/or hypochromic red blood cells when MCV and MCHC are within reference intervals. Thrombocytosis is also frequently present in cases of both absolute and functional iron deficiency. The pathogenesis of thrombocytosis seen in absolute iron deficiency is poorly understood. In functional deficiency, it is likely secondary to inflammatory cytokines that are known to stimulate thrombopoiesis.[21]

Because reticulocytes have been recently produced, they most accurately reflect the current iron status of the bone marrow.[36,45] Because changes in reticulocyte indices may occur before changes in mature erythrocyte indices, they offer increased sensitivity in detecting iron deficiency. The most extensively studied reticulocyte indices include reticulocyte hemoglobin content (CHr) and reticulocyte mean cell volume (MCVr), both of which decrease with iron deficiency.[9,36,40,45] Other indices reported may include reticulocyte hemoglobin concentration, percentage of hypochromic reticulocytes, percentage of reticulocytes with low/high CHr, and percentage of microcytic/macrocytic reticulocytes.

RBC Morphological Findings

Alterations in RBC morphology are commonly noted in iron-deficient animals. Hypochromic RBCs have a narrow rim of lightly stained hemoglobin and a larger than normal area of central pallor. Keratocytes, schistocytes, and target cells are also frequently present in iron deficiency. Tear-drop-shaped RBCs called *dacryocytes* and folded RBCs are commonly noted on blood films in iron-deficient camelids.[29] The mechanism responsible for these shape alterations is uncertain. When iron deficiency anemia is the result of hemorrhage, reticulocytosis is frequently present in the early stages.

Portosystemic Shunts

Portosystemic shunts (PSS) are vascular connections between the portal and systemic circulation that divert blood around the liver. These vessels may occur congenitally or arise secondary to portal hypertension associated with chronic hepatic disease. Most affected dogs have mild microcytosis and hypochromasia, but are not anemic.[28] Although iron metabolism is clearly affected in dogs with PSS, it is most likely secondary to sequestration rather than absolute iron deficiency. This is evidenced by a normal or reduced TIBC accompanied by a variable transferrin saturation, normal to increased serum ferritin concentrations, and increases in liver iron stores after experimental PSS induction. In spite of the fact that the microcytosis and anemia of PSS appear to be functional, hepcidin mRNA has been shown to be unchanged after PSS correction.[10] Further research is needed to fully characterize the mechanism of iron dysregulation in PSS.

OTHER MICROCYTIC OR HYPOCHROMIC ANEMIAS

Microcytic or hypochromic anemias have been associated with sideroblastic anemia and refractory anemia with ringed sideroblasts. Refractory anemia with ringed sideroblasts is a type of myelodysplastic syndrome (see Chapter 67).[12]

Sideroblastic Anemias

The hallmark of sideroblastic anemias is the presence of ≥5 large iron aggregates within RBCs (i.e., siderocytes) or erythroid precursors (sideroblasts), or the presence of iron distributed in a ring around the nucleus of erythroid precursor cells (i.e., ringed sideroblasts). Sideroblastic anemia in dogs is rare, and association with clinical disease has primarily been seen in dogs with inflammatory diseases.[51] The hemogram in these dogs is characterized by moderate to severe nonregenerative anemia that is frequently hypochromic and sometimes microcytic, in spite of elevated serum iron concentrations. There are also dysplastic features in the erythroid lineage of the bone marrow.

COPPER DEFICIENCY

Copper deficiency can develop in ruminants eating forage from copper-deficient soils. Secondary copper deficiency can occur when copper intake is adequate, but use is impaired by high dietary molybdenum or sulfate. Affected animals generally have a microcytic hypochromic anemia; however, normocytic anemias have been reported in experimental copper deficiency in dogs.[3] Microcytosis and hypochromasia are the result of functional iron deficiency. Functional iron deficiency results from a decrease in production of the copper-containing proteins ceruloplasmin, hephaestin, and cytochrome oxidase that are required for iron transport.[25] Hephaestin is involved in release of iron from intestinal enterocytes. Ceruloplasmin facilitates mobilization of iron from the liver and tissues other than enterocytes.[53] Pigs with acute experimental copper deficiency have low serum iron, but pigs with chronic experimental copper deficiency develop increased serum iron concentration and have many sideroblasts present.[25] This may be due to a deficiency of the copper-containing enzyme cytochrome oxidase in erythroid precursor cells. Cytochrome oxidase is involved in reducing ferric to ferrous iron in mitochondria for use in heme synthesis.

IRON OVERLOAD

Iron overload can result from acute iron toxicity or chronic accumulation that results in hemosiderosis or hemochromatosis. Iron overload can result in saturation of transferrin, leading to increased levels of non-transferrin-bound iron. Non-transferrin-bound iron can freely interact with cells, resulting in the production of hydroxyl radicals and subsequent organ damage.[35]

Acute Iron Toxicity

Acute iron toxicity results from administration of large doses of iron to animals (the dose depends on the species). Parenteral administration of large amounts of iron to ruminants results in respiratory distress, icterus, and central nervous system signs.[39] In dogs, oral intake of >20 mg/kg of iron can result in gastrointestinal distress, followed by cardiovascular shock, icterus, vomiting, bloody diarrhea, and hypovolemic shock.[1] Excess iron given orally can override the normal mechanisms that regulate intestinal absorption. Hepatotoxicity has been documented in neonatal foals after ingestion of a nutritional supplement containing ferrous fumarate iron at a concentration lower than that required to induce acute iron toxicity in adult horses.[30]

Hemosiderosis and Hemochromatosis

Hemochromatosis refers to accumulation of iron in tissue with associated organ injury and dysfunction, while hemosiderosis refers to accumulation of iron within tissues without evidence of organ injury or dysfunction. Hemosiderosis occurs locally within tissues at sites where hemorrhage has occurred and in a more disseminated way during conditions such as chronic hemolytic conditions.

Primary Hemochromatosis

Primary (hereditary) hemochromatosis has been described in related Salers cattle, captive black rhinoceros, and certain birds.[2,27,34] Affected Salers cattle had a history of stunted growth, poor hair coat, and weight loss, with iron accumulation in multiple tissues and increased serum iron and percent saturation of transferrin.

Secondary Hemochromatosis

Secondary hemochromatosis is most frequently associated with hemolytic anemias, repeated blood transfusions, parenteral iron administration, or dietary iron overload. Dietary iron overload does not appear to be a problem in animals except in a few wild animal species housed in captivity.[26] Hemolytic anemias are associated with increased iron absorption. When the hemolysis is chronic, increased intestinal absorption results in iron accumulation within hepatocytes as well as within macrophages. Liver fibrosis eventually develops. Secondary hemochromatosis associated with blood transfusions was reported in a dog given regular transfusions over a long period of time.[44]

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CHAPTER 28

The Porphyrias—Disorders of Defective Heme Synthesis

ANDREA A. BOHN

Normal Heme Synthesis
Abnormal Heme Synthesis
General Consequences of Defective Heme Synthesis
Testing
Enzymatic Steps of Heme Synthesis and Associated Porphyrias
  δ-Aminolevulinic Acid Synthase
  δ-Aminolevulinic Acid Dehydratase
  Hydroxymethylbilane Synthase
  Uroporphyrinogen III Synthase
  Uroporphyrinogen Decarboxylase
  Coproporphyrinogen Oxidase
  Protoporphyrinogen Oxidase
  Ferrochelatase
Animal Porphyrias
  ALAD-Deficient Porphyria
  Acute Intermittent Porphyria
  Congenital Erythropoietic Porphyria
  Porphyria Cutanea Tarda
  Erythropoietic Protoporphyria

Acronyms and Abbreviations

ADP, ALAD-deficient porphyria; AIP, acute intermittent porphyria; ALAS, δ-aminolevulinic acid (ALA) synthase; ALAD, ALA dehydratase; CEP, congenital erythropoietic porphyria; CPO, coproporphyrinogen oxidase; DSH, domestic short-haired; EPP, erythropoietic protoporphyria; HCP, hereditary coproporphyria; HEP, hepatoerythropoietic porphyria; HMBS, hydroxymethylbilane synthase; PBG, porphobilinogen; PCT, porphyria cutanea tarda; PPO, protoporphyrinogen oxidase; UROD, uroporphyrinogen decarboxylase; UROS, uroporphyrinogen synthase; UV, ultraviolet; VP, variegate porphyria.

Porphyrias are a complex group of rare but important diseases that arise due to defective heme production. Because defects can arise at any of the eight enzymatic steps responsible for the production of heme and there are nine different genes encoding those enzymes, porphyrias manifest in a variety of ways, most commonly recognized by veterinarians because of cutaneous photosensitization in cattle or because of pink, red, or brownish discoloration of teeth, bones, soft tissues, or urine in multiple species, including cattle. This chapter will provide a basic description of the disorders associated with defective heme production including an update of what has been reported in the veterinary literature.

NORMAL HEME SYNTHESIS

The synthetic pathway for heme originates in mitochondria, progresses into the cytosol, and then returns to mitochondria for the final stages (see Figure 20.2 in Chapter 20). There are eight different enzymatic steps involved in heme formation (Table 28.1). The first enzymatic reaction is the rate-limiting step. All other enzymes are normally more active than the first, with each step progressing to formation of the next heme precursor without significant production of side products. Precursors are listed in Table 28.1 and discussed in more detail in the following text.

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TABLE 28.1 Steps of heme biosynthesis, including precursors, enzymes, and the porphyria diseases corresponding to the associated enzymatic deficit.

Heme Precursors	Enzyme	Porphyria
Succinyl-CoA + Glycine		
↓	ALAS	
δ-aminolevulinic acid		
↓	ALAD*	ADP
Porphobilinogen		
↓	HMBS	AIP
Hydroxymethylbilane		
↓	UROS	CEP
Uroporphyrinogen III		
↓	UROD	PCT HEP
Coproporphyrinogen III		
↓	CPO	HCP
Protoporphyrinogen IX		
↓	PPO	VP
Protoporphyrin IX		
Fe^{2+} ↓	FECH*	EPP
Heme		

*Inhibited in lead toxicity

Enzymes: ALAS, δ-aminolevulinic acid (ALA) synthase; ALAD, ALA dehydratase; HMBS, hydroxymethylbilane synthase; UROS, uroporphyrinogen III synthase; UROD, uroporphyrinogen decarboxylase; CPO, coproporphyrinogen oxidase; PPO, protoporphyrinogen oxidase; FECH, ferrochelatase.

Porphyrias: ADP, ALAD-deficient porphyria; AIP, acute intermittent porphyria; CEP, congenital erythropoietic porphyria; PCT, porphyria cutanea tarda; HEP, hepatoerythropoietic porphyria; HCP, hereditary coproporphyria; VP, variegate porphyria; EPP, erythropoietic protoporphyria.

Heme is incorporated into various protein matrixes to create the hemoproteins, including hemoglobin, myoglobin, cytochromes, catalases, peroxidases, and endothelial nitric oxide synthase. Tissues that produce the most heme are bone marrow and liver. Approximately 80% of overall heme production occurs in bone marrow for hemoglobin (in nucleated erythrocytes and reticulocytes, since mitochondria are necessary for synthesis). Approximately 15% of overall heme production occurs in the liver, largely for incorporation into microsomal cytochrome P450 enzymes, but also catalase and other cytochromes.

ABNORMAL HEME SYNTHESIS

Heme synthesis enzyme defects are predominantly recognized in either erythropoietic or liver tissue. Hence, the different porphyria diseases are often classified as either erythrocytic or hepatic (or in combination). Porphyrias are often also categorized as either acute neurovisceral or cutaneous, depending on symptoms.

Porphyrins are a class of macrocyclic aromatic compounds composed of four pyrrole rings connected by methine bridges. All porphyrin compounds absorb light intensely at 400–410 nm and can cause cell damage and discoloration of tissues. During normal heme synthesis, porphyrins are not produced in significant amounts, except for the last precursor, protoporphyrin IX, into which iron is inserted for the final product of heme. The other heme precursors are not porphyrins and their chemical structures do not absorb light energy or fluoresce. However, if these precursors do not go on to make heme, they can build up to toxic concentrations themselves or undergo alternative reactions, which result in pathologic porphyrin products. The major porphyrins derived from aberrant heme synthesis are shown in Figure 28.1.

GENERAL CONSEQUENCES OF DEFECTIVE HEME SYNTHESIS

Porphyria is often suspected because of reddish-brown discoloration of tissues and/or cutaneous lesions of photosensitivity. When porphyrins are present early in life, they are particularly noticeable in the reddish-orange staining of the teeth and dentine. Dentine discoloration can also be evident in adults. Pink, reddish-orange, to brownish discoloration of urine is often readily visible and may be the presenting complaint. Urine that contains precursors may not be discolored initially, but

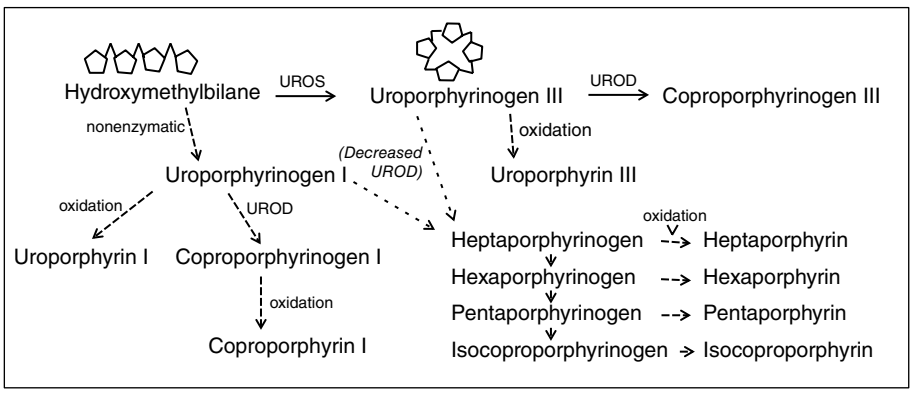


FIGURE 28.1 Pathways for aberrant porphyrin production when UROS or UROD activity is deficient (dashed and dotted lines) for heme synthesis.

become so when substances conjugate and oxidize over time. Urine reagent strip blood pads should remain negative, as their reaction requires heme iron. Animals may show signs of photosensitivity or be poor-doing.

Deficiencies in the activity of all but the first three heme synthesis enzymes are to some degree associated with cutaneous lesions (although not in all cases, depending on which enzyme and species affected). Cutaneous lesions are due to the photosensitive nature of porphyrins. Porphyrins absorb ultraviolet (UV) light energy, causing transition to an excited energy state. The excited porphyrin may directly react with cellular structures or transfer the energy to molecular oxygen to form reactive oxygen species which act as powerful oxidants for many biologically important compounds.[2]

Photohemolysis is not likely to play a significant role in the hemolytic anemia associated with some porphyrias. Although in vitro exposure to UV light resulted in lysis of erythrocytes from human erythropoietic porphyria cases, this affect was not thought to occur in vivo.[13,15] High concentrations of porphyrins are thought to alter the erythrocytes in some unknown way, which results in their early removal from circulation.[18] A direct effect is supported by finding decreased erythrocyte survival time in cows with erythropoietic porphyria, despite no exposure to sunlight.[18]

The underlying mechanism of the microcytosis described in many cases of porphyria has not been elucidated, but microcytosis is not necessarily surprising, since porphyrias are associated with decreased heme production and altered iron metabolism.

Acute neurovisceral signs (fatigue, abdominal pain, nausea, vomiting, constipation, neuropathies, or mental disturbances) have only been described for humans, although seizures have occasionally been reported in cattle.[6,25] The cause of these neurovisceral effects is not well understood, but it is thought that δ-aminolevulinic acid (ALA) and/or porphobilinogen (PBG) are neurotoxic.

With porphyrias, disease manifestations and severities and patterns of porphyrin and precursor accumulation can be widely variable, even within named disease entities. In affected people or animals, patterns of accumulation can fluctuate episodically and severity of clinical signs can vary with age, either improving or worsening over time. Potential contributors to the complexity of the porphyria disease processes, many of which are poorly understood, include: the high number of different genetic defects associated with all of the different heme synthesis enzymes; potential defects in transcription factors or other heme-synthesis-related substances or pathways; altered regulation of heme biosynthesis by accumulated substances; external influences; and species differences.

TESTING

Suspicion of the presence of porphyrins can be substantiated by their characteristic reddish fluorescence when exposed to UV light. When a Wood's lamp or other UV light that includes the Soret band (400–410 nm) is shone on teeth, urine, or blood-containing porphyrins, an intense pink or red fluorescence is seen. This is virtually pathognomonic of porphyria, but not necessarily the type.

It is beyond the scope of this chapter to delve into the different testing methods for porphyrias. Definitive diagnosis of specific porphyria disease entities requires measuring products of defective heme synthesis in urine, blood, or feces (excretory routes are dependent on water solubility), measuring the activity of enzymes important for heme synthesis, and/or enzyme genetic analysis for specific known mutations or characterization of new ones. Unfortunately, other than a few genetic tests (see Section "Animal Porphyrias"), no known veterinary diagnostic laboratory offers testing for porphyria (some screening test methodologies are described in Kaneko[19]). There are specific human diagnostic laboratories that perform porphyria testing, but they may not accept animal samples. A source for these laboratories is the American Porphyria Foundation's website: https://porphyriafoundation.org (For patients; About porphyria; Diagnosis or For healthcare professionals; Diagnosis and testing). If one finds a laboratory willing to assess veterinary samples, consider also submitting comparable samples from an unrelated, healthy animal of the same species. Once collected, samples need to be protected from light exposure. Measuring the different heme precursors and porphyrins to determine which predominate is useful in supporting a disease diagnosis, but may not be definitive.

ENZYMATIC STEPS OF HEME SYNTHESIS AND ASSOCIATED PORPHYRIAS

In order to provide a comprehensive overview of the porphyrias, this section will provide a brief description of each of the eight different enzymatic steps in heme synthesis (see Table 28.1) and their associated porphyrias as seen in humans. Porphyrias that have been described in animals will be more thoroughly discussed in the next section. In humans, hundreds of different genetic defects have been detected across the different enzymes.[5]

δ-Aminolevulinic Acid Synthase

δ-Aminolevulinic acid synthase (ALAS) is a mitochondrial enzyme and the rate-limiting enzyme in the synthesis of heme. ALAS catalyzes the condensation reaction of glycine and succinyl-CoA to form ALA. There are two different genes encoding two different ALAS enzymes: ALAS1 is expressed ubiquitously and ALAS2 is expressed only in erythroid precursors. Although no mutations in ALAS1 have been identified, ALAS1 plays a role in some other porphyria diseases. In liver, ALAS1 is inducible as a response to increased demand (e.g., induction of P450 enzyme synthesis) or to excess accumulation of heme biosynthetic intermediates.[5] Heme insufficiency also leads to increased ALAS1

activity because feedback inhibition is lacking. This overexpression of hepatic ALAS1 is associated with neurovisceral signs. Diseases due to abnormal ALAS2 activity have only been identified in people: X-linked sideroblastic anemia with decreased ALAS2 activity (microcytosis possible) and a gain of function mutation in ALAS2 leading to excess protoporphyrin production and subsequent photosensitivity and liver damage.[27]

δ-Aminolevulinic Acid Dehydratase

δ-Aminolevulinic acid dehydratase (ALAD) is a cytosolic enzyme that catalyzes the condensation reaction of two molecules of ALA to form PBG, which contains a pyrrole ring structure. There is only one ALAD gene, but two different promoters: one "housekeeping" and the other erythroid-specific. ALAD-deficient porphyria (ADP) is extremely rare in humans and only one suspect case has been reported in animals. In ADP, ALAD activity is markedly deficient, resulting in high ALA and coproporphyrin III excretion in urine and elevated erythrocyte protoporphyrin. Clinical manifestations are similar to other acute neurovisceral porphyrias, with potentially severe neurological symptoms.[4,5,27]

Since lead inhibits ALAD, lead toxicity can result in increased urinary excretion of ALA and clinical manifestations similar to ADP.[5]

Hydroxymethylbilane Synthase

Hydroxymethylbilane synthase (HMBS) is also known as porphobilinogen deaminase (PBGD) and was previously called uroporphyrinogen I synthase. It is a cytosolic enzyme that, like ALAD, has only one gene, but different housekeeping and erythroid-specific promoters. HMBS combines four molecules of porphobilinogen into a linear tetrapyrrole called hydroxymethylbilane.

Human acute intermittent porphyria (AIP) is typically associated with approximately 50% decreased HMBS activity; it is autosomal dominant and the most common acute porphyria in humans. Most people with the defect do not exhibit clinical signs. However, if liver ALAS1 activity is induced by concurrent conditions or inducing substances, nonspecific neurovisceral manifestations can result. During an attack, urine may become dark red but erythrodontia (reddish discolored teeth) is only seen in extremely rare homozygous cases.[10] The most significant finding for tentative diagnosis is an elevated urinary PBG concentration. AIP has also been reported in cats.[10,11]

Enzymatic activity of HMBS can also be inhibited by high concentrations of protoporphyrinogen and coproporphyrinogen, which likely play a role in other hepatic porphyria diseases.[4,5]

Uroporphyrinogen III Synthase

Uroporphyrinogen synthase (UROS) was previously called uroporphyrinogen III cosynthase. It is a cytosolic enzyme that also has different housekeeping and erythroid-specific promoters. UROS facilitates the transformation of the linear tetrapyrrole, hydroxymethylbilane, into a tetrapyrrole ring structure called uroporphyrinogen III, the only isotype able to progress in the heme biosynthetic pathway.

Congenital erythropoietic porphyria (CEP) is an autosomal recessive disorder caused by markedly decreased UROS activity, which results in accumulation of nonenzymatically formed, nonphysiologic and pathogenic porphyrin isomers (see Figure 28.1). It is rare in humans, but one of the more commonly described porphyrias across different animal species.

In humans, CEP manifests as chronic, severe blistering cutaneous photosensitivity, mild to marked hemolytic anemia, and possible discoloration of teeth and urine.

Uroporphyrinogen Decarboxylase

Uroporphyrinogen decarboxylase (UROD) is a cytosolic enzyme that facilitates decarboxylation of uroporphyrinogen (containing eight carboxyl groups), to coproporphyrinogen (containing four carboxyl groups). A deficiency in UROD activity causes the most common form of porphyria in humans, porphyria cutanea tarda (PCT), as well as the less common but more severe hepatoerythropoietic porphyria (HEP), which has complex patterns of porphyrin accumulation. There are a few reports of suspected UROD deficiency in sheep and pigs; however, definitive diagnoses have not been made.[26,30] PCT may or may not have an inherited mutation in the UROD gene and relies on acquisition of other susceptibility factors to cause liver UROD inhibition. HEP is due to homozygous or compound mutations of the UROD gene, which markedly decrease both liver and erythrocyte UROD activity. PCT typically exhibits milder cutaneous photosensitivity only in adults, while HEP is more similar to CEP with severe photosensitivity and possible anemia and discoloration of teeth and urine, often manifesting in early childhood. Decreased UROD activity results in high levels of urine uroporphyrin I and heptacarboxyl porphyrin III with isotype III porphyrins predominating overall (see Figure 28.1). High fecal and urine isocoproporphyrin concentration is unique to PCT and HEP.[5]

Coproporphyrinogen Oxidase

Coproporphyrinogen oxidase (CPO) is a mitochondrial enzyme, which catalyzes a side chain modification to convert coproporphyrinogen III to protoporphyrinogen IX.[4] Hereditary coproporphyria (HCP) is an autosomal dominant hepatic porphyria associated with half-decreased CPO activity. HCP manifests very similar to AIP except that cutaneous lesions similar to PCT can occasionally occur.[5] In this disease, large amounts of coproporphyrin, mostly type III, are excreted in urine and feces. During acute attacks, urinary ALA, PBG, and uroporphyrin are also increased. There are no documented cases of HCP in animals.

Protoporphyrinogen Oxidase

Protoporphyrinogen oxidase (PPO) is a mitochondrial enzyme that oxidizes protoporphyrinogen IX to

protoporphyrin IX. Mutations in PPO are causative for variegate porphyria (VP), an autosomal dominant disorder that manifests in either acute neurovisceral attacks similar to AIP, cutaneous photosensitivity similar to PCT, or both.[4] It has not been reported to occur in animals. Excretory products are similar to CPO with the addition of high fecal protoporphyrin concentrations. Plasma porphyrin analysis is effective in diagnosing VP.[5]

Ferrochelatase

Mitochondrial ferrochelatase (FECH) facilitates the insertion of iron into the protoporphyrin heme backbone. Erythropoietic protoporphyria (EPP) is caused by mutations of the ferrochelatase gene and subsequent decreased enzymatic activity. In humans, heredity is complex, as an autosomal dominant disorder that requires the presence of a second molecular defect.[5,27] The disorder is autosomal recessive in cattle.[5,16] Protoporphyrin is the only heme pathway intermediate that significantly accumulates in EPP, predominantly accumulating in bone marrow reticulocytes, with excess amounts detectable in circulating erythrocytes, plasma, bile, and feces.[4,5] Diagnosis is often based on the high accumulation of protoporphyrin in erythrocytes.[27] The protoporphyrin is free, in contrast to the zinc-complexed protoporphyrin associated with other porphyrias, although assays typically do not make a distinction.[5] Humans with EPP have an acute, painful, erythemic, nonblistering photosensitivity that can be lifelong. Mild microcytic nonregenerative anemia is frequently reported.[5,27]

ANIMAL PORPHYRIAS

Reports of naturally occurring porphyria in animals implicate five of the enzymes involved in heme biosynthesis, two of these are only suspected and not proven (Table 28.2). Definitively diagnosing specific porphyrias can be difficult, given the scarcity and/or difficulty of enzymatic and genetic testing combined with the low incidence of these diseases. Measuring intermediates of heme formation and porphyrins may provide supporting evidence for the different porphyrias, but can be challenging to perform and results can be confusing. Enzyme activity and/or genetic analysis are usually necessary for definitive diagnosis. Porphyria is typically suspected in animals because of cutaneous photosensitivity and/or discoloration with subsequent fluorescence of tissues or urine. Acute hepatic porphyrias without those features would be very difficult to detect in animals, given the nonspecific neurovisceral signs associated with them. Being mindful of often variable and incomplete testing between investigations, this section is a review of porphyrias as reported in animals.

TABLE 28.2 Porphyrias reported in animals.

Disorder*	Enzyme defect*	Major accumulated substances	Reported animals
ADP	ALAD	ALA in urine	Suspect cat
AIP	HMBS	ALA, PBG, URO III in urine; URO I and III in teeth	Cats
CEP	UROS	URO I, COPRO I in urine and RBC; COPRO I in feces	Cattle, swine, cats, hedgehog, rabbit, fox squirrels
PCT	UROD	URO, COPRO, heptaporphyrins in urine	Sheep, possibly swine
EPP	FECH	Protoporphyrin IX in feces and RBC	Cattle, dogs, possibly chickens

* See acronyms, Table 28.1.
ALA, δ-aminolevulinic acid; PBG, porphobilinogen; URO, uroporphyrin; COPRO, coproporphyrin; PROTO, protoporphyrin.

ALAD-Deficient Porphyria

A cat that presented with frequent seizures and vague gastrointestinal signs was suspected to have ADP after lead toxicity was ruled out.[36] Suspicion was based on consistently elevated ALA concentrations in urine. Urine porphyrin concentration was not elevated as is typically seen in humans with ADP; no enzymatic or genetic analysis was performed. Findings in this cat were also consistent with a different rare human disorder associated with decreased ALAD activity, hereditary tyrosinemia.[5,12] A diagnosis of ALD in this cat cannot be considered definitive.

Acute Intermittent Porphyria

AIP has been recently diagnosed in four lines of cats as well as two additional cats.[10,11] Unlike in humans with AIP, which present with neurovisceral symptoms, these cats were identified because of erythrodontia and brownish urine, similar to CEP. Teeth and bone fluoresced under UV light. Urinary porphyrins were elevated as with CEP; however, patterns of individual heme precursors and porphyrins were more consistent with AIP. Type I porphyrin isomers predominate in CEP, whereas in these cats (as in humans), high concentrations of urinary uroporphyrin and coproporphyrin types I and III as well as ALA and PBG were detected. In contrast to humans where PBG concentration elevates mostly during acute attacks, levels were constitutively elevated in cats.[10] Porphyrin concentrations were also constitutively elevated in liver and spleen tissue, bone, teeth, and erythrocytes. Enzyme assays detected decreased activity of HMBS but not of UROS; HMBS activity was decreased by half in liver and by half or more in erythrocytes. Genetic analysis revealed unique mutations of the HMBS gene in each of the cat lineages and each additional cat, with no relevant mutations of the UROS gene. Similar to humans, disease inheritance

was autosomal dominant except for in one cat lineage where it was recessive. Mild regenerative anemia and microcytosis were observed in several of the cats. Neurovisceral manifestations were not appreciated. These cats easily could have been diagnosed with CEP without enzymatic and genetic testing; previous cases of porphyria in animals may have been misclassified. DNA mutation testing for AIP is currently available for cats.[a]

Congenital Erythropoietic Porphyria

CEP has been proven in a domestic short-haired (DSH) cat and has been suspected in other cats, typically presenting for erythrodontia. In the proven case, similar to humans, there were marked increases in uroporphyrin I and coproporphyrin I in urine, plasma, and erythrocytes and their precursors; urinary ALA and PBG were not increased.[9] UROS activity in erythrocytes was <1%. Mutations of the UROS gene were characterized and shown to decrease the enzyme's activity and stability. This cat was not anemic, but increased polychromasia and basophilic stippling were observed. A family of Siamese cats was also thought to have CEP.[14] These cats had discolored and fluorescent urine and tissues, as well as increased concentrations of uroporphyrin, coproporphyrin, and protoporphyrin in all tissues evaluated. Marked elevations in concentrations of urinary uroporphyrin I and fecal coproporphyrin I were detected. This marked predominance of the type I isomers is the strongest evidence for CEP. These cats may have had mild photosensitivity, as evidenced by seasonally periodic episodes of nasal and ocular discharge. They were generally listless, likely correlating with severity of anemia. These cats also had renal disease, but it was uncertain if or how that was related to the porphyria. In a few other case reports in which CEP was only speculative, cats had discolored bones and teeth that fluoresced, but no biochemical or genetic testing was performed.[1,33] CEP in cats differs from humans and cattle in that cutaneous lesions are not seen. Consistent with other species, anemia appears variable. DNA mutation testing for CEP is available for cats.[a]

In cattle, CEP was first reported in South African Shorthorns, but more commonly affects Holstein-Friesians.[3,17,34] Occasional listings of other affected breeds, including Jamaica Red and Black, Ayrshire, and Hereford cattle, refer to isolated, decades-old reports.[17,34] A more recent case report of CEP in a Longhorn calf in the United Kingdom theorizes Shorthorn ancestry.[17] Another recent suspected case was reported in a Gir calf.[28]

Cattle with CEP are often detected due to purplish pink to brownish red discoloration of teeth, brownish-colored bones, and amber to red–brown-colored urine. Pink to red florescence confirms porphyrins as the cause of the discoloration. Cattle with CEP are also often detected due to lesions of the nonpigmented areas of the skin. Severity of cutaneous lesions varies between individuals, from nonexistent to exhibiting erythema, denudation, vesicle formation, or necrosis.[7,17,20,29] Severity is related to the amount of porphyrin accumulation, coat color and density, and the amount of sun exposure.[7,20] The hemolytic anemia associated with CEP varies in severity between individual cattle and can be present at birth. CEP can cause retarded growth and lack of condition. Clinical signs are mitigated by keeping affected cattle out of sunlight.

Biochemically, high concentrations of uroporphyrin I and coproporphyrin I are present in urine and erythrocytes, predominantly metarubricytes. A high amount of coproporphyrin I is also excreted in feces. Porphyrin concentrations tend not to be significantly elevated in plasma, but elevated erythrocyte protoporphyrin concentrations have been reported in cattle.[17,20]

Activity of UROS is not typically measured, but has been shown to be decreased in cattle with CEP.[23,31] A decrease in activity of approximately 20% was reported, although activity was figured on a per-gram of hemoglobin basis using hemolysates. Since it was not taken into account how many reticulocytes with higher activity were present versus mature red cells and given that CEP is a recessive disorder, enzyme activity is likely lower than what was reported.[23,31]

Definitive causative mutations of the UROS gene have not been identified in cattle; however, genetic analysis of a family of Danish Holstein cattle was supportive of a mutation affecting UROS. These cattle had photodermatitis, discoloration of tissues, and a high concentration of bone uroporphyrin consistent with decreased UROS activity.[3]

Data regarding porphyrias in pigs is incomplete. Pigs with discoloration of bones and soft tissues have been sporadically observed in slaughterhouses. Cases can often be traced to a particular sow or boar and typically cease through breeding and removing newborns with discolored deciduous teeth.[34] Both autosomal dominant and recessive inheritance have been suggested in different reports. It is difficult to determine how many of the early swine cases suspected to have CEP were accurately diagnosed, since diagnosis was predominantly based on physical characteristics. At least in one early study, urine porphyrins were measured and found to be elevated, with the majority being the type-I isomer, which is supportive of CEP in that study.[29] Pigs have not exhibited photosensitization, but may exhibit poor growth and variable anemia. Disease severity can fluctuate with age. The most recent report of pig porphyria appears to be from 1995, within which the authors suspected a mixed porphyria of UROS and UROD activity deficiencies (see in the following text).[30]

Porphyria was diagnosed in a 6-month-old hedgehog that presented for pink urine.[37] The urine, teeth, feet, and spines fluoresced under UV light. There were marked increases of uroporphyrin (90–95% type I isomers) and coproporphyrin in the urine. Urine and feces from this hedgehog and its nonclinical mother also showed a pattern of porphyrin intermediates with a high proportion of highly carboxylated porphyrins in comparison to two normal hedgehogs that had only fully decarboxylated intermediates.

A New Zealand white rabbit was noted to have pink-tinged teeth on necropsy (the cause of death was determined to be septicemia from pasteurellosis) and its teeth

[a]http://research.vet.upenn.edu/penngen

and bone fluoresced pink.[32] Collected urine contained elevated amounts of uroporphyrin and coproporphyrin compared to a control rabbit's urine, while ALA and PBG concentrations were similar between the two rabbits. Porphyrin levels, especially hexaporphyrin, were also increased in liver tissue.

Markedly low UROS activity appears to be ubiquitous in fox squirrels, which do not exhibit clinical disease.[24] In comparison to gray squirrels, fox squirrels all have less UROS activity and have browner bones and teeth, which fluoresce pink. Erythropoiesis is increased, but the squirrels are not anemic.

Porphyria Cutanea Tarda

A flock of German blackface sheep contained a few poor-doing individuals with anemia and light brown discolored urine.[26] Consistent with PCT, these individuals were heterozygous for a UROD point mutation and had high levels of porphyrins in urine, predominantly uroporphyrins, coproporphyrins, and heptaporphyrins. No mutations were detected in the UROS gene. Activity of UROD and UROS were not measured, which could have definitively confirmed if the porphyria was due to decreased UROD activity.

UROD deficiency might have been present in a Belgian Landrace pig condemned at slaughter due to mahogany-colored tissues. Tissues from this pig contained increased concentrations of multiple decarboxylation products of uroporphyrinogen, typical of UROD deficiency, although a preponderance of isomers I in comparison to isomers III suggests there may have also been decreased UROS activity.[30]

Erythropoietic Protoporphyria

EPP is most common in Limousin cattle, but also described in the Blonde d'Aquitaine breed.[25] Cattle exhibit photosensitization, and seizures have been reported.[6,16,25] Cutaneous lesions most commonly occur on the head (especially ears, muzzle, periocular) and dorsum. Crusting, exudation, ulceration, or alopecia may be seen. The animal may be painful, pruritic, or photophobic. Animals can do better if kept out of sunlight and photosensitivity can decrease as the animal ages. Cattle are not anemic, and discoloration of teeth and urine do not occur.[20]

EPP is characterized by markedly high concentrations of protoporphyrin in erythrocytes and feces. Whole blood fluoresces because of high protoporphyrin levels in reticulocytes and plasma. Nonsymptomatic heterozygotes can be detected because of increased total porphyrins in plasma and erythrocytes. The aberrant ferrochelatase protein resulting in decreased activity is an autosomal recessive trait.[8] Genetic testing can be requested through the North American and Canadian Limousin Foundations and Australian Limousin Breeders' Society.[b,c,d]

[b]https://nalf.org/management/dna-genomics/
[c]http://www.limousin.com/about-us/dna-testing/
[d]https://www.limousin.com.au/member-services/dna-testing

Chickens with elevated protoporphyrin levels consistent with EPP were detected at a poultry-meat-processing plant in Japan.[35] Several broiler chickens with dark green to black livers were collected for analysis. Interestingly, all chickens collected were female; they had mild growth retardation with mildly enlarged livers. Protoporphyrin IX concentration was markedly elevated in liver, bone marrow, and feces when compared to unaffected chickens. Ferrochelatase activity was not measured and genetic analysis was not performed. Protoporphyrin pigments were detected in lung as well as liver on histologic examination. The chickens came from several different farms, but all were progeny from a common breeder farm.

No inherited form of protoporphyria has ever been described in dogs; however, a spontaneous, transient form of the disease was described in a breeding colony of German Shepherd dogs.[21] These dogs had hepatobiliary lesions with no evidence of cutaneous photosensitivity. High concentrations of protoporphyrin IX and low ferrochelatase activity were measured in the affected dogs' livers. All dogs that survived made a full clinical recovery; a cause was not determined. A case of congenital EPP with associated progressive pigmentary hepatopathy, microcytosis, and chronic dermatopathy was recently reported in a young dog.[22] This dog had markedly high protoporphyrin concentrations in plasma, erythrocytes, feces, and liver, with a predominance of metal-free protoporphyrin.

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CHAPTER 29

Hereditary Erythroenzymopathies

URS GIGER

Phosphofructokinase Deficiency
Canine Phosphofructokinase Deficiency
Pyruvate Kinase Deficiency
Canine Pyruvate Kinase Deficiency
Feline Pyruvate Kinase Deficiency

Acronyms and Abbreviations

AMP, adenosine monophosphate; BFU-E, burst-forming unit-erythroid; CFU-E, colony-forming unit-erythroid; DNA, deoxyribonucleic acid; DPG, diphosphoglycerate; EDTA, ethylenediaminetetraacetic acid; G6PD, glucose-6-phosphate dehydrogenase; L-M-P-PFK, liver, muscle, platelet phosphofructokinase; L-M1/M2-R-PK, liver, muscle, red blood cell pyruvate kinase; PFK, phosphofructokinase; PK, pyruvate kinase.

As the committed erythroid precursor cells proliferate and develop into mature RBCs in the bone marrow, their cellular metabolism undergoes many changes. And while the burst-forming unit-erythroid (BFU-E) and colony-forming unit-erythroid (CFU-E) cells are geared to massively expand the erythroid cell line, which is under the control of erythropoietin, major metabolic and structural shifts occur, particularly during the later erythroid stages, to achieve the highly specialized functions of mature RBCs (see Chapters 19, 20, and 21).[42] To that end, during rubricyte and metarubricyte (also known as normoblasts) stages until the reticulocyte stage, heme and alpha- and beta-globin syntheses are in full force to produce hemoglobin, reaching one-third of a mature RBCs mass.

Heme synthesis occurs in both mitochondria and cytoplasm involving several enzymatic steps (see Chapter 20). Hereditary enzymatic defects in heme synthesis, also known as porphyrias, cause tissue (bone and teeth) accumulation and urinary as well as fecal excretion of a variety of heme precursors and porphyrins, and can lead to hematological, neurological, gastrointestinal, and cutaneous signs. Porphyrias and their classification and characterization to the molecular level in cats, pigs, sheep, and cattle are described in Chapter 28.

While sickle cell disease and thalassemias are the most common RBC disorders in humans, interestingly, no hemoglobinopathies causing anemia have thus far been documented in domestic animals.

Hereditary methemoglobinemias, either due to a cytochrome b5 (methemoglobin) reductase deficiency or a yet unknown mechanism, have been observed in dogs, cats, and even rhesus monkeys, and are typically associated with cyanosis and erythrocytosis rather than anemia (see Chapter 26).[23,34,36,41]

Glucose-6-phosphate dehydrogenase (G6PD) of the hexose-monophosphate shunt is the most common hereditary (and only X-chromosomal) RBC enzyme deficiency in humans (Figure 29.1); these patients are generally not anemic, but can develop hemolytic crises with oxidative stress (drugs, infections, fava beans [favism]).[2] While in a large canine survey only a single Weimaraner dog was found to have a reduced G6PD activity without any hematological abnormalities,[78] an American Saddlebred colt with G6PD had anemia with eccentrocytosis.[41,66] Furthermore, horses with RBC flavin adenine dinucleotide deficiency have both eccentrocytosis (attributable to severe deficiency in glutathione reductase activity) and methemoglobinemia (attributable to cytochrome b5 reductase deficiency); the dual enzyme deficiency occurs because flavin adenine dinucleotide is a required cofactor for both enzymes.[31]

Although various erythrocytic membrane defects have also been described affecting cytoskeletal proteins,

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such as spectrin and band 4.1, others remain undefined and may involve enzymatic pumps to maintain electrolyte and calcium homeostasis as suspected for stomatocytosis in the Alaskan Malamute and Miniature and Middle Schnauzers (see Chapter 30).[84]

Devoid of a nucleus and mitochondria, the metabolism of the mature mammalian RBCs is fairly restricted, and its energy is solely generated by anaerobic glycolysis, also known as the Embden–Meyerhof pathway (Figure 29.1, see Chapter 21).[42] Metabolism of one molecule of glucose to two molecules of lactate leads to the net production of two molecules of ATP. The rate of glycolysis depends on the need for ATP of RBCs to maintain the shape, deformability, membrane transport, and

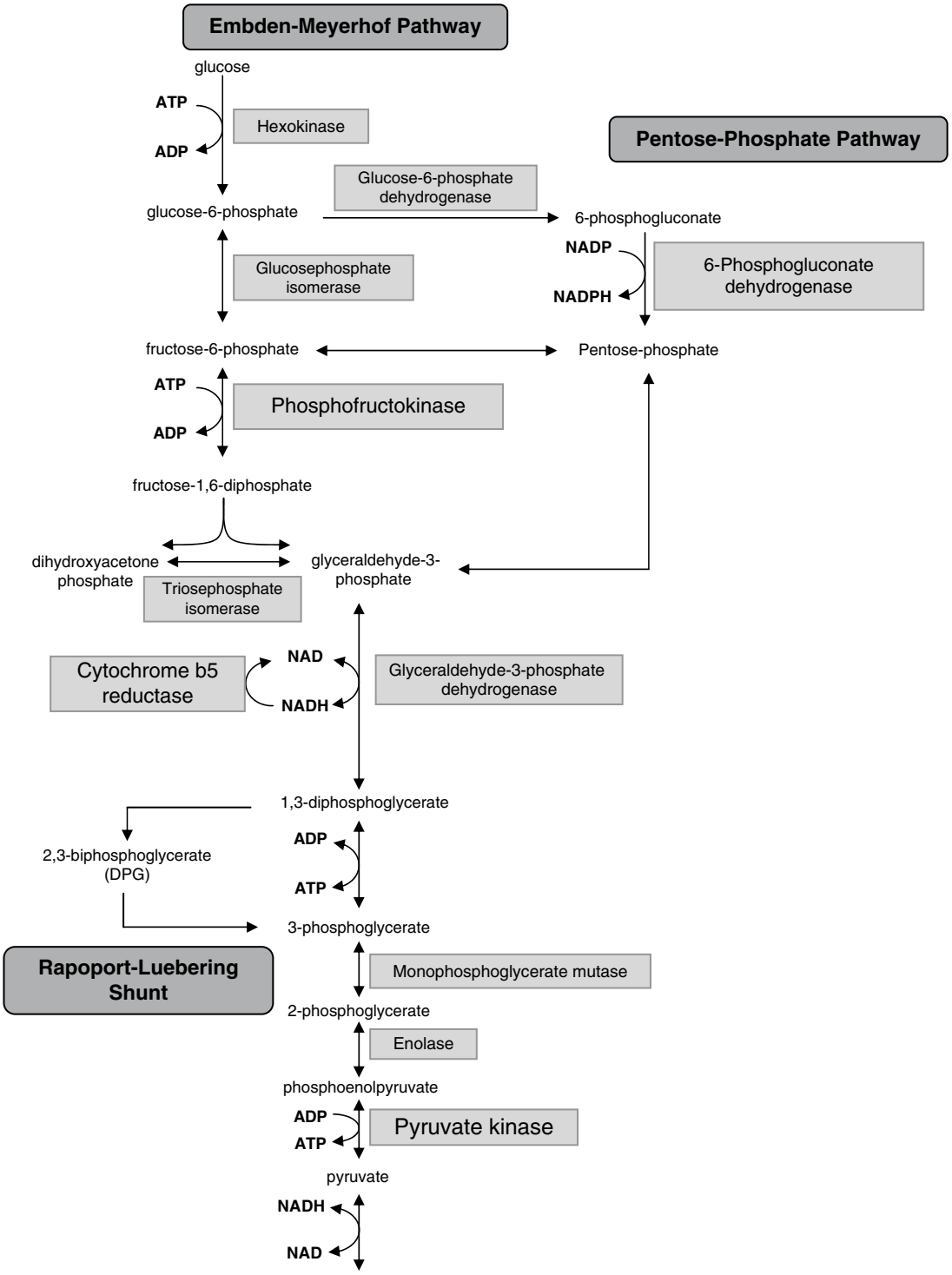


FIGURE 29.1 Embden–Meyerhof (anaerobic glycolytic) pathway with hexose-monophosphate and Rapoport–Luebering shunts in RBCs (simplified). ATP, adenosine triphosphate; ADP, adenosine diphosphate.

metabolic functions, such as phosphorylation and synthesis of purines, pyrimidines, and glutathione. Over a dozen enzymes are involved in glycolysis, which are associated with the two ancillary pathways, unique to RBCs, the pentose-phosphate (hexose-monophosphate) pathway and Rapoport–Luebering shunts. Some of these enzymes exist in different isoforms allowing for cell- and tissue-specific expression and regulation. The key regulatory enzyme of anaerobic glycolysis is phosphofructokinase (PFK). Whereas ATP is the most important inhibitor of PFK activity, inorganic phosphate, AMP, and ADP action is stimulatory. In addition, glucose-1,6-biphosphate and fructose-2,6-biphosphate are activators. Under maximally activating conditions for the enzyme PFK, an enzyme distal in glycolysis, pyruvate kinase (PK), becomes rate-limiting (Figure 29.1).

It is, therefore, not surprising that a deficiency of either PFK or PK activity will lead to RBC malfunction and premature RBC destruction, thereby causing hemolytic anemia. Although both erythroenzymopathies impair the same metabolic pathway, their metabolic and hematological abnormalities and clinical presentations are distinctly different and can vary between species. Subsequently, both erythroenzymopathies will be reviewed separately and contrasting features between the two disorders as well as affected species will be highlighted.

PHOSPHOFRUCTOKINASE DEFICIENCY

Phosphofructokinase (EC 2.7.1.11) catalyzes the regulatory phosphorylation step of fructose-6-phosphate to fructose-1,6-biphosphate. There are three isoforms of PFK, referred to as muscle (M-PFK), liver (L-PFK), and platelet (P-PFK), that are encoded by three different genes.[86] The active PFK enzyme is a homotetramer or heterotetramer composed of one or more isoforms; each of these combinations provides unique enzyme kinetic properties. Their expression is cell- and tissue-specific and developmentally regulated. Skeletal muscle contains exclusively M-PFK homotetramers.[60] Human RBCs express equal amounts of M- and L-PFK, whereas in canine RBCs, the M-PFK isoform predominates in a ratio of 86:14 over P-PFK.[86] Furthermore, during myogenesis[8] and erythropoiesis as well as postnatal development,[32] the isoform composition in these cells changes from L-PFK to M- and P-PFK. Deficiency of M-PFK has been associated with hemolysis and myopathy in humans and dogs, whereas in horses and Holstein calves, PFK deficiency apparently did not cause any hematologic abnormalities[83] and will, therefore, not be discussed here.

Muscle-type PFK deficiency, also known as Tarui–Layzer syndrome, is a rare genetic disorder in humans characterized by metabolic myopathy and a well-compensated hemolytic disorder.[26,69] PFK-deficient humans exhibit exercise intolerance, muscle weakness, and muscle cramping on exertion. Because of the residual half-normal PFK activity in affected RBCs, contributed by L-PFK isozymes and lack of alkaline fragility of human RBCs, overt clinical signs of hemolysis occur rarely. In fact, human patients can overcompensate their hemolytic component, and develop a mild erythrocytosis, because PFK-deficient erythrocytes do not readily release oxygen from hemoglobin. The ensuing tissue hypoxia accelerates erythropoiesis. Several mutations in the M-PFK gene have been described in human patients.[61]

Canine Phosphofructokinase Deficiency

Canine M-PFK deficiency was first described as an autosomal recessive trait in English Springer Spaniels in 1985 and was the first defect characterized at the molecular level for a common inborn error in dogs.[10,11,13,14,24,75] PFK-deficient spaniels have a nonsense mutation in the last exon of the M-PFK gene.[76,77] The resulting change from a tryptophan codon to a stop codon causes truncation of the M-PFK protein by 40 amino acids, leading to rapid degradation and complete deficiency of the M-PFK enzyme.[59,77] Cocker Spaniels,[20] Whippets,[9] a Mexican Street Dog and mixed breed dogs with PFK deficiency have the same mutation, while the Wachtelhund[45] has a different M-PFK mutation. Affected dogs completely lack PFK activity in muscle and have 8–22% of control PFK activity in RBCs due to residual P- and L-PFK expressions (other tissues appear less affected, as they express higher levels of other PFK isoforms).[16,32,35,38,86] The metabolic block at the PFK step results in deficiency of ATP and diphosphoglycerate (DPG) in RBCs, reduced blood lactate production, and accumulation of sugar phosphates and glycogen in muscle; hence, also the term glycogenosis or glycogen storage disease type VII.[11,12,16] This metabolic pattern is also known as a metabolic crossover, indicating the specific metabolic block in a pathway.

In erythroenzymopathies, the mechanism of accelerated lysis is generally not known, but it is assumed to be caused by energy depletion. In dogs, the compensated hemolytic disorder of PFK deficiency is accentuated by life-threatening hemolytic crises. A unique mechanism has been documented to be responsible for intravascular lysis, namely, an increased alkaline-induced hemolysis.[10] Although incompletely understood and possibly related to an aberrant RBC calcium homeostasis, canine RBCs are more alkaline fragile than cells from other species.[43,87] The RBC calcium homeostasis is disturbed in PFK deficiency, which results in an energy imbalance that accelerates adenine nucleotide pool depletion. Ca^{2+}-calmodulin activation of RBC AMP deaminase contributes to this metabolic dysregulation and limits ATP, thereby likely contributing to increased RBC lysis.[70] In vivo, the intracellular pH of RBCs is determined by organic phosphate and chloride anions. Because PFK-deficient RBCs have markedly decreased DPG concentrations, chloride ions move in and increase RBC pH.[10,12,31] Thus, PFK-deficient RBCs start lysing at about pH 7.4 (Figure 29.2a). Consequently, even minor systemic alkalemia or hyperthermia can induce intravascular hemolysis. Alkalemia is associated with any form of hyperventilation.[10,14]

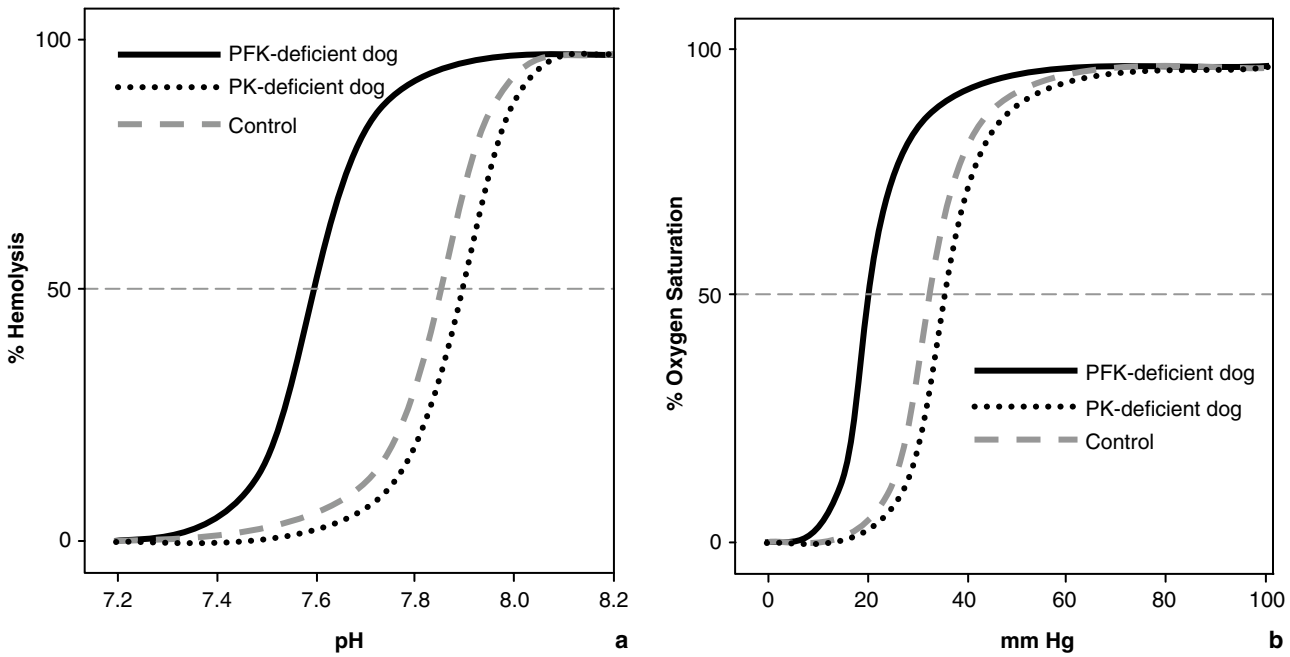


FIGURE 29.2 (a) Alkaline fragility of RBCs from a phosphofructokinase (PFK)-deficient English Springer Spaniel and pyruvate kinase (PK)-deficient Beagle compared to a healthy control dog. RBCs were incubated at various pH values for 1 hour at 37°C 24 hours after collecting the blood samples. Note that RBCs from PFK-deficient dog are very alkaline fragile, while those from PK-deficient dogs behave like controls; (b) Hemoglobin–oxygen dissociation curve of RBCs from PFK-deficient English Springer Spaniel and PK-deficient Beagle. Note the severe left shift of the curve of the PFK-deficient cells (higher hemoglobin–oxygen affinity, P_{50} = 17 mmHg), while PK-deficient canine RBCs are slightly right-shifted (lower hemoglobin–oxygen affinity, P_{50} = 32 mmHg) compared to canine control blood (28 mmHg).

The low DPG content of PFK-deficient RBCs markedly increases oxygen affinity and this is reflected in a left shift of the hemoglobin–oxygen dissociation curve.[10,11] The relative tissue hypoxia impairs, for instance, muscle and other tissue metabolism and stimulates renal erythropoietin synthesis and erythropoiesis. Therefore, aside from the occasional hemolytic crises associated with anemia, the chronic hemolytic disorder can be fully compensated with a robust regenerative response even at normal hematocrit. However, erythrocytosis as seen in PFK-deficient humans does not occur in affected dogs.

Sporadic intravascular hemolytic crises associated with pigmenturia, anemia, or jaundice are the hematological hallmark findings of PFK deficiency in dogs.[10,14,37] These episodes are induced by hyperventilation-associated events such as excessive panting and barking, strenuous exercise, and high environmental temperatures. Thus, guests or a new pet at the patient's home, a visit to the veterinary clinic, boarding, and training for field trialing can precipitate hemolysis. These crises can be first observed at a few months of age, although some animals may not experience any problems until several years of age, or they may be completely missed until the owner looks for them. During these episodes, which generally last one to several days, affected animals can have pale to severely icteric mucous membranes, are lethargic, inappetent, and often febrile, and splenomegaly may be noted. Thus, PFK deficiency can readily be confused with immune-mediated and other acquired hemolytic anemias. Pigmenturia is characterized by hemoglobinuria during crises, whereas hyperbilirubinuria is persistently strong. The anemia may become life-threatening with hematocrits as low as 5%, but is always macrocytic, hypochromic, and strongly regenerative with corrected and absolute reticulocyte counts of 4–25% and 200,000–1,200,000/μL, respectively. The apparent half-life of chromium-labeled PFK-deficient RBCs is 16 days compared to 20–28 days for normal canine cells. Besides polychromasia, marked anisocytosis and normoblastosis (but no poikilocytosis) are observed on microscopic examination of a blood smear. Yet unexplained, leukocytosis and hyperglobulinemia also may be present. The plasma may be discolored because of hemoglobinemia and hyperbilirubinemia. Transient hyperkalemia is due to lysis of high potassium containing reticulocytes and young RBCs.[10,14]

The debilitating exertional metabolic myopathy typically seen in human PFK-deficient patients is less commonly observed and frequently milder in affected dogs. PFK-deficient dogs may develop muscle signs on exertion, even only for a short distance.[3,15,16,57,58] In fact, muscle cramping in one of the limbs even after mild exercise is the most dramatic presentation.[15] Affected dogs may suddenly refuse to run and have high serum creatine kinase activity. Despite a complete lack of PFK activity in muscle, clinical signs of myopathy in deficient dogs are generally mild and rare, presumably due to the high oxidative rate of canine skeletal muscle.[79] Nevertheless, major impairments in oxidative and anaerobic muscle

metabolism have been demonstrated experimentally.[3,57,58] Aside from the acute signs of cramping, affected dogs may only show clinically mild exercise intolerance and intermittent muscle wasting. The serum creatine kinase activity is normal to slightly increased, except during crises. PFK-deficient skeletal muscle accumulates slightly more glycogen and occasionally amylopectin, an abnormal glycogen.[15,33] Interestingly, a couple of Whippets with PFK deficiency also showed clinical signs of a cardiomyopathy probably resulting from the M-PFK isozyme deficiency in heart muscle.[9]

PFK deficiency is an autosomal recessive trait[11] and occurs commonly in the English Springer Spaniel breed.[21,49] Since the first description in 1985,[10] hundreds of cases have been documented, but the gene frequency remains unknown. A randomized survey of American Kennel Club (AKC)-registered championed and bred English Springer Spaniels in 1998 revealed that 4% of field trial and 2.7% of show (bench) English Springer Spaniels were still carriers. Based on extensive pedigree analyses, a common ancestor goes at least back to the 1960s, if not earlier. PFK deficiency also has been reported in American Cocker Spaniels,[20] a Mexican Street Dog and several mixed breed dogs and Whippets.[9] As they all had the same disease-causing DNA mutation, these animals must have a common ancestor. By contrast, the rare PFK-deficient Wachtelhunds have a different M-PFK mutation.[45]

PFK deficiency may be suspected based upon signalment, family history, typical clinical signs, and suggestive laboratory test abnormalities. While the pigmenturia is generally marked (i.e., orange to black), it may not be noted by the owners, particularly with a female dog, and other signs may be mild unless the dog is rigorously exercised. A definitive diagnosis requires proof of the PFK mutation or reduced RBC PFK enzyme activity. A simple PCR-based DNA test accurately identifies PFK-deficient dogs (homozygotes, affected; two mutant alleles) and carriers (heterozygotes; one mutant and one normal allele) in the English Springer Spaniel, American Cocker Spaniel, a Mexican Street Dog and Whippet, as well as the mixed breed dogs (also Wachtelhund, albeit a breed-specific mutation).[9,19,20,45,77] EDTA-anticoagulated blood, but also a drop of blood dried on special filter paper (Guthrie paper), buccal swabs with a cytobrush, and even sperms from a semen bank or formalized tissues can be suitable sources for DNA extraction and PCR testing. Whereas the PCR test and differentiation are simple and permit a diagnosis of deficient, carrier, and normal animals from the first day of life, the PFK enzyme activity test is cumbersome and not accurate until two months of age. Normal and affected neonatal dogs express large quantities of L-PFK in RBCs, which increases the overall PFK activity.[32] As the PCR-based test is mutation-specific, the measurement of PFK enzyme activity may, however, be indicated in other breeds and species, where the same disease is suspected. The PCR-based PFK test is recommended for use as a screening test for English Springer Spaniels (1) with suspicious clinical signs, (2) related to affected or carrier dogs, (3) used for breeding, and (4) prior to training for field trialing. In Whippets a Mexican Street Dog and American Cocker Spaniels, PFK deficiency seems to be more isolated, and, thus, general screening is not recommended.

PFK-deficient dogs can have a normal life expectancy, if crises-inducing situations are avoided.[14] Dogs experiencing a hemolytic crisis are provided supportive care including, if needed, type-matched blood transfusions and in case of repeat transfusions cross-matched blood. Fever and excessive panting or barking should be avoided whenever possible. Diamox, a carbonic anhydrase inhibitor, may acidify the blood, and aspirin and dipyrone may counter the fever accompanying hemolysis, thereby preventing further intravascular lysis during an acute crisis; however, they have not been proven effective in clinical practice. Similarly, because of disturbed purine metabolism, adenine could be considered as treatment, but efficacy and safety data are lacking.[70] Despite massive intravascular hemolysis, hemoprotein-induced acute nephropathy has not been observed. Nevertheless, hemolytic dogs should be well-hydrated. Affected dogs recover from these crises within days. The management of the cardiomyopathy seen in PFK-deficient Whippets may represent one of the breed-specific complications limiting their life expectancy.[9]

Finally, experimental bone marrow transplantation has successfully corrected the hematological abnormalities and abolished the hemolytic crises. Moreover, the corrected RBCs also normalized the hemoglobin–oxygen affinity and, thereby, positively affected oxygenation of muscle and aerobic muscle function.[57,58] Because of the morbidity- and mortality-associated bone marrow transplantations even when a dog leukocyte antigen (DLA)-matched donor (PFK carrier or normal) sibling is available and the relatively mild signs, when preventative measures are instituted, bone marrow transplantation cannot be recommended for this inborn error of metabolism.

PYRUVATE KINASE DEFICIENCY

Pyruvate kinase (EC 2.7.1.40) catalyzes the ATP-generating conversion of phosphoenolpyruvate to pyruvate and is, therefore, an important regulator in the terminal glycolytic pathway (Figure 29.1). There are two different genes coding by alternative splicing a total of four developmentally dependent and tissue-specific PK isoforms.[44,56,64,65] The PKLR gene encodes RBC (R-PK) and liver (L-PK) isoenzymes, whereas the PKM gene generates the muscle (M1-PK) and M2-PK isoforms. R-PK is expressed almost exclusively in mature RBCs and has a different first exon making the amino-terminal sequence longer when compared to that of L-PK. By contrast, erythroid precursors express M2-PK and switch to R-PK isoforms during differentiation to mature RBCs.[56] Erythrocytic PK deficiency causing hemolytic anemia has been described in humans, dogs, cats, and mice.[48,61,62,80]

Pyruvate kinase deficiency is the most prevalent hereditary nonspherocytic hemolytic anemia caused by a

glycolytic enzymopathy.[61,81] Many mutations in the PKLR gene have been found to cause R-PK deficiency in human patients; some of them are common in certain ethnic (Amish) and geographical regions.[6,61] Most of the patients, however, are compound heterozygotes with two different mutant alleles. Clinical signs in PK-deficient humans are very variable, ranging from a mild compensated hemolytic disorder to severely anemic patients who become transfusion-dependent, may develop iron overload (particularly when receiving regular transfusions), and die during early childhood. Splenectomy has been shown to be helpful in some human patients with severe anemia; iron chelation and bone marrow transplantation are other options.[26,81] While clinical manifestations of PK deficiency in dogs are similar to humans, PK-deficient cats show signs more like PFK-deficient dogs.

Canine Pyruvate Kinase Deficiency

In 1971, erythrocytic PK deficiency was first recognized in Basenji dogs.[71] It served as the classic example of an inborn error of metabolism in veterinary medicine, despite the fact that the biochemical derangements turned out to be more complex, making a clinical diagnosis more difficult. R-PK deficiency occurred most frequently in Basenjis in the past,[18,71,72,89] but has recently been more frequently reported in Beagles[17,30,46,67] and West Highland White Terriers[5,45–47,74] and occasionally in Cairn Terriers,[71] Labrador Retrievers,[46] Pugs,[46] Miniature Poodles, Dachshunds,[50] Chihuahuas, and American Eskimo Toy dogs. In Basenjis, a single base-pair deletion in exon 5 of the R-PK gene has been identified causing a frameshift and severe truncation of the protein.[89,90] By contrast, a six base insertion ambiguously positioned at the 3′-end of exon 10 in the R-PK sequence was found in West Highland White Terriers.[74] This insertion results in the in-frame addition of two amino acids, threonine and lysine, and is likely also the causative mutation in Cairn Terriers,[70] a related breed. In the Labrador Retrievers, a missense, and in Beagles and Pugs, different missense mutations have been found,[46] while in the other breeds, a disease-causing mutation has yet to be confirmed. Despite the varied mutations in R-PK, the biochemical changes and clinicopathologic manifestations of PK deficiency in any of the reported canine breeds appear identical.

The R-PK deficiency in RBCs of affected dogs can be demonstrated by agarose gel electrophoresis, immunoblotting, and immunoprecipitation studies.[18,39,40] However, all affected dogs express M2-PK in RBCs, which increases total RBC PK activity of affected dogs, and, therefore, is misleading. For instance, the Miniature Poodles with nonspherocytic anemia and osteosclerosis may well have had R-PK deficiency,[68] despite high RBC PK activity in vitro. By contrast, M2-PK expression is very rarely observed in human patients. This M2-PK isozyme, which is normally present in erythroid precursor and white blood cells, appears to be heat-labile and dysfunctional in vivo, because affected RBCs show a severe metabolic block at the PK step.[18] Besides the accumulation of proximal glycolytic metabolites in RBCs, the DPG content is also increased. The resulting right shift of the hemoglobin–oxygen dissociation curve allows for improved oxygen delivery, thereby ameliorating the clinical signs of severe anemia in PK-deficient dogs.

PK-deficient dogs frequently present for evaluation at a few months to a couple of years of age because of intermittent weakness and exercise intolerance. They appear to adapt very well to the severe anemia, presumably due to the favorable tissue oxygenation. They may also be serendipitously found when observing very pale mucous membranes in a clinically asymptomatic puppy. The hematocrit ranges persistently between 12% and 28%; the anemia is highly regenerative with corrected reticulocyte counts of 10–90%. Echinocytes have been documented.[71] In contrast to canine PFK-deficient RBCs, canine PK-deficient RBCs have normal alkaline fragility and readily release oxygen (Figure 29.2). The apparent erythrocyte half-life is only 5.8 days compared to 19–28 days in normal dogs.[18] Hepatosplenomegaly, due to extramedullary erythropoiesis and hemosiderin deposition, may also be noted, in fact some dogs may develop hemochromatosis.[4,71] Affected dogs die because of anemia or hepatic failure between 1 and 9 years of age, with Basenjis and Labrador Retrievers having a more severe form than Beagles and West Highland White Terriers.

Interestingly, all PK-deficient dogs develop progressive myelofibrosis and osteosclerosis that remain unexplained and do not occur in other species with PK deficiency.[4,5,17,18,30,45,47,50,70,71,73] The osteosclerosis may be identified by radiography of long bones as early as at 1 year of age and completely obstructs the marrow cavities by 3 years in Basenjis, but may occur later in other breeds.

A diagnosis of PK deficiency is strongly suggested by the concurrent occurrence of a highly regenerative severe anemia and osteosclerosis in a dog. Generally, routine screening tests rule out or are not supportive of acquired anemias and the anemia is persistent and appears well-tolerated. PCR-based PK tests are available for several breeds (https://www.vet.upenn.edu/research/academic-departments/clinical-sciences-advanced-medicine/research-labs-centers/penngen/tests-worldwide). These DNA tests are accurate to detect affected and carrier dogs in these breeds. However, because they are mutation-specific, they cannot be used for other breeds. In other breeds, cumbersome PK enzyme activity assays using agarose gel electrophoresis, immunologic, and heat-lability methods specific for R-PK are required due to M-PK expression. PK carriers do not express M-PK and, therefore, have intermediate (approximately 50% of normal) RBC PK activity. Unfortunately, the PK activity assays are not very accurate in detecting carriers, as the carrier and normal ranges overlap.[18] PK deficiency has most commonly been reported in the Basenji, West Highland White Terrier, and Beagle breeds, but the PK mutation frequency remains unknown in all canine breeds.

Affected dogs are managed symptomatically in clinical practice. Because of the excellent DPG-facilitated oxygen release from hemoglobin, PK-deficient dogs adapt well despite severe anemia. They rarely need transfusions except in their terminal stages. Bone marrow transplantation, performed experimentally,[88]

corrects the hemolytic anemia and halts the osteosclerosis and hemosiderin deposition, whereas splenectomy has not been shown to be effective in slowing the hemolysis and anemia in dogs.

Feline Pyruvate Kinase Deficiency

In the early 1990s, erythrocytic pyruvate kinase deficiency was identified and characterized in domestic short-haired cats and Abyssinian, Somali, and other breeds.[7,22] A 13 base-pair deletion at the 3′-end of exon 5 in the R-PK cDNA, is caused by a splicing defect and results in a severe reduction in RBC PK enzyme activity.[7,27,54] In cats there is no anomalous M2-PK expression in RBCs and no osteosclerosis, which are observed in all PK-deficient dogs. Since the original observations of the PK-deficient Abyssinian and Somali cats, over 3000 have been screened; approximately 12% are affected and 24% are carriers, making this one of the most frequent causes of anemia in these breeds. Since then, many more PK-deficient purebred and domestic short-haired cats have been reported from Europe,[27–29,52,53,63,85] Australia,[1,55] and Japan.[54] Interestingly, a specific pathogen-free research colony of domestic cats also carries this same PK mutation and was responsible for an unexplained mild to moderate intermittent anemia.

Affected cats have chronic intermittent hemolytic anemia that might not first be recognized until advanced age. The anemia is intermittent, mild to severe with PCV ranging from 5 to 35%. It is slightly macrocytic-hypochromic and slightly to strongly regenerative (reticulocyte count 45,000–290,000/μL).[25,52,53] Moreover, PK-deficient cats frequently have a marked lymphocytosis and hyperglobulinemia, which remains unexplained. Many of these features are similar to cats with increased RBC osmotic fragility with splenomegaly in the same breeds.[51,82] However, PK-deficient cats typically have a greater regenerative response and only mild splenomegaly and normal or only mild osmotic fragility (Figure 29.3). Extrahepatic biliary obstruction due to bilirubin calculi is seen in affected cats.[9,29] It should be noted that many deficient cats remain completely asymptomatic for years, and the associated anemia may not be the primary reason for a PK-deficient cat's illness.

PK-deficient cats frequently do not require treatment, just like PK- and PFK-deficient dogs. However, during a crisis, deficient cats may need to be transfused with AB-blood-type compatible blood. In case of severe splenomegaly or persistently severe anemia, splenectomy appears to reduce the severity of the hemolytic crises.[25]

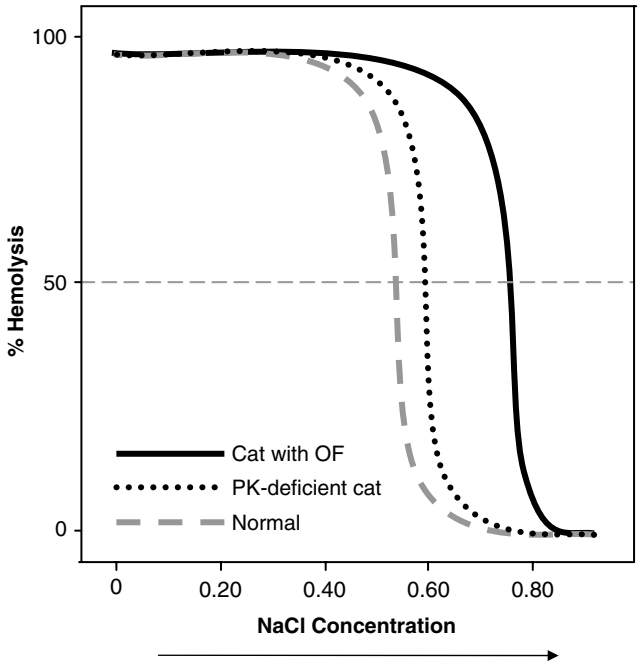


FIGURE 29.3 Erythrocyte osmotic fragility (OF) curve from a PK-deficient Abyssinian cat and an Abyssinian with increased OF and splenomegaly. RBCs were exposed in vitro to different saline concentrations from 0% (water) to 0.85% (physiological saline) at room temperature for 30 minutes.

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CHAPTER 30

Erythrocyte Membrane Defects

MUTSUMI INABA and JOANNE B. MESSICK

Hemolytic Anemias Caused by Hereditary Red Cell Membrane Defects
Hereditary Spherocytosis
  Hereditary Band 3 Deficiency in Cattle (Band 3$^{Bov.Yamagata}$) and Other Animal Species
  Spectrin and Ankyrin Deficiencies in Animals
Hereditary Elliptocytosis
  Hereditary Protein 4.1 Deficiency in Dogs (HE in Dogs)
  Spectrin Anomaly with Elliptocytosis in a Dog
Hereditary Stomatocytosis
  Hereditary Stomatocytosis in Dogs and Cats
  HSt in Miniature and Standard Schnauzers
  HSt in Chondrodysplastic Alaskan Malamute Dwarf Dogs
  Familial Stomatocytosis-Hypertrophic Gastritis in Dogs
  Hemolytic Anemia with Increased Erythrocyte Osmotic Fragility in Cats
Diagnostic Approaches to Erythrocyte Membrane Defects
  Other Testing for Diagnosis of Red Cell Membrane Disorders
Membrane Transport Defects
  Amino Acid Transport Deficiency in Animals
  High Membrane Na,K-ATPase Activity in Dogs

Acronyms and Abbreviations

DHS, dehydrated hereditary stomatocytosis; GLAST, glutamate aspartate transporter; HE, hereditary elliptocytosis; HPP, hereditary pyropoikilocytosis; HS, hereditary spherocytosis; HSt, hereditary stomatocytosis; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; OHS, overhydrated hereditary stomatocytosis; RBC, red blood cell; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

To survive repeated passages through the microcirculation, RBCs must be very durable and flexible, undergoing marked reversible deformation under these high-shear-stress conditions. The RBCs do not fragment despite these contortions and maintain a constant membrane surface area as they traverse circulatory beds.[56] These important properties are determined by three major components of their membranes: a lipid bilayer, integral or transmembrane proteins, and a membrane skeletal network (Figure 30.1). A lipid bilayer provides a permeability barrier between the cytosol and the external environment. Major constituents of the membrane skeleton mechanically support the plasma membrane and are organized into a lattice-like meshwork that is linked with both integral membrane components and cytoskeletal elements. Some of the transmembrane proteins embedded within the lipid bilayer ensure selective permeability to maintain RBC homeostasis. Accelerated destruction of RBCs (hemolytic anemia) may occur when these properties of the RBC membrane are affected or deficient by some genetic defects. This chapter reviews the current progress in the investigation of inherited red cell membrane disorders in animals.

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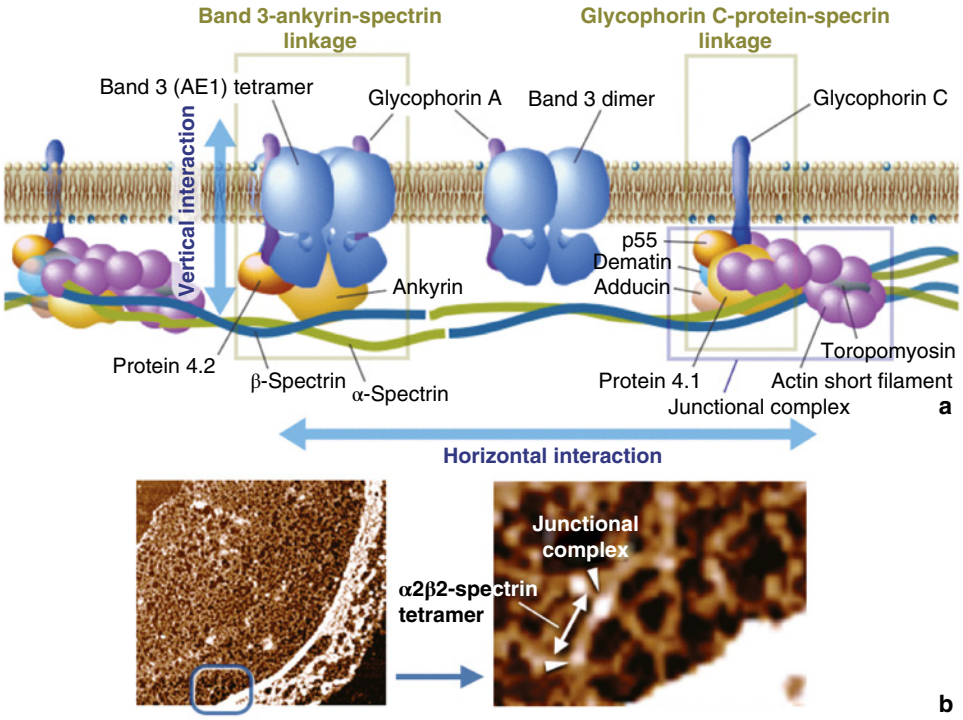


FIGURE 30.1 A schematic diagram illustrating structural and functional organization of RBC membrane proteins. Membrane protein–protein associations are divided into two categories: (a) vertical interactions involving band 3–ankyrin–spectrin linkage and glycophorin C–protein 4.1R–spectrin linkage; (b) horizontal interactions involving the spectrin heterodimer contact (tetramer formation) and association of spectrin tetramer at the distal ends with F-actin at the junctional complex consisting of actin, 4.1R, and adducin.

HEMOLYTIC ANEMIAS CAUSED BY HEREDITARY RED CELL MEMBRANE DEFECTS

The membrane protein–protein and protein–lipid interactions are critical determinants of red cell morphology and mechanical stability, as evidenced by the numerous hereditary RBC disorders in humans attributed to mutations of the membrane.[2,22,55,57,58,82] These interactions are divided into two categories (Figure 30.1): (1) vertical interactions involving the band 3–ankyrin–spectrin and glycophorin C–protein 4.1R–spectrin binding, both of which attach the spectrin–actin network to the plasma membrane and stabilize the lipid bilayer, and (2) horizontal interactions involving spectrin dimer–dimer association (tetramer formation), and contact of the distal ends of spectrin with F-actin by the aid of protein 4.1R and adducin within the junctional complex. In general, the loss of vertical linkage between membrane skeleton and lipid bilayer causes decreased membrane cohesion leading to membrane loss (e.g., hereditary spherocytosis (HS)). Weakening of horizontal linkages between skeletal proteins results in decreased membrane mechanical stability, leading to membrane loss (e.g., hereditary elliptocytosis (HE) and pyropoikilocytosis).[2,22,55,56] The severity of the anemia is directly related to the extent of the membrane surface area loss in both of these defects; loss of membrane not only compromises the ability of the RBC to deliver oxygen to the tissues, but also results in premature removal from the circulation by the spleen. The reader is directed to Chapter 20 for details of the structural organization of the normal RBC membrane.

HEREDITARY SPHEROCYTOSIS

The cardinal features of HS are hemolytic anemia of varying severity, spherocytosis, increased RBC osmotic fragility (OF), and splenomegaly.[67] In human medicine, HS is classified as mild, moderate, or severe based on hemoglobin levels and reticulocyte counts.[2,22,55,56,63] A mild HS is distinguished by a well-compensated hemolytic anemia, minimal numbers of spherocytes on the peripheral blood smear, and only a slight reticulocytosis, whereas these features are more remarkable with moderate and severe forms of the disease. The pathophysiology of HS involves two major factors: intrinsic membrane defect, and selective sequestration of HS cells in the spleen. Hence, anemia can be significantly improved by splenectomy in human HS patients [2,63]. Typical forms of HS need to be distinguished from other hemolytic anemias having spherocytes, such as immune-mediated hemolytic anemia by Coombs testing as well as unstable hemoglobin and oxidative damage by Heinz body screening. HS is now considered to be a disorder of vertical interactions of the membrane proteins, although the primary molecular defects are heterogeneous including deficiencies or dysfunctions of spectrin, ankyrin, band 3, and protein 4.2.[2,24,55–57,63,82] Consequently, the lipid bilayer is destabilized, leading to membrane

vesiculation and surface area loss, and spherocyte formation.[22,63]

Hereditary Band 3 Deficiency in Cattle (Band 3$^{Bov.Yamagata}$) and Other Animal Species

Band 3 (anion exchanger 1, AE1) is the most abundant transmembrane protein in mammalian RBCs,[62] accounting for about 25% of the total membrane proteins. Hereditary band 3 deficiency in Japanese Black cattle (band 3$^{Bov.Yamagata}$) is associated with HS and is inherited by an autosomal dominant trait.[31] Homozygous affected animals totally lack band 3 due to an R664X nonsense mutation of the AE1 gene (*SLC4A1*) and show mild to moderate, chronic hemolytic anemia (hematocrit, 25–35%), acidosis, and growth retardation. Carrier cattle heterozygous for the R664X mutation also have spherocytosis and impaired anion transport activity due to partial deficiency of RBC band 3 (about 30%). However, the hemolysis is well-compensated. In heterozygous animals, mutant band 3 is rapidly degraded in the endoplasmic reticulum without translocation to the plasma membrane and has a dominant-negative effect on the expression of wild-type band 3.[32,33]

Band 3 has two major functions:[8,36,62,79,80] (1) it mediates a rapid Cl^-/HCO_3^- exchange across the plasma membrane, increasing up to fivefold the capacity of the blood to carry CO_2 from tissues to lungs and maintaining blood acid–base homeostasis together with the isoform expressed by α-intercalated cells of the renal collecting duct; and (2) it also participates in maintaining mechanical properties of RBC membranes by forming the band 3–ankyrin–spectrin complex. Various mutations leading to disorders and partial deficiency of RBC band 3 associated with abnormal RBC morphology (spherocytosis and ovalocytosis) have been reported in humans.[34,37,58] Only one case of complete deficiency[67] and an aberrant nearly total deficiency[62] in humans, both of which were associated with life-threatening hemolytic anemias, have also been reported to date. The most surprising finding, therefore, is that cattle with total lack of band 3 survive and may thrive in adulthood. They suffer from extremely severe hemolytic anemia shortly after birth and exhibit jaundice and splenomegaly. Although the mortality rate is high during this period, beyond this crisis period, jaundice subsides and hemolysis becomes modest.

RBC membranes from homozygotes of band 3$^{Bov.Yamagata}$ are deficient in spectrin, ankyrin, actin (by 20–50%), and protein 4.2, resulting in a distorted and disrupted membrane skeletal network (Figure 30.2). They are extremely unstable and demonstrate the spontaneous loss of surface area by invagination, vesiculation, and extrusion of exosome-like microvesicles, thereby leading to the formation of spherocytes with irregular contours, gouging, and pitting (Figure 30.3). RBCs from homozygous and heterozygous cattle show significantly increased osmotic fragility with 50% hemolysis at 0.75% and 0.65–0.70% NaCl, respectively (normal, 0.45–0.55% NaCl) (Figure 30.4). These findings are consistent with a remarkably decreased surface

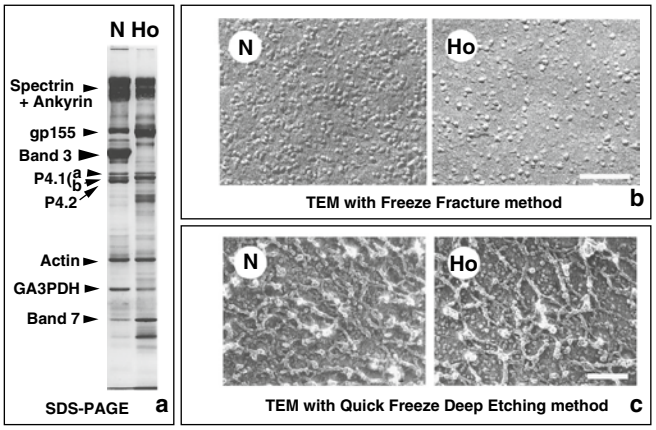


FIGURE 30.2 Abnormal RBC membrane structures in bovine band 3 deficiency (band 3$^{Bov.Yamagata}$). (a) SDS-PAGE profiles of RBC membrane proteins from the normal (N) and the homozygote for R664X mutation (Ho). A 66-kDa protein only found in the homozygote was albumin. gp155 indicates a transmembrane protein characteristic to ruminant RBCs[28]; (b) Markedly reduced number of intramembrane particles in homozygous RBCs (Ho) compared to normal RBC on electron micrographs by the freeze fracture method; (c) Disrupted membrane skeletal network in band-3-deficient RBCs visualized by the quick freeze deep etching method. The membrane skeletons in band-3-deficient RBCs (Ho) are disrupted and distorted with filaments of uneven length and width compared with the well-organized normal RBCs (N). Scale bars = 0.1 μm.

area/volume ratio of RBCs from both homozygotes and heterozygotes due to the mechanical instability of their RBC membranes. Despite these severe changes, the affected animals show no reticulocytosis and no noticeable intravascular hemolysis. However, the protein 4.1a/4.1b ratio, which is a good marker of RBC aging,[27,30] is remarkably reduced and paralleled by increased erythropoiesis. These demonstrate the functional importance of band 3–ankyrin–spectrin association in maintaining mechanical stability of the membrane,[45] but not in assembly of the membrane skeletal architecture.

Total deficiency of band 3 also results in defective Cl^-/HCO_3^- exchange.[31] The Cl^- influx into the RBCs from homozygotes requires approximately 2 hours to reach transmembranous equilibrium even at 37 °C (less than several seconds in normal RBCs). Band 3 deficiency causes mild acidosis with decreases in the HCO_3^- concentration and total CO_2 in the blood. However, these values remain in the normal range. As CO_2 in blood rarely reaches saturation, it is suggested that the additional CO_2-carrying capacity facilitated by band 3 is probably not as critical as has been believed previously,[35,36,78,79] except under high-stress conditions such as vigorous exercise or high altitude.

Transgenic mice with complete band 3 deficiency,[64,76] as well as only the case of total band 3 deficiency in human,[67] have similar RBC features. These studies demonstrate that band 3 indeed contributes to RBC membrane organization, CO_2 transport, and acid–base homeostasis. In band 3 knockout mice, glycolytic enzymes that normally exist as multienzyme complexes

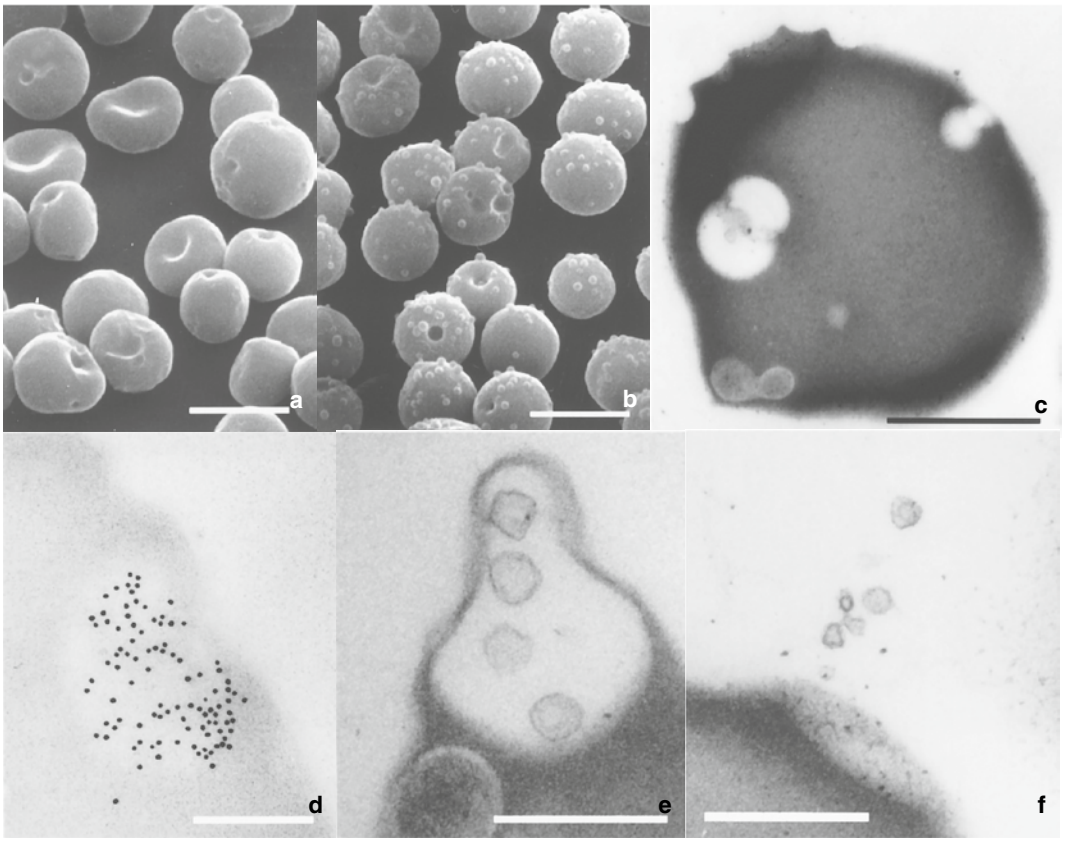


FIGURE 30.3 Morphologic anomaly in bovine band 3 deficiency. (a) and (b) Scanning and (c)–(f) transmission electron micrographs of RBCs from a homozygous animal of band $3^{Bov.Yamagata}$. (a) The proband RBCs look like potatoes and greatly vary in size, principally being spherocytic and stomatocytic with irregular contours; (b) When blood is allowed to stand for several hours at ambient temperature, numerous small globules are observed on the surface; (c)–(f) Marked endocytosis-like invagination, exocytosis-like projections, fusion of vesicles inside the cell, and extrusion of microvesicles, leading to surface area loss; (d) The vesicles in the cytoplasm contain plasma proteins as determined by immunoelectron micrography using antibovine albumin antibodies. Scale bars = 5 μm (a) and (b), 1 μm (c), and 0.2 μm (d)–(f).

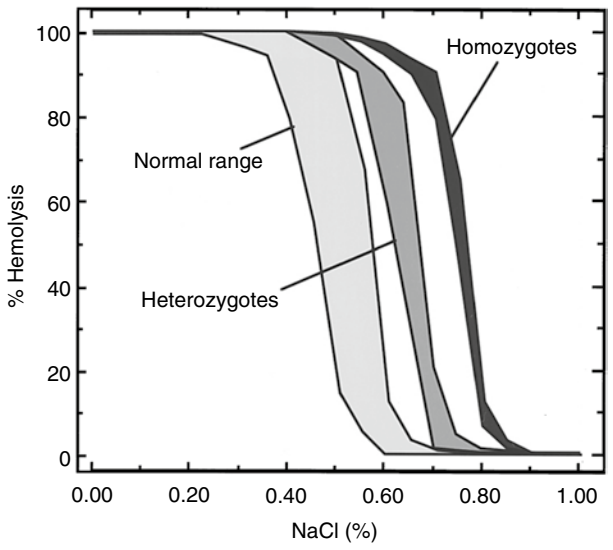


FIGURE 30.4 Increased RBC osmotic fragility in bovine band 3 deficiency. Data for normal cattle (n = 15) as well as homozygotes (n = 4) and heterozygotes (n = 8) of band 3 deficiency.

on the inner surface of RBC membranes are not membrane-associated but distributed throughout the cytoplasm. The assembly of some glycolytic enzymes on the membrane is likely a general phenomenon of mammalian RBCs and stability of these interactions depends primarily on band 3.[9]

The use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to explore alterations in protein profiles of the RBC membrane and molecular genetic techniques has greatly increased our understanding of the pathophysiology of HS. A recent report described the application of proteomics to explore the cause of a presumed RBC membrane protein deficiency in a Jack Russell Terrier presenting with hereditary hemolytic anemia.[5]

Spectrin and Ankyrin Deficiencies in Animals

Several mutations that affect components of the RBC membrane skeleton have also been reported in mice.[4,87] These include spectrin deficiencies in house[22,46] and deer mice[71] as well as ankyrin deficiency in nb mice[87] with

moderate to life-threatening hemolytic anemia and fragile short-lived spherocytes. A quantitative spectrin deficiency with an autosomal dominant inheritance was reported in Dutch Golden Retrievers.[74] The affected dogs had markedly reduced RBC spectrin concentration that was 50–65% of unaffected dogs and exhibited increased RBC osmotic fragility. However, the disorder was not associated with either spherocytosis or elliptocytosis. The exact mechanism for this occult spectrin deficiency without morphological anomaly remains to be clarified.

HEREDITARY ELLIPTOCYTOSIS

The principal lesion of human HE involves heterogeneous defects in horizontal membrane protein interactions (Figure 30.1) resulting in a mechanically unstable membrane (e.g., abnormal spectrin structure affecting the spectrin heterodimer contacts/dimerization as previously described or deficiency/dysfunction of protein 4.1).[22,55,58,82] Common HE is morphologically characterized by elliptocytes and rod-shaped RBCs in some patients. While the majority of the cases of HE are asymptomatic, a severe form, hereditary pyropoikilocytosis (HPP), characterized by hemolytic anemia and jaundice is also recognized. The red blood cell morphologic findings in these cases resemble that seen in severe burn patient including poikilocytes, fragments or schistocytes, microspherocytes, and rare elliptocytes.[66]

Ovalocytosis, spherocytic HE, is a rare condition in which both oval RBCs lacking central concavity and spherocytes are present on the blood film. It is likely that elliptocytes and poikilocytes are permanently stabilized in their abnormal shape. The weakened horizontal connections facilitate reorganization of the skeleton, which follows axial deformation of cells by a prolonged shear stress.[55,58,82] Although camelid RBCs are elliptocytic by nature, the molecular basis and physiologic importance of this unique feature remain unknown.

Hereditary Protein 4.1 Deficiency in Dogs (HE in Dogs)

Canine HE was discovered by Smith et al.[75] The proband exhibited elliptocytosis, membrane fragmentation, microcytosis, and poikilocytosis without anemia (Figure 30.5). RBC mechanical stability was markedly decreased, and osmotic fragility was remarkably increased. The proband RBC membranes were deficient in protein 4.1R (4.1Ra and 4.1Rb). The parents of the proband had decreased amounts (approximately 50% of normal) of protein 4.1R and some elliptocytes.

Protein 4.1R binds to both spectrin and actin to form spectrin–4.1R–actin ternary complex through its 10-kDa spectrin–actin binding domain (SABD)[12]. The SABD contains amino acid sequences encoded by a 21-amino acid erythroid-specific exon (exon 16) and a 59-amino acid constitutive exon.[10] Immunoblotting of RBC membrane proteins from the proband showed the presence of a small amount of 76-kDa polypeptide lacking the 21-amino acid segment and a very faint band of normal 4.1R. Reverse transcription (RT)-PCR analysis and sequencing of cloned reticulocyte 4.1R cDNA consistently showed that mRNA lacking exon 16 was the predominant form with only a small quantity of normal mRNA. Therefore, the functional defect in the HE dog is likely due to the combined influences of two factors: a quantitative deficiency of protein 4.1R and a failure to activate efficiently expression of an alternatively spliced exon 16 in the SABD during erythropoiesis.[11] The primary cause leading to inefficient erythroid-specific alternative splicing has not been defined.

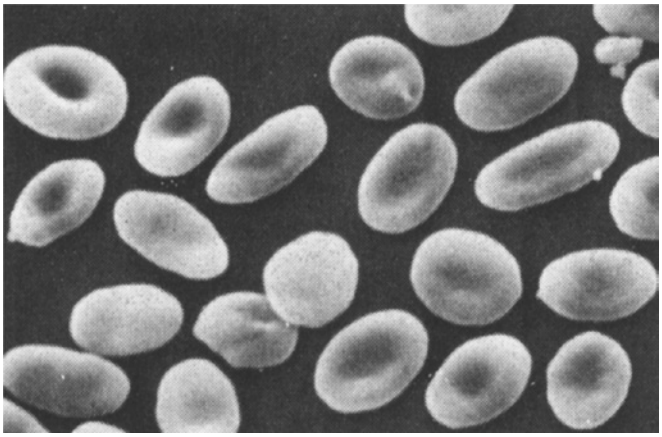


FIGURE 30.5 Scanning electron micrograph of RBCs from the proband with hereditary elliptocytosis. The proband RBCs reveal biconcave elliptocytes. (**Source:** Reproduced from Smith et al.[75] with permission)

Spectrin Anomaly with Elliptocytosis in a Dog

An elliptocytosis was reported in a mixed-breed dog.[14] Although the elliptocytosis was asymptomatic and was not associated with hemolytic anemia, the dog's RBCs had decreased deformability and stability when subjected to shear stress. This is consistent with nonhemolytic HE in humans due to structural abnormality in either the α- or β-spectrin. Molecular analysis revealed that the elliptocytosis was probably due to T2110M mutation in β-spectrin, a region of spectrin that is critical for the self-association of spectrin dimers. This would be expected to result in reduced formation or reduced stability of tetramers.

HEREDITARY STOMATOCYTOSIS

Stomatocytes are uniconcave or bowl-shaped RBCs in suspension but have a slit-like appearance, an artifact on dried blood films (Figure 30.6). Two distinct phenotypes having abnormalities in membrane cation permeability leading to changes in RBC volume have been identified in humans: dehydrated hereditary stomatocytosis (DHS) and overhydrated hereditary stomatocytosis (OHS).[2,55] DHS is associated with a well-compensated

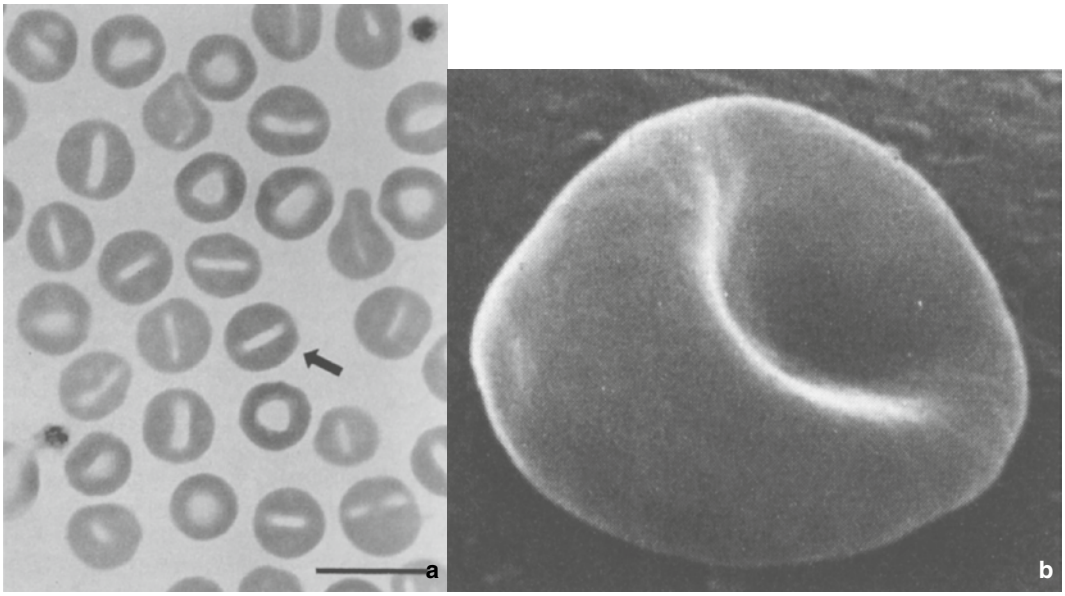


FIGURE 30.6 Erythrocyte morphology of canine hereditary stomatocytosis (hydrocytosis). (a) Numerous stomatocytes (arrow) with a slit-like appearance are present on a Wright–Giemsa-stained blood smear from a Miniature Schnauzer. (**Source:** Reproduced with permission[7]) Scale bar = 10 μm; (b) Scanning electron micrograph of a typical stomatocyte from an affected Alaskan Malamute. (**Source:** Reproduced from Pinkerton et al.[65] with permission)

hemolytic anemia with less than 10% stomatocytes usually seen on blood smears. By contrast, stomatocytes are a major feature of RBC morphology in OHS. An important characteristic of these stomatocytosis patients is a marked predisposition to thrombocytosis and development of hypercoagulability after splenectomy.[2,22]

OHS (hydrocytosis) is a heterogeneous group of disorders in humans characterized by moderate to severe hemolytic anemia with stomatocytes, an elevated mean corpuscular volume (MCV), and a reduced mean corpuscular hemoglobin concentration (MCHC). The principal lesion involves a remarkable increase of Na^+ influx into RBCs, resulting in a marked increase of intracellular Na^+ and water content and a corresponding decrease of K^+.[54,94] The molecular basis of this permeability defect is unknown. Human OHS is frequently associated with reduced contents of a 31-kDa integral membrane protein stomatin, which may function in regulation of cation transport and a stretch- or pressure-sensitive system, in the RBC membrane from OHS patients.[17,19,44,77] It is reported that stomatin–actin association is necessary for maintaining the structure and modulating the function of stomatin in RBCs.[88]

Hereditary Stomatocytosis in Dogs and Cats

Hereditary stomatocytosis (HSt) is recognized in dogs and cats with undefined etiologies. All disorders appear to be transmitted as autosomal recessive traits.

HSt in Miniature and Standard Schnauzers

Stomatocytosis has been reported in Miniature and Standard Schnauzers (Figure 30.6a) without clinical signs of disease.[6,7,23] Hereditary stomatocytosis in Schnauzers is characterized by macrocytosis, relatively high packed cell volume, remarkably decreased MCHC, and increased osmotic fragility. Erythrocyte survival is only slightly shortened. Affected Miniature Schnauzers are of normal stature. Stomatin is not deficient from the erythrocyte membrane in affected Standard Schnauzers.[59]

HSt in Chondrodysplastic Alaskan Malamute Dwarf Dogs

In Alaskan Malamutes, chondrodysplasia (short-limbed dwarfism) occurs along with stomatocytosis.[17,18] The affected Malamutes have macrocytosis, decreased MCHC, increased osmotic fragility, shortened RBC survival, reticulocytosis, erythroid hyperplasia, and increased iron turnover (Figure 30.6b).[65] Although heterozygous carriers have minor changes in their RBCs, they have a normal RBC life span and no dwarfism. RBC Na^+ concentration and water content are increased in affected dogs.

The pathogenesis of stomatocyte formation in Malamutes and Schnauzers is attributed to an increase in monovalent cation and, consequently, increased water content of RBCs as is reported in human HSt. However, the exact nature of membrane defects leading to the changes in RBC indices in these breeds is obscure.

Familial Stomatocytosis-Hypertrophic Gastritis in Dogs

Familial stomatocytosis-hypertrophic gastritis is a multiorgan disease with hemolytic anemia, HSt, and hypertrophic gastritis described in the Drentse Patrijshond breed.[72,73] The main clinical signs are diarrhea, jaundice, and ataxia and paresis of the pelvic limbs. Pathologic

findings involve hypertrophic gastritis, progressive liver disease, renal cysts in aged subjects, and polyneuropathy.[72]

Erythrocytes from affected dogs show increased osmotic fragility. In contrast to Malamutes and Schnauzers with stomatocytosis, RBCs from affected Patrijshonds have normal MCV, slightly increased cell water with basically normal content, and fluxes of Na and K, suggesting a different mechanism for stomatocyte formation.[73] Affected dogs have normal RBC membrane protein profile, but a decrease of phosphatidylcholine with altered fatty acid composition and simultaneous increase of sphingomyelin in both RBCs and plasma. It is suggested that this polysystemic disease is a disorder of lipid metabolism in which defective membrane function and RBC shape change are induced by abnormal phospholipid composition of the plasma. This is supported by a shortened half-life of RBCs from normal dogs after transfusion into dogs with this syndrome.[73] Stomatocytic shape change may be attributed to membrane surface loss, presumably due to abnormal phospholipid composition of the bilayer. The exact relation between anomaly in phospholipid and hypertrophic gastritis and the primary cause of this syndrome are unknown.

Hemolytic Anemia with Increased Erythrocyte Osmotic Fragility in Cats

A hereditary RBC defect is suspected in Abyssinian and Somali cats with Coombs negative hemolytic anemia.[41,42] The affected cats exhibited recurrent anemia (hematocrit, 15–25%); severe splenomegaly with extramedullary hematopoiesis, hemosiderosis, congestion, and lymphoid hyperplasia; loss of body mass; macrocytosis; and a few stomatocytes. The anemia was variably regenerative. The osmotic fragility of their RBCs is markedly increased. Splenectomy partially corrected the anemia and prevented hemolytic crises, but long-term survival remains unknown. More recently, a similar condition was described in domestic short-haired and cats of other breeds (Ocicat and Siamese). A moderate to severe macrocytic hypochromic anemia was described in these cats with red cells showing increased osmotic fragility. Most of these cats also had moderate to severe splenomegaly with marked extramedullary hematopoiesis.[81]

DIAGNOSTIC APPROACHES TO ERYTHROCYTE MEMBRANE DEFECTS

Genetic aberrations of RBC membranes described in the earlier text are feasibly surveyed by evaluating RBC morphology, osmotic fragility, and RBC parameters. The OF test[3,61] is a simplified means to estimate the surface area/volume ratio of RBCs. It is most valuable in the diagnosis of HS, but is also useful in evaluation of most forms of HE and overhydrated HSt (hydrocytosis). False positives may be detected in other conditions associated with spherocytosis, such as immune-mediated hemolytic anemia. Several laboratory tests, such as Coombs test and Heinz body screening, may be required to eliminate a possibility that hemolytic anemia and abnormal RBC shapes result from extrinsic factors. As exemplified for bovine HS in Figure 30.2a, SDS-PAGE analysis followed by some immunochemical, biochemical, and biophysical techniques often provides insights into primary defects of membrane skeletal and integral proteins resulting in HS, HE, and HPP.

Other Testing for Diagnosis of Red Cell Membrane Disorders

In 2011, the International Council for Standardization in Haematology (ICSH) guidelines for diagnosis of nonimmune hereditary red cell membrane disorders suggested various screening tests that might be considered.[40] The best screening test for these disorders is osmotic gradient ektacytometry. This instrument monitors the extent of RBC shape change within a particular osmotic gradient under a known shear stress; the deformability profiles that are generated are well correlated to specific red cell membrane disorders. The major limitation of this procedure is the limited availability of the instrumentation and testing must be performed within 48 hour of sample collection. Another diagnostic approach is direct mutational analysis of the various genes responsible for these disorders. However, this is a case-by-case consideration for identifying a putative genetic mutation, particularly when family history is not helpful. Next generation sequencing (NGS) technologies for use in the diagnosis of red cell membrane disorders also have recently been reported.[1,40] After identifying the protein gene mutation using NGS in the patient, other family members can be tested by PCR and sequencing to confirm the inherited mutation.

MEMBRANE TRANSPORT DEFECTS

Deficiency and dysfunction of membrane transport systems may affect RBC homeostasis. Particularly, defects in transport of amino acids involved in glutathione metabolism have been reported to generate hemolytic anemia when RBCs are exposed to extrinsic factors including oxidants.

Amino Acid Transport Deficiency in Animals

Red blood cell glutathione deficiency, inherited as an autosomal recessive trait, occurs in Finnish Landrace sheep.[83] Affected animals are not anemic, but have shortened RBC life span.[84] This is possibly caused by increased oxidant sensitivity as exemplified by Heinz bodies. Affected sheep are more likely to become anemic after the administration of oxidants in vivo.[86] RBCs from the affected animals are defective in the transport system for various amino acids including cysteine.[47,92,93] Consequently, cysteine uptake and glutathione synthesis are limited, and glutathione concentrations in RBCs are decreased to approximately 30% of normal. The transport deficiency appears to develop during reticulocyte maturation.[85]

A similar defect of amino acid transport is found in about 30% of thoroughbred horses,[16] but seems to cause no clinical signs. The lesion appears to result in increased amino acid levels and glutathione deficiency in some cases.

High Membrane Na, K-ATPase Activity in Dogs

Although canine reticulocytes have a considerable amount of membrane Na, K-ATPase (Na/K-pump) activity, its activity is rapidly lost during maturation into mature RBCs.[26,50] Proteolytic degradation[26] and extrusion of vesicles (exosomes)[38,43] are likely involved in this process. As a consequence, dogs usually have RBCs with low K^+ and high Na^+ concentrations (LK RBCs).[60] However, some Japanese Shiba and mongrel dogs have HK RBCs with high K^+ and low Na^+ concentrations, because the Na, K-ATPase protein and its activity are retained in mature RBCs.[26,49,53] This HK phenotype representing immaturity of erythroid precursor cells[26,29,43,50,51] is inherited in an autosomal recessive manner[48,49,53] and has also been found in Japanese Akitas[13] and several breeds of Korean dogs.[21] Although dogs with HK RBCs are not anemic, their RBCs have shortened life spans,[51] increased osmotic fragility, increased MCV, and normal mean corpuscular hemoglobin (MCH) values, suggesting an increase in RBC water.[49] The genome-wide linkage analysis identified the mutations in the translocator protein 2 (TSPO2) gene as the molecular cause for HK RBC trait.[39] The defect of cholesterol-binding membrane protein TSPO2 causes the delays in multifaceted events during terminal erythroid differentiation and cell-cycle progression,[39] resulting in immature RBC phenotypes in HK dog RBCs.[29,43,50] Due to the leak of K^+ from RBCs, blood from HK dogs may cause pseudohyperkalemia in vitro after storage or on delaying plasma or serum separation.[13]

Canine RBCs possess a high-affinity Na^+-dependent transport system for glutamate and aspartate,[15,25] which resembles the kinetic and pharmacologic properties of the glutamate aspartate transporter (GLAST) in the brain.[68–70] The increased concentration gradients of Na^+ and K^+ across the membrane produced by the presence of Na, K-ATPase with high activity accelerate transport of glutamate and aspartate into HK RBCs.[25] The concentration of reduced glutathione is increased five to seven times that of normal because the feedback inhibition of γ-glutamylcysteine synthetase by glutathione is released by glutamate accumulated in these cells at about 90 times that in normal RBCs.[48] Some variant dogs of HK phenotype that lack the increase of RBC glutathione have been reported,[20,69] suggesting that several independent mutations have emerged in these breeds.[69]

The accumulation of glutathione in the canine HK RBCs only provides improved protection against oxidative damage induced by acetylphenylhydrazine, but these RBCs are more susceptible to oxidative damage induced by 4-aminophenyl disulfide,[52] onions,[47,89] and sodium n-propylthiosulfate,[91] one of the hemolytic thiosulfate compounds isolated from onions.[90] The increased glutathione concentration accelerates the generation of superoxide through its redox reaction with the aromatic disulfide,[52] but the exact mechanism by which HK RBCs are more sensitive to the thiosulfates remains to be clarified.

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CHAPTER 31

Congenital Dyserythropoiesis

DOUGLAS J. WEISS

Pathogenesis
Poodle Macrocytosis
Inherited Selective Malabsorption of Cobalamin Clinical Condition
Congenital Dyserythropoiesis, Polymyopathy, and Cardiac Disease in English Springer Spaniels
Congenital Anemia, Dyskeratosis, and Progressive Alopecia in Polled Hereford Calves

Acronyms and Abbreviations

DNA, deoxyribonucleic acid; RDW, RBC distribution width.

Congenital conditions associated with dysplastic features in RBCs and bone marrow include Poodle macrocytosis,[3,11] inherited selective malabsorption of cobalamin,[5,6,10] congenital dyserythropoiesis, polymyopathy, and cardiac disease in English Springer Spaniels,[7] hereditary stomatocytosis in Alaskan Malamutes (see Chapter 30), and congenital anemia, dyskeratosis, and progressive alopecia in polled Hereford calves.[12,13] In general, these conditions are characterized by dysplastic changes in RBCs or erythroid precursor cells associated with ineffective erythropoiesis.[15]

PATHOGENESIS

Recent studies in human medicine have identified more than 40 mutations in genes specifically involved in DNA repair, maintenance of genomic stability, and ribosome and telomere biology that are involved in bone marrow failure syndromes.[1,9] Mutations in ribosomal proteins are associated with a variety of specific human conditions.[9] These various ribosomal mutations appear to affect cells primarily through the p53 signaling pathway resulting in p53-dependent cell cycle arrest and apoptosis.[9] Aldehyde dehydrogenase 2 deficiency has been identified in people with Fanconi anemia.[14] Aldehyde dehydrogenase 2 is a mitochondrial enzyme that is essential for the metabolism of aldehydes. Aldehydes are genotoxic, specifically causing DNA cross-linking that may lead to hematopoietic failure. Both ribosomal mutations and many mutations in genes involved in DNA repair predispose patients to develop myelodysplastic syndromes or acute leukemias.[1]

POODLE MACROCYTOSIS

Poodle macrocytosis is a familial condition, affecting both sexes, that occurs mostly in Toy and Miniature Poodles.[3,11] Affected dogs do not have associated clinical signs, are not anemic, and do not have reticulocytosis. The most distinctive feature in the blood is marked macrocytosis with mean cell volumes varying between 85 and 105 fL.[3,11] Metarubricytosis is a frequent finding and some of these cells have nuclear-cytoplasmic asynchrony. Additionally, Howell–Jolly bodies are increased in number and tend to be large and multiple (Figure 31.1). Bone marrow is characterized by marked dyserythropoiesis and the morphologic changes resemble those associated with vitamin B_{12} deficiency. Features of dyserythropoiesis include megaloblasts, binucleation, multinucleation, irregular nuclear shapes, nuclear fragmentation, nuclear-cytoplasmic asynchrony, and nuclear bridging (Figure 31.2).[3] Nuclear bridging is a unique feature seen in Poodle macrocytosis that is rarely seen in other congenital or acquired types of dyserythropoiesis

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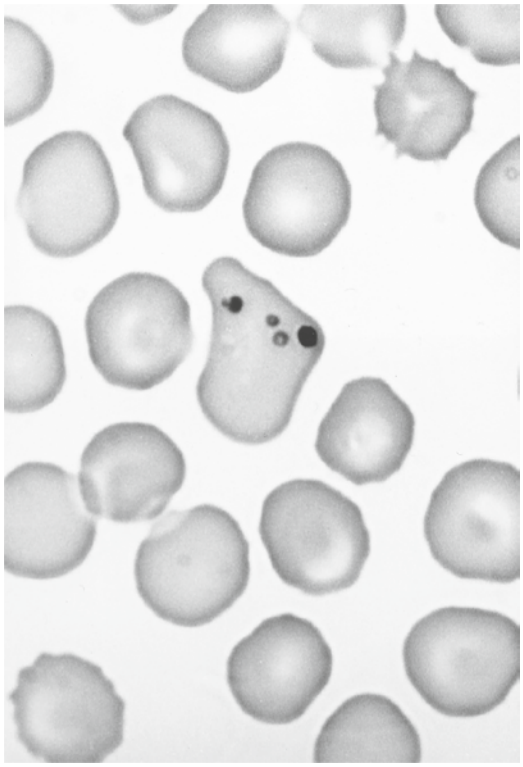


FIGURE 31.1 Blood from a dog with poodle macrocytosis. Notice the presence of an RBC with multiple large Howell–Jolly bodies.

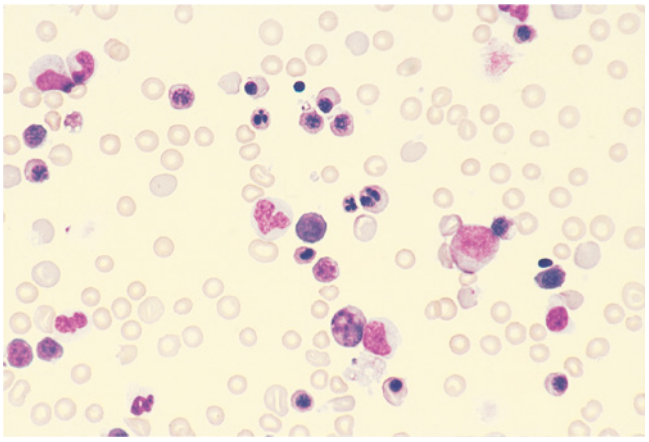


FIGURE 31.2 Erythroid precursor cells in a bone marrow aspiration smear from a dog with Poodle macrocytosis. Notice the presence of misshapen and fragmented nuclei in rubricytes.

or in myelodysplastic syndromes (Figure 31.3). Mitotic figures are increased in number. The nature of the hematopoietic defect has not been investigated. Bone marrow cytologic abnormalities in Poodle macrocytosis have some similarity to those seen in congenital dyserythropoietic anemia type I of children as well as those in Hereford cattle with congenital anemia, dyskeratosis, and progressive alopecia.[6,12,13] The mutated gene in congenital dyserythropoietic anemia type I of children has been identified as CDAN1.[8] The cellular function of this protein remains unknown.

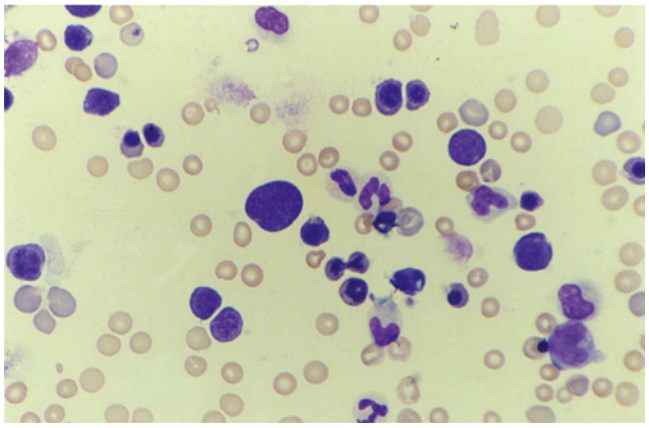


FIGURE 31.3 Bone marrow aspiration smear from a dog with Poodle macrocytosis. Notice the presence of a rubricyte with nuclear bridging (center of photo).

INHERITED SELECTIVE MALABSORPTION OF COBALAMIN

An inherited selective malabsorption of cobalamin (vitamin B_{12}) has been identified in Giant Schnauzers and has been reported in Border Collies, in a Beagle, and a cat.[4,5,10] Cobalamin is obtained in all species by absorption from the intestinal tract. Cobalamin can be produced by microorganisms in the gastrointestinal tract of ruminants, but must be liberated from digestion of foodstuffs by other species. Cobalamin is essential as a cofactor for the activity of the enzymes methylmalonyl-CoA mutase and methionine synthase.[2] Deficiency of methylmalonyl-CoA mutase results in methylmalonic acidemia and aciduria.[2] Deficiency of methionine synthase causes homocysteinemia and inhibits conversion of 5-methyltetrahydrofolate to tetrahydrofolate.[2] Tetrahydrofolate is an essential cofactor for enzymes of purine and pyrimidine synthesis.[2] Resultant inhibition of nucleic acid synthesis is most noticeable as inhibition of hematopoiesis in adult animals.[4,5] Inhibition of nuclear maturation typically results in a nonregenerative anemia, with megaloblastosis, neutropenia, and hypersegmented neutrophils.[4,5] Although humans and nonhuman primates develop a macrocytic anemia with cobalamin deficiency, the anemia in dogs, rodents, horses, pigs, and ruminants is normocytic and normochromic.[4,5] Both large and small RBCs are present in the blood of dogs with cobalamin deficiency.[4,5] This results in an increase in red cell distribution width (RDW), but the mean cell volume remains within the reference interval.

Clinical Condition

Inherited selective malabsorption of cobalamin in Giant Schnauzers is an autosomal recessive condition caused by lack of a receptor for intrinsic factor–cobalamin complex (i.e., cubilin) on the brush border of the small intestine and renal tubular epithelial cells.[4,5] Affected dogs develop anorexia, lethargy, and cachexia by 2–3 months of age. Blood cell morphology is characterized initially

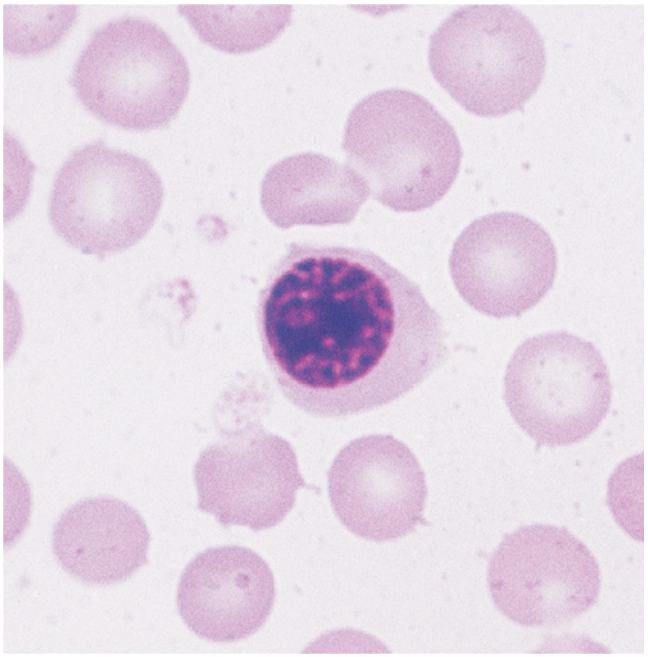


FIGURE 31.4 Blood from a dog with selective malabsorption of cobalamin. Notice the presence of a megaloblast exhibiting an immature nucleus and a fully hemoglobinized cytoplasm.

by a mild neutropenia followed by development of a chronic nonregenerative normocytic normochromic anemia, acanthocytosis, and increased RDW.[4,5] RBC morphology is characterized by many small RBCs, ovalocytes, and occasional megaloblasts (Figure 31.4). Occasional hypersegmented neutrophils and giant platelets are also present. Bone marrow granulocytes are characterized by hypersegmented neutrophils and giant bands and metamyelocytes. Erythroid cells in marrow are characterized by nuclear-cytoplasmic asynchrony and abnormal nuclear chromatin clumping.

Diagnosis of cobalamin deficiency is best made by determining serum cobalamin concentrations.[4,5] Low serum cobalamin concentration appears to be the earliest indicator of cobalamin deficiency. Increased serum methylmalonic acid and serum total homocysteine concentrations can also be measured.

Affected dogs respond well to daily injections of vitamin B_{12}.[4,5] Alternatively, one megadose of 1 mg of vitamin B_{12} is sufficient to maintain an affected dog in remission for 1 month. Complete resolution of hematologic signs and growth retardation occurs with parenteral administration of cyanocobalamin.

CONGENITAL DYSERYTHROPOIESIS, POLYMYOPATHY, AND CARDIAC DISEASE IN ENGLISH SPRINGER SPANIELS

Congenital dyserythropoiesis, polymyopathy, and cardiac disease have been described in three related English Springer Spaniels.[7] Clinical signs included regurgitation, stunted growth, stiff gait, skeletal muscle wasting, cardiomegaly, megaesophagus, and generalized muscle atrophy that was most pronounced in temporal muscles. All dogs had a mild to moderate microcytic normochromic nonregenerative anemia with marked metarubricytosis. Alterations in RBC morphology included spherocytes, schistocytes, dacryocytes, codocytes, and vacuolated RBCs. Bone marrow was characterized by erythroid hyperplasia and dyserythropoiesis. Features of dyserythropoiesis included lobulated nuclei, marked binucleation, arrested mitotic figures, and cytoplasmic vacuolization. The hematologic condition remained stable, but the polymyopathy was slowly progressive.

CONGENITAL ANEMIA, DYSKERATOSIS, AND PROGRESSIVE ALOPECIA IN POLLED HEREFORD CALVES

Hereford calves with congenital anemia, dyskeratosis, and progressive alopecia have a congenital syndrome characterized by dyserythropoiesis and progressive alopecia associated with dyskeratotic hyperkeratosis.[12,13] A familial pattern has been demonstrated which is consistent with a simple autosomal recessive mode of inheritance.

Clinically, the condition is slowly progressive, but is frequently fatal.[12] Iron, cobalamin, and folate deficiencies were ruled out, as causes and viral agents were not identified in affected calves. Affected calves have a macrocytic normochromic anemia with a mild reticulocytosis. Dyserythropoiesis in bone marrow is characterized by multinuclearity, nuclear bridging, irregular nuclear shapes, and irregular chromatin patterns. These dysplastic changes resemble those associated with Poodle macrocytosis and congenital dyserythropoiesis type I in humans.[2,6]

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CHAPTER 32

Anemia Associated with Oxidative Injury

ERICA BEHLING-KELLY and ASHLEIGH NEWMAN

Pathophysiology of Oxidative Injury
  Visually Evident Oxidation
  Visually Occult Oxidation
Specific Causes of Oxidative Injury in Domestic Species
  Feline Species
    Increased Susceptibility of Feline Hemoglobin
    Acetaminophen (Paracetamol)
    Diabetes Mellitus
    Propylene Glycol
    Hyperthyroidism
    Lymphoma
    Phenazopyridine
    Methylene Blue
    Benzocaine
    Onions
  Canine Species
    Plants of the Allium Family (Onions/Garlic/Chives)
    Acetaminophen (Paracetamol)
    Skunk Musk
    Zinc
    Naphthalene
    Diabetes Mellitus
    T Cell Lymphoma
    Vitamin K and Vitamin K Antagonists
    Benzocaine
  Equine Species
    Red Maple Leaves
    Garlic and Onions
    Phenothiazine
    Lymphoma
  Bovine/Ovine Species
    Selenium/Copper Deficiency
    Copper Intoxication
    Brassica
    Onions/Garlic/Chives
    Oak Leaves and Shrubs
  Miscellaneous Species
    Avian
    Reptiles
    Mink

Acronyms and Abbreviations

FDA, Food and Drug Administration; G6PD, glucose-6-phosphate dehydrogenase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; HB, Heinz body; HK, high potassium; MB, methylene blue; NAPQI, *N*-acetyl-*para*-benzoquinoneimine; PAP, *para*-aminophenol; PDB, paradichlorobenzene; PG, propylene glycol; PS, phosphatidylserine; SAMe, *S*-adenosylmethionine; SMCO, *S*-methylcysteine sulfoxide; SOD, superoxide dismutase; TBARs, thiobarbituric acid reactive substances.

PATHOPHYSIOLOGY OF OXIDATIVE INJURY

RBCs are highly specialized cells that cyclically perform the critical function of oxygen transport in a wide range of potentially hostile environments. Oxygen carried by the RBC is a strong oxidant, since it can generate highly reactive derivatives, such as superoxide free radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) by reacting with iron. These oxidants are constantly produced, and the inability to synthesize proteins de novo limits the RBC's arsenal to preformed enzymes including superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx), and reduced glutathione (GSH) (see Chapter 21). When these anti-oxidant systems

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are overwhelmed, oxidative damage to RBCs occurs, which can either be seen grossly and/or microscopically or can be occult. Oxidative modification of the RBC membrane and oxidant-induced clustering of band-3 alter the rigidity and therefore the circulating life span of RBCs.[50,49]

Visually Evident Oxidation

Heinz bodies (HBs), eccentrocytes, and methemoglobin are all visible consequences of RBC oxidative injury. When present in the context of an anemia, a causative association is supported. In the context of a nonanemic animal, it is plausible that the erythropoietic drive is compensating for a shortened RBC life span. This compensation may cause a mild reticulocytosis. In 1890, Dr. Robert Heinz first described round structures protruding from the surface of RBCs of humans and animals exposed to certain types of coal tar drugs.[28] These structures, which were associated with RBC destruction, were subsequently called Heinz bodies and can be found on the surface of RBCs in several species secondary to oxidative injury. Heinz bodies are formed by denatured clumps of precipitated hemoglobin on the inner surface of the RBC (Figure 32.1). These structures can more easily be seen with new methylene blue (MB) staining (Figure 32.2). RBCs plagued by this precipitation of hemoglobin have decreased deformability, which presumptively leads to hemolysis and/or accelerated removal, as extrapolated from ex vivo filtration studies.[12,34]

Eccentrocytes (alternatively referred to as erythrocyte hemighosts) are erythrocytes characterized by the concentration of hemoglobin on one side of the cell leaving a pale or clear portion on the other side (see Chapter 24).[9]

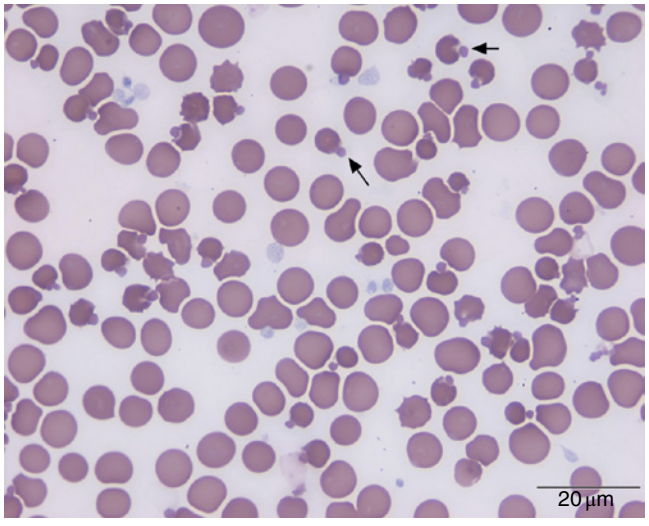


FIGURE 32.1 Photomicrograph of a blood smear from a horse with red-maple-leaf-induced Heinz-body hemolytic anemia. 100× objective, Wright–Giemsa stain. The arrows indicate prominent Heinz bodies along the margins of the RBCs. Note the prominent anisocytosis. (Case material provided by Dr. Laura Snyder)

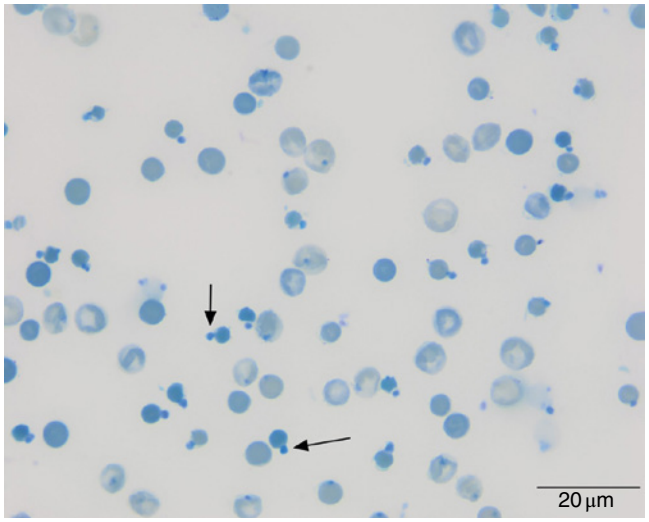


FIGURE 32.2 Photomicrograph of a blood smear from a horse with red-maple-leaf-induced Heinz-body hemolytic anemia. 100× objective, new methylene blue stain. The arrows indicate prominent Heinz bodies along the margins of the RBCs. (Case material provided by Dr. Laura Snyder)

The term eccentrocytes was first applied in dogs given acetylphenylhydrazine, a drug used to treat polycythemia.[25] Two theories exist regarding the formation of these cells. First, direct damage to the RBC membrane with adhesion of the opposing faces of the membrane has been proposed.[9] Disulfide-linked polypeptide aggregates have been identified in the RBC membranes from patients with the quintessential eccentrocytic disease, glucose-6-phosphate dehydrogenase (G6PD) deficiency. The majority of the links were intramolecular and concentrated on cytoskeletal anchoring proteins. Intermolecular aggregates were more spectrin-associated.[21] Second, the membrane lesion may form secondary to precipitation of oxidized hemoglobin and bystander oxidation of the membrane.[57]

Methemoglobin is formed by the oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) in the heme groups and can be seen alone or in combination with HBs and/or eccentrocytes. Methemoglobinemia is grossly visible by the brown discoloration of blood, which can be confirmed by placing a drop of blood on a white piece of paper.[20]

Visually Occult Oxidation

Many diseases with an alternative primary etiology causing an anemia have a contributory oxidative component. Eryptosis, a form of programed cell death that results in accelerated clearance of RBCs occurs in the context of immune-mediated anemias and oxidative stress. Oxidative stress can result in the activation of Ca^{2+} channels in the membrane and Ca^{2+} entry into the cell. The inward flux of Ca^{2+} ultimately leads to membrane scrambling, exposure of phosphatidylserine (PS) on the outer leaflet, and cell shrinkage due to water loss. In people, a plethora of diseases including diabetes, hepatic failure, cancer, chronic kidney disease, hemolytic

disease, and inflammation all present with concurrent evidence of oxidative stress and increased numbers of eryptotic RBCs.[6]

SPECIFIC CAUSES OF OXIDATIVE INJURY IN DOMESTIC SPECIES

Feline Species

Increased Susceptibility of Feline Hemoglobin

Feline hemoglobin is structurally different from that of other mammalian species. Cats possess two species of hemoglobin, based on differences in the β-chain. Feline hemoglobin also has eight sulfhydryl groups, as opposed to the two on human hemoglobin and that of other domestic species.[54] The sulfhydryl groups contribute to a lower oxygen affinity and resistance to allosteric inhibitors, but also render feline hemoglobin more susceptible to oxidation.[12,18]

The feline spleen is non-sinusoidal and is relatively inefficient at removing HBs from RBCs because of the large pores in splenic pulp venules, which allow RBCs to enter without deformation. This is thought to contribute to the routine presence of low numbers of small HBs in feline blood.

Because RBCs may circulate relatively unimpeded in the feline spleen, splenic destruction of RBCs is thought to not be the major mechanism for the development of anemia in cases of oxidative stress in cats. Thus, additional mechanisms are likely present, such as increased rigidity of the RBC membrane. Alterations in surface charge distribution and hemoglobin electrophoretic migration patterns have been documented in the context of oxidative injury, but the contribution of these alterations to accelerated clearance is not fully elucidated.[12]

Acetaminophen (Paracetamol)

Acetaminophen toxicity is the leading cause of acute liver failure in people in the United States and Canada. In people and most mammals, hepatotoxicity occurs when GSH is depleted and glucuronidation and sulfation pathways are saturated, leading to metabolism of acetaminophen by P450 enzymes that generate a highly reactive metabolite, *N*-acetyl-*para*-benzoquinoneimine (NAPQI). NAPQI forms protein adducts by reacting with sulfhydryl groups on cysteine and lysine residues. Protein adduct formation on mitochondrial proteins precipitates oxidative stress, mitochondrial dysfunction, and ultimately cell death.[37]

Dogs and cats with acetaminophen toxicity are unique, because they tend to develop methemoglobinemia with signs of cyanosis, depression, facial edema, dyspnea, and vomiting occurring 2–4 hours post intoxication.[3] Oxidative hemolytic anemia is usually seen 48–72 hours post intoxication.[35] Cats were once thought to have increased susceptibility to acetaminophen toxicity due to low hepatic levels of a specific uridine diphosphate-glucuronosyltransferase.[23] However, feline acetaminophen metabolism is primarily by sulfation, with glucuronidation playing a minimal role (less than 1.5%).[35] Sulfation pathways are less robust, allowing accumulation of toxic metabolites.

The metabolite responsible for the hematological manifestations of acetaminophen toxicity has not been definitively identified. NAPQI was hypothesized to be the culprit responsible for oxidizing both hemoglobin and ferrous iron resulting in HB and methemoglobin formation, respectively. However, the RBC lacks the P450 enzymes required to generate this metabolite, and the mechanism by which this metabolite would escape from hepatocytes and enter the RBC remains unclear, given both its electrophilic nature and propensity to react with mitochondrial proteins.

More recent work found that a minor acetaminophen metabolite, *para*-aminophenol (PAP), can react with oxyhemoglobin in a redox fashion.[35] In experiments incubating canine and feline RBCs with PAP or NAPQI ex vivo, PAP elicited formation of methemoglobin, whereas NAPQI and the parent compound had no effect.[35] Additionally, the lack of hepatic N-acetyltransferase activity in the dog and limited activity in the cat may contribute to increased formation of the PAP metabolite.[35]

There is no safe dose of acetaminophen for cats. Toxic doses as low as 10 mg/kg of body weight have resulted in death in cats. In one case report, even exposure to an empty bottle that previously held acetaminophen resulted in classic symptoms of toxicity.[3]

Diabetes Mellitus

Oxygen-radical-mediated damage has been recognized in both humans and animals with diabetes mellitus. Oxidative damage is caused by the formation of radicals secondary to glucose auto-oxidation, compromised antioxidant systems, oxidized plasma lipoproteins, alterations in inflammatory mediators, as well as changes in antioxidant nutrient status.[13] Cats with diabetic ketoacidosis can have significantly more HBs than cats with nonketotic diabetes and healthy controls.[13] In one study, the percentage of HBs in diabetic cats showed a moderate positive correlation with plasma ß-hydroxybutyrate concentration and an inverse correlation with serum chloride concentration and RBC glutathione concentrations.[13] In this study, the ketoacidotic cats were mildly anemic.

Cats with diabetes mellitus may become severely hypophosphatemic. Hypophosphatemia may impair phosphorylation of glucose to glucose-6-phosphate, preventing glucose entry into the pentose monophosphate shunt and thus impair formation of GSH; decreased GSH could further result in increased HB formation.[1]

Propylene Glycol

Although propylene glycol (PG) itself is "generally recognized as safe" by the FDA and is nontoxic in vitro to RBCs, it has been proven to be deleterious to feline

RBCs.[12] Experimental feeding of PG to cats at a dose of 1.6 g/kg, roughly equivalent to the 6–13% previously used in semi-moist cat food, for 5 weeks resulted in formation of HBs and decreased RBC life span, but not the development of anemia. Feeding cats a higher dose, 8 g/kg/day for 3 weeks, resulted in a mild anemia with punctate reticulocytosis and bone marrow erythroid hyperplasia.[11] Despite the absence of anemia in the majority of exposed cats, the species-specific evidence of RBC oxidative injury pushed the FDA's Center for Veterinary Medicine to prohibit the use of PG in cat food.

Hyperthyroidism

To the authors' knowledge, only one study has looked at the association between hyperthyroidism and the development of HBs in cats.[10] In this study, 15 out of 21 cats with hyperthyroidism had an increased percentage of HBs and their presence usually did not result in overt anemia.[10]

Lymphoma

One study investigated the relationship between lymphoma and HB formation.[10] In this study, 15 out of 17 cats with lymphoma had increased numbers of HBs.

Phenazopyridine

Use of phenazopyridine (Pyridium®), alone or in combination with other drugs, is contraindicated in cats, because of the high risk of dose-related methemoglobinemia and HB formation.[26]

Methylene Blue

MB is used in veterinary medicine to therapeutically reduce methemoglobin, or less commonly as a urinary antiseptic.[17,46,47] Intravenous administration of single or limited doses of MB to treat methemoglobinemia did not result in anemia despite an increase in the number of HB-containing RBCs.[46] Methemoglobinemia due to a cytochrome B5 reductase deficiency was responsive to treatment with methylene blue.[47]

Benzocaine

Benzocaine is an ethyl ester of *p*-aminobenzoic acid that is used as a local anesthetic in several over-the-counter medications (e.g., Lanacane®). To our knowledge, only one case of benzocaine intoxication has been reported in cats.[59]

Onions

Onions (*Allium cepa*) are considered to cause oxidative damage through thiosulfate compounds that form disulfide bonds with hemoglobin or GSH.[61] Onion intoxication is far less common in cats than dogs, probably due to the more finicky eating habits of cats. However, baby food, commonly fed to sick anorexic cats, may contain onion powder causing HB formation and sometimes anemia. The rate and degree of HB formation vary depending on the concentration of onion powder in the baby food.[61]

Canine Species

Plants of the Allium Family (Onions/Garlic/Chives)

All types of onions (i.e., raw, cooked, or dehydrated) can be toxic to dogs.[29] In onions, *n*-propyl disulfide, sodium *n*-propyl thiosulfate, sodium *trans*-1-propenyl thiosulfate, and sodium *cis*-1-propenyl thiosulfate are thought to be the agents responsible for oxidative injury.[61,60] Ingestion of garlic (*Allium sativum*) and Chinese chive (*Allium tuberosum*) can also result in oxidative injury.[60] The oxidative agent present in garlic is sodium 2-propenyl thiosulfate.[60] In cases of natural intoxication with garlic and/or Chinese chive, eccentrocytosis seems a more prominent feature of anemia than HB formation.[60] In dogs, there seems to be a correlation between the animal's weight and the development of HBs in cases of onion/garlic/Chinese chive intoxication, as HB anemia is mostly seen in small breeds. So-called high potassium (HK) breeds (e.g., Akitas, Japanese mongrels, Shibas, Jindos, and Chinese Shar-Peis) are more susceptible to onion-induced oxidative damage than non-HK dogs.[61]

Acetaminophen (Paracetamol)

Acetaminophen can result in both hepatic damage (zone-3 hepatocellular necrosis) and oxidative injury (eccentrocytes, HBs, and methemoglobinemia) in dogs. The toxic dose in dogs is around 200 mg/kg body weight.[48] Clinical signs may include tachycardia, tachypnea, blue-gray or pale brown mucous membranes, facial edema, as well as brown urine and blood.[5] The mechanisms for RBC oxidative damage are discussed in the feline section. Given the low therapeutic window and availability of alternatives, this medication should be avoided.

Skunk Musk

Spraying with skunk musk is a rare cause of HB anemia and methemoglobinemia in dogs.[5] Skunk musk contains thiols which may potentially react with oxyhemoglobin, forming methemoglobin thiol radicals and hydrogen peroxide.[5] Both thiol radicals and hydrogen peroxide are highly reactive substances that may react with sulfhydryl groups on hemoglobin, resulting in the formation of HBs.

Zinc

Most cases of zinc-induced hemolytic anemia are related to ingestion of zinc-containing material. Skin ointments, pennies minted in the USA after 1982, and metallic objects such as toys or nuts coated with zinc represent the three most common sources of exposure.[5,24] Most dogs with zinc intoxication are young (less than 2 years of age), presumably since younger animals are more

likely to ingest foreign objects than older animals. Vomiting secondary to gastrointestinal irritation is the most common clinical presentation. In one study,[24] smaller dogs (mean weight 6 kg) were more likely to be intoxicated than larger dogs. Smaller dogs have smaller pylori, making it more difficult for objects to pass from the stomach into the small intestine, allowing more time for erosion by gastric acid, thus freeing more zinc for absorption. A dose-related effect may also be involved.[24] The pathophysiology of zinc-induced hemolysis in veterinary medicine is not completely understood, but inhibition of GR and enzymes of the hexose-monophosphate pathway probably play a role. Not all dogs with zinc intoxication develop RBC oxidative injury with formation of HBs or eccentrocytes.[24]

Naphthalene

Naphthalene, derived from coal tar, can be found in mothballs, driveway sealants, and toilet bowl deodorizers.[16] Naphthalene is metabolized by the liver to α-naphthol, ß-naphthol, α-naphtholquinone, and ß-naphtholquinone.[16] The metabolite responsible for the hemolytic crisis and HB anemia is α-naphthol.

Naphthalene intoxication seems relatively common in human medicine, but, to our knowledge, is rare in veterinary medicine with only one case reported in a dog.[16]

Diabetes Mellitus

Pathophysiology and mechanisms for oxidative injury to RBCs in dogs with diabetes mellitus are similar to those in cats. Similar to cats, dogs with diabetic ketoacidosis are at greater risk for oxidative damage than nonketoacidotic animals.[9] In a retrospective analysis, 8/33 (24%) dogs with diabetes mellitus had eccentrocytosis, versus 36.8% of the dogs with diabetic ketoacidosis.[35]

T Cell Lymphoma

In a retrospective study, several dogs with T-cell lymphoma had eccentrocytes indicative of RBC oxidative injury. The mechanism for oxidative injury is unclear and detailed information regarding the stage of lymphoma and drug history were not provided in the study.[9]

Vitamin K and Vitamin K Antagonists

Both vitamin K administration and vitamin K antagonist intoxication have been reported as causes of oxidative injury in dogs.[9,19] In cases of vitamin K administration, both vitamin K_3 and vitamin K_1 may result in HB formation, with vitamin K_3 being more toxic than vitamin K_1.[11] Vitamin K_3 reacts with hemoglobin and oxidizes oxyhemoglobin to methemoglobin with production of an intermediate, the semiquinone radical.[19] Semiquinone can combine with O_2 to produce H_2O_2 and contribute to RBC membrane damage. In the study by Caldin et al.,[9] more dogs that were intoxicated with vitamin K antagonists had eccentrocytosis than dogs with vitamin K administration. It is therefore possible that HBs present in dogs treated with vitamin K were present prior to vitamin K administration. Different explanations are possible for oxidative injury in cases of vitamin K antagonist intoxication. First, vitamin K antagonists themselves may have oxidative potential. It is also possible that an additive to the drug has oxidant properties. Finally, pathological abnormalities caused by vitamin K antagonism (e.g., acidosis caused by blood loss) may lead to oxidative damage.[9] In conclusion, both vitamin K administration and vitamin K antagonist intoxication can cause oxidative damage to RBCs, resulting in the formation of HBs and/or eccentrocytes.

Benzocaine

Rare cases of HB anemia/methemoglobinemia have been reported in dogs secondary to benzocaine-containing products.[27,15]

Equine Species

Red Maple Leaves

Dried red maple (*Acer rubrum*) leaves can induce toxicosis with oxidative injury to the RBC membrane and hemoglobin, resulting in hemolytic anemia characterized by HBs, eccentrocytes, and/or methemoglobinemia.[4] There does not seem to be a sex, breed, or age predisposition for this condition and most cases seem to occur either during the summer or fall.[4] Clinical signs may include tachypnea, tachycardia, icterus, cyanosis, scleral petechiation, and brownish discoloration of urine and blood. Signs appear as soon as 24 hours after ingestion with a peak of 72 hours after ingestion.[22] In one study of 32 horses with red maple leaf intoxication, 19 horses did not survive. However, HBs or eccentrocytes were noted in only 33% of affected animals, and no relationship was found between hemoglobin or methemoglobin concentration and mortality.[4] Studies have identified putative toxic metabolites and a role for microbial metabolism in their production.[2,7]

Garlic and Onions

Garlic can be used as an antifungal ingredient in livestock feed in order to prevent mycotoxin contamination.[40] Horses fed freeze-dried garlic at a dose of 0.2 g/kg of body weight or more showed signs of oxidative injury to RBCs with anemia and presence of HBs; these changes occurred 4 days after initial feeding and were associated with sweating.[40] Complete recovery occurred within 5 weeks after cessation of garlic supplementation. Horses that graze on pastures containing wild onions have also developed HB hemolytic anemias.[41]

Phenothiazine

Phenothiazine was one of the first broad-spectrum veterinary anthelmintics. Phenothiazine is degraded in the gastrointestinal tract to phenothiazine disulfide, an oxidative

metabolite.[23] Clinical signs associated with intoxication in horses may include tachycardia, anorexia, dullness, pale mucous membranes, and opaque brown urine. The toxic dose seems to be 30 g or more. Anemia usually develops over a period of 6–13 days after administration.

Lymphoma

To our knowledge, there is only one case report of HB anemia associated with lymphoma in a Quarter Horse filly.[45] In a recent review of equine lymphoma, none of the anemic horses had morphological evidence of oxidative injury, but the majority had evidence of agglutination, suggesting an immune-mediated component.[36] The contribution of oxidative injury to increased RBC destruction has not been studied in horses with lymphoma.

Bovine/Ovine Species

Selenium/Copper Deficiency

Selenium deficiency with or without concurrent copper deficiency can be seen in cattle and sheep.[53,38] In cattle, selenium deficiency can lead to a marked decrease in the selenium-containing enzyme GPx, whereas copper deficiency may result in HB anemia because SOD is a copper-containing enzyme. SOD protects RBCs from oxidative damage caused by accumulation of superoxide.[55] If both selenium and copper deficiencies are present concurrently, a cumulative effect may be present because both enzymes are required to detoxify superoxide free radicals. Cattle grazing on St. Augustine grass displayed a high incidence of HBs and an associated anemia that responded to selenium supplementation.[38]

Copper Intoxication

In sheep, copper toxicosis may also result in hemolytic anemia due to oxidative damage. Sheep are thought to be more susceptible to copper intoxication than other species due to lower rates of biliary excretion.[51] The copper:molybdenum ratio in the diet has also been implicated as a determinant of hepatic copper levels in sheep.[39] When liver copper increases, there is increased hepatocyte turnover enhancing both cell division and apoptosis.[20] When the rate of hepatocyte apoptosis and loss exceed replacement, copper is released into circulation resulting in intravascular hemolysis. Copper intoxication is also possible in cattle, but is less common than in sheep.[55] There have been multiple case reports of copper toxicity causing HB hemolytic anemia and hemoglobinuric nephrosis in Boer goats.[8,14] A breed sensitivity has been proposed, but no genetic cause of the increased incidence has been found.

Brassica

Forage brassicas (cabbage, kale, forage rape) constitute a feed with high nutritional value, allowing a reduction in the purchase of concentrates.[43] However, brassicas contain sulfur compounds such as *S*-methylcysteine sulfoxide (SMCO) which is first converted in the rumen to thiosulfonate. Thiosulfonate is further metabolized to n-propyl disulfides, dipropyl disulfides, and dipropenyl disulfides. All three disulfides are oxidizing agents, with dipropenyl disulfides being the most powerful.

When cattle are fed brassica-type plants, HB numbers peak at 10 days to more than 4 weeks after the initiation of feeding. In extreme cases, the hematocrit may fall to 30–50% of initial values.[43] The severity and rapidity at which the anemia develops is proportional to the amount of *Brassica* in the diet. Total recovery is achieved when *Brassica* is completely removed from the diet. RBC parameters return to reference intervals 3–4 weeks after complete removal of the plants.

Onions/Garlic/Chives

Onions, garlic, and other plants of the *Allium* family contain SMCO. Cattle are less susceptible to onion toxicity than dogs and cats, but are more susceptible than sheep and goats.[44] Ruminal fermentation of SMCO leads to the formation of oxidative metabolites.[44] The results of studies in cattle are discordant. One study looking at onion feeding in cattle showed that feeding up to 25% cull onions on a dry-matter basis resulted in HBs and mild decreases in RBCs, hemoglobin, and packed cell volume, but did not result in clinical anemia. In other studies, anemias have been life-threatening. Cows fed approximately 20 kg onions/cow/day for 6 weeks developed methemoglobinemia and a hemolytic anemia that was fatal in five cows, and cows consuming vegetable waste have also developed clinical anemia.[31,44] Similar to the horse, sheep grazing on pastures containing wild onions have developed HB hemolytic anemia.[32,52]

Oak Leaves and Shrubs

Oak trees and shrubs are present in most countries, and ingestion of immature leaves or freshly fallen acorns is the most common source of intoxication. Immature leaves and acorns are rich in tannic acid that is hydrolyzed to phenolic compounds including gallic acid. Gallic acid is hypothesized to be further metabolized to pyrogallol and resorcinol.[42] Pyrogallol can trigger oxidation of hemoglobin to methemoglobin,[42] resulting in oxidative damage to RBCs. In studies administering oak leaves or commercial tannic acid orally to calves, the response differed. Calves fed oak leaves developed renal disease characterized by hematuria and glucosuria with pyrogallol detected in serum as early as 3 hours post administration, whereas calves given high doses of tannic acid developed methemoglobinemia.[42]

Miscellaneous Species

Avian

Crude oil contains polycyclic aromatic hydrocarbons (PAHs) that cause oxidative HB hemolytic anemia in oiled sea birds. The cause-and-effect relationship has

been documented by oral dosing of birds with crude oil, which has recapitulated the severe anemias documented in birds exposed to the PAHs via environmental oil spills.[33,56]

Reptiles

Snakes are highly sensitive to acetaminophen and develop a severe methemoglobinemia. Comparison of metabolic pathways in liver homogenates showed that snakes lack phenol-type glucuronidation and N-acetyltransferase activity.[30]

Mink

Mink orally dosed with propylene glycol develop a strongly regenerative anemia characterized by both eccentrocytosis and a marked increase in the number of HB.[58]

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CHAPTER 33

Anemia Caused by Rickettsia, Mycoplasma, and Protozoa

SUSAN FIELDER, ROBIN W. ALLISON, and JAMES H. MEINKOTH

Rickettsia
- Ehrlichiosis
  - Canine Monocytic Ehrlichiosis
  - Canine Granulocytic Ehrlichiosis
- Anaplasmosis
  - Granulocytic Anaplasmosis
  - Granulocytic Anaplasmosis in Dogs
  - Granulocytic Anaplasmosis in Horses
  - Granulocytic Anaplasmosis in Ruminants
  - Erythrocytic Anaplasmosis in Ruminants

Hemotrophic Mycoplasmas (Hemoplasmas)
- Canine Hemotrophic Mycoplasmas
- Feline Hemotrophic Mycoplasmas
- Swine Hemotrophic Mycoplasmas
- Ruminant Hemotrophic Mycoplasmas
- Camelid Hemotrophic Mycoplasmas

Protozoa
- Theileriosis
  - Ruminants
  - Equines
- Babesiosis
  - Canines
  - Ruminants
  - Equines
  - Felines
- Feline Cytauxzoonosis
- Trypanosomiasis
- Sarcocystosis
- Haemoproteus
- Plasmodium

Acronyms and Abbreviations

CNS, central nervous system; FeLV, feline leukemia virus; FIV, feline immunodeficiency virus; IMHA, immune-mediated hemolytic anemia.

A seemingly ever-increasing number of rickettsial, mycoplasmal, and protozoal agents are being recognized as the cause of anemia in veterinary species. The anemia is often hemolytic in nature; however, with some organisms the anemia is nonregenerative resulting from either cytokine suppression of hematopoiesis or bone marrow pathology. Of importance to the veterinary hematologist is that many of these organisms can be visualized on blood films, providing a definitive diagnosis.

RICKETTSIA

The order *Rickettsiales* contains many organisms that cause significant hematologic disease in veterinary species. Many of the genera in this order have undergone reclassification. The order now contains two families, *Anaplasmataceae* and *Rickettsiaceae*. The *Anaplasmataceae* are intracellular parasites that grow within a cytoplasmic vacuole in the host cell as opposed to the *Rickettsiaceae* which infect the host cell cytoplasm or nucleus and are not bounded by a vacuole.

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Ehrlichiosis

Dogs may be infected with a variety of ehrlichial organisms including *Ehrlichia canis*, *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, Panola Mountain *Ehrlichia sp*., and *Ehrlichia muris*. Marked cross-reactivity of the resulting antibody response on serologic assays and to some degree cross-reactivity on molecular testing can lead to difficulty in definitively identifying the causative agent unless sequencing of a PCR amplicon is performed for confirmation.[1,42] Severe clinical disease is most commonly seen with *E. canis* infection, while infection with *E. ewingii* is generally milder and infection with *E. chaffeensis* causes inapparent to mild disease. Despite causing milder disease, infection with *E. ewingii* and *E. chaffeensis* is much more common in areas with high vector tick populations, and this must be considered in interpreting serologic test results.[42]

Canine Monocytic Ehrlichiosis

E. canis is the most significant cause of canine monocytic ehrlichiosis worldwide, as far as clinical disease severity. Transmission is by tick vectors (primarily *Rhipicephalus sanguineus*) and direct blood inoculation. Organisms replicate in cells of the mononuclear phagocyte system and can be seen in lymphocytes, monocytes, or macrophages.[55] Within mononuclear cells, organisms multiply by binary fission within a cytoplasmic vacuole. They appear by light microscopy as round, variably sized, basophilic cytoplasmic inclusions termed morulae that have a punctate, granular appearance (Figure 33.1). Parasitemia in peripheral blood is extremely low and transient following initial infection, so they are rarely found on routine examination of peripheral blood smears from affected animals.

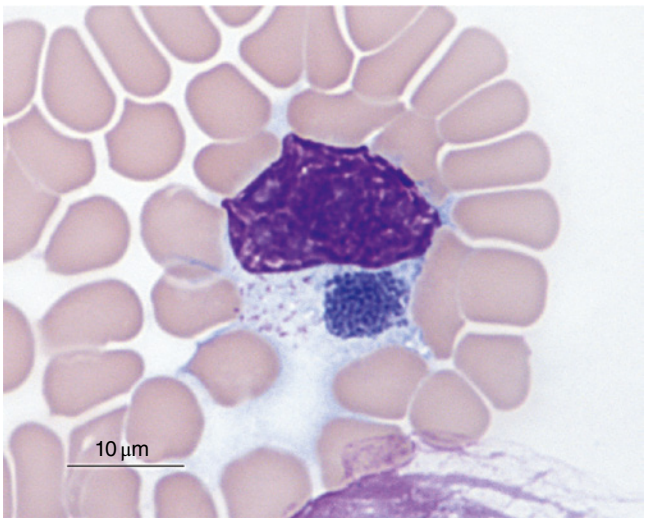


FIGURE 33.1 Blood film from a dog with *E. canis* infection. A morula is present in a disrupted large granular lymphocyte. The morula has the distinctive punctate, granular appearance. Aqueous Romanowsky stain.

Prolonged examination of buffy coat preparations increases identification, but is still generally unrewarding.[1] Morulae may be identified on cytologic preparations of tissue aspirates (lymph node, spleen, bone marrow, respiratory wash) during acute disease; however, diagnosis is most often confirmed by a combination of serology and PCR.

Hematologic findings depend on the stage of infection. Acute infection is associated with thrombocytopenia, which may be marked and associated with clinical bleeding (e.g., epistaxis), and nonregenerative anemia. Leukopenia and neutropenia are less consistent. Following acute infection, there is a subclinical phase during which anemia typically resolves but thrombocytopenia may be persistent. Dogs that develop the severe chronic phase typically have profound pancytopenia. Bone marrow aspirates obtained in these animals are hypocellular with marked reductions of all hematopoietic cell lines.[13] German Shepherd dogs are particularly prone to severe clinical disease.[42]

Canine Granulocytic Ehrlichiosis

The causative agent of granulocytic ehrlichiosis is *E. ewingii*.[2] Transmitted by *Amblyomma americanum*, *E. ewingii* has been reported from the Southern and Southeastern United States.[32] Clinical disease is considerably milder than that caused by *E. canis* and generally consists of nonspecific clinical findings such as fever, lethargy, and anorexia.[42] Polyarthritis is one clinical manifestation of infection that occurs in a minority of infected dogs, but should prompt consideration of this disease in endemic areas.

Thrombocytopenia, often marked, is the most common hematologic change in most reports, and provides an index of suspicion for this disease in endemic areas. Anemia is also a common finding. Most reports describe a relatively nonspecific mild to moderate nonregenerative anemia.[32] However, a recent review reported immune-mediated hemolytic anemia (IMHA) in approximately 15% of cases.[61] These dogs were extensively tested as being negative for co-infections leading the authors to suggest infection with this organism may be an underappreciated cause of immune-mediated hemolysis.[61] While there is some evidence to suggest *E. ewingii* infection may be persistent, such infections are not associated with a chronic-phase pancytopenia as is seen with *E. canis*.[61]

Morulae of *E. ewingii* are found predominantly in neutrophils and rarely in eosinophils. Morulae are similar in appearance to those of *E. canis*, although they sometimes have a "packeted" appearance rather than fine punctate granulation typical of *E. canis*. Organisms are seen in the peripheral blood only transiently during acute infection (approximately 2 weeks), but parasitemia is often high enough to allow diagnosis by microscopy.[1,32] Organisms may be identified in neutrophils of synovial fluid samples in dogs with polyarthritis. No specific serologic test for *E. ewingii* exists, but infection can be confirmed by appropriate PCR testing which is commercially available.[32,61]

Anaplasmosis

Historically, members of the genus *Anaplasma* infect RBCs of ruminants causing hemolytic anemia. However, with the reclassification of several *Ehrlichia spp*. as *Anaplasma spp*., members of this genus may infect RBCs (*Anaplasma marginale*), granulocytes (*Anaplasma phagocytophilum*), or platelets (*Anaplasma platys*). Anemia is not a main feature of *A. platys* infection and is not discussed in this chapter.

Granulocytic Anaplasmosis

A. phagocytophilum now includes the organisms previously described as *Ehrlichia phagocytophila*, *Ehrlichia equi*, and the human granulocytic ehrlichiosis (HGE) agent.

Granulocytic Anaplasmosis in Dogs

A. phagocytophilum infection in dogs is widespread. In the United States, it occurs most commonly in the Upper Midwest and along the Pacific coast.[33] Clinical signs are nonspecific and include fever, anorexia, depression, lymphadenopathy, and splenomegaly.[33] Some dogs may manifest either a neutrophilic polyarthritis or acute central nervous system (CNS) signs, including seizures.

Thrombocytopenia, mild to severe, is the most common hematologic abnormality and occurs in the majority of cases.[33] Mild to moderate anemia is also common.[33] Anemia is typically nonregenerative, but sporadic reports of immune-mediated hemolysis, as evidenced by regenerative anemia and spherocytosis and/or a positive Coombs test, have been reported in some cases.[18,47]

Morulae are similar to those of *E. ewingii* and are present at detectable levels during the acute phase of infection (approximately 4–14 days) in many cases.[16] Morulae may also be found in neutrophils upon examination of cytologic preparations of synovial fluid of dogs manifesting polyarthritis.

Granulocytic Anaplasmosis in Horses

A. phagocytophilum infection in horses has been reported from many states in the USA as well as Canada, Brazil, and Europe.[44] It can be transmitted by *Ixodes pacificus* and *Ixodes scapularis* and by direct blood inoculation from infected animals.[60] It causes a febrile illness characterized by anorexia, limb edema, petechiation, icterus, and reluctance to move. Clinical disease is less severe in horses under 4 years of age. The predominant hematologic finding is a profound thrombocytopenia, but variable neutropenia and mild normocytic, normochromic anemia may also be present.[29,67] The parasitemia may be high with morulae readily seen in peripheral blood neutrophils during acute infection.

Granulocytic Anaplasmosis in Ruminants

A. phagocytophilum causes the disease known as tick-borne fever, the most common tick-borne disease of animals in Europe. Disease is most common in cattle and sheep. Disease in cattle commonly manifests as abortion with reduced milk yield and as respiratory disease. In sheep, young lambs have a febrile illness, while abortions may be seen in ewes. Unlike erythrocytic anaplasmosis in ruminants, anemia is not a prominent feature of the clinical disease.

Erythrocytic Anaplasmosis in Ruminants

Anaplasmosis is a significant cause of disease characterized by marked hemolytic anemia in cattle.[41] Two species are described: *A. marginale* is widespread and *Anaplasma centrale* occurs in South America and South Africa. Wild ruminants are reservoirs, but do not usually manifest disease.

A. marginale infection causes fever and mild to marked hemolytic anemia in cattle. Decreased milk production and abortion add to economic loss. Numerous tick species serve as biological vectors and mechanical transmission may also occur via flies or medical procedures (needles, dehorning, etc.). The hemolysis is primarily extravascular; hemoglobinemia and hemoglobinuria are not seen. Disease is most pronounced in older animals; calves 6–9 months old are relatively resistant and disease is mild, unless they are splenectomized. *A. centrale* is generally less pathogenic than *A. marginale*, causing mild or inapparent disease in cattle, sheep, and goats.

Organisms are visible in erythrocytes during acute infection. They are round, 0.5–1 μm, basophilic bodies often present on the periphery of RBCs (Figure 33.2). They resemble Howell–Jolly bodies, from which they must be differentiated. *A. centrale* organisms are similar, but not peripherally located in the RBCs. Although long thought to occur only in RBCs, *A. marginale* has been shown to invade microvascular endothelial cells both in vivo and in vitro.[17]

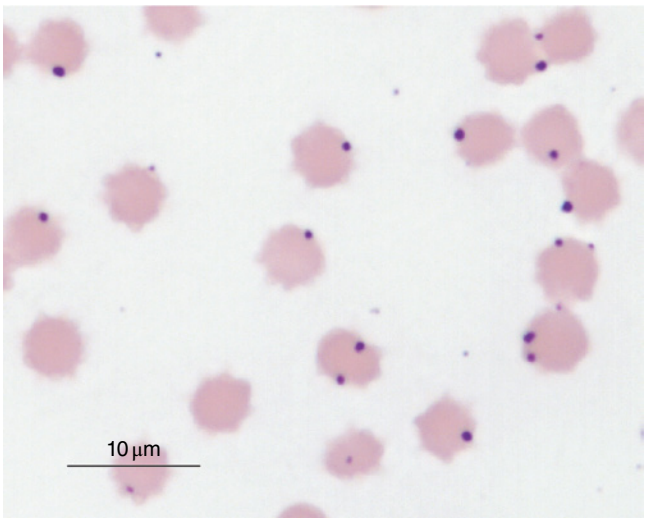


FIGURE 33.2 Blood film from a steer experimental infection with *A. marginale*. Marked parasitemia with more than 50% of erythrocytes containing inclusions. Note the marginal position of most organisms. Aqueous Romanowsky stain.

After infection, parasitemia increases until the hemolytic crisis, frequently with more than 50% of the RBCs infected.[41] After development of the anemia, parasite numbers decline due to removal of infected RBCs. Cattle that survive acute infection develop lifelong persistent infections. Persistent carriers are immune to clinical disease upon reinfection, but act as important reservoirs for infection of ticks and mechanical transmission to other animals.

Anaplasma ovis causes anaplasmosis in sheep and goats. The disease occurs in tropical and subtropical regions throughout the world. Morphologically, *A. ovis* is similar to *A. marginale*.

HEMOTROPHIC MYCOPLASMAS (HEMOPLASMAS)

Epierythrocytic parasites previously known as *Haemobartonella* and *Eperythrozoon* have been reclassified to the genus *Mycoplasma* and renamed, some with the designation *Candidatus* indicating they are incompletely described. As a group, these hemoplasmas have worldwide distribution, infect a wide variety of vertebrate animals, and share similar characteristics and morphologic features.[53] On routine blood films they are small basophilic round, rod, or ring-shaped organisms that appear on RBCs individually or in chains. Organisms are typically found in depressions and invaginations in RBC membranes and appear connected to the membrane by delicate fibrils.[77]

Hemoplasmas vary in their ability to cause clinically apparent disease, but infected animals may remain carriers despite antibiotic therapy; parasitemia may re-emerge in times of stress. Hypoglycemia secondary to glucose consumption by the bacteria has been reported in heavily parasitized pigs, sheep, llamas, and calves, however, rapid bacterial glycolysis *in vitro* may also cause artifactually decreased blood glucose concentrations.[14] Historically, diagnosis has relied upon detection of hemoplasmas on blood films. Organisms are generally found associated with the RBC membrane, but sometimes may be free in the plasma (Figure 33.3). Hemoplasmas dissociate from erythrocytes and die after a variable time in EDTA.[57] In aged samples, dead organisms can appear as pale pink clumped granular material in the background of the blood film (Figure 33.4). Development of sensitive PCR assays capable of discriminating between various hemoplasmas has greatly enhanced diagnosis of these parasites, and has led to the identification of several new *Mycoplasma* species.

Canine Hemotrophic Mycoplasmas

Two hemoplasmas, *Mycoplasma haemocanis* (previously *Haemobartonella canis*) and "*Candidatus* Mycoplasma haematoparvum," infect domestic canines. *M. haemocanis* causes acute hemolytic anemia in dogs that are splenectomized, immunocompromised, or have concurrent infections.[52] These dogs usually have sufficient circulating parasites to be easily demonstrated on blood films. Anemia may be severe; spherocytes may be observed and Coombs test results may be positive. By contrast, healthy dogs typically develop chronic, asymptomatic infections with sporadic, low-grade parasitemia.[52]

Transmission is presumed to occur via arthropod vectors and has been experimentally demonstrated with the brown dog tick, *R. sanguineus*. Transmission of organisms may also occur via blood transfusion from asymptomatic carriers, but the recipient must be splenectomized or otherwise severely compromised for hemolytic disease to occur.[52]

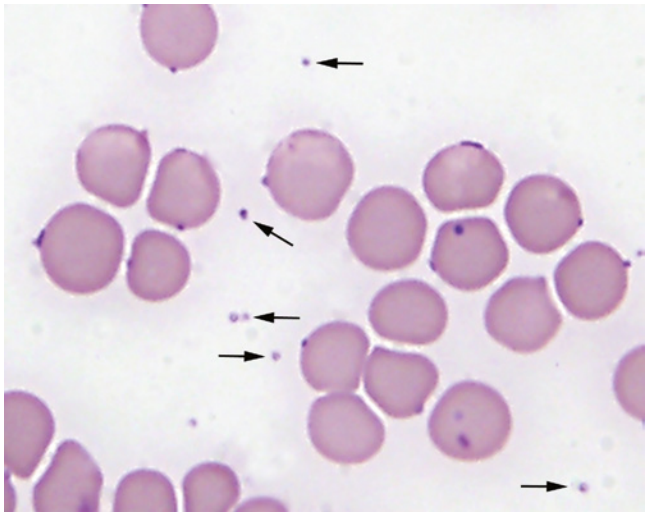


FIGURE 33.3 Blood film from a cat with *M. haemofelis* infection. Organisms are visible on the surface of erythrocytes and free in the background (arrows). Aqueous Romanowsky stain.

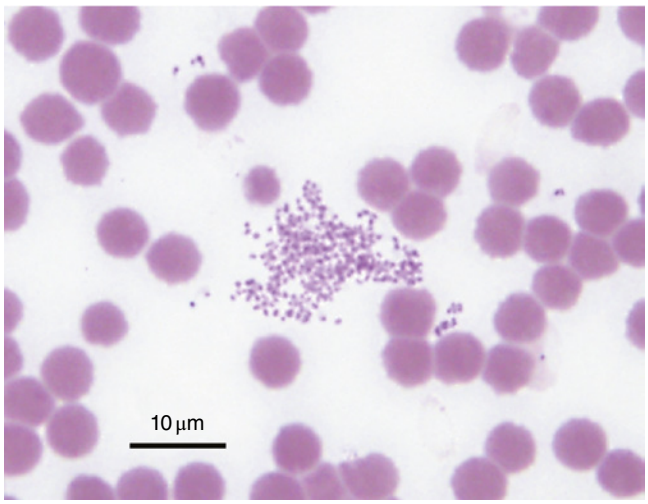


FIGURE 33.4 Blood film from a cat with *M. haemofelis* infection prepared from aged blood. Organisms have detached from erythrocytes and are degenerating, appearing as clumps of pink granular material in the background. Aqueous Romanowsky stain.

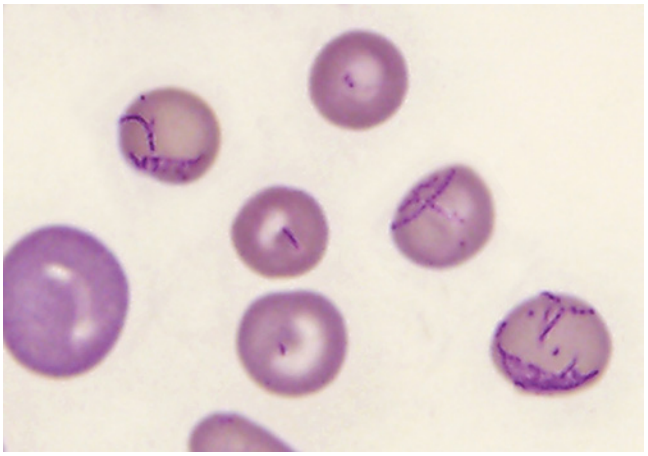


FIGURE 33.5 Blood film from a dog with *M. haemocanis* infection. Organisms are visible as chains of cocci on the surface of erythrocytes. Aqueous Romanowsky stain, 1000×.

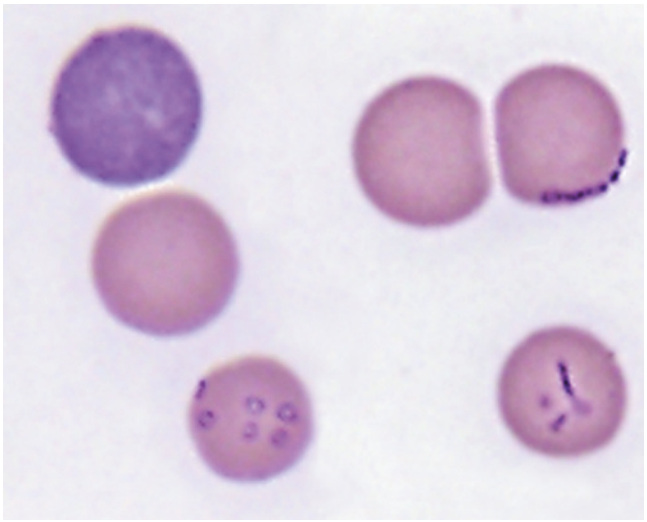


FIGURE 33.6 Blood film from a cat with *M. haemofelis* infection. Organisms are visible as chains of cocci and individual ring forms on the surface of erythrocytes. Aqueous Romanowsky stain, 1000×.

On routine blood films, *M. haemocanis* organisms are quite pleomorphic, but have a tendency to form long chains across the surface of the RBCs, sometimes with "violin bow" morphology. Individual organisms are 1–3 µm and can appear round, rod-shaped, or in ring form[77] (Figure 33.5).

"*Candidatus* Mycoplasma haematoparvum" was originally identified in a California dog with a T-cell lymphoproliferative disease that had undergone splenectomy and blood transfusions, but has since been identified in nonanemic dogs in numerous regions of the world.[3,71] Molecular characterization found the organism to be most similar to "*Candidatus* Mycoplasma haemominutum" in cats. These organisms are smaller than *M. haemocanis* or *Mycoplasma haemofelis* (<0.3 µm), and therefore are more difficult to appreciate on blood films.

Feline Hemotrophic Mycoplasmas

Three hemoplasmas, *M. haemofelis* (previously *Haemobartonella felis*), "*Candidatus* Mycoplasma haemominutum," and "*Candidatus* Mycoplasma turicensis," are currently known to infect domestic cats. *M. haemofelis* was previously described as the large variant or Ohio strain to differentiate it from the small variant or California strain that was later named "*Candidatus* M. haemominutum."[53]

M. haemofelis causes an acute hemolytic anemia characterized by variable numbers of circulating RBC parasites. Unlike *M. haemocanis*, disease occurs in immunocompetent hosts; however, retrovirus infection (feline leukemia virus (FeLV) or feline immunodeficiency virus (FIV)) may be a predisposing factor in some cases. Hemolysis is primarily extravascular and results in a regenerative anemia that may be severe. Erythrocyte agglutination may be present and Coombs test results are frequently positive. Parasitemia is cyclic, with a gradual increase in parasite numbers over a period of days followed by a sudden decline; infected erythrocytes may disappear from circulation in as little as 2 hours.[35] Cats that recover remain carriers despite treatment, with variable low-grade parasitemia.

Transmission is presumed to occur by arthropod vectors such as fleas, but experimental evidence of such transmission is lacking. Transmission has been accomplished by blood transfer and is suspected to occur through biting and fighting activity.[70] Iatrogenic transmission can occur via blood transfusions and screening of potential blood donors using PCR-based DNA tests is recommended.[34] Vertical transmission from queens to kittens may also occur.

Morphology of *M. haemofelis* on routine blood films is similar to that described for *M. haemocanis*, with round (<0.8 µm), rod (up to 1.5 µm), and ring forms observed singly, in clusters, and short chains (Figure 33.6).

"*Candidatus* Mycoplasma haemominutum" is reported to be about half the size of *M. haemofelis*, hindering visualization on blood films. Prevalence investigations utilizing PCR-based DNA tests indicate there is substantial subclinical infection in domestic cats, supporting the experimental evidence that "*Candidatus* M. haemominutum" is less pathogenic than *M. haemofelis*.[72] However, there is evidence that immunosuppressed cats or those coinfected with *M. haemofelis*, FIV, or FeLV may develop clinically significant anemia.

"*Candidatus* Mycoplasma turicensis" is relatively common, widely distributed, and often associated with "*Candidatus* M. haemominutum" co-infection.[72] Like "*Candidatus* M. haemominutum," disease in cats infected with "*Candidatus* Mycoplasma turicensis" was most often observed in immunosuppressed cats infected with FIV or FeLV.[79]

Swine Hemotrophic Mycoplasmas

Two hemoplasmas, *Mycoplasma suis* and *Mycoplasma parvum* (previously *Eperythrozoon suis* and *Eperythrozoon*

parvum), are known to infect swine.[25] *M. suis* has a worldwide distribution and causes hemolytic anemia accompanied by icterus in neonatal pigs, feeder pigs, and pregnant sows. Splenectomy of latently infected animals results in a marked increase in parasitemia, as does immunosuppressive therapy. On routine blood films, *M. suis* may appear as small (approximately 1 µm) cocci, rods, or ring forms occurring individually, in chains, or in clusters. Similar to *M. haemofelis*, parasitemia is frequently transient. PCR-based DNA tests are proving sensitive and specific for the diagnosis of *M. suis* in the absence of demonstrable parasitemia.[37]

M. parvum is a coccoid or ring-shaped organism approximately half the size of *M. suis*. Infection in splenectomized pigs resulted in few clinical signs even at peak bacteremia.[25]

Ruminant Hemotrophic Mycoplasmas

Two hemoplasmas, *Mycoplasma wenyonii* and "*Candidatus* Mycoplasma haemobos" are currently known to infect cattle. *M. wenyonii* occurs worldwide and is typically thought of as an opportunistic pathogen, resulting in clinical anemia in splenectomized or otherwise compromised cattle. Parasitemia without anemia has been reported associated with fever and mammary, scrotal, or hind limb edema.[54,68] The mode of transmission for *M. wenyonii* is unknown, but presumed to occur via vectors such as lice, flies, ticks, and mosquitoes. Iatrogenic infection from blood transfer by contaminated needles and equipment may also occur.

On blood films, *M. wenyonii* organisms are small and pleomorphic but frequently coccoid or ring-shaped, and may be found free in the background in large numbers.[68] Parasitemia is transient, however, and PCR-based DNA tests may be more sensitive than microscopy for detection of latent infections.[76]

"*Candidatus* Mycoplasma haemobos" is considered nonpathogenic and has been detected in several countries in dairy cattle. "*Candidatus* Mycoplasma haemobos" is often seen as a co-infection with *M. wenyonii* and does not appear to cause significant disease.[36]

Mycoplasma ovis (previously *Eperythrozoon ovis*) infects sheep and goats. While frequently considered relatively nonpathogenic, the severity of hemolytic anemia with acute infection is variable depending upon age, nutrition status, concurrent worm burden, and possibly *M. ovis* strain differences.[53,56] Fatal disease occurs more often in lambs than in adults. Natural transmission may occur via arthropod vectors such as mosquitoes and ticks, and iatrogenically by blood transfer. On blood films, *M. ovis* organisms are coccoid, rod, or ring-shaped, 0.5–1 µm in diameter, and are frequently seen free in the background.

Camelid Hemotrophic Mycoplasmas

"*Candidatus* Mycoplasma haemolamae," originally described as an eperythrozoon-like parasite, affects llamas, alpacas, and camels. Anemia varies from mild to severe, with more severe clinical signs found in younger animals that are stressed or have other concurrent diseases; adults may be asymptomatic. Heavy parasitemia has been identified in crias as young as 1 day of age, suggesting vertical transmission in utero. On blood films, "*Candidatus* M. haemolamae" organisms are coccoid to ring-shaped, 0.5–1 µm in diameter, and are often free in the background[62] (Figure 33.7). Asymptomatic carriers have been identified using a DNA-based PCR assay.[73]

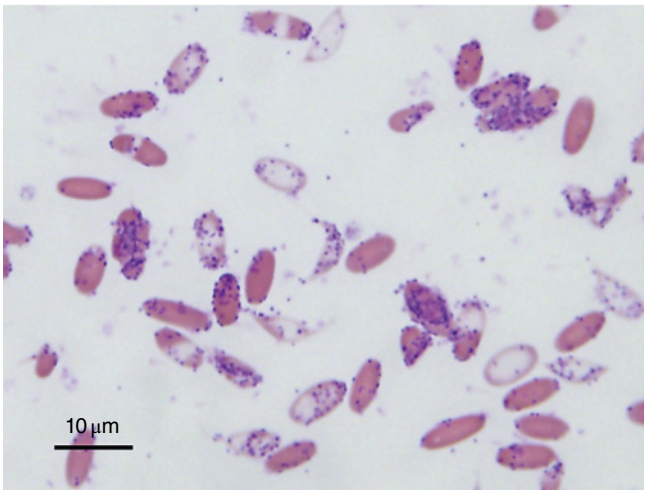


FIGURE 33.7 Blood film from a llama with "*Candidatus* M. haemolamae" infection and iron deficiency. Large numbers of coccoid organisms are present on the surface of erythrocytes and free in the background. Aqueous Romanowsky stain.

PROTOZOA

Members of two groups of apicomplexan protozoal erythroparasites, the *Babesias* and *Theilerias*, have undergone reorganization subsequent to genetic sequencing and phylogenetic studies. Historically, morphology and features of their life cycles defined them. Neither genera form pigment in the parasitized cell (as opposed to *Plasmodium* and *Hemoproteus*). Both are transmitted by ixodid ticks, but transovarial tick transmission occurs with *Babesia* species and not with *Theileria* species.[49] Additionally, *Babesia* sporozoites directly invade erythrocytes, while *Theileria* sporozoites first invade lymphocytes or macrophages to form schizonts.[49]

Theileriosis

Ruminants

There are multiple *Theileria* species that affect domestic and wild ruminants primarily in Africa, Europe, Australia, and Asia. The life cycle of these parasites involves a schizont stage, typically within lymphocytes, in addition to piroplasms within RBCs.[49] An interesting feature of the life cycle of some *Theileria* is the reversible transformation and clonal expansion of lymphocytes that occurs as a direct result of infection.[26]

Theileria parva is the most economically important, causing the rapidly fatal bovine disease East Coast fever in Africa.[10] The schizont stage of *T. parva* is the major cause of pathology. Anemia is typically mild and nonregenerative, but may become severe as erythroid precursors in bone marrow are infected. *Theileria annulata* causes tropical theileriosis in cattle from the Mediterranean to Asia.[10] Both the schizont stages and erythrocytic piroplasms are pathogenic, with severe hemolytic anemia and icterus a common occurrence. *Theileria hirci* (also known as *Theileria lestoquardi*) causes a similar hemolytic disease in sheep and goats in Asia and Africa. Several organisms have been described in oriental theileriosis (*Theileria sergenti*, *Theileria orientalis*, *Theileria buffeli*) and at this time it is unclear if these represent different subspecies or strains of the same parasite.[39] Oriental theileriosis is often benign, but hemolytic disease has been reported with some cases.[69] Many other *Theileria* species have been described, including *Theileria mutans*, *Theileria verifera*, *and Theileria taurotragi* in cattle and *Theileria cervi* in deer and elk, but seem to be less pathogenic under most circumstances.[39]

On routine blood films, theilerial piroplasms are pleomorphic, occurring as round, rod, comma, piriform, signet-ring, and racquet shapes, with occasional tetrad (Maltese cross) formation (Figure 33.8). The particular species cannot be determined based on morphology. Schizonts may be identified in lymphocytes from lymph node biopsies or aspirates.

Equines

The small piroplasm formerly described as *Babesia equi* has been renamed *Theileria equi*.[48] *T. equi* causes fever, hemolytic anemia, and icterus in horses in tropical and subtropical regions of the world. It is not yet endemic in the United States despite the presence of appropriate tick vectors and recognition of sporadic cases. The anemia varies from mild to severe, and subclinical infections may be detected by serology. *T. equi* piroplasms are small (1–2 μm) round to oval signet-ring structures within RBCs. Dividing stages may appear piriform in Maltese-cross arrangement.[48]

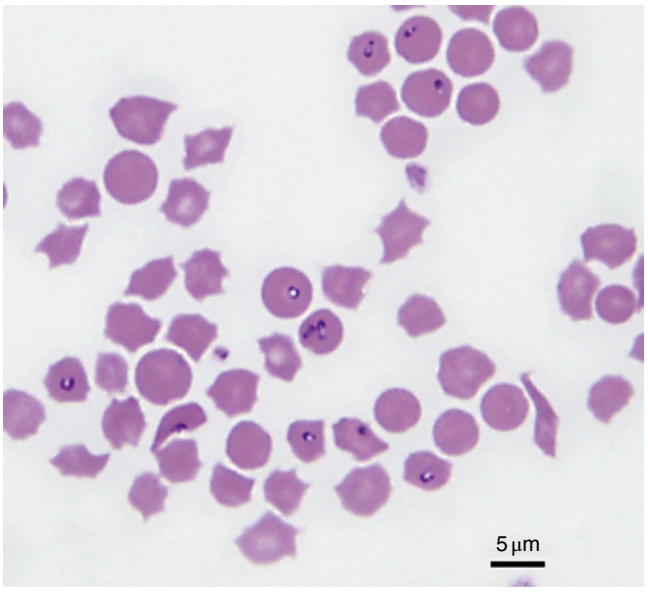


FIGURE 33.8 Blood film from a white-tailed deer with *T. cervi* infection. Small round to oval "signet-ring" piroplasms are present within erythrocytes. Aqueous Romanowsky stain.

Babesiosis

In contrast to *Theilerias*, pathogens of the genus *Babesia* are limited to RBCs within the host. Sporozoites injected into the host directly attach to, penetrate, and multiply within erythrocytes.[80] They are classified morphologically as large (>3 μm) or small (<3 μm). Organisms exit by rupturing the host RBC and then invade new RBCs. Hematologic findings are primarily hemolytic anemia, although thrombocytopenia is also common with many *Babesia* infections.

Canines

Large Canine Babesia Large canine *Babesia* appear as large (up to 5 μm), single, or paired piriform bodies (Figure 33.9). Large Babesia have historically been called *Babesia canis*. The organisms appear as large (up to 5 μm), single, or paired piriform bodies and based on differences in clinical syndromes, vectors, serologic tests, and cross-immunity were previously divided into subspecies (*B. canis canis*, *B. canis vogeli*, and *B. canis rossi*). Recently, they have been identified as three distinct species: *B. canis*, *B. vogeli*, and *B. rossi*.[65]

B. vogeli is the large *Babesia* found in the United States and also occurs in Africa, Europe, Asia, and Australia. It

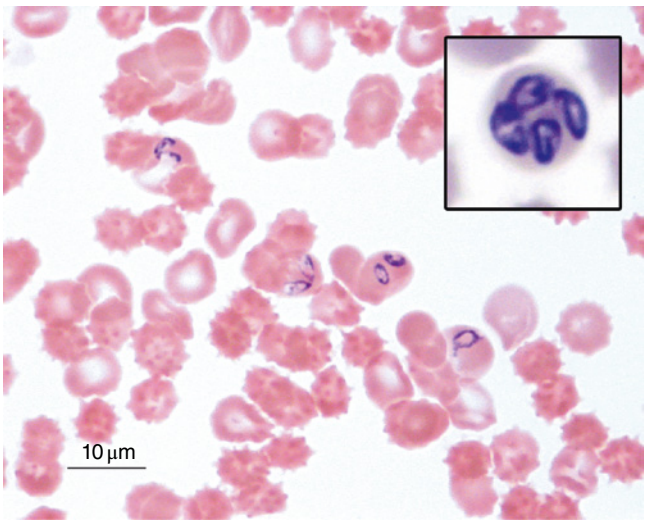


FIGURE 33.9 Blood film from a dog with *B. canis* infection. This dog did not have evidence of hemolysis (note lack of polychromasia) and probably represents a recurrent parasitemia in a chronically infected dog with subsequent immunosuppression from concurrent illness. Note the characteristic paired, piriform organism in erythrocytes. Inset: Erythrocyte containing four organisms. Aqueous Romanowsky stain.

causes relatively mild disease and is endemic in many populations of kenneled Greyhound dogs.[11] Infections are often subclinical with clinical disease most common in pups. Hematologic manifestations include a hemolytic anemia and thrombocytopenia. Parasites are rarely seen in the peripheral blood of subclinically affected animals and diagnosis generally is made by serology.

B. rossi[65] is the most virulent strain and is found in Africa. It causes a severe, hemolytic anemia which can be both intravascular and extravascular. Thrombocytopenia is also common. This parasite can induce a wide spectrum of disease manifestations unrelated to hemolysis, referred to as "complicated" babesiosis.[38]

B. canis is found in Europe and is of moderate pathogenicity. A fourth, genetically distinct, large *Babesia* has been identified in immunocompromised dogs in the Eastern United States. Based on molecular analysis, this organism is most similar to Babesia bigemina.[9,66]

Small Canine Babesia *Babesia gibsoni* has long been recognized as a problem of dogs in Asia, Africa, and Europe and has now been identified as an emerging infection of dogs in the USA.[8] In the United States, the majority of the affected dogs are American Pit Bull Terriers (APBT) or dogs that have been bitten by Pit Bulls.[43] Acutely, the organism causes a hemolytic anemia and thrombocytopenia.[8,51] Surviving dogs remain subclinical carriers and the incidence of such carriers is quite high in certain populations of APBTs.[43]

B. gibsoni can be transmitted between dogs by tick vectors, direct blood inoculation, or transplacental transmission.[31,51] In most endemic areas, *B. gibsoni* is transmitted by *Haemaphysalis* ticks.[8] It is not known if there is a competent tick vector in the United States. Clinical signs are nonspecific and generally relate to the hemolytic anemia. The anemia is regenerative; spherocytosis and positive direct Coombs test results suggest an immune-mediated component. Marked thrombocytopenia is a common finding and may occur prior to and last longer than parasitemia and/or anemia.[51] Parasitemia is detectable in the acute phase of infection, but is generally low. Parasites are small (1–2.5 µm) and pleomorphic.[43] The most common shape is a signet-ring, although elongated forms are also seen (Figure 33.10). Splenectomized animals show more severe clinical signs, more profound anemia, and a greater parasitemia than in animals with an intact spleen.[51] Serologic tests and PCR are useful for detecting chronically infected animals and have been used in prevalence studies.[7]

Babesia conradae is a small *Babesia* identified in a group of dogs (Greyhounds and Greyhound mixes) from California with a history of coyote fighting.[21] These dogs were anemic (regenerative), leukopenic, and thrombocytopenic and had multiple co-infections with *M. haemocanis*, "*Candidatus* Mycoplasma haematoparvum," and *E. canis*.[24]

Babesia vulpes (previously *Theileria annae* and *Babesia microti*-like) has been identified in the RBCs of dogs and foxes in North America and Europe. Foxes are thought to be subclinical carriers, while dogs are more severely affected with hemolytic anemia, thrombocytopenia, and azotemia.[5]

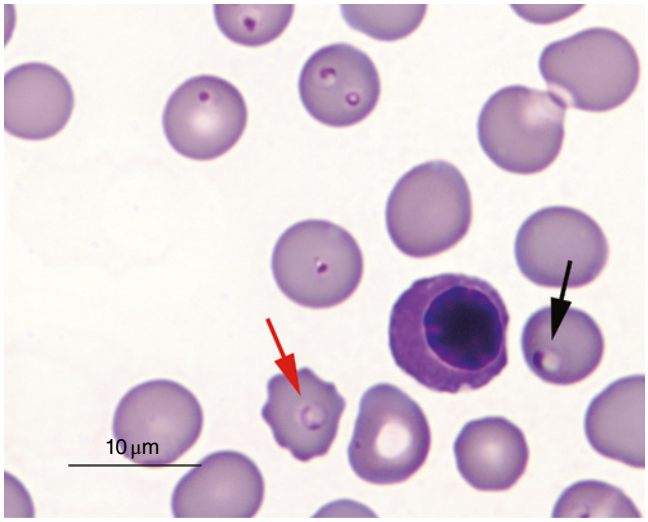


FIGURE 33.10 Blood film from an American Pit Bull Terrier with *B. gibsoni* infection. This dog suffered an acute hemolytic crisis following splenectomy for removal of a splenic tumor. Note the varying sized organisms, many of which have the classic signet-ring appearance. In some, the nuclear material is punctate (black arrow), while in others the nuclear material is spread along the periphery of the ring (red arrow). Aqueous Romanowsky stain.

Ruminants

There are four widely recognized species of *Babesia* in cattle: *Babesia bovis*, *Babesia bigemina*, *Babesia divergans*, and *Babesia major*. *B. divergans* and *B. major* are reported in cattle in Europe.[74] *B. bovis* and *B. bigemina* are the most widespread and of most economic significance. *Rhipicephalus* ticks serve as vectors, although iatrogenic transmission by direct blood transfer also occurs.

B. bigemina is a large piroplasm often appearing as the classical pair of piriform organisms. *B. bovis* is a small piroplasm frequently seen as a single round body. Both organisms cause an acute febrile illness and hemolytic episode. Calves infected at less than 8 months of age often develop subclinical disease due to innate resistance.[22] Release of replicating parasites from red cells causes intravascular hemolysis with resultant hemoglobinemia and hemoglobinuria. Increased osmotic fragility of all RBCs may result in massive terminal hemolysis despite reduced parasitemia.[22] Marked accumulation of *B. bovis*-parasitized RBCs in small cerebral capillaries can result in ischemia. The associated CNS signs are termed "cerebral babesiosis."

Two species, *Babesia ovis* and *Babesia motasi*, cause disease in sheep and goats in Africa, Asia, and Europe.[74] *B. ovis* is a small oval piroplasm, while *B. motasi* is larger and is commonly seen as paired, piriform organisms.

Equines

Babesia caballi is the only true equine *Babesia* species, since *B. equi* has been properly reclassified as *T. equi*. It is a large, piriform organism often appearing in pairs with an acute angle. It is tick transmitted, and transovarial

transmission may occur through several generations of ticks. The distribution of *B. caballi* includes Africa, the Americas, Asia, and Europe. Despite the presence of potential tick vectors, it is not a significant disease in the USA. Disease induced by *B. caballi* is generally much less severe than that of *T. equi*. Pronounced clinical signs and severe anemia are uncommon. Hemoglobinuria is not typical. The parasitemia is generally extremely low, even in splenectomized animals.[23] Foals are immune for the first 6–9 months of life from passive immunity and innate factors.

Felines

Babesia felis is a small piroplasm reported regularly in cats from coastal regions of South Africa.[59] No vector has been identified, although it is assumed to be tick-borne. It is most common in young adult cats, which may have severe regenerative anemia, hyperbilirubinemia, and agglutination. The parasitemia is variable and may be extremely high.

Feline Cytauxzoonosis

Cytauxzoon felis is a hemoparasite of wild and domestic cats. Historically most common in the South Central and Southeastern United States, its geographic distribution appears to be constantly expanding. The disease is tick-borne, most efficiently transmitted by *A. americanum*.[63] Bobcats, and possibly other wild felids, as well as domestic cats serve as a reservoir host.[40]

Cytauxzoon piroplasms are found in erythrocytes with schizogony occurring in macrophages of various tissues. The schizogonous phase precedes erythroparasitemia and is responsible for the marked clinical disease with parasitized intravascular macrophages becoming greatly enlarged and obstructing the lumen of vessels. Clinical signs include profound depression, dehydration, marked fever, and icterus. Anemia, generally nonregenerative, is a common finding that develops relatively late in the course of disease. Leukopenia and/or thrombocytopenia commonly precede or occur concurrently with anemia.

Diagnosis is usually confirmed by identification of piroplasms on peripheral blood films. While piroplasms are extremely pleomorphic, the characteristic shape most easily differentiated from artifact is the signet-ring. This is a small ring (approximately 1–3 μm) with a round or linear thickening of nuclear material at one point of the ring[78] (Figure 33.11). Clinical signs precede parasitemia, so organisms may not be present at the time of presentation. Parasitemia tends to increase as infection progresses.

Schizont-containing macrophages can be identified in impression smears of many tissues including lung, spleen, liver, lymph nodes, and bone marrow, and may also be observed on peripheral blood films late in the disease course. Parasitized macrophages become greatly enlarged and have a large, prominent nucleolus (Figure 33.12). Identification of schizonts in tissue aspirates can confirm a diagnosis in patients that are not yet parasitemic. PCR may also be used to confirm infection

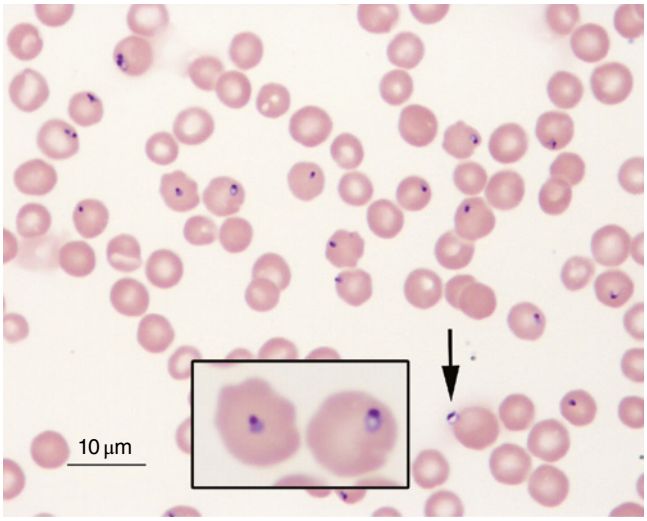


FIGURE 33.11 Blood film from a cat with terminal cytauxzoonosis. In fatal cases, parasitemia typically progressively increases with time. Organisms are variably sized and somewhat pleomorphic. Only some demonstrate the characteristic signet-ring shape. One extracellular piroplasm is present (arrow). Inset: Higher magnification of two piroplasms. In the larger one on the right, the inner portion of the ring can be seen to be lighter than the erythrocyte. Aqueous Romanowsky stain.

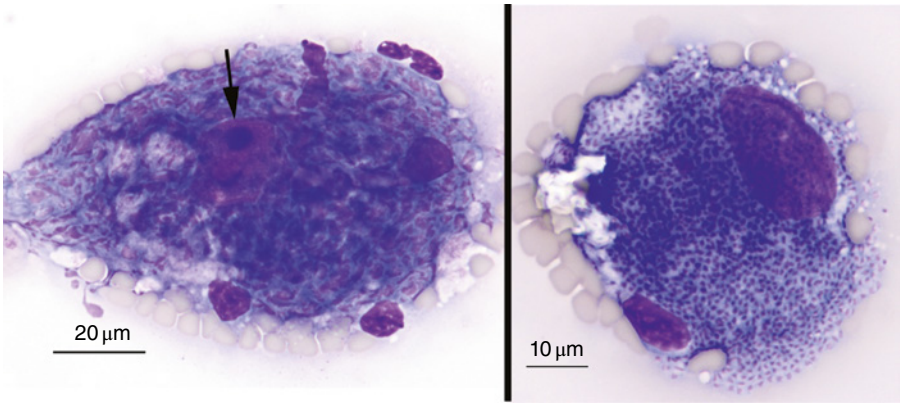


FIGURE 33.12 Large mononuclear cells with *C. felis* schizonts form the feathered edge of a blood film of a cat with cytauxzoonosis. The schizont on the left is less mature. Nuclear material is visible as variably sized, amorphous purple areas within the basophilic schizont. The schizont on the right, from the same blood film, is more mature. Nuclear material has transformed to fine punctate inclusions of developing merozoites. Aqueous Romanowsky stain.

with parasite DNA being detectable by this method several days prior to visualization by microscopy.[63]

The prognosis for cats with cytauxzoonosis has historically been grave with mortality rates approaching 100%. Successful treatment using a combination of atovaquone and azithromycin has been reported, but mortality rates are still high.[19] Survival of cats unrelated to treatment and identification of a high prevalence of asymptomatic carriers in hyperendemic regions have been reported and suggest the presence of less virulent strains in certain regions.[12,50,64] Recovered cats remain persistently parasitemic and may serve as a source of infection of other domestic cats.[63]

Trypanosomiasis

Trypanosomes are flagellated protozoal organisms. They are not a significant cause of anemia in animals in North America.

Trypanosoma cruzi, etiologic agent of Chagas disease, has been reported in dogs in several states.[6] The organisms may be found in the peripheral blood in the acute stages of infection; however, anemia is not a significant finding. Organisms are fusiform, approximately 15–20 µm in length, with a centrally located nucleus and small basophilic subterminal kinetoplast (Figure 33.13).

Trypanosoma theileri is a larger organism (up to 100 µm in length) that occurs in cattle worldwide. This organism is fairly prevalent, but is considered nonpathogenic.[28]

Anemia in animals is caused by a variety of African trypanosomes including *Trypanosoma congolense* and *Trypanosoma vivax* in cattle; *Trypanosoma brucei and T. congolense* in goats and sheep; *T. brucei* in horses and donkeys; and *T. brucei and T. congolense* in dogs.[20] Anemia is most often identified in chronic disease and the pathogenesis is complex.[4] In acute disease, RBC membranes are damaged directly by hemolysins released from trypanosomes. Extravascular RBC removal also occurs in the spleen, bone marrow, and lungs secondary to coating of erythrocytes with trypanosomal antigens.[20]

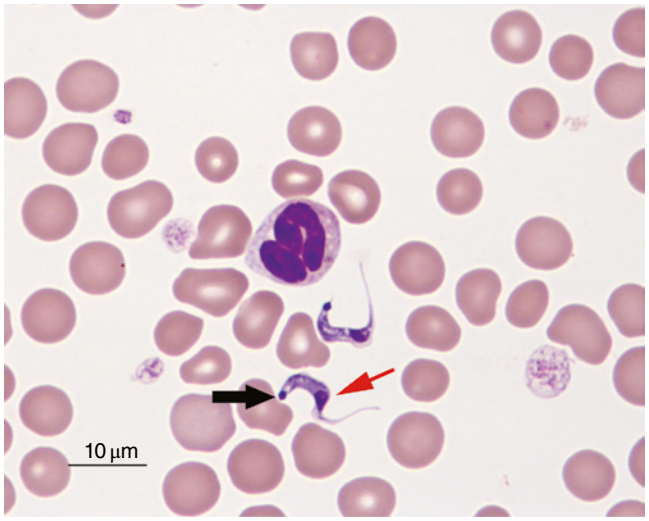


FIGURE 33.13 Blood film from a dog with *T. cruzi* infection. This dog was treated with multiple immunosuppressive agents for immune-mediated hemolytic anemia. Organisms were visible on follow-up blood films after resolution of anemia. Note the dark, subterminal kinetoplast (black arrow) and larger purple nucleus (red arrow). Aqueous Romanowsky stain.

Sarcocystosis

While several different species of *Sarcocystis* have been reported to cause anemia in ruminants including cattle, sheep, and goats, hematologic disease appears to be minimal in comparison to the large numbers of animals infected.[27] There is no parasitic stage of *Sarcocystis* that infects erythrocytes, thus there are no specific hematologic findings. Anemia related to sarcocystosis is thought to be due to hemolysis. In experimental infections, it reaches a nadir at about 1 month post inoculation. Second generation meronts are maturing in endothelium at that time, suggesting that microangiopathy due to vasculitis may play a role in the anemia. Calves inoculated with *Sarcocystis cruzi*, the most pathogenic species for cattle, developed a severe anemia attributed to extravascular immune-mediated hemolysis, and had evidence of a consumptive coagulopathy attributed to endothelial cell damage and disseminated intravascular coagulation.[30] The anemia is reportedly minimally regenerative, possibly due to superimposed anemia of inflammatory disease.[45]

Haemoproteus

Many different species of *Haemoproteus* can affect birds and reptiles. In general, they are considered relatively nonpathogenic, although co-infection with other hemoparasites may have detrimental effects in wild populations. Clinical disease seems to be associated with the tissue stages of parasite development, rather than the RBC stages. Anemia may develop in parasitized animals that are ill, injured, or immunocompromised, with a few reports of mortality associated with anemia occurring in young quail and pigeons. Parasitemia is frequently observed in raptors undergoing rehabilitation for illness or injury, and the magnitude of parasitemia often decreases as birds recover.[58]

Haemoproteus gametocytes are generally present within erythrocytes, although extraerythrocytic forms may occasionally be seen on blood films (Figure 33.14). Gametocytes vary in appearance depending on their stage of development, and may be round, oval, or elongated.[15,46] Large forms typically encircle the erythrocyte nucleus without displacing it. Gametocytes usually contain refractile yellow to brown iron pigment (hemozoin), a result of hemoglobin digestion ("malarial pigment").

Plasmodium

Parasites of the genus *Plasmodium* are the cause of malaria in humans and many animal species. Numerous different *Plasmodium* species have been identified. In veterinary medicine, malarial parasites are most often

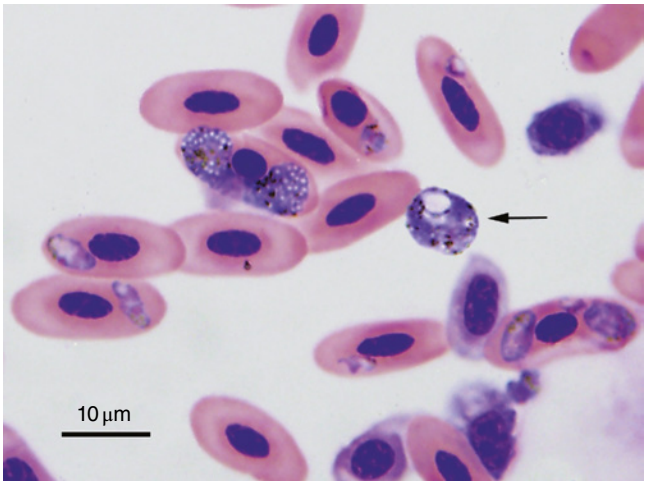


FIGURE 33.14 Blood film from an owl with *Haemoproteus* infection. Several different stages of intracellular gametocytes containing yellow–brown hemozoin pigment are visible, which surround but do not displace the nucleus. One extracellular form is present (arrow). Wright's stain.

encountered in birds and reptiles. Some species of birds seem more susceptible to clinical disease (canaries, penguins, ducks, pigeons, raptors, domestic poultry), while others may be asymptomatic carriers. Reported disease manifestations include hemolytic anemia, leukocytosis, lymphocytosis, hepatomegaly, splenomegaly, and death.[15]

Gametocytes contain yellow-brown iron pigment (hemozoin), similar to that found in *Haemoproteus* gametocytes. Gametocytes may be elongated or round depending on the particular species; round forms tend to displace the host cell nucleus, unlike *Haemoproteus* (Figure 33.15). Schizonts may be found in peripheral blood RBCs, thrombocytes, and leukocytes. They have a variable appearance depending upon the stages of the developing merozoites, which may appear fused or as distinct basophilic structures. Trophozoites are round to oval with an eccentric nucleus, resulting in a signet-ring appearance. Features of *Plasmodium* useful to differentiate it from *Haemoproteus* are presence of schizonts in peripheral blood, developing parasite stages within leukocytes and thrombocytes in addition to RBCs, and displacement of the host cell nucleus.[15] However, there are significant differences in the appearance of these parasites in blood cells; depending upon the particular *Plasmodium* species observed, schizonts may only be observed within RBCs, and the host cell nucleus may or may not be displaced. Those interested in detailed morphologic descriptions of avian haemosporidia are referred to the comprehensive monograph by Valkiunas.[75] Recently developed DNA-based PCR assays should prove helpful to better understand and classify the various morphologic variants.

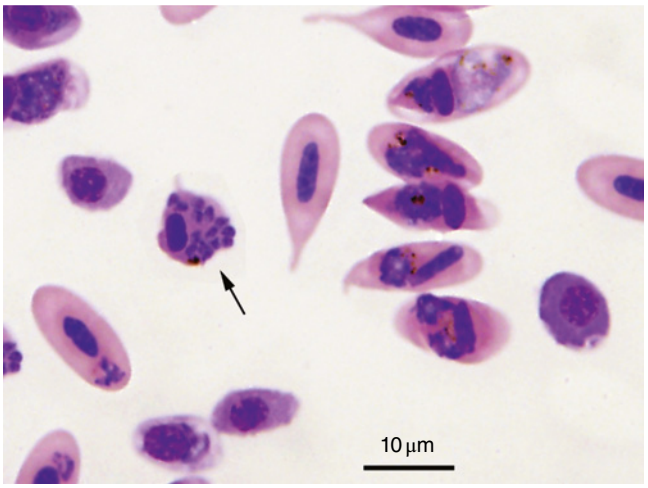


FIGURE 33.15 Blood film from a golden eagle with *Plasmodium* infection. Developing gametocytes containing yellow–brown hemozoin pigment displace erythrocyte nuclei. One erythrocyte contains developing merozoites (arrow). Wright's stain.

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CHAPTER 34

Anemia Associated with Bacterial and Viral Infections

GEORGE M. BARRINGTON and DEBRA C. SELLON

Clostridium Perfringens Infections (Yellow Lamb Disease, Bacillary Hemoglobinuria, Clostridial Myonecrosis)
Clostridium Haemolyticum or C. Novyi Type D Infections (Bacillary Hemoglobinuria, Red Water Disease)
Corynebacterium Pseudotuberculosis Infections
Leptospirosis
Equine Infectious Anemia

Acronyms and Abbreviations

AGID, agar-gel immunodiffusion; C-ELISA, competitive enzyme-linked immunosorbent assay; EIA, equine infectious anemia; EIAV, equine infectious anemia virus.

Many bacterial and viral infections can directly or indirectly cause anemia. This chapter's primary topics are the anemias associated with infections by *Clostridium*, *Corynebacterium*, *Leptospira*, and equine infectious anemia virus (EIAV) (Table 34.1). Anemias caused by feline viruses (i.e., feline leukemia virus and feline immunodeficiency virus), rickettsial bacteria (e.g., *Rickettsia*, *Ehrlichia, Anaplasma*), or mycoplasmal bacteria (e.g., *Mycoplasma haemocanis*, *Mycoplasma haemofelis*) are described in Chapters 18, 33, 53, and 60, respectively.

Most anemias associated with bacterial and viral infections are caused by the inflammatory reaction (i.e., anemia of inflammatory disease, see Chapter 39). Infections may cause more severe anemias if they result in hemorrhage (vascular damage, thrombocytopenia), RBC fragmentation (vasculopathy, coagulopathy), or generalized bone marrow damage (marrow necrosis or replacement, see Chapters 16 and 17).

Infectious agents, their products, or the animal's specific response to the agent may cause an anemia via three major processes: (1) damaged circulating RBCs causing intravascular or extravascular hemolysis, (2) damaged erythroid precursors causing erythroid hypoplasia, and (3) damaged hematopoietic or supportive stromal cells causing general marrow hypoplasia.

CLOSTRIDIUM PERFRINGENS INFECTIONS (YELLOW LAMB DISEASE, BACILLARY HEMOGLOBINURIA, CLOSTRIDIAL MYONECROSIS)

Yellow lamb disease occurs in spring nursing lambs of Northern California and Oregon[23] and in lambs and calves in Canada, Australia, New Zealand, and South Africa.[33] Hemolytic anemias are not described in most enteric diseases caused by *C. perfringens* type A.

Clinical signs include depression, fever, weakness, pale mucous membranes, anemia, hemoglobinuria, icterus, and diarrhea. Death may occur within 6–12 hours of onset of illness especially if icterus and hemoglobinuria are present.[23,33] Changes in blood are consistent with massive intravascular hemolysis.[6,33,48]

The α-toxin causes hydrolysis of membrane phospholipids (Figure 34.1) and resultant lysis of RBCs, WBCs, platelets, endothelial cells, and myocytes.[43] Studies have

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TABLE 34.1 Bacterial and viral infections that cause anemia.[a]

Organism	Disease	Susceptible animals
Clostridium perfringens type A	Yellow lamb disease, enterotoxemic jaundice, or yellows in lambs; clostridial myonecrosis in horses	Lambs, calves, captive Siberian ibex, horses
Clostridium haemolyticum (or *C. novyi* type D)	Bacillary hemoglobinuria, red water disease	Cattle, sheep
Corynebacterium pseudotuberculosis	(Experimental infection)	Sheep
Leptospira interrogans serogroups	Leptospirosis	Calves, lambs, pigs, perhaps rhinoceros, possibly dogs
Equine infectious anemia virus	Equine infectious anemia, swamp fever	Horses, ponies, donkeys, mules

[a] Feline leukemia virus, feline immunodeficiency virus, rickettsial bacteria (e.g., *Rickettsia, Ehrlichia, Anaplasma*), and mycoplasmal bacteria (*Mycoplasma haemocanis, Mycoplasma haemofelis*) not included.

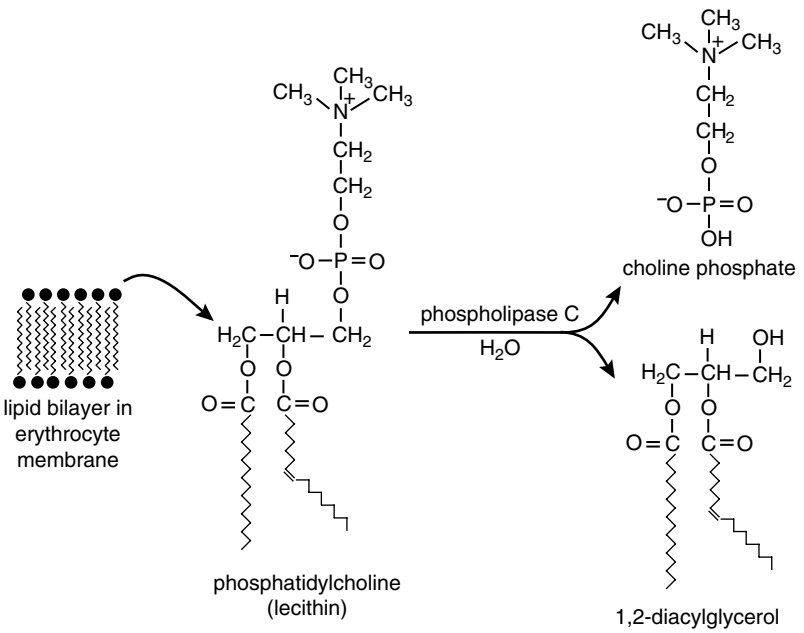


FIGURE 34.1 Action of phospholipase C on the RBC membrane. Phospholipase C catalyzes the hydrolysis of two RBC membrane lipids—phosphatidylcholine and sphingomyelin—that are the predominant phospholipids in the outer hemileaflet of the RBC membrane. The reaction shows hydrolysis of phosphatidylcholine into its hydrophilic portion (choline phosphate) and hydrophobic portion (1,2-diacylglycerol). In a similar reaction, phospholipase C catalyzes the hydrolysis of sphingomyelin to ceramide and sphingosine 1-phosphate (not shown).

demonstrated that α-toxin activates the sphingomyelin metabolic system as well as phospholipase C leading to hemolysis.[26,27,34,35] Furthermore, sensitivity to α-toxin hemolysis varies between species and is proposed to depend on the phospholipid composition of cell membranes.[14] A recent study demonstrated that α-toxin can impair erythroid differentiation in vitro;; however, inhibitory effects on erythroid differentiation were not detected in vivo, but may have been masked by a concurrent increase in plasma erythropoietin.[45]

C. perfringens type A is a part of intestinal microbiota in animals, but increased bacterial growth has been associated with highly proteinaceous diets,[46] overfeeding,[44] and starchy foods.[42] After production by the bacteria, α-toxin is absorbed by intestinal mucosa, enters the blood, and damages cell membranes. Major postmortem findings include evidence of massive intravascular hemolysis, renal hemoglobin casts, destruction of capillary walls leading to intestinal mucosal necrosis, centrilobular hepatic necrosis, and serosal, petechial, and ecchymotic hemorrhages. In calves, abomasal hemorrhage and ulceration may be present. Small intestine may be hyperemic, but rapid autolysis may obliterate the lesions; large, Gram-positive bacilli may be found in the mucosa and submucosa.[32]

A diagnosis of yellow lamb disease is confirmed by detecting α-toxin in intestinal contents or blood from fresh carcasses via serum-neutralization tests in mice and guinea pigs.[5] Polymerase chain reaction assays can detect the α-toxin gene (also known as cpa gene) of *C. perfringens* type A.[10]

Horses with *C. perfringens* infections such as clostridial myonecrosis may develop immune-mediated intravascular and extravascular hemolysis.[1,9,30,48]. The inciting factor in immune-mediated erythrocyte destruction is unconfirmed, but it has been postulated that

toxin-induced damage to erythrocyte membranes may alter surface antigens resulting in an autoimmune response.[30]

CLOSTRIDIUM HAEMOLYTICUM OR *C. NOVYI* TYPE D INFECTIONS (BACILLARY HEMOGLOBINURIA, RED WATER DISEASE)

Bacillary hemoglobinuria is a fatal disease that occurs naturally in cattle and experimentally in sheep. It can be a seasonal disease (summer and early fall) and is associated with liver fluke migration. It is endemic in poorly drained or swampy areas of the Gulf Coast states, Western United States, South America, Wales, Eastern Europe, and New Zealand.[44,46]

Bacillary hemoglobinuria is rarely recognized antemortem because the severe hemolysis causes rapid deterioration and death. When seen, clinical signs include malaise, reluctance to move, arched back, bloody diarrhea, fever, rapid and shallow breathing, and blood-tinged froth from nostrils. The classic port-wine urine of hemoglobinuria is uncommon.[40] If the affected animal survives the initial crisis, icterus and a regenerative anemia can develop. In experimental infections, severe anemia and death are seen within 3 days of inoculation. Calves and ewes develop fever, a gradual to precipitous drop in hematocrit values, mild to moderate leukocytosis, and increased aspartate transaminase activity (either due to RBC or hepatocyte damage).[28]

RBC damage is caused primarily by a β-toxin, a phospholipase C (lecithinase), that hydrolyzes phosphatidylcholine and sphingomyelin (Figure 34.1). The β-toxin is not related serologically to the phospholipase of other clostridial species.[41] The β-toxin is produced by two clostridial organisms, *C. haemolyticum* and *C. novyi* type D. Other minor hemolysins are reported as products of *C. haemolyticum*.[22]

After spores are ingested, organisms cross the intestinal mucosa and are transported to liver and other organs in macrophages. Spores may persist in Kupffer cells for a long time. Anaerobic conditions, such as those induced by hepatic biopsy and the presence of liver parasites, stimulate growth of organisms and production of toxins.[25] Absorption of β-toxin into blood leads rapidly to intravascular hemolysis, damaged endothelial cells, icterus, hemoglobinuria, and death.[40]

Necropsy findings may include pale and icteric mucous membranes, petechial and ecchymotic subcutaneous hemorrhages, copious amounts of red-tinged abdominal and thoracic fluid, hemoglobinuria, renal hemoglobin casts, congested or hemorrhagic lymph nodes, extensive edema, and bloody intestinal contents. Characteristic liver lesions are foci of hepatic necrosis surrounded by a reddened reaction zone.[44]

Diagnosis is usually made from the combination of clinical signs (rapid deterioration and death) and gross and microscopic postmortem findings. Numerous Gram-positive bacilli may be found in liver, spleen, blood, and abdominal fluid, but their presence must be interpreted with caution. Fluorescent antibody testing or immunohistochemistry for detection of the organism in fresh liver tissue can be performed.[18] Confirmation of bacillary hemoglobinuria can be made by demonstration of *C. haemolyticum* in the liver of affected animals by culture or polymerase chain reaction to detect the *C. haemolyticum* β-toxin gene.[25]

CORYNEBACTERIUM PSEUDOTUBERCULOSIS INFECTIONS

C. pseudotuberculosis causes caseous lymphadenitis and other forms of abscessation in sheep and goats. A distinct, species-specific biotype of the organism causes "pigeon fever" with cutaneous or internal abscessation in horses. *C. pseudotuberculosis* produces an exotoxin that has sphingomyelin-specific phospholipase D activity. When experimentally given intravenously in sheep, the exotoxin causes an acute, severe intravascular hemolytic anemia.[15,19] The exotoxin causes alterations in the phospholipid composition of RBC membranes leading to spherostomatocyte formation and pitting of RBC membranes.[3] Natural infections with *C. pseudotuberculosis* may not cause a hemolytic anemia due to the production of antitoxin against the phospholipase D during early stages of the disease. The anemia observed in affected animals is likely anemia of inflammatory disease.

LEPTOSPIROSIS

Leptospirae are saprophytic spirochetes that live in moist habitats throughout the world and infect humans, most domestic animals, and many wild animals.[17] In domestic animals, leptospirosis with hemolytic anemia is seen primarily in calves, lambs, and pigs.[4,47] A leptospiral infection may be a cause of acute hemolytic anemias of black rhinoceros.[13] Many serovars of *Leptospira* are reported to cause anemia in dogs, but the mechanism for the anemia is not understood.[16]

When the hemolytic state is present, the clinical signs and clinicopathologic abnormalities can include fever, depression, anorexia, anemia, hemoglobinemia, hemoglobinuria, icterus, neutrophilia, and petechial or ecchymotic hemorrhages. In experimental infections in calves, RBC fragility was increased.[31] Several explanations for the hemolytic process have been proposed; experimental evidence in lambs indicates involvement of an IgM cold agglutinin.[2,12] A study by Lee et al. suggested that sphingomyelin H (SphH) is a novel hemolysin which functions as a direct pore-forming protein among pathogenic *Leptospira*.[21] Direct membrane damage of sheep erythrocytes and mammalian cells is due to SphH in vitro;; further studies are necessary, however, to confirm the pathogenic role of SphH in animal models.

In lambs, major necropsy lesions include hemoglobinuria, icteric membranes and tissues, severe renal

tubular necrosis with casts, and periacinar hepatocellular necrosis.[11] A hemolytic anemia caused by leptospirosis is confirmed by finding leptospiral spirochetes in urine or other fluids by direct dark-field microscopic examination, culture of organisms with specific media, animal inoculation, and by serologic testing. Either ELISA or agglutination tests can detect IgM agglutinins within 7–9 days of onset of disease.[47] Diagnosis should be based on finding at least a fourfold increase in titer to either *pomona* or *icterohaemorrhagiae* serogroups over the course of infection.

EQUINE INFECTIOUS ANEMIA

Equine infectious anemia (EIA) is caused by EIAV, which is classified in the family Retroviridae and the Lentivirus genus.[24,36] This disease of equids occurs worldwide, but is most common in hot, humid climates (see Chapters 18 and 37).

The virus is spread via the bites of hematophagous flies (tabanids, deer flies) or iatrogenically through blood-contaminated fomites. It persists in mononuclear phagocytes and cannot be eliminated from an infected horse.[38] Intermittent episodes of fever, dependent edema, anemia, and thrombocytopenia are associated with emergence of novel antigenic variants of EIAV with associated upregulation of viral replication.[37] Most horses ultimately develop a broad immune response that controls viral replication and these infected horses show few if any recognizable clinical signs (inapparent carrier horses). By contrast, a few infected horses are unable to control viral replication and develop classic clinical signs of "swamp fever" that include fever, weight loss, persistent anemia and thrombocytopenia, and hypergammaglobulinemia.[24]

During episodes of viremia and fever, clinicopathologic abnormalities may include anemia, thrombocytopenia, and either neutropenia or neutrophilia. In acute disease, thrombocytopenia is the most consistent hematologic abnormality and is a result of both decreased platelet survival and decreased platelet production. Platelets are believed to be removed from circulation by tissue macrophages as a response to surface binding of complement or immune-complexes. Increased inflammatory cytokines (tumor necrosis factor-α, transforming growth factor-β, interferon-α) suppress megakaryocytopoiesis and dampen the bone marrow response that should occur in response to increased platelet destruction.

The anemia of EIA is a result of decreased RBC life span and decreased RBC production.[24,37] In acute disease, it is possible, but uncommon, for horses to experience intravascular hemolysis. The association of EIAV envelope glycoprotein with RBC membranes activates complement and causes a C3-fraction coating of RBCs and resultant hemolysis.[7,8,39] Infected horses may be Coombs positive. Morphologic changes in RBCs may include anisocytosis due to macrocytosis, spherocytes (difficult to detect in equine blood), and

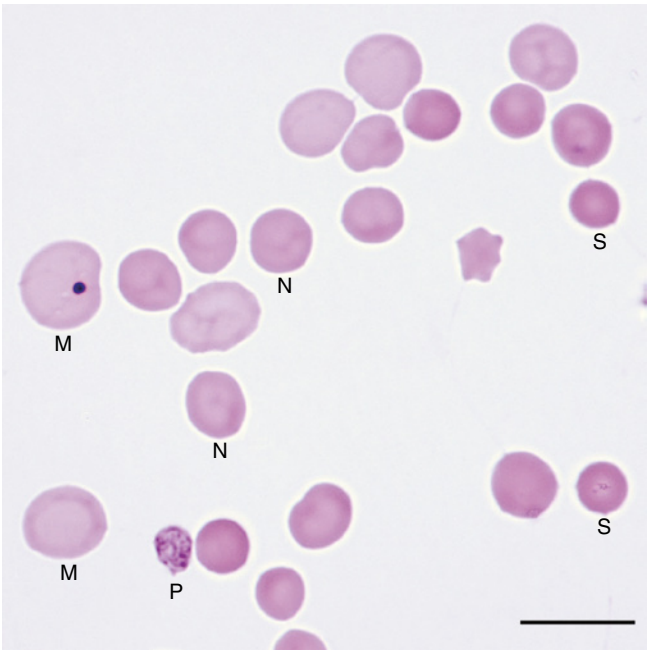


FIGURE 34.2 Blood film from a horse with acute hemolytic anemia due to EIA. The blood film contains macrocytes (M; one contains a Howell–Jolly body), two possible spherocytes (S), normocytes (N), and a platelet (P). Modified Wright stain; bar, 10 μm.

sideroleukocytes (Figure 34.2). Specific immune responses are not required for the development of anemia because foals with severe combined immune deficiency become profoundly anemic after experimental infection.[29] The pathogenesis of the progressive anemia observed in chronically infected horses is likely multifactorial with decreased RBC production, extravascular hemolysis, and anemia secondary to chronic inflammatory disease (see Chapter 39).

Because of the persistent nature of EIAV infection, detection of serum antibody to the virus confirms the diagnosis. Most infected horses are seropositive within 45 days of infection.[24] The Coggins test, an agar-gel immunodiffusion (AGID) assay which detects antibody against the virus's group-specific core protein, p26, has been the most widely used diagnostic test for EIA. Competitive ELISA (C-ELISA) are widely available and are considered more sensitive but less specific than the AGID assay. Discrepancies between AGID and C-ELISA results are most often resolved by western immunoblot assay to better characterize antibody responses against specific viral proteins.[20]

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CHAPTER 35

Immune-Mediated Anemia in the Dog

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Pathogenesis
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- Secondary IMHA
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- Marrow Involvement
- Cold Reactive Antibodies

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- Clinical Signs
- Secondary Complications

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- Thromboprophylaxis
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Acronyms and Abbreviations

AIHA, autoimmune hemolytic anemia; ALT alanine transaminase; ANA, antinuclear antibody; DAT, direct antiglobulin test; DIC, disseminated intravascular coagulation; HIVIG, human intravenous immunoglobulin; IL, interleukin; IMHA, immune-mediated hemolytic anemia; IMT, immune-mediated thrombocytopenia; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; NRIMA, nonregenerative immune-mediated anemia; PIMA, precursor-targeted immune-mediated anemia; PRCA, pure red cell aplasia; PTE, pulmonary thromboembolism; SLE, systemic lupus erythematosus; Th2, type 2 T helper cells; Tregs, regulatory T cells.

Immune-mediated hemolytic anemia (IMHA) is a common cause of severe and often life-threatening anemia in dogs. IMHA can be classified as either primary (idiopathic or autoimmune) if no triggering factor is identified, or secondary if an underlying cause is suspected to have triggered the immune-mediated process (Figure 35.1). Distinction between primary and secondary IMHA is therapeutically important, because secondary IMHA may show poor treatment response and recurrence if the underlying cause is not recognized and eliminated.

PATHOGENESIS

Primary IMHA

Primary (or nonassociative) IMHA, a classical autoimmune disorder with no recognized underlying cause, is the most frequent form of IMHA in dogs. Primary IMHA is also commonly known as autoimmune hemolytic anemia (AIHA). The condition typically affects young adult and middle-aged animals (median age about 6 years), and is most common in Cocker Spaniels, English

* deceased

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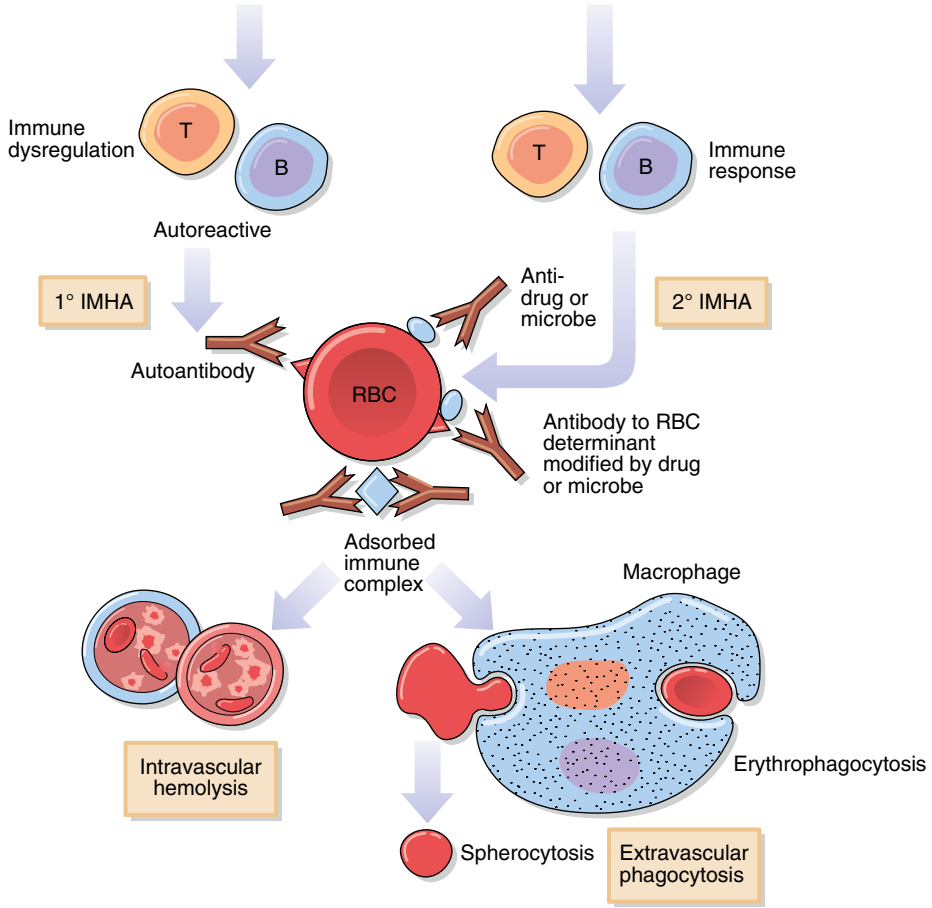


FIGURE 35.1 Pathogenesis of IMHA. IMHA may be primary (autoimmune) or secondary to an underlying triggering cause. In primary IMHA, predisposing factors interact to allow activation of self-reactive T and B cells and production of autoantibody specific for an antigen of the RBC membrane. In secondary IMHA, the erythrocyte can be an "innocent bystander" in an immune response that generates immune complexes that nonspecifically adsorb to the RBC surface, or generates antibodies that may be directed against RBC membrane components antigenically similar to non-RBC antigens associated with the underlying disease, or against membrane components modified by the underlying disease, or against an antigen such as a drug or microbe attached to the RBC membrane. The RBC may be destroyed following complement fixation and formation of the "membrane attack complex" (intravascular hemolysis), or interact with phagocytic cells that express surface Fc and complement receptors, in which case the RBC may be entirely removed by erythrophagocytosis (extravascular hemolysis), or suffer partial loss of its cell membrane to form a spherocyte.

Springer Spaniels, Poodles, Miniature Schnauzers, Maltese Terriers, Hungarian Vizslas, Airedale Terriers, and Old English Sheepdogs, although it can occur in any breed.[5,23,37,39,50,66,68] Breed predispositions and transmission through pedigrees strongly suggest that there is an inherited susceptibility to IMHA, and a strong association between several DLA-79 gene mutations and multiple immune-mediated diseases, including IMHA, has been demonstrated in dogs.[13] Both susceptibility and protective haplotypes of class II loci of the major histocompatibility complex have been identified in English Cocker Spaniel and Springer Spaniel dogs.[31] Neutered dogs (both male and female) appear to be more prone to IMHA than intact dogs of either sex.[6,50,68]

There is much interest in unraveling the complex immunopathogenesis of autoimmune diseases in order to develop novel targeted immunotherapies. Since the canine disease shares many aspects with human IMHA and experimental rodent models of the disease, investigations of spontaneously arising canine primary IMHA have been conducted. Classically, an autoantibody-mediated disease would reflect an immune system imbalance where a lack of function of natural regulatory T cells (Tregs) would permit inappropriate activation of type 2 T helper cells (Th2). This in turn activates autoreactive B lymphocytes by providing stimulatory signals allowing B-cell activation, clonal proliferation, and immunoglobulin class switch, resulting in the production of IgG or IgM erythrocyte-specific autoantibodies. However, the most detailed study of immune function in canine IMHA failed to identify a simple reduction in the proportions of Tregs or other T-cell subsets or in production of the signature cytokine associated with Treg function, interleukin (IL)-10. Instead, an elevation in pro-inflammatory cytokines (IL-2, IL-6, CXCL8, and tumor necrosis factor-α) was found in the affected dogs.[60] This finding is not unexpected and is likely to be relatively nonspecific, with

other studies reporting no difference in pro-inflammatory cytokine profiles between dogs with IMHA and dogs with bacterial sepsis.[27] It remains possible that other T cell subsets (Th1, Th17) may play a role in the immunopathogenesis of canine primary IMHA, but this has yet to be explored.

Secondary IMHA

Secondary (or associative) IMHA is suspected to develop in response to a triggering process, including various medications and a range of infectious, inflammatory, and neoplastic diseases. It can affect dogs of any age or breed, and can be suspected in patients with a signalment atypical for primary IMHA, such as in geriatric animals. In many instances, direct causation cannot be established. While the hemolytic anemia associated with canine babesiosis has been clearly shown to have an immune-mediated component, the link between other infectious agents such as *Mycoplasma haemocanis*,[67] bartonellosis, and rickettsial organisms is not as well established.[14] Neoplasia, heartworm infection, medications, and vaccinations have also been implicated in the pathogenesis of IMHA, but definitively established causation has not been shown.[14] Secondary IMHA has also been reported following bee stings and elapid snake bites.[44,45] Interestingly, a seasonal increase in incidence has been found for IMHA in dogs in some areas (e.g., spring and summer in Southern California), potentially suggesting some as yet identified environmental or infectious factor.[33,68]

Mechanism of Pathogenesis

The mechanism underlying typical cases of IMHA is antibody-mediated cytotoxic (Type II) destruction of circulating RBCs. Although most cases of IMHA share this common mechanism, the disease is otherwise highly variable among individuals. In primary IMHA, both the pattern of immunoglobulin and complement involvement in RBC destruction, as well as the site of antibody attachment to RBC membranes, vary widely between patients. Although the most common immunoglobulin type involved in canine primary IMHA is IgG, less commonly IgM may also be implicated, along with variable involvement of complement, or both IgG and IgM combined.[23] Dogs with a combination of IgG and IgM anti-RBC autoantibodies tend to have lower hematocrits, more spherocytes, and more autoagglutination.[23] Antibodies have been reported to attach to various components of the RBC membrane, particularly glycophorins and the band 3 anion transport protein, but also to a range of other proteins, including calpain, complement component 3, and peroxiredoxin 2.[62] Given the range of different immunoglobulins and antigenic targets involved, it is probably overly simplistic to think of IMHA as a single disease syndrome: rather, it is in actuality a heterogenous mix of different diseases with different presentations, prognoses, and responses to therapy.

Antibody attachment to RBC membranes triggers cell destruction by several mechanisms. Complement fixation and formation of membrane attack complexes directly damage RBC membranes. The osmotic effects of hemoglobin draw extracellular water into the cytoplasm, resulting in swelling and rupture of the RBC while still in circulation ("intravascular hemolysis") (Figure 35.2). In the absence of direct RBC lysis, antibody attachment to cell membranes can still lead to an accelerated rate of destruction of affected RBCs by tissue macrophages within the mononuclear phagocytic system, a process that occurs outside of the circulation ("extravascular hemolysis"). Mononuclear phagocytic system destruction of RBCs is mediated by Fc and complement receptors on the macrophage surface, receptors which bind the Fc component of the antibodies and/or complement attached to the RBC membranes. Since the mononuclear phagocytic system is located throughout the body, extravascular hemolysis can occur in many organs, but typically is most pronounced in the spleen and liver.

In some patients with high levels of anti-RBC antibodies, individual antibodies can bind to multiple different RBCs simultaneously, causing agglutination. Patients that exhibit significant RBC agglutination at body temperature may be susceptible to an increased

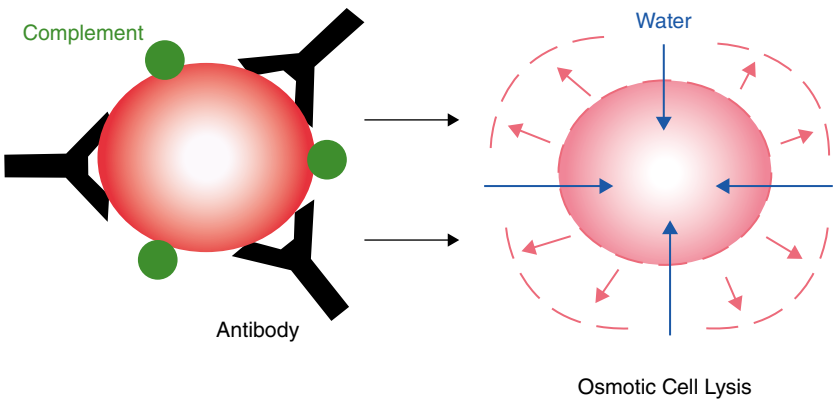


FIGURE 35.2 Intravascular hemolysis. Antibody attachment, complement fixation, and formation of membrane attack complexes directly damage RBC membranes. The osmotic effects of hemoglobin draw extracellular water into the cytoplasm, resulting in swelling and rupture of the RBC while still in circulation ("intravascular hemolysis").

rate of extravascular hemolysis, since clumping of RBC within the vasculature may slow their passage through vessels and facilitate their removal by the mononuclear phagocytic system.

Marrow Involvement

Primary IMHA is caused by antibodies directed against circulating mature RBCs, with the marrow typically mounting a healthy regenerative response to the resultant anemia. By contrast, if antibodies are directed against membrane components that are present only on marrow erythroid precursors, and not on mature RBCs, nonregenerative anemia can develop without peripheral hemolysis (see Chapter 38). This condition is termed precursor-targeted immune-mediated anemia (PIMA) or nonregenerative immune-mediated anemia (NRIMA), and dogs with PIMA/NRIMA often have hypercellular bone marrows with erythroid hyperplasia, erythroid precursor erythrophagocytosis, and maturation arrest, with or without myelofibrosis.[35,57] Some affected dogs have bone marrow changes consistent with pure red cell aplasia (PRCA), where all stages of marrow RBC precursor are reduced or absent. Both conditions can have an immune-mediated pathogenesis, with PRCA being a more severe form of disease, and the two being best differentiated by the increase in early erythroid precursors and erythrophagocytosis seen in PIMA/NRIMA, with few erythroid precursors present in PRCA. If antibodies are directed against cell membrane components that are present both on mature RBCs and their marrow precursors, the patient will present with the rapid RBC loss seen with typical IMHA, but will have an inappropriately poor regenerative response. These so-called "poorly regenerative IMHA" patients have inappropriately low reticulocyte counts in response to anemia, and are a relatively common manifestation of canine IMHA (typically, about 30% of cases, but reported to have a prevalence as high as 60% in some studies).[5,48,54,68]

Interestingly, in some dogs with IMHA, lack of regenerative responses appears to be due to a functional iron deficiency (a mechanism comparable to "anemia of chronic disease").[53] Acute phase proteins such as C-reactive protein are markedly elevated in some dogs with IMHA and, since anemia of chronic disease is thought to be a functional iron deficiency mediated by acute phase proteins (hepcidin in particular), it should perhaps not be surprising that this mechanism may affect erythropoiesis in IMHA patients.[53] Microcytosis is also seen in association with myelofibrosis, a potential characteristic of IMHA, PIMA/NRIMA, and PRCA,[69,71] and may represent altered regulation in iron homeostasis.[58]

In secondary IMHA, antibodies may be directed against the RBC membrane, but alternatively they may be directed against a foreign antigen (such as a drug or virus) that is attached to the RBC membrane, against normal RBC membrane components that are antigenically similar to non-RBC antigens associated with the underlying disease process, or against membrane components that are normally hidden but are exposed by the underlying disease.

Cold Reactive Antibodies

Typical IMHA is caused by antibodies that exert their effects at body temperature, so-called warm reactive antibodies. Some dogs, however, have anti-RBC antibodies that are only reactive at much lower temperatures. Although such cold reactive antibodies may be associated with minimal harmful effects, their presence can potentially cause specific unique clinical syndromes, and can also arguably lead to a false positive diagnosis of IMHA if agglutination testing is performed only at cold temperatures.[66] Anti-RBC antibodies that are reactive at cold temperatures can uncommonly cause agglutination within the vasculature of the extremities, particularly in colder weather, and cause some degree of intravascular hemolysis within the extremities, manifesting as transient hemoglobinemia and hemoglobinuria.[43]

CLINICAL PRESENTATION

Clinical Signs

Clinical signs typically associated with IMHA reflect the presence of both anemia (lethargy, weakness, collapse, pale mucous membranes, and a hemic heart murmur) and compensatory responses caused by tissue hypoxia and stimulation of the sympathetic nervous system (tachypnea, tachycardia, and bounding pulses).[37,48,50,59] Hemic heart murmurs are low-grade systolic murmurs thought to be associated with reduced blood viscosity, and are typically not auscultated until the hematocrit falls to below approximately 20%. Some dogs may also show clinical signs of an ongoing immunological or inflammatory process such as anorexia and, uncommonly, low-grade fever or lymphadenopathy.[37,48] Surprisingly, although the mononuclear system within the spleen and liver is usually the main site of RBC destruction, organomegaly is only variably present in animals with IMHA.[37,50]

Extravascular hemolysis with phagocytosis of antibody-coated RBCs by the mononuclear phagocytic system can overwhelm hepatic ability to process the excess bilirubin associated with the breakdown of hemoglobin within macrophages. Therefore, hyperbilirubinemia, bilirubinuria, and tissue jaundice are often seen during acute severe episodes of IMHA (Figure 35.3).[37,50] Profound hyperbilirubinuria can, rarely, cause neurological signs due to excessive bilirubin entering the brain through the blood–brain barrier ("kernicterus").[63] Since intravascular hemolysis is relatively uncommon (reported in around 5% of canine IMHA cases), hemoglobinemia and hemoglobinuria are observed infrequently.[59] Hemoglobinuria can cause a dark brown to near black urine discoloration colloquially known as "port wine" urine (Figure 35.4). Hemoglobinuria can also sometimes cause a reddish urine discoloration that mimics true hematuria.

Dogs with slowly progressing anemia due to subacute or chronic IMHA may not show significant clinical



FIGURE 35.3 Jaundice. Severe jaundice in a Schnauzer with IMHA.



FIGURE 35.4 Hemoglobinuria. Tubes containing urine samples from two consecutive days from a dog with IMHA. The bottom tube is from the first day, and demonstrates the dark discoloration typical of the hemoglobinuria seen with intravascular hemolysis. By the second day, the intravascular hemolysis had resolved, and hemoglobinuria had cleared (top tube).

signs despite the presence of severe anemia. In these patients, the extra bilirubin released by RBC breakdown occurs at a rate slow enough to allow the liver to process the bilirubin, and jaundice does not necessarily occur.[37] In those non-jaundiced patients with suspected IMHA, definitive establishment of the presence of hemolysis can be very challenging.

Secondary Complications

Thromboembolic disease may develop in a portion of dogs with IMHA, and is particularly common in those animals with acute severe anemia receiving high-dose glucocorticoid therapy, especially those with marked leukocytosis.[40] Pulmonary thromboembolism (PTE) is the most well-recognized manifestation of this complication,[6,17,37] but emboli may also effect the kidneys, spleen, heart, or lymph nodes.[6,68] Many dogs with IMHA appear to be prothrombotic, as evidenced by high levels of activated platelets, increased levels of tissue factor and phosphatidylserine positive microparticles, and a range of altered hemostatic and thromboelastographic markers suggestive of hypercoagulability.[4,12,16,17,32,54,70] Risk factors for development of these complications are reported as thrombocytopenia, hyperbilirubinemia, hypoalbuminemia, elevated serum alkaline phosphatase, a negative Coombs test, cage confinement, recumbency, and the presence of indwelling catheters.[6] Pulmonary thromboembolism should always be suspected in those anemic patients that suddenly develop severe and persistent dyspnea,[17] although other causes of dyspnea such as cardiogenic pulmonary edema or acute bacterial pneumonia should also be considered.

Disseminated intravascular coagulation (DIC) has also been reported as a complication of IMHA,[6,54] but clinically significant DIC is probably uncommon, and associated hemorrhage is rare. Concurrent immune-mediated thrombocytopenia (IMT) ("Evans syndrome") or PTE can both cause some of the laboratory abnormalities that are typically associated with DIC, such as thrombocytopenia, decreased fibrinogen and antithrombin, and increased fibrin degradation products and D-dimers,[6,12,15,17] possibly leading to an overdiagnosis of DIC in IMHA patients.[46] Transient ascites has also been reported, possibly due to acute cor pulmonale secondary to PTE, or alternatively due to either serositis or extramedullary hematopoiesis within the peritoneal cavity.[3,37]

DIAGNOSIS

Diagnostic testing performed in a patient with IMHA can serve many purposes. Determination of an appropriate treatment plan as well as prognosis can be highly reliant on knowing whether the IMHA is a primary or secondary disease process, understanding the unique disease manifestation, and recognizing associated complications. Specific diagnostics that are indicated will vary from individual to individual dog, and may include first demonstrating that IMHA is present, followed by determining the type of IMHA, identifying underlying or concurrent diseases, and recognizing when specific treatment interventions are needed.

Hematology

Hematology in patients with IMHA typically reveals a moderate to severe anemia, which is most commonly regenerative, with anisocytosis, polychromasia, spherocytosis, a high corrected reticulocyte percentage, a high absolute reticulocyte count, and, sometimes, increased numbers of nucleated RBCs.[37] In some cases, reticulocyte counts may be inappropriately low,[5,50] either because of the presence of antibodies directed against RBC precursors, due to functional iron deficiency, or

because the anemia is peracute. The bone marrow typically takes between 3 and 5 days to mount a strong regenerative response to anemia, so reticulocyte counts prior to that time will be normal or "pre-regenerative" in dogs with peracute IMHA.

White cell and neutrophil counts are often moderately to markedly increased,[37,39,40,48,50,54] probably in response to both nonspecific bone marrow stimulation and the pro-inflammatory processes associated with RBC breakdown.[34,60] Occasionally, white cell counts can be high enough to mimic myelogenous leukemia,[48] a reaction sometimes called a "leukemoid response."[40] Platelet counts are often normal, unless the animal also has IMT or platelet consumption secondary to PTE or DIC.[5,6,15,37,39,46,50,54,59] Evans syndrome may affect up to approximately 20% of dogs with IMHA,[6,59] but the frequency of Evans syndrome may be overestimated if the effects of PTE or DIC on platelet counts are not considered as an alternative diagnosis to IMT.[15,46] True Evans syndrome is most likely in those IMHA patients with marked thrombocytopenia.[15,46] IMHA may also occur concurrently with immune-mediated neutropenia and, rarely, all three cytopenias may occur in one patient.[28]

Agglutination

Examination of blood smears in dogs with IMHA may reveal microscopic autoagglutination (clumping) of RBCs. Such agglutination can also form very large rafts of tightly clumped RBCs that, when a blood or microhematocrit tube containing anticoagulated blood is closely inspected, are visible to the naked eye as multiple red speckles. Similar speckles can however be created by rouleaux formation. Rouleaux are stacked arrays of loosely adhered RBCs that have a higher tendency to form in the presence of systemic inflammatory diseases (high acute-phase proteins). Rouleaux can occur in normal dogs as well, and will disperse with saline washing. Clinicians should therefore perform a saline washing process to differentiate rouleaux from genuine autoagglutination (Figure 35.5).

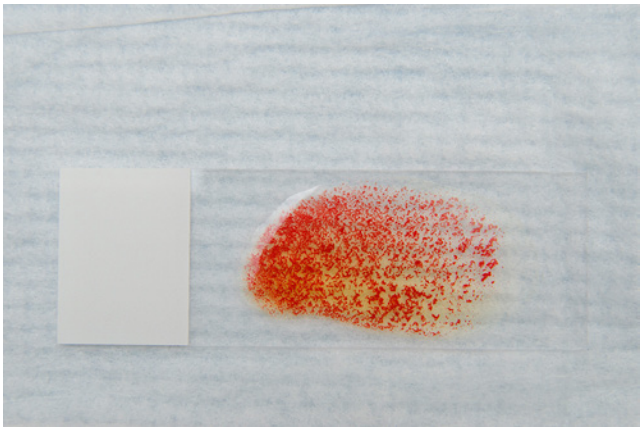


FIGURE 35.5 Saline slide agglutination. Positive saline slide agglutination, denoted by obvious red speckles against a white background, in a jaundiced dog with IMHA.

A simple in-house "slide agglutination test" can be performed by adding one drop of anticoagulated whole blood to two to four drops of saline on a microscope slide, and then swirling the slide against a white background for approximately 1 minute to observe for the persistence of gross RBC speckles (Figure 35.6). A cover slip can then be placed over the slide to facilitate microscopic examination for microagglutination. Care should be taken to perform the test using blood and saline that are warmed to at least room temperature, in order to avoid detection of cold agglutination of dubious clinical relevance. Cell washing techniques using repeated centrifugation and multiple (typically three) saline washes have been reported to decrease the diagnostic sensitivity and increase the diagnostic specificity of testing for agglutination.[7]

The presence of autoagglutination after saline washing is strongly suggestive of a diagnosis of IMHA, and also suggests that the condition is more likely to be acute and severe. An absence of autoagglutination, however, does not rule out IMHA, and negative autoagglutination has been reported in some studies to be a common result in dogs with IMHA.[23,37,54]

Autoagglutination can affect hematology results generated by automated analyzers. Small clumps of agglutinated RBCs may be recorded as a single cell, and large clumps may not even be recorded as RBCs resulting in an artifactually high mean corpuscular volume or a lowering of the calculated hematocrit. Since the hemoglobin within all RBCs is still measured by the analyzer, this leads to an erroneously high estimation of mean corpuscular hemoglobin concentration (MCHC). When agglutination is suspected to be the cause of a lower hematocrit, a manual PCV, which is not affected by RBC clumping, should be performed on well-mixed, anticoagulated blood.

Spherocytosis

Cell morphology can also be used to aid in the diagnosis of IMHA. Spherocytes are small spherical erythrocytes that, when present in large numbers, strongly suggest a diagnosis of either primary or secondary IMHA. The presence of only a small number of spherocytes, such as one to two per high-powered field, should not be overinterpreted as evidence of IMHA, especially since other hemolytic conditions can also cause spherocytosis. The absence of spherocytes does not exclude a diagnosis of IMHA, since spherocytes are only variably present in IMHA patients.[5,7,23,37,50,54,68] Spherocytes are formed when tissue macrophages remove a piece of RBC membrane without cell destruction or a significant loss of cytoplasm. The RBC volume remains normal, and RBCs are deformed into a spherical shape with a smaller diameter and loss of central pallor (Figure 35.7).

Ghost Cells

"Ghost cells" are RBCs that have ruptured in the circulation as part of intravascular hemolysis, and that have lost some or all of their hemoglobin content as

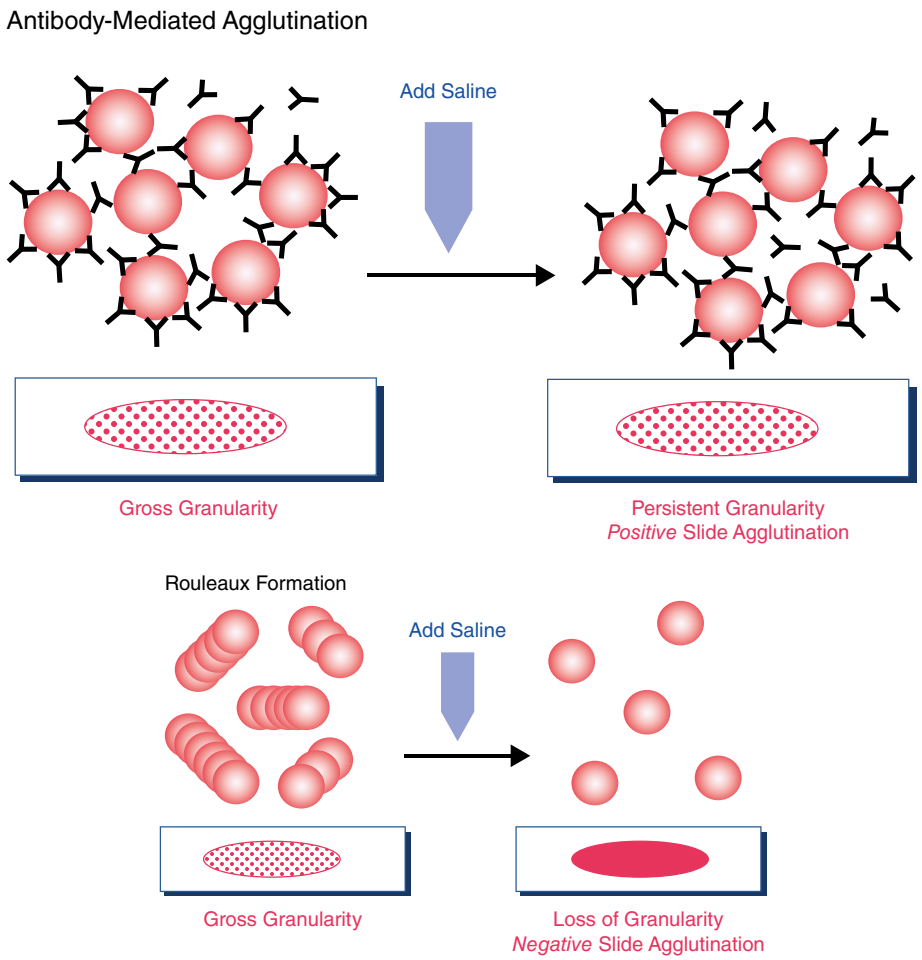


FIGURE 35.6 Saline slide agglutination test. In some dogs with IMHA, antibodies can bind to multiple different RBCs simultaneously, causing agglutination. These agglutinated RBCs are visible as red speckles. Similar speckles can be created by rouleaux, a weak nonimmune linear stacking of RBCs. A saline slide agglutination test is used to differentiate rouleaux from autoagglutination. Two to four drops of saline are added to one drop of anticoagulated (EDTA) blood on a microscope slide at room temperature, and the slide is swirled against a white background. Agglutination is seen grossly as persistent speckles despite dilution with saline (rouleaux, in contrast, disperse with saline), and microscopically as nonlinear clumps of RBCs.

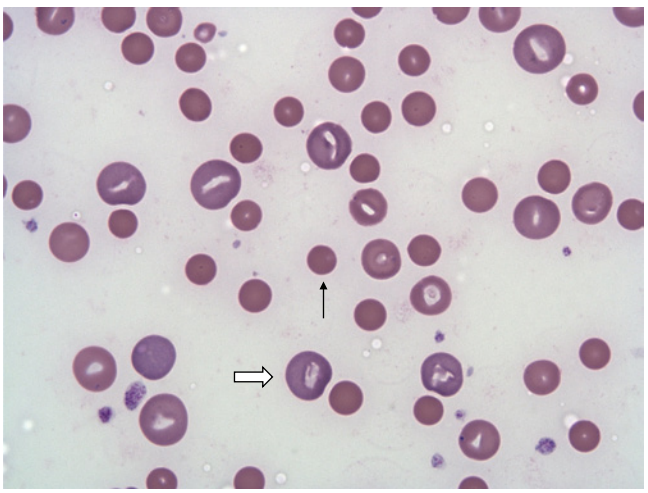


FIGURE 35.7 Blood smear. Typical blood smear from dog with IMHA, demonstrating spherocytosis (small RBCs without central pallor, denoted by black arrow), and the polychromasia and anisocytosis seen with the regenerative forms of IMHA (larger, bluer RBCs, denoted by hollow arrow).

part of this process. The residual RBC membranes linger and appear as empty erythrocytes. Ghost cells can be seen with intravascular IMHA, but also with nonimmune causes of intravascular hemolysis such as zinc toxicity. Ghost cells are only of diagnostic significance in blood smears that are made shortly after blood collection, since prolonged storage of anticoagulated blood prior to performance of blood smears can lead to a marked artefactual increase in the presence of ghost cells.

Other RBC Changes

Beyond revealing direct evidence of immune-mediated RBC lysis, careful examination of RBC morphology on blood smears may also suggest an underlying cause of either immunological or nonimmunological hemolysis. Diagnostically useful RBC abnormalities include detection of organisms such as *M. haemocanis* or *Babesia* species (which may cause secondary IMHA), Heinz bodies or eccentrocytes (suggesting hemolysis secondary to

oxidative damage), and schistocytes (suggesting a microangiopathic hemolytic process such as DIC, hemangiosarcoma, or caval syndrome).

Serum Biochemistry/Urinalysis

Serum biochemistry and urinalysis are often normal in dogs with IMHA. Potential abnormalities that may be seen in some patients include mild, moderate, or even marked elevation of liver enzymes (thought to indicate hepatic hypoxia secondary to severe anemia)[50] and variable hyperglobulinemia. Since serum protein and albumin are usually normal in IMHA patients, hypoproteinemia and hypoalbuminemia could support occult blood loss rather than hemolysis, or the presence of another illness. Mild to marked hyperbilirubinemia and bilirubinuria may be seen in dogs with acute severe hemolytic anemia,[50] but many dogs with IMHA will have normal bilirubin levels.[37,39,54]

Since the liver is usually able to cope with all but the transient overwhelming bilirubin loads produced by acute severe hemolysis, severe hyperbilirubinemia for more than 3–5 days, even in the markedly anemic animal, usually indicates the presence of concurrent hepatic disease or biliary obstruction. An exception to this may be the patient who is receiving multiple ongoing transfusions that potentially increase the hemolytic load.

Intravascular Hemolysis

Hemoglobinemia and hemoglobinuria are typically uncommon, transient events that indicate the presence of severe intravascular hemolysis.[37] Hemoglobinemia can be detected clinically in centrifuged whole blood samples as pink to red supernatant serum or plasma, and can lead to falsely elevated MCHC. While hemoglobinuria will test positive for "blood" on urine dipsticks, unlike hematuria, RBCs will not be detected on urine sediment examination in dogs with hemoglobinuria, and centrifuged urine samples will not separate into two layers of infranatant RBCs and supernatant urine.

Immunologic Testing

Specific immunological testing can be used to support a tentative diagnosis of IMHA. The most widely used test is the direct antiglobulin test (DAT) or Coombs test, which detects antibodies and/or complement bound to RBC membranes (see Chapter 143).[7,49] A standard DAT as provided by many laboratories may use a polyvalent mix of antibodies directed against IgG, IgM (to a variable extent), and complement, and is performed at body temperature. The end point of the typical DAT test is RBC agglutination (Figure 35.8). The addition of antibodies that detect and bind to IgG, IgM, or complement on the RBC membrane serves to facilitate the agglutination of RBCs in samples that would otherwise not have true autoagglutination. This process would be expected to increase test sensitivity compared to slide agglutination, albeit with a potential decrease in test specificity. Modifications of the routine screening DAT that have been shown to increase its diagnostic value include running the test at different temperatures and a wide range of titers, and using individual monovalent antibodies against IgG, IgM, and complement factor C3 as well as a standard polyvalent antibody/complement mix.[49,66] Positive DAT results at 4 °C; however, may arguably be of dubious diagnostic significance unless the patient has clinical signs consistent with cold-antibody-type agglutination or intravascular hemolysis.[66]

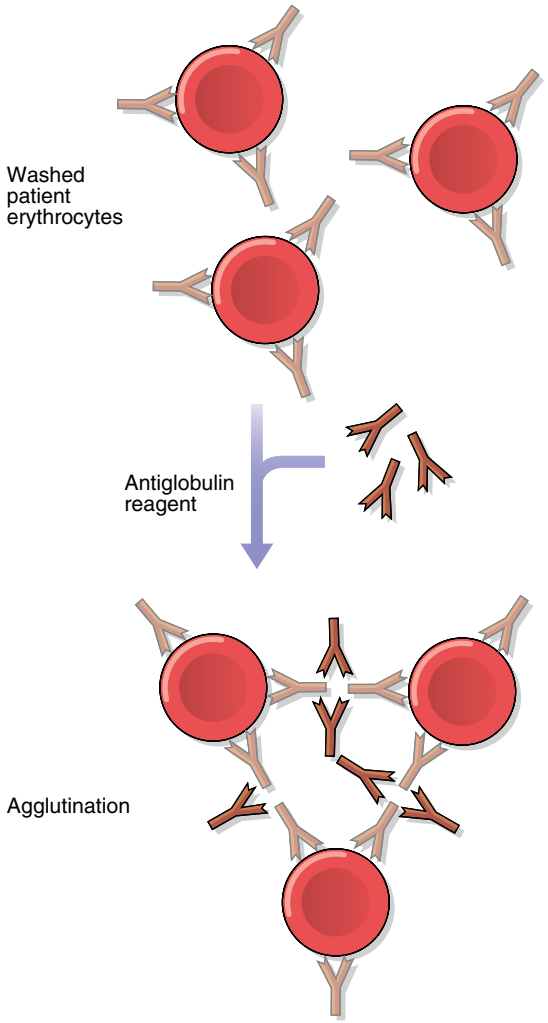


FIGURE 35.8 Principle of the Coombs test. A suspension of washed patient RBCs with membrane immunoglobulin or complement attached in vivo is incubated with an antiglobulin reagent that cross-links these immunoreactants, forming a lattice that appears grossly as an agglutinate of RBCs.

Strictly interpreted, a positive DAT result strongly supports a diagnosis of IMHA, while a negative test result suggests a nonimmunological cause of hemolysis.[7] Depending on the methodology used by the laboratory, the diagnostic accuracy of the DAT can vary from mediocre to high:[7,49] without careful attention to test methodology, false negative and false positive results can occur relatively commonly.[66] Veterinarians should therefore be aware that since IMHA can potentially occur in the presence of a negative DAT[37,50,54,59,68]

and, conversely, since a positive test does not absolutely prove the presence of IMHA, sometimes a tentative diagnosis must be made based on clinical judgement despite the presence of an apparently discrepant DAT result.

Various other immunological tests for detecting anti-RBC antibody have been reported, including an enzyme-linked immunosorbent assay, a direct enzyme-linked antiglobulin test, and flow cytometric detection of membrane-bound antibodies (see Chapter 143).[2,23] In-house rapid test kits are now also becoming available for performance of a DAT in tube-based or immunochromatographic format. Such tests provide a simple "positive/negative" result and can have value in an emergency setting or where traditional DAT testing is not available. Regardless of whether a DAT or an alternative test for anti-RBC antibody is used, clinicians should be aware that a positive result merely records the presence of antibody, and does not determine whether IMHA is primary or secondary. Interestingly, in some dogs with IMHA, Coombs tests (and other measures of anti-RBC antibody such as slide agglutination) can remain positive for many months following apparent clinical remission with immunosuppressive therapy.[7,9] Arguably, patients with persistently positive Coombs tests are at a greater risk of disease relapse if therapy is tapered too soon.

Rarely, IMHA (with or without IMT) will be merely one component of systemic lupus erythematosus (SLE), a multisystemic immunological disturbance. Measurement of serum antinuclear antibody (ANA) may therefore be indicated in those patients displaying evidence of the concurrent involvement of more than one body system, such as IMT, glomerulonephritis, polyarthritis, polymyositis, or immune-mediated skin disease. By contrast, ANA is probably not indicated (and is often negative) in those patients suspected to have uncomplicated IMHA.[54,68]

Screening for Secondary IMHA

Since IMHA can be the result of secondary causes, confirmation of a diagnosis of IMHA is only the first diagnostic step. Primary IMHA can only be diagnosed with absolute certainty once potential underlying causes have been thoroughly investigated and eliminated. This can present a diagnostic dilemma, because a complete search for these underlying causes can be expensive and, in the case of primary IMHA, ultimately fruitless. However, treatment for IMHA is unlikely to be effective if the underlying cause is not addressed. Failure to respond to therapy may indicate the need for more extensive diagnostic investigation.

Standard screening tests for underlying disease which ideally should be performed in all animals with IMHA include hematology (including careful examination of a blood smear), serum biochemistry, urinalysis, heartworm testing in endemic areas, and thoracic and abdominal radiography.[14] Blood smear examination and serologic and/or PCR testing for hemotropic organisms are also often indicated. *M. haemocanis*, an uncommon cause of significant anemia in dogs, can cause hemolysis in splenectomized dogs, immunosuppressed dogs, and dogs that have been transfused from a donor harboring the organism. Babesiosis should be considered as a potential cause of IMHA in particular breeds, geographic areas, and clinical circumstances. In North America, e.g., *Babesia canis vogeli* (also known as *Babesia vogeli*) should be considered in Greyhounds and dogs that have received transfusions from Greyhounds, *Babesia gibsoni* should be considered in Pit Bull Terriers and other fighting breeds, or dogs that have been bitten by or received transfusions from Pit Bull Terriers, and *Babesia conradae* should be considered in Californian dogs. Since arguably rickettsial diseases may also predispose to secondary IMHA, testing for rickettsial species may also be indicated in endemic areas, as may testing for bartonellosis or leishmaniosis. Further tests that might be considered in patients at risk for occult neoplasia (especially lymphoproliferative disease) include abdominal ultrasonography, lymph node aspiration cytology, and bone marrow analysis.

Marrow Analysis

Bone marrow analysis (aspiration cytology and/or core biopsy histopathology) is also typically indicated in dogs that are believed to have the nonregenerative forms of IMHA, or dogs that have suspected IMHA with concurrent neutropenia and thrombocytopenia ("pancytopenia"). Pure red cell aplasia is characterized by a relative or complete lack of RBC precursors within the marrow, whereas cytological or histopathological evidence of an erythroid "maturation arrest" (preponderance of immature precursors, with an absence of more mature RBC precursors) suggests PIMA/NRIMA. Marrow cytology and/or histopathology may also reveal macrophages phagocytosing erythrocytes or RBC precursors ("erythrophagocytosis"), plasma cell hyperplasia, and myelofibrosis.[69] In such patients, when available, techniques such as immunofluorescent or immunoperoxidase staining of marrow samples may confirm the presence of antibodies directed against RBC precursors. Underlying causes of secondary IMHA, such as lymphoid neoplasia, may also uncommonly be detected by bone marrow analysis.

Diagnostic Algorithm

Given that false positive and false negative results have been reported with practically all tests that have been associated with a diagnosis of IMHA, a diagnostic algorithm based on multiple test results has been recently suggested that categorizes the likelihood of a final diagnosis as "diagnostic," "supportive," "suspicious," or "not IMHA."[14] In an anemic dog, a definitive diagnosis is based on the presence of at least two indicators of immune-mediated destruction (such as spherocytes, a positive slide agglutination, or positive Coombs test) and at least one indicator of hemolysis (jaundice/hyperbilirubinemia/marked bilirubinuria, hemoglobinemia/hemoglobinuria, or ghost cells), in the absence of other obvious causes of anemia. A suspicious diagnosis of

IMHA is based on the presence of only one indicator of immune-mediated destruction in the absence of indicators of hemolysis, while a supportive diagnosis has more indicators of immune-mediated destruction or hemolysis, but does not meet the strict criteria required for a definitive diagnosis.[14] In pragmatic clinical terms, clinicians may also need to use an appropriate response to immunosuppressive therapy as a guide to a final diagnosis in patients that are suspected to have IMHA, but that do not meet initial criteria for a definitive diagnosis.

THERAPY

Veterinarians have been successfully treating individual dogs with IMHA for many years. Unfortunately, overall survival rates for canine IMHA patients are in general unsatisfactory, with many published studies reporting long-term survival rates of not much better than 50%.[5,6,15,18,21,25,29,39,48,50,59,68] Deaths (naturally occurring or euthanasia) occur either during initial hospitalization, or at a later date due to disease recurrence or owner intolerance of long-term medication.[68]

Since effective treatment cannot proceed without a correct diagnosis, a thorough workup is always recommended during the initial management of IMHA, with particular emphasis on detecting and potentially eliminating underlying triggers for secondary IMHA. Given a working diagnosis of primary IMHA, standard therapy during an initial crisis will typically include immunosuppressive doses of glucocorticoids with or without other immunosuppressive agents, transfusion as needed, and medications designed to prevent PTE ("thromboprophylaxis"). Even if an underlying cause for secondary IMHA has been identified and removed, immunosuppressive therapy may still be indicated during the initial treatment phase.

Glucocorticoids

Glucocorticoid therapy is a mainstay of both the emergency management and the chronic treatment of IMHA. Oral prednisolone (or prednisone) can be initiated at a starting dose of approximately 2–3 mg/kg once daily, although lower doses may be considered in large breed dogs, which seem to be particularly susceptible to steroid side effects. For larger dogs, prednisolone doses can be reduced to 1–1.5 mg/kg daily, or to 50–60 mg/m^2 daily, or "capped out" at a total dose of 50–60 mg daily. Injectable dexamethasone (0.2–0.4 mg/kg) is a reasonable alternative to oral prednisolone in dogs that cannot tolerate oral medications, though there is minimal evidence that intravenous steroid therapy hastens recovery. Commonly encountered glucocorticoid side effects include polydipsia, polyuria, polyphagia, and hyperventilation. If drug doses are not tapered over the first weeks to months of therapy, eventual signs of iatrogenic hyperadrenocorticism such as alopecia, abdominomegaly, hepatomegaly, muscle wasting, weakness and predisposition to infection, hypertension, diabetes mellitus, and pancreatitis are likely to occur. Adjunctive therapy (additional immunosuppressive agents, or splenectomy) may enable more rapid glucocorticoid dose reduction.

Immunosuppressive Agents

Immunosuppressive therapy with drugs such as azathioprine (2 mg/kg orally once daily), mycophenolate mofetil (8–12 mg/kg orally twice daily), microemulsified ("modified") cyclosporine (5–10 mg/kg orally twice daily), or leflunomide (2 mg/kg once daily) can also be commenced concurrently with prednisolone.[48,50,59,61,65,68] Some clinicians prefer to begin these drugs at the same time as prednisolone, especially in dogs with severe or immediately life-threatening IMHA. Others prefer to start therapy with prednisolone alone, and to then add in another immunosuppressive agent if prednisolone is ineffective after approximately 1–2 weeks of therapy, or if steroid side effects are unacceptable. Although azathioprine, mycophenolate, and cyclosporine are all available in injectable forms, there is minimal evidence that intravenous administration hastens recovery during crises. Cyclophosphamide, which was historically recommended in dogs with severe IMHA, has been associated with increased mortality rates, and is not recommended.[5,21,37]

Azathioprine (a purine analog antimetabolite), leflunomide (a pyrimidine synthesis inhibitor), and mycophenolate mofetil (a purine inhibitor) all target B and T lymphocytes. Azathioprine is usually well-tolerated, although hepatotoxicity, typically characterized by a rapid rise in serum alanine transaminase (ALT) that resolves with drug discontinuation, is seen in about 15% of treated dogs.[64] Azathioprine may also cause a mild nonregenerative anemia in some patients, which rarely affects the patient clinically. Other potential side effects, such as pancreatitis and severe myelosuppression, are uncommon to rare. Mycophenolate mofetil is often well-tolerated in dogs, but gastrointestinal signs, especially diarrhea, can occur commonly.[65] Gastrointestinal side effects have the potential to be severe, but are often dose-dependent. Limited literature is available on the use of leflunomide for the treatment of IMHA in dogs. The most commonly reported side effects associated with the use of leflunomide include diarrhea and lethargy, with unexplained hemorrhage and hepatopathies reported infrequently.[19,51]

Cyclosporine works by inhibiting calcineurin, which is needed for T-lymphocyte activation.[1] It is typically well-tolerated in dogs and, since it is not myelosuppressive, is likely the drug of choice for poorly regenerative forms of IMHA. Unlike azathioprine and mycophenolate, cyclosporine is approved for use in small animals (for management of atopic dermatitis and, in some countries, anal furunculosis), and therefore comes in a range of veterinary capsule and liquid formulations that allow for accurate dosing in small dogs without the need for drug compounding. Cyclosporine bioavailability varies widely between formulations, and only the

microemulsified form has sufficient bioavailability to be suitable for use in canine IMHA patients. Given that bioavailability is unpredictable, even with the microemulsified formulation, cyclosporine doses should be adjusted based on subsequent therapeutic drug monitoring (whole blood drug levels or pharmacodynamic monitoring of T-cell function). Cyclosporine can cause gastrointestinal disturbances, gingival hyperplasia, and, uncommonly, hepatotoxicity.

All canine IMHA patients receiving immunosuppressive therapy are at increased risk for bacterial infections (including cystitis, pneumonia, and pyoderma), and are also at risk for unusual and potentially severe infectious diseases such as toxoplasmosis, neosporosis, and opportunistic fungal infections. For this reason, clinicians should avoid overly aggressive immunosuppression unless patients have particularly severe forms of IMHA. The use of cyclosporine, arguably the most potent of the immunosuppressive drugs, without therapeutic drug monitoring, and the utilization of so-called "triple therapy" (a combination of a glucocorticoid and two other immunosuppressive drugs given concurrently) are particularly associated with an increased risk of infection.[10,20] Once immunosuppressive therapy is commenced, clinicians should be particularly vigilant for eruptions of underlying infectious diseases (particularly babesiosis and mycoplasmosis) that may in fact have been the underlying cause of secondary IMHA, but that were missed during the initial diagnostic investigation.

Dogs with Evans syndrome may also be treated with a single intravenous bolus of vincristine (0.02 mg/kg),[15,46] although vincristine is not indicated in dogs with IMHA alone. Vincristine is extremely corrosive if extravasated. Single vincristine doses are otherwise typically well tolerated, except in breeds that are prone to the MDR1 (ABCB1) gene mutation (such as Collies, Shetland Sheepdogs, Australian Shepherds, and Longhaired Whippets), in which the mutation should be tested for before starting the drug.

Supportive Care

Canine IMHA patients with severe anemia will be suffering from generalized tissue hypoxia, and will benefit from reducing oxygen demand by instituting strict cage rest until anemia responds to transfusion and/or drug therapy. Hemoglobin oxygen saturation is already near maximal, and supplementation with oxygen therefore is expected to be of minimal benefit, with the potential exception of patients suspected to have PTE. Since patients with IMHA have a normal blood volume, intravenous plasma or crystalloid or colloid fluid therapy is of little benefit and may contribute to volume overload.

Thromboprophylaxis

Since dogs with IMHA are prone to PTE, thromboprophylaxis is indicated during the initial treatment phase, except in those dogs with low platelet counts due to concurrent IMT.[61] Anticoagulant therapy with a heparin-based protocol is typically recommended.[4,24] Unfractionated heparin (typically administered as a starting dose at 150–200 U/kg subcutaneously four times daily, or as a constant rate infusion) can be utilized, ideally with dosing adjustments based on an inhibition of factor Xa assay when available,[4,24] or based on attaining a prolongation of partial thromboplastin times by at least 1.5 times baseline values. Alternatively, low-molecular-weight forms of heparin such as dalteparin (150 U/kg subcutaneously three times daily) or enoxaparin (0.8–1 mg/kg subcutaneously four times daily) may allow safer and more effective anticoagulation, particularly if Xa assays cannot be performed.[47] Newer human oral anticoagulants, such as rivaroxaban (0.9 mg/kg orally once daily), are also just starting to be used in IMHA dogs to prevent PTE.[42] Antiplatelet therapy, such as oral clopidogrel (1–4 mg/kg once daily) and/or low-dose aspirin (1–2 mg/kg once daily), can also be considered in addition to anticoagulant therapy, and can be safely and easily continued as part of long-term management.[41,68]

Transfusion

Cage rest and drug therapy alone can be successful in most dogs with non-life-threatening IMHA. However, initial response to therapy can sometimes be delayed (up to a week or more). Until response is seen, transfusion may be needed in dogs with life-threatening acute and marked anemia (signs of severe compromise, such as collapse, nystagmus or stupor, or a PCV of less than about 12%). Transfused RBCs often have a very short life span (days or even hours) in dogs with IMHA, and transfusions can in theory increase the rate of hemolysis ("add fuel to the fire"). Transfusions should therefore be avoided when possible in stable dogs with IMHA. However, in those IMHA patients that are severely compromised, blood transfusions are life-saving, and should not be withheld.

Since IMHA patients are typically normovolemic, and do not need additional plasma, packed red blood cells are preferable to whole blood, although whole blood may be an acceptable alternative when packed cells are not available. Cross-matches are typically difficult to interpret in dogs with autoagglutinating IMHA, and in such patients, universal or type-matched donors should be used when possible. Multiple transfusions, potentially as often as every day or two, may be needed in very severely affected dogs. Volume overload after transfusion can become a significant risk in dogs that have already recently received high volumes of blood or other fluids. In these dogs, blood transfusions should be given slowly (maximum rate of about 5 mL/kg/hour). Fresh packed red blood cells (less than 7–10 days old) are preferable to products that have been stored for a longer period,[22] and appear to be associated with a lower incidence of post-transfusion hemolysis.[36] Bovine purified polymerized hemoglobin can be considered as a means of providing several days of oxygen-carrying support for the severely anemic patient, but is not an available or financially feasible option for most cases.

Plasma transfusions are of minimal therapeutic benefit in dogs with IMHA.

Additional Therapeutic Options

Unfortunately, some dogs with severe IMHA, despite appropriate standard drug therapy and multiple transfusions, succumb to severe anemia during the first weeks of treatment. Additional treatment options which may be used in a refractory crisis include human intravenous immunoglobulin, therapeutic plasma exchange (plasmapheresis), splenectomy, and liposomal clodronate.

Human intravenous immunoglobulin (HIVIG) is a pooled preparation of immunoglobulin obtained from the plasma of multiple healthy blood donors. HIVIG as a 6–12 hour infusion at doses ranging from 0.5 to 1 g/kg variably causes rapid and sometimes sustained remission of IMHA,[30,55] although definitive proof of efficacy is lacking.[56,72] The proposed major mechanism of action of HIVIG in IMHA patients is that the infused antibodies bind to and overwhelm available macrophage Fc receptor sites, leaving no receptors left to bind antibody-coated cells.[30,55] The use of HIVIG in dogs is associated with few side effects,[56] although there is some concern that treated dogs have a higher incidence of PTE.[55] Because of the high risk of adverse immunologic reactions, previously treated dogs should not receive a second dose of HIVIG.[30,55]

Therapeutic plasma exchange can be a very effective method of rapidly removing unbound anti-RBC antibodies from the circulation,[8] and can also be used to reduce hyperbilirubinemia in dogs with kernicterus.[63] Therapeutic plasma exchange, although expensive and currently of limited availability, has been reported to reduce the severity of autoagglutination and decrease transfusion dependency in canine IMHA patients.[52]

Splenectomy is potentially a particularly effective treatment for IMHA because many different splenic elements contribute to the mechanisms reducing circulating RBC numbers: anti-RBC antibody production (plasma cells), antibody-coated RBC destruction (mononuclear phagocytic system), and RBC sequestration (splenic vasculature).[11] Splenectomy is the treatment of choice for some humans with chronic IMHA, with higher reported remission rates than medical therapy alone. Many human IMHA patients (60–80%) subsequently maintain adequate RBC counts without further medical therapy. Splenectomized patients that do require further treatment frequently demonstrate an improved response to medical therapy. Splenectomy has therefore been recommended early in the course of chronic human IMHA. Splenectomy has not been thoroughly clinically evaluated in a large group of canine IMHA patients, although case series reporting splenectomy in dogs with IMHA showed some promising results.[11,26] Early splenectomy in canine IMHA is not currently strongly recommended, because rates of remission are unpredictable and medical therapy is often better tolerated than in people. Splenectomy may be indicated in canine patients that are refractory to glucocorticoids and immunosuppressive agents, particularly if associated drug side effects are unacceptable. Life-threatening post-splenectomy complications in people (overwhelming infection, DIC) are uncommon in the dog. The most commonly reported post-splenectomy complication in dogs, erythrocyte parasitemia (mycoplasmosis or babesiosis), usually responds well to medical therapy. Persistent IMHA post-splenectomy indicates ongoing erythrocyte destruction by the non-splenic mononuclear phagocytic system.

Liposomal clodronate has marked macrophage-depleting properties, and may have potential rapid benefits comparable to effects of splenectomy.[38] When clodronate, a bisphosphonate, is incorporated into liposomes, it is then preferentially phagocytosed by cells in the mononuclear phagocytic system, causing rapid apoptosis and depletion of macrophages. Liposomal clodronate has been used with apparent therapeutic benefit in a small number of dogs with IMHA.[38] Although the drug is commercially available for experimental purposes, it is not currently available for medical use.

Follow-Up Monitoring

During initial treatment of IMHA, the hematocrit should be monitored at least daily until an appreciable response to immunosuppressive therapy is observed, and the hematocrit rises above approximately 20% in dogs. The hematocrit should then be monitored at least weekly until the anemia resolves completely. Given an adequate initial treatment response, drug therapy should be gradually tapered to acceptable maintenance doses that can be given safely long-term without significant side effects. Once well-tolerated maintenance doses of glucocorticoids and other immunosuppressive drugs are attained, therapy should arguably be continued for at least several months, with monthly monitoring of the hematocrit. Presuming that an underlying cause of IMHA has not been identified and eliminated, tapering therapy too rapidly increases the risk of a life-threatening relapse. Once anemia has been in remission for approximately 3–6 months, therapy can be cautiously withdrawn. The hematocrit should be monitored regularly over the months following cessation of therapy to enable early detection of potential relapses. Uncommonly, affected dogs may need ongoing immunosuppressive therapy for years, or even for life, to prevent severe relapse of IMHA. Exposure to potential triggers that were strongly suspected to have caused an initial episode of secondary IMHA in an individual patient, such as medications or vaccinations, should be avoided whenever possible. In general, medications and vaccinations probably do not need to be avoided in patients with a history of primary IMHA.

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CHAPTER 36

Immune-Mediated Anemia in the Cat

ASHLEIGH NEWMAN and TRACY STOKOL

Pathogenesis
Clinical Signs
Diagnosis
  Agglutination
  Direct Coombs Test
  Other Clinical Pathologic Findings
  Bone Marrow Aspiration
Features of IMHA
  Demographics
  Hematologic and Coagulation Findings
  Biochemical Findings
  Treatment, Response, and Survival
Triggers of Secondary/Associative IMHA
  Infectious Agents
  Inflammatory Disease
  Neoplasia
  Vaccines and Drugs
    Miscellaneous Causes
Cold Agglutinin Disease
Red Blood Cell Group Incompatibilities

Acronyms and Abbreviations

ACVIM, American College of Veterinary Internal Medicine; C3, complement component 3; CAD, cold agglutinin disease; Fc, fragment crystallizable region of the immunoglobulin heavy chain; FeLV, feline leukemia virus; FIP, feline infectious peritonitis; FIV, feline immunodeficiency virus; Hct, hematocrit; IgG, immunoglobulin G; IL, interleukin; IgM, immunoglobulin M; IMHA, immune-mediated hemolytic anemia; MDS, myelodysplastic syndrome; MHC, major histocompatibility complex; PCR, polymerase chain reaction; PCV, packed cell volume; PIMA, precursor-targeted immune-mediated anemia; PRCA, pure red cell aplasia; PTU, propylthiouracil; RBC, red blood cell; Th, T helper cell; Treg, regulatory T cell.

Immune-mediated hemolytic anemia (IMHA) is an uncommon cause of anemia in cats,[29] and occurs less commonly in cats than dogs.[19] It is usually caused by the binding of antibodies to mature RBC membranes, causing their accelerated and premature removal from the circulation by mononuclear phagocytes. In some cases, the immune attack is thought to be directed against erythroid precursors in the bone marrow, resulting in a nonregenerative anemia from ineffective erythropoiesis or pure red cell aplasia (PRCA, see Chapter 38).[8,38,45] If the initiating cause of IMHA is unknown, the disease has been traditionally called primary or idiopathic IMHA or autoimmune hemolytic anemia. When IMHA is believed to have been initiated by an underlying disease (e.g., infectious agents, neoplasia) or drugs, it has been called secondary IMHA. The American College of Veterinary Internal Medicine (ACVIM) released a consensus statement on the diagnosis of IMHA in dogs and cats in 2019. In this consensus statement, it was proposed to use new nomenclature of "non-associative" and "associative," to replace primary and secondary IMHA, respectively.[19] This nomenclature appropriately reflects the possibility that all triggers may not have been definitively ruled out, and that while a comorbidity may have been identified, this does not necessarily prove causation. A retrospective study of 107 cats with IMHA showed that approximately two-thirds of cases are primary/non-associative and one-third are secondary/associative,[39] which is contrary to the older literature.[18,37] Hemolytic transfusion reactions and neonatal isoerythrolysis due to blood group incompatibilities are also variants of immune-mediated anemia, and will be addressed at the end of this chapter.

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PATHOGENESIS

IMHA is a type II immune complex hypersensitivity reaction.[6] Antibodies, usually IgG or IgM, react with antigens on the RBC membrane. Once bound to RBCs, certain types of Ig classes can activate complement. Erythrocytes are then destroyed through Fc- and complement-receptor-mediated phagocytosis by macrophages primarily in the spleen, liver, and bone marrow (i.e., extravascular hemolysis), and/or through direct complement-mediated lysis (i.e., intravascular hemolysis) (Figure 36.1). In cats, hemolysis is primarily extravascular, with RBC agglutination present in many cases.[28,39] Intravascular hemolysis has only been reported in cats with immune-mediated anemia secondary to blood group incompatibilities.[13,20,26]

While the basic pathogenesis of IMHA is still not completely understood, it is generally considered to be

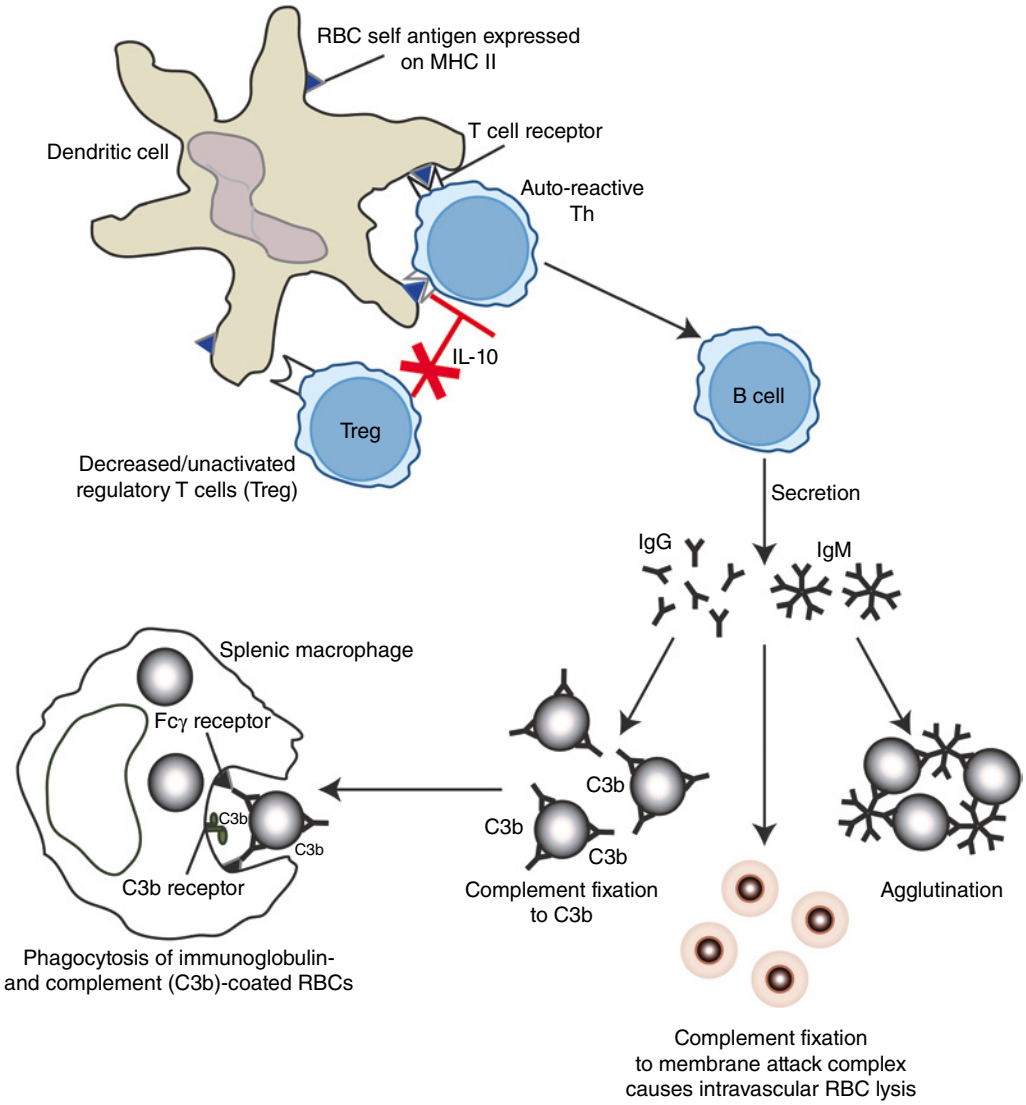


FIGURE 36.1 Proposed pathogenesis of IMHA based on studies in humans and murine models. A self-RBC antigen, which was previously cryptic or inefficiently or not processed, is expressed on the surface of dendritic or other antigen-presenting cells in conjunction with major histocompatibility type II (MHC II) proteins. The self-RBC antigen and MHC II (not shown) are recognized by autoreactive T helper cells (Th). Concurrently, regulatory T cells (Treg), which normally keep the immune system in check through secretion of anti-inflammatory cytokines, such as IL-10, are suppressed, leading to proliferation of autoreactive Th cells. Both Th1 and Th2 cells can activate B cells, which secrete immunoglobulins (IgG and/or IgM) that specifically recognize the RBC self-antigen. Complement activation by IgM or certain IgG classes leads to deposition of C3b on RBCs. Macrophages in the spleen, liver, and bone marrow recognize the Fc portion of immunoglobulin and bound C3b through specific receptors and remove the immunoglobulin- and C3b-coated RBCs prematurely from the circulation. Formation of the membrane attack complex of complement (C6-9) on RBCs causes them to lyse intravascularly. Alternative mechanisms by which Th cells can be activated are processing of a foreign peptide that cross-reacts with RBC antigens or microbial superantigens that cause hyperstimulation of the immune system. (Sources: Barcellini[6]; Barros et al.[7])

a loss or failure of self-tolerance.[6,7] Based on studies in humans and rodent models, T cell dysregulation is thought to be crucial for IMHA. Autoreactive helper T cells (Th), including pro-inflammatory T cells that secrete interleukin (IL)-17, react to RBC antigens[6,46] that were either cryptic or not efficiently processed or presented on dendritic cells.[7] These antigens can be intrinsic to the RBC ("self" antigens) or foreign proteins (e.g., drugs or infectious agents) that bind to or modify RBCs rendering them antigenic, or that express antigenic determinants that mimic those on RBCs ("molecular mimicry"). Regulatory T cells (Treg), which normally inhibit autoreactive Th cells, may be concurrently suppressed.[6,7] Specific RBC antigens responsible for IMHA in cats have not been identified and the role of Treg or Th in cats with IMHA has not been elucidated.

CLINICAL SIGNS

Clinical signs in cats with IMHA are primarily attributable to the anemia and consist mostly of lethargy and inappetence or anorexia.[8,28,37] Some cats may demonstrate vomiting, pica, weight loss, obstipation, and polydipsia.[8,28,37,49] On physical examination, cats have pale pink or rarely icteric mucous membranes, tachycardia, tachypnea, and heart murmurs and gallop rhythms.[8,28,49] Some cats have splenomegaly, hepatomegaly, or lymphadenopathy.[28,37] Cats with secondary/associative IMHA may display additional signs related to their underlying disease.

DIAGNOSIS

Current diagnostic criteria for IMHA are aimed at documenting an underlying immune-mediated pathogenesis for the anemia, which includes agglutination and a positive direct Coombs test.[19] In the absence of these findings, the diagnosis of IMHA depends on a positive response to immunosuppressive therapy.[8,38,43,48] Unlike dogs, spherocytes are not included in the diagnostic criteria for IMHA in cats.[19] This is because spherocytes are difficult to detect in cats, due to their smaller erythrocytes, which generally lack central pallor.

IMHA typically results in a moderate to severe anemia with a hematocrit (Hct) or PCV ranging from 6–22% (average, 9–13%).[28,29,37,39] The anemia may be regenerative or nonregenerative, although the latter appears to be more common. In a recent retrospective study of 107 cats with IMHA, the anemia was nonregenerative in 56% of cats with primary/non-associative IMHA, with a median absolute reticulocyte count of 32.5 × 10^3/μL. Cats with secondary/associative IMHA had a similar median absolute reticulocyte count of 38.6 × 10^3/μL.[39] In another study, 58% of 19 cats with primary/non-associative IMHA had a nonregenerative anemia.[28] Lack of regeneration may be due to duration, presence of concomitant disease, or targeting of erythroid precursors, rather than mature RBCs (Figure 36.2).

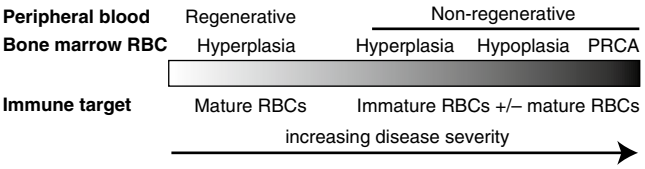


FIGURE 36.2 The IMHA continuum. The disorder can manifest anywhere on a spectrum of disease, ranging from a regenerative anemia with bone marrow erythroid hyperplasia to a nonregenerative anemia with no to few erythroid precursors in the marrow, that is, PRCA (see Chapter 38).

Agglutination

Agglutination can be detected microscopically on a Wright's-stained blood smear by the presence of three-dimensional RBC aggregates, which are often most obvious at the base of the smear. Agglutination can be differentiated from rouleaux formation with a saline agglutination or dispersion test or "in-saline agglutination test" (see Chapter 35).[19,41]

The ACVIM consensus statement recommends using warmed or room temperature saline for washing or saline agglutination tests, based on reports of persistent agglutination in normal dogs at 4 °C.[19] As for cats, one study used 4 °C for detection of persistent agglutination in cats with and without anemia (n = 60 per group).[41] None of the nonanemic cats had persistent agglutination at 4 °C in this study, but some anemic cats without IMHA agglutinated, indicating agglutination at this temperature is not specific for IMHA. It is worth noting that agglutination can occur as a consequence of EDTA-associated binding of a nonpathogenic antibody to RBCs.[36] In this report, there was no other clinical or hematologic evidence of hemolysis, emphasizing the importance of using multiple diagnostic criteria to reach a diagnosis of IMHA.

Direct Coombs Test

The direct Coombs test, also known as the direct antiglobulin test or DAT, detects antibody and complement bound to the surface of patient RBCs. This is in contrast to the indirect antiglobulin test, which detects unbound antibody in serum, and is infrequently used in veterinary medicine.[43] While flow cytometry can also be performed to detect antibody and/or complement binding in dogs, there are no reports of its use in cats at this time[19,43] (for more on the Coombs test and its methodology, see Chapters 35 and 43).

The sensitivity of the Coombs test for the diagnosis of IMHA in cats varies from 27%[39] (using >1:16 Coombs positive as a cutoff) to 82%[41] (positive result regardless of titer). With nonregenerative variants, 50% of cats in two studies had a positive Coombs test, supporting concurrent peripheral and intramedullary destruction of erythrocytes.[8,38] Specificity varies, depending on the use of cutoff titers and temperature of analysis, but ranges from 93% (77/83) in nonanemic cats[2] to 95% (89/94) in anemic or nonanemic cats[41] with polyvalent antisera at

37 °C. This indicates that positive reactions can be seen in anemic cats that do not fulfill criteria for IMHA, and that the Coombs test alone should not be relied upon for a diagnosis. Additionally, the Coombs test does not differentiate between primary/non-associative and secondary/associative IMHA.[28,41]

Other Clinical Pathologic Findings

Clinical pathologic results indicating intravascular hemolysis, including ghost cells in a freshly made blood smear, can also be used to support a diagnosis of IMHA, as long as other causes of intravascular hemolysis, such as oxidant injury, are excluded.[19] Ghost cells can be artifacts of smear preparation, therefore a diagnosis of intravascular hemolysis should not solely be based on the presence of these cells in a blood smear, without hemoglobinemia and/or hemoglobinuria as additional support. Intravascular hemolysis is usually a feature of blood group incompatibilities.[13,20,26]

Hyperbilirubinemia or icterus, in the absence of liver disease or cholestasis, including bile sludging in cats, can also be provided as support for IMHA, particularly if there is only a positive saline agglutination test or Coombs test.[19]

Bone Marrow Aspiration

A bone marrow aspirate is not indicated in a regenerative IMHA; however, it should be considered if the anemia is persistently nonregenerative. Up to 40% of cats with IMHA continue to have a nonregenerative anemia 5 days after beginning immunosuppressive therapy, indicating that duration alone is not a contributing reason for lack of regeneration in up to one-third of cats.[39] A bone marrow aspirate is recommended in such cases to determine the status of the erythropoietic response and to help identify or exclude underlying causes, such as primary or infiltrative bone marrow neoplasia and myelodysplastic syndrome (MDS, see Chapter 67). In the absence of bone marrow neoplasia or other causes that could inhibit erythropoiesis, lack of regeneration is usually attributed to targeting of erythroid precursors in the marrow, called precursor-targeted immune-mediated anemias or PIMA.[30] This precursor targeting can result in ineffective erythropoiesis, decreased production, or lack of production of RBCs (PRCA). Ineffective erythropoiesis and PRCA are reported to occur with similar frequency in cats with nonregenerative forms of IMHA.[8,45] Ineffective erythropoiesis is characterized by erythroid hyperplasia in a typically hyperplastic marrow. Erythroid maturation may be left-shifted (more immature than mature precursors) or incomplete, where maturation only appears to proceed to a specific stage. Incomplete maturation has been called "maturation arrest,"[45] a term which should be avoided, because there is no evidence that there is a true arrest in maturation. By contrast, an absence or marked decrease in erythroid precursors would be consistent with PRCA (see Chapter 38). Phagocytized erythroid precursors in marrow macrophages, known as rubriphagocytosis, provide additional cytologic support for intramedullary destruction of erythroid precursors.[30] Stainable iron within macrophages may be seen in all forms of immune-mediated anemia in cats due to increased RBC turnover or lack of erythroid production.[8,9,38,39,45] A small cell lymphocytosis in marrow is a common finding in cats with IMHA, particularly those with PIMA, and may argue for an immune-mediated pathogenesis.[8,38,39] Lymphoid hyperplasia has been described, as both distinct follicles or a diffuse infiltrate, in the bone marrow of cats with primary/non-associative and secondary/associative IMHA with higher proportions in the former (30%, 14/47) than the latter (6%, 1/18).[39] Lymphocytes constituted a median of 20% of bone marrow cells in the latter study, with higher numbers being reported in cases of PRCA (up to 45%).[38]

The main differential diagnosis for a cat with a nonregenerative anemia and ineffective erythropoiesis is MDS. In MDS, there is marked dysplasia in erythroid or other hematopoietic cells,[10] whereas only mild to moderate erythroid dysplasia is seen in cats with PIMA. Myelofibrosis is seen in just over half of MDS cases,[9] whereas it was only documented in 7% (2/28)[45] and 25% (2/8)[8] of cats, with nonregenerative IMHA in two different studies. Lastly, the presence of siderocytes or sideroblasts[10] argues for an MDS, as these are considered uncommon in cats with PIMA in the authors' experience. Ultimately, however, the distinction of PIMA from MDS may rely on response to immunosuppressive therapy.

FEATURES OF IMHA

Demographics

In a retrospective study of 107 cats with IMHA, those with primary/non-associative were significantly younger (median 4.2 years old) than cats with secondary/associative IMHA (median 6.8 years old).[39] A young age predilection (<4 years old) in primary/non-associative IMHA is further supported by other studies.[8,28,38] While some case series have shown a male predominance,[8,28,37] this is not a consistent finding.[38,39]

Hematologic and Coagulation Findings

Unlike dogs with regenerative IMHA,[15,23] inflammatory leukograms are not a common finding in cats.[28,39] In two different studies, 15% and 32% of cats with primary/non-associative IMHA had a lymphocytosis,[28,39] which consisted of B cells in one cat.[39] The lymphocytosis resolved rapidly with treatment in the latter study. Cats with primary/non-associative IMHA had higher median lymphocyte counts (median 2.4 × 10^3/μL, range 0.4–20.0 × 10^3/μL) than those with secondary/associative IMHA (1 × 10^3/μL, 0.1–17.4 × 10^3/μL).[39] While this may be due to their younger age and an epinephrine response, antigenic stimulation could also be occurring. The latter hypothesis is supported by increased lymphocytes in the bone marrow of cats with IMHA.[8,38,39]

Cats with primary/non-associative IMHA may also have other cytopenias. Thrombocytopenia is reported in 42–67% and neutropenia in 17–53% of cats with primary/non-associative IMHA, including both regenerative and nonregenerative cases.[8,28,39] Marked thrombocytopenia (<30,000/μL) has been documented in one cat with primary/non-associative IMHA,[28] and two cats with PRCA,[8] although both thrombocytopenia and neutropenia are not common features of PRCA.[8,38] These studies did not state how many cats were pancytopenic.

Coagulation data was only provided in one study of cats with primary/non-associative IMHA.[28] In this study, the prothrombin time (PT) and activated partial thromboplastin time (aPTT) were prolonged in 30% (3/10) of these cats.[28] Based on prolongation of the PT and aPTT ± thrombocytopenia, disseminated intravascular coagulation was diagnosed in one cat and suspected in two other cats, the incidence of which is lower than reported for dogs with IMHA.[15] Thrombosis is rarely definitively documented in cats, being identified ultrasonographically in hepatic or portal vessels in one of 107 cats (<1%) with IMHA.[39] By contrast, thrombosis is a known complication of IMHA in dogs.[17]

Biochemical Findings

Biochemical results largely reflect the consequences of anemia, including hypoxic liver injury (increased transaminase activity)[8,28] and excessive bilirubin production.[28] Hyperbilirubinemia (median 0.7 mg/dL, range 0.2–1.9 g/dL) was documented in 68% of cats with primary/non-associative IMHA in one study.[28] Bilirubin concentrations are lower in cats with nonregenerative versus regenerative forms of IMHA.[39] Serum albumin concentrations were significantly lower in cats with secondary/associative IMHA (mean 2.7 ± 1.1 g/dL) versus primary/non-associative IMHA (3.2 ± 0.7 g/dL).[39] This may reflect a negative acute phase response in cats with other disorders. Hyperglobulinemia (median 4.1 g/dL, range 2.8–8.6 g/dL) was documented in 53% of cats with primary/non-associative IMHA.[28] In a study that compared cats with primary/non-associative and secondary/associative IMHA, globulin concentrations were similar between the two groups.[39] Hyperglobulinemia further supports the hypothesis of antigenic stimulation in some cats with IMHA.

Treatment, Response, and Survival

The mainstay of treatment for primary/non-associative IMHA is immunosuppressive doses of glucocorticoids.[28,39] This can be used alone or in combination with other immunosuppressive agents, such as cyclosporine, mycophenolate, or chemotherapeutics such as chlorambucil or cyclophosphamide.[5,39] Before initiating immunosuppressive therapy, a thorough evaluation for underlying disease should be performed (see the next section titled "Triggers of Secondary/Associative IMHA"). Blood transfusions are frequently given to symptomatic cats (53–93% in two studies[8,28]) and multiple transfusions may be required over a short time frame. Other common adjunctive therapies include crystalloid fluids, histamine or H_2-receptor antagonists, and doxycycline or other antibiotics.[28,39]

One study provided data on the early response rate of cats with nonregenerative primary/non-associative IMHA. Within the first 5 days of treatment, a regenerative response was seen in 45% (18/40), the anemia remained nonregenerative in 40% (16/40), 8% (3/40) died or were euthanized, and 8% (3/40) of the cats did not have reticulocytes reassessed. For those cats that survived beyond 5 days, 52% (25/48) attained a Hct ≥ 25% within 1 month of discharge. Of the nonresponsive cats, 48% (11/23) died or were euthanized within 1 month.[39] These data are in line with other studies, which show that most cats have a regenerative response or a Hct or PCV ≥ 25% within 1 month of treatment.[8,28]

Relapses, as documented by a decrease in Hct or PCV, can occur while immunosuppressive therapy is being tapered or in some cases as long as 7 or 10 months after discontinuation of all medications.[8,28] Reported relapse rates are 27–31%, occurring from 56 to approximately 300 days after diagnosis, and are often a reason for euthanasia.[8,28] The survival time for cats with primary/non-associative IMHA was longer than those with secondary/associative IMHA with median survival times of 516 days versus 14 days, respectively (not including death or euthanasia during hospitalization), indicating comorbidities are a negative prognostic indicator.[39]

TRIGGERS OF SECONDARY/ ASSOCIATIVE IMHA

Evaluation for potential triggers or comorbidities in cats with IMHA should include a thorough history with attention to drug exposure and travel, and a search for infectious, inflammatory, and neoplastic disorders. In the 2019 ACVIM consensus statement, the authors critically and systematically evaluated data from published veterinary studies to determine the level of evidence for presumed triggers of IMHA. High- and intermediate-level evidence indicates the trigger likely induces IMHA, whereas low-level evidence indicates the trigger is unlikely to result in IMHA or there is insufficient information to answer this question.[19]

Infectious Agents

Any recent or recurrent infection could be a possible contributing factor to the development of IMHA.[19] There is a high level of evidence for *Mycoplasma haemofelis* (Chapter 33) inducing IMHA, hence, the frequent incorporation of empirical doxycycline therapy pending PCR results.[8,19,28,39,42,50] Genetic testing for *M. haemofelis* is recommended in all cats with IMHA.[19] Experimental studies suggest the anemia likely results from the parasite damaging RBCs and unmasking cryptic antigens.[19,50] As for other less pathogenic *Mycoplasma* species, there is negligible to low level or no evidence that *Candidatus*

Mycoplasma haemominutum and *Candidatus Mycoplasma turicensis* induce IMHA in cats, respectively.[19,42] There is low to intermediate level of evidence[19] that *Mycoplasma gatae*, a nonhemotropic mycoplasma, induces IMHA based on a case report of a cat with inflammatory joint disease and Coombs-positive anemia.[47]

There is intermediate-level of evidence that *Babesia felis* causes IMHA in cats and that adjunctive immunosuppressive therapy is not necessary in infected cats.[19] Primaquine phosphate is the treatment of choice.[4] Genetic testing is recommended for cats from endemic regions with compatible clinical signs (see Chapter 33).[19]

During the 1970s and 1980s, 43–60% of cats with IMHA were feline leukemia virus (FeLV)-positive,[18,37] whereas the prevalence of FeLV infection in IMHA cats is now much lower (approximately 3%).[21,25] Regardless, FeLV and feline immunodeficiency virus (FIV) testing are recommended in all sick cats per the American Association of Feline Practitioners.[19] Cats positive for FeLV have a higher risk of anemia (typically nonregenerative),[25] thrombocytopenia, and neutropenia.[21] While some studies document intermediate-to-high level of evidence for FeLV inducing IMHA, the consensus was that there is a low of evidence for an association overall.[19] See Chapter 60 for more information on FeLV.

There is negligible evidence that FIV, feline infectious peritonitis (FIP), *Leishmania*, or localized bacterial infections induce IMHA in cats, however, additional mechanistic studies are needed.[19,21,25,35,39]

Inflammatory Disease

Reported underlying inflammatory conditions in cats with secondary/non-associative IMHA include pancreatitis, cholangitis, glomerulonephritis, chronic interstitial nephritis, urinary tract infection, suspected pyelonephritis, and polyarthritis.[18,39,49] A significant association between pancreatitis and IMHA in cats was documented in one study of 155 sick cats, with 27% of cats (3/11) with IMHA having pancreatitis,[49] interpreted as low-to intermediate-level of evidence.[19] However, the overall prevalence of concurrent IMHA and pancreatitis was low in that study (2%).[49]

Neoplasia

In a retrospective study of 107 cats with IMHA, 15% of all cases and 46% of secondary/associative IMHA cases had underlying neoplasia.[39] While neoplasia, predominantly that of hematopoietic origin including lymphoma, MDS, acute myeloid leukemia, histiocytic sarcoma, and multiple myeloma, has a relatively high prevalence in cats with IMHA,[18,27,28,37,39] overall there is negligible to low-evidence for a cause and effect relationship.[19]

Vaccines and Drugs

There are no studies directly evaluating a link between vaccination and IMHA in cats, unlike in dogs where there is mixed evidence supporting or negating an association.[19] In one study of 19 cats diagnosed with primary/non-associative IMHA, 16% of cats were vaccinated in the 4 weeks prior to initial presentation.[28]

TABLE 36.1 Mechanisms of drug-induced secondary immune-mediated hemolytic anemia.

Hapten	Antibodies primarily recognize the drug, which is bound to RBC proteins. The RBCs are subsequently destroyed as an innocent bystander
Immune complex	Antibodies (usually IgM) primarily recognize the drug–RBC protein complex
Autoimmune	Anti-RBC or anti-erythropoietin antibodies are induced by the drug (due to probable cross-reactivity). Hemolysis can proceed in the absence of the inciting drug
Adsorption	Drugs can induce the nonspecific binding of immunoglobulins, complement components, and other proteins to RBC membranes. Although this can result in a positive Coombs test, RBC survival is usually unaffected

Sources: Cunningham and Silberstein[6]; Packman[33]

There are various proposed mechanisms of how drugs may induce IMHA (see Table 36.1).[16,33] Although any drug has the potential to cause IMHA, reports in cats are scarce.[19] There is intermediate to strong evidence that propylthiouracil (PTU), a drug used to treat hyperthyroidism, is a trigger for IMHA.[3,19,34] Carbimazole, another medication for hyperthyroidism, was implicated as the trigger in a single case report.[32] Warfarin toxicity reportedly resulted in a weak positive Coombs anemia in a single cat.[18] Aside from PTU, the prevalence of drug-induced IMHA in cats is low or potentially underreported.[19] However, as with other categories of triggers, the lack of evidence does not preclude the possibility of a drug or toxin triggering IMHA.[19]

Miscellaneous Causes

There is a report of five closely related, FeLV- and FIV-negative, 6–7-week-old British Shorthair kittens with a moderate to severe regenerative anemia and a non-neoplastic, nonpainful, multicentric lymphadenopathy.[1] IMHA was suspected based on the absence of hemorrhage, the presence of many splenic and hepatic erythrophagocytic macrophages on histologic examination, and grossly visible agglutination in one case. However, a direct Coombs test was negative in the two cats in which it was run. Additionally, immunosuppressive treatment with corticosteroids was unsuccessful in two cats.[1] Further research is needed on this rare condition.

Other immune-mediated conditions that have been associated with secondary IMHA include presumptive graft-versus-host disease in a cat after bone marrow transplantation[14] and systemic lupus erythematosus.[31] The cat in the latter report was Coombs-negative; however, a regenerative anemia with hyperbilirubinemia with a predominance of unconjugated bilirubin was present.[31]

Cold Agglutinin Disease

The term "cold agglutinin" gets its name from the finding of persistent agglutination in a Coombs test at 4 °C.[40] While typically cold reactive antibodies are thought to be nonpathogenic, given their presence in low titers in healthy animals,[2,18] there are cases in which they are pathogenic and indicative of cold agglutinin disease (CAD), a rare form of IMHA.[40]

In cats, there are rare reports of CAD, although it is possible that the condition is underreported or underrecognized.[11,22,50] Three kittens from two separate litters of Birman kittens from the same queen developed tail-tip necrosis and were diagnosed with presumptive neonatal isoerythrolysis involving cold-reactive agglutinins based on the queen and the second male cats' blood types. Coombs testing on the kittens was not performed.[11] An anemic cat with ear-tip cyanosis and histopathologic evidence of thrombosis of small superficial vessels was suspected to have CAD based on a positive saline agglutination test in refrigerated heparinized blood diluted 1:1 in saline. The clinical signs in this cat resolved after removal of a lead foreign body.[22] Cold agglutinins have also been identified with experimental *M. haemofelis* infections and were suspected to contribute to the anemia in these cats.[50] Persistent agglutination at 4 °C can be seen in healthy nonanemic cats,[2,18] and should not be interpreted as a pathologic finding without other clinical or laboratory supportive evidence.

RED BLOOD CELL GROUP INCOMPATIBILITIES

Three main blood types are present in cats: A, B, and AB.[12,24] In addition, cats may express the *Mik* antigen, the inheritance of which is unknown.[44] Immune-mediated RBC destruction through intravascular and extravascular hemolysis occurs in cats exposed to incompatible blood types through transfusions or via colostrum ingestion with neonatal isoerythrolysis.[12,13,20,26] These hemolytic reactions do not require prior sensitization because cats develop naturally occurring alloantibodies against RBC antigens by 2–3 months of age[12,20,44] (see Chapters 96 and 107 for more information).

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CHAPTER 37

Immune-Mediated Anemia in Ruminants and Horses

JENIFER R. GOLD

Immune-Mediated Hemolytic Anemia
- Pathogenesis of Immune-Mediated Hemolytic Anemia
- Clinical Features of Immune-Mediated Hemolytic Anemia
- Diagnosis of Immune-Mediated Hemolytic Anemia
- Treatment of Immune-Mediated Hemolytic Anemia
- Prognosis for Immune-Mediated Hemolytic Anemia

Neonatal Isoerythrolysis
- Pathogenesis of Neonatal Isoerythrolysis
- Clinical Features of Neonatal Isoerythrolysis
- Diagnosis of Neonatal Isoerythrolysis
- Prevention and Treatment of Neonatal Isoerythrolysis
- Prognosis for Neonatal Isoerythrolysis

Equine Infectious Anemia
- Pathogenesis of Equine Infectious Anemia
- Clinical Features of Equine Infectious Anemia
- Diagnosis of Equine Infectious Anemia
- Treatment and Prevention of Equine Infectious Anemia

Piroplasmosis/Babesiosis
- Pathogenesis of Piroplasmosis
- Clinical Signs of Piroplasmosis
- Diagnosis of Piroplasmosis
- Treatment of Piroplasmosis
- Control of Piroplasmosis

Acronyms and Abbreviations

AGID, agar gel immunodiffusion test; AIHA, autoimmuine hemolytic anemia; CR1, complement receptor 1; CR3, complement receptor 3; cELISA, competitive enzyme-linked immunosorbent assay; EIA, equine infectious anemia; EIAV, equine infectious anemia virus; EMA, equine merozoite antigens; Fcγ receptor, fragment crystallizable gamma receptor; Hgb, hemoglobin; JFA, jaundiced foal agglutination (test); MAC, membrane attack complex; NI, neonatal isoerythrolysis; qPCR, quantitative polymerase chain reaction; RAP, apical merozoite protein rhoptry; USDA, United States Department of Agriculture.

Immune-mediated hemolytic anemia (IMHA) is an immunohematologic syndrome where destruction of RBCs is accelerated by the attachment of antibody, with or without complement, to the erythrocyte (type II hypersensitivity reaction). Horses and cattle have no breed or gender predilection for IMHA, with occurrence seen less frequently than in dogs but considered to be an important cause for anemia.[3,6,8,10,16,28,30,36,41,45] IMHA can either be primary (idiopathic) or secondary. Primary IMHA occurs when antibodies are directed against the unaltered cell surface antigens. Examples of primary IMHA are autoimmune disease, neonatal isoerythrolysis (NI), or incompatible blood transfusions. Secondary IMHA occurs when antibodies are formed against substances that antigenically alter the surface of red cell.[10,17,36] Affected horses most commonly have secondary IMHA with causes reported to include neoplasia, *Rhodococcus equi* infection in foals, *Clostridium sp*., drug administration, and infections (Table 37.1). IMHA is an infrequent cause of anemia in cattle with both primary and secondary IMHA reported (Table 37.1).

This chapter provides an overview of the major causes of immune-mediated anemia in ruminants and horses, with focus on immune-mediated hemolytic disease, neonatal isoerythrolysis, equine piroplasmosis, and equine infectious anemia (EIA).

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TABLE 37.1 Causes of immune-mediated hemolytic anemia (IMHA) in equine and ruminants.

	Horses	Cattle
Primary IMHA	Incompatible blood transfusion[2,8,22,27] Neonatal isoerythrolysis [4,11,12,18,21,23,37] Autoimmune hemolytic anemia (idiopathic)[3,8,28,36,45]	Incompatible blood transfusion[2,14,22] Neonatal isoerythrolysis[19,34,37] Autoimmune hemolytic anemia (idiopathic)[6,10,14,16,30,41]
Secondary IMHA infectious	*Clostridium perfringens*[31,44] *Clostridium sp.*[1] Equine infectious anemia[26] Injection abscesses[1] Leptospirosis[7] Piroplasmosis[20,35,39,45–47] *Rhodococcus equi*[15] *Streptococcus spp.*[13]	Anaplasmosis[14] *Salmonella dublin*[17] Pneumonia[14] Trypanosomiasis[9] *Theileria*[14,30,40]
Drugs/toxins	Penicillin[24,33] Sulfonamides[37] *Pistacia*[5,42]	Foot and mouth vaccine[30] Sulfonamides[30]
Neoplasia	Lymphosarcoma,[25,32] melanoma, other neoplasia[27]	
Other systemic diseases	Protein-losing enteropathy[29] Purpura hemorrhagica[13]	Mastitis[30] Metritis[30]

IMMUNE-MEDIATED HEMOLYTIC ANEMIA

Primary IMHA resulting from an idiopathic loss of self-tolerance to RBC surface antigens is a rare condition in large animals.[2,3,28] Secondary IMHA in equine and ruminants tends to be associated with infection, exposure to drugs or toxins, or neoplasia. Drug-induced hemolytic anemia is a type II hypersensitivity reaction in which destruction of RBCs occurs by one of three mechanisms: (1) the drug and antibodies combine to active complement and destroy the bystander RBC; (2) the drug acts as a hapten, a molecule that is not inherently immunogenic but binds to a carrier protein and covalently binds to RBC; or (3) the drug modifies the RBC membrane such that antibody coats the RBC (see Chapters 12 and 15).[38] A number of equine cases of IMHA associated with penicillin as a hapten exist and trimethoprim–sulfamethoxazole as a hapten has been noted in horses and cows.[24,30]

RBC destruction secondary to infectious disease is also a type II hypersensitivity reaction. For example, lipopolysaccharide and hemagglutinin subunits of EIA bind to RBCs resulting in antibody coating and binding CE complement (see Chapter 34).[26,39]

Pathogenesis of Immune-Mediated Hemolytic Anemia

Destruction of RBCs in IMHA is due to the removal of the antibody-coated RBCs by macrophages primarily in the spleen and liver (extravascular hemolysis) or less frequently mediated by complement (intravascular hemolysis). Specifically, RBC bound with IgG is cleared from circulation via cells of the fixed mononuclear phagocytic system expressing a specific Fcγ receptor and most predominantly in the spleen. Immunoglobulin-coated RBCs can fix C3 and are cleared by macrophages with CR1 and CR3. Alternatively, some immunoglobulin bound to RBCs activates the entire complement cascade with formation of the membrane attack complex (MAC) and results in intravascular lysis of RBCs.[8,37] Autoantibodies are further classified as warm or cold reactive. Warm RBC autoantibodies react strongly at 37 °C, are associated with severe anemia, and are predominantly IgG. Cold RBC autoantibodies react strongly at 4 °C and tend to be of the IgM class.[2] A special subset of cold autoantibodies that are active at 30 °C are thought to cause a cryopathic or cold hemagglutinin disease, in which IgM agglutinates in the peripheral circulation of the extremities (distal limbs, ears, etc.) and causes ischemic necrosis and gangrene. This form of IMHA has been reported in Friesian heifers as a consequence of *Salmonella dublin* infection.[17] Cold RBC autoantibodies that react only at 10–15 °C appear to not cause pathology, as they have been demonstrated in the serum of healthy horses.

Clinical Features of Immune-Mediated Hemolytic Anemia

Primary and secondary IMHA are clinically indistinguishable. Onset of hemolytic disease is variable depending on the rate of destruction, severity of anemia, and on the underlying disease process. Common clinical signs include fever, obtundation, icterus, and hemoglobinuria. With severe profound hemolysis (PCV < 15%), clinical signs include: tachycardia, tachypnea, cold extremities, pale mucous membranes, muscle weakness, and heart murmurs. Some animals may have an apparent splenomegaly based on ultrasound and transrectal palpation findings.

Diagnosis of Immune-Mediated Hemolytic Anemia

Diagnosis of IMHA is based upon multiple clinicopathological criteria: (1) RBC morphology changes consistent

with RBC destruction; (2) anemia; (3) autoagglutination; (4) a positive direct antiglobulin result (i.e., Coombs test) in a test validated for use in the horse or ruminant; and (5) elimination of other underlying causes of anemia.

Red cell morphology changes that have been described include marked poikilocytosis and echinocytes (type III > type II). Anemia of IMHA is usually quite severe with PCVs of <20%, but is typically regenerative as erythropoiesis is not affected. Unlike blood-loss anemia, horse with hemolytic anemia may develop very low levels of reticulocytosis.

Confirmation of anti-RBC antibody is done by use of a direct antiglobulin (Coombs) test (see Chapter 143). The patient's washed RBCs are incubated with antiserum to equine IgG, IgM, and complement. Specific identification of antibody-coated RBCs can be done using flow cytometry which detects low levels of cell-bound antibody. Flow cytometry can assess the therapeutic response and can monitor reduction of RBC-bound and platelet-bound IgG, IgM, and IgA in horses.[27]

Additional hematologic changes in IMHA include leukocytosis with neutrophilia, hyperfibrinogenemia, and hyperbilirubinemia associated pigmenturia. Urinalysis may indicate hemoglobinuria.

Further evaluation should focus on the identification of the underlying disease process. Transrectal palpation, abdominocentesis, and ultrasound examination should be performed for detection of intra-abdominal abscesses and neoplasia. Auscultation, thoracic radiographs, endoscopy, and ultrasonography of the chest should be done to determine lower airway disease or presence of thoracic masses. Transtracheal wash and/or thoracocentesis may be needed. Endoscopy of the upper airways and guttural pouches is recommended to look for potential *Streptococcus sp.* and lymphadenopathy. Additional laboratory tests included serology of various infectious diseases, particularly EIA, and bacterial culture. Lymphosarcoma has been associated with IMHA in horses, dogs, and humans, but not documented in cattle.[32]

Treatment of Immune-Mediated Hemolytic Anemia

Once IHMA diagnosis is made, therapy should include discontinuation of any drugs associated with IMHA. If a primary disease process or infection is involved, antimicrobial therapy may necessitate switching of the class of antimicrobials in use. Additional therapies to treat the underlying disease process should also be instituted.

Immunosuppressive doses of corticosteroids are indicated with the purpose of stabilizing RBCs, decreasing production of antibodies, the avidity of the antibody for RBCs, and the rate of clearance of antibody-coated RBC. It is important to note that corticosteroids may be less effective for treating IMHA with intravascular hemolysis mediated by IgM and complement.[8,27] Additionally, in horses chronically infected with EIA, steroid immunosuppression may result in recrudescence of the virus.[8,26] Horses with suspected IMHA should test negative for EIA prior to initiation of therapy. Dexamethasone can be used as a dose (0.1–0.2 mg/kg IV q 24 h), or prednisolone (1–2 mg/kg IV, IM or PO q 24 h). Once the PCV stabilizes at greater than 20%, tapering of the steroid dose to 0.1–0.2 mg/kg daily can be initiated. The PCV should be monitored until it normalizes. Treatment may be necessary for several weeks. In cases that are nonresponsive to corticosteroids, azathioprine can be used (3 mg/kg PO q 24 h). Cyclophosphamide and vincristine have also been used.[30,27] Blood transfusion is indicated if anemia becomes life-threatening; however, compatible blood donors may be difficult to find, and transfusion may exacerbate the hemolytic process. Splenectomy is utilized as a treatment modality in refractory cases of IMHA in dogs, but has not been described in large animals.

Blood transfusion in cattle is difficult and not commonly used because blood groups are complex and difficult to match (see Chapters 96 and 107). However, successful blood transfusion and treatment of a year-old Holstein heifer with IMHA has been reported.[16]

Prognosis for Immune-Mediated Hemolytic Anemia

The overall prognosis for horses with IMHA is dependent upon the underlying disease process. Horses with multiple recurrences of hemolysis are at high risk for having neoplasia and should be carefully examined. Reports of IMHA in cattle are too limited to make a reliable estimation of prognosis.

NEONATAL ISOERYTHROLYSIS

Pathogenesis of Neonatal Isoerythrolysis

Neonatal isoerythrolysis is caused by maternal alloantibodies directed against specific surface blood-group antigens on the affected foal's RBCs (see Chapter 96 for more detail).

In cows, sensitization occurs with vaccination for anaplasmosis and babesiosis. These vaccines contain large quantities of RBCs obtained from infected calves and induce antibodies against the A and F blood systems. If the sensitized cow is mated to a sire with these blood groups, the calf may be at risk of developing NI.[19,30,34,37]

Clinical Features of Neonatal Isoerythrolysis

Typically, foals and calves are normal at birth with signs developing as early as 5 hours after birth, but usually at 12–48 hours of age. Signs vary from subacute to peracute depending on the degree of anemia and rate of RBC destruction. Foals may present with lethargy, weakness, tachypnea, tachycardia, pale mucous membranes, icterus, pigmenturia, and hemodynamic shock. Death may occur if hemolysis is severe. Additionally, hypoxemia and tissue hypoxia may induce neurologic disorders, metabolic acidosis, and multiple organ failure.[12] Extreme elevations of unconjugated bilirubin can lead to bilirubin encephalopathy or kernicterus in foals.[18] Fulminant hemolysis in calves can trigger fatal

disseminated intravascular coagulopathy.[41] Mule foals may develop concurrent thrombocytopenic purpura.[4,23]

Diagnosis of Neonatal Isoerythrolysis

Neonatal isoerythrolysis should be considered in a newborn foal or calf presenting with weakness and icterus in the first few days of its life. It should be considered in a multiparous mare or mare with a previous history of foals with NI, but should also be considered even in a maiden mare. It should be considered in a cow vaccinated for anaplasma or babesia.

Anemia is the primary clinicopathologic finding in neonatal foals or calves with NI, with the PCV typically <20%. A decrease in the normal 3:1 ratio of PCV to hemoglobin may be noted due to the presence of free hemoglobin (Hgb) in serum. Other clinicopathologic findings include: hemoglobinemia and hemoglobinuria due to intravascular hemolysis and hyperbilirubinemia (20–40 mg/dL). If toxic hepatopathy occurs, liver enzymes, bile acids, and blood ammonia levels may be elevated.[4,8] In a retrospective study of NI, four out of 15 foals with NI had failure of passive transfer.[4,8]

Diagnosis of NI requires demonstration of maternal alloantibody binding to foal RBCs. Lytic assays are considered more accurate as alloantibodies act more strongly as hemolysins. Agglutination tests may be hindered by rouleaux formation of equine blood. Hemolytic assays involve the mare's serum mixed with foal RBCs and complement (pooled rabbit serum). If the serum contains alloantibodies, hemolysis results in pink/red supernatant and decreased RBC mass after centrifugation.[2,8] Similar hemolytic assays with appropriate bovine reagents can be performed in calves.[10,40]

The jaundiced foal agglutination (JFA) test consists of serial dilutions of the mare's colostrum in the presence of 5% RBC suspension from the foal. Agglutination is the end point of a positive test result (agglutination at dilutions of 1:16 or greater is considered significant). The JFA test is reported to correlate well with the hemolytic test and does not require an exogenous source of complement.[11] The Coombs test can be used to demonstrate the presence of antibodies or complement bound to foal RBC, but is not specific for the diagnosis of NI.[43]

Differential diagnosis of NI in foals presenting with weakness and icterus include septicemia, hypoxia, neonatal encephalopathy, equine herpesvirus-1, and leptospirosis. Potential causes of anemia in neonates include hemothorax, rib fracture, umbilical bleeding, and external hemorrhage.

Prevention and Treatment of Neonatal Isoerythrolysis

Prevention of NI is twofold: (1) determining whether the foal is at risk, and (2) protecting the foal from exposure to alloantibody. Determination of risk requires blood typing of the mare before parturition. Mares that test negative for Aa, Qa, Ua, or Pa should be considered at risk for making alloantibodies when exposed to antigen. Stallions bred to Qa/Aa-negative mares can also be blood-typed to determine if they are Qa/Aa-positive. Alternatively, the mare's serum can be screened for the presence of anti-RBC antibodies within 30 days before parturition using a panel of blood-typed RBCs or tested against the sire's RBCs. Before the foal is allowed to nurse, either a hemolytic test or JFA test should be performed.[4,8,12] In calves, screening of serum and colostrum of vaccinated cows with a saline agglutination test has been reported to be helpful in predicting the occurrence of NI.[19]

If the mare's colostrum has been determined to contain alloantibodies and the foal is positive for the specific antigen, the foal should be muzzled and not allowed to nurse for the first 30–48 hours postpartum. The mare's udder should be stripped regularly during this time to remove all colostrum and maintain milk production. Colostrum from an alternative source should be administered to the foal.

Foals that are diagnosed with NI should be immediately prevented from further exposure. Foals with mild anemia may only require exercise restriction to prevent clinical signs of NI. Use of corticosteroids to reduce the rate of clearance of sensitized RBCs may be beneficial. Broad-spectrum antibiotics are recommended, particularly with the use of corticosteroids for the prevention of secondary sepsis. In foals with clinical signs of NI, supportive therapy with oxygen insufflation may improve oxygen saturation. Weak and recumbent foals need supplemental nutrition, intravenous fluid therapy, and nursing care.[4,12]

In cases of severe anemia, a blood transfusion is needed. In addition to clinical signs (weakness, acidosis, tachypnea), parameters indicative of blood transfusion include: a PCV of <10–15%, RBC count $<3 \times 10^6$ cells/μL, and Hgb concentration ≤5 g/dL. The choice of donor is critical as the donor RBCs must be free of the specific reactive antigen. Donor whole blood should be from an Aa/Qa-negative universal blood donor. Suitable donors should be cross-matched (donor RBCs with mare or foal serum). The optimal choice for donor is the mare, but the mare's RBCs need to be washed to remove alloantibody-containing plasma. The foal's sire is the least desirable donor, as it has the same RBC antigenic factors as the foal. If a cross-matched donor is not available, a gelding that has had no history of receiving blood or blood products can be used. In the case of mule foals, because the offending antigen is the unique donkey antigen, RBCs from any horse could potentially be used for transfusion. Transfused RBCs have a 2–4-day life span in circulation. One to four liters of whole or washed RBCs are sufficient to induce clinical improvement. Depending on the foal's bone marrow response to anemia or continued hemolysis, additional transfusions may be needed.[2,4,12]

Prognosis for Neonatal Isoerythrolysis

Prognosis for NI depends on the degree of alloantibody antigenicity. Cases with acute anemia, hypoxemia, and onset of seizures have a guarded prognosis. Foals that continue to have hemolysis after transfusion develop secondary degenerative hepatopathy or have signs of kernicterus having a grave prognosis.

EQUINE INFECTIOUS ANEMIA

EIA is an important and reportable disease of horses and can be characterized by IMHA. Horses of all ages and breeds are susceptible to infection with equine infectious anemia virus (EIAV). Despite the development of a sensitive and specific federal regulatory test (Coggins test) and institution of regulatory guidelines for infected horses, EIA is still present in the United States, with outbreaks occurring sporadically throughout the country.[26]

Pathogenesis of Equine Infectious Anemia

Equine infectious anemia is caused by a nononcogenic retrovirus of the *Lentivirus* genus that infects macrophages (see Chapter 34). Natural infection occurs from the transmission of infected blood through the interrupted feeding of hematophagous arthropods (primarily *Tabanus spp*. and *Chrysops spp*.). Upon infection, the virus multiplies in the host macrophages, incorporates into the genome, and persistently infects the host. Viral replication has been found to occur at all times even during subclinical phases at reduced rates.[26]

Clinical Features of Equine Infectious Anemia

Three clinical forms of EIAV disease have been described and include: acute, subacute to chronic, and chronic inapparent (carrier). In acute infection, the virus invades the host macrophages and undergoes massive replication. Clinical signs of acute EIA are seen within 7–30 days after exposure and manifest as fever, depression, anorexia, and mucosal petechiae. Thrombocytopenia is common during initial infection and leukopenia with lymphocytosis and monocytosis also may occur. Anemia is not seen in this stage.[8,26]

In the subacute to chronic form of EIA disease, horses infected for longer than 30 days develop signs of weight loss, edema of the limbs and ventral abdomen, cyclical fevers, anemia, and icterus. Horses also can develop ataxia, abortion, colic, liver disease, and renal disease associated with viral infection. During the subacute to chronic form of disease, the PCV, RBC count, and Hgb levels decrease with hemolysis (see Chapter 34). Horses may succumb to infection either in the acute or subacute to chronic stage of disease, but most recover. Some horses have periodic recrudescence of the virus during periods of stress or treatment with corticosteroids, but a majority of horses remain clinically inapparent carriers of EIAV. No hematological abnormalities are detected in these horses.

Diagnosis of Equine Infectious Anemia

The Coggins test is still primarily utilized to diagnose EIA. The Coggins test is an agar gel immunodiffusion (AGID) test and detects serum antibodies against the EIAV gag p26 protein. The test is sensitive and specific but may provide false-negative results during the acute stage of EIA infection. Foals that have ingested colostrum from infected dams may have a false-positive test. Repeat testing of suspected horses after 10–14 days and of suspected foals after 6 months of age (as maternal antibody levels decrease) is advocated. Three other tests have been approved by the United States Department of Agriculture (USDA). These tests provide for more rapid testing and are more sensitive than the AGID, but are less specific and should be confirmed with the AGID.[26]

Treatment and Prevention of Equine Infectious Anemia

There is no treatment for EIAV infection. Individual states and federal government require the humane destruction and/or quarantine of EIAV-infected horses. State regulatory guidelines vary, but a physical quarantine of a 200-yard distance between affected horses and noninfected horses is required.

PIROPLASMOSIS/BABESIOSIS

Equine piroplasmosis or babesiosis is an infectious disease caused by two intraerythrocytic, apicomplexan equine-specific hemoprotozoa; *Babesia caballi and Theileria* (formerly *Babesia*) *equi*. These parasites and their tick vectors are endemic to most countries with tropical, subtropical, and temperate climates. Despite North America and Canada being considered free of the disease, repeated outbreaks have occurred in the United States.[40] Recently, a new species of piroplasmosis was identified in North America known as *Theileria haneyi*; however, the exact pathogenesis and virulence are unknown at this time.[20]

Horses that recover from the disease become inapparent carriers and are potential reservoirs, thus, creating a management issue in endemic and nonendemic areas. Untreated horses are lifelong carriers of *T. equi*, however, horses can clear *B. caballi*. Virulence of piroplasmosis varies greatly depending upon geographic location and potentially genetic differences.[46] The natural vectors for *T. equi* in North America include: *Dermacentor variabilis* and *Boophilus microplus*. Vectors for *B. caballi* include: *Dermacentor nitens*, *Dermacentor albipictus*, and *Amblyomma cajennense*. Transmission from dam to fetus had been reported. An outbreak in Florida and Texas was iatrogenic caused by contaminated blood transfusions as well as tick transmission.[40,47]

Pathogenesis of Piroplasmosis

The life cycles of *B. caballi* and *T. equi* are different. *B. caballi* invades erythrocytes directly, whereas the initial developmental stage of *T. equi* occurs in leukocytes. Asexual reproduction occurs and the numbers of merozoites increase. Erythrocytes lyse, which can result in both intravascular and extravascular hemolytic anemia. The hemolytic anemia is frequently accompanied by thrombocytopenia and coagulopathies. Severe disease can result in systemic inflammatory response syndrome, hypercoagulability, and multiorgan system dysfunction and death.[47]

Clinical Signs of Piroplasmosis

Clinical signs develop in horses exposed to infected ticks within 10–30 days for *B. caballi* and 12–19 days for *T. equi*. Clinical signs in horses that are via iatrogenic means (e.g., horses that receive a blood transfusion from an infected horse or syringes and needles reused without sterilization) vary depending upon the parasite dose, immune status of the horse, as well as the route of infection.[8,47] Clinical signs seen are lethargy, fever, petechiae, edema, and hemoglobinuria. Colic, respiratory, and neurologic signs have also been noted. Horses that survive the disease usually show few or mild clinical signs, but recrudescence can occur under stressful conditions with *T. equi*.

Horses infected with *T. equi* develop antibodies against equi merozoite antigens (EMAs) within 7–10 days of infestation.[8] Horses infected with *B. caballi* have an immune response to the apical merozoite protein rhoptry (RAP)1, which is less well understood.[9] Carrier horses are immune from reinfection, but coinfection with both parasites is possible.

Diagnosis of Piroplasmosis

In the acute stages of disease, diagnosis via a blood smear is feasible. Due to a low parasite load in the chronic stages of disease, identification visually is not possible. Therefore, serologic tests are used. The diagnostic method of choice is the EMA 1 and EMA 2 cELISA. ARAP1 cELISA can also be utilized.[8,47] Immunofluorescent tests are used in conjunction with the cELISA for disease confirmation. Duplex real-time quantitative PCR is available for *T. equi*.[8]

Treatment of Piroplasmosis

Treatment of piroplasmosis during the acute phase of the disease is supportive care; intravenous fluids, nonsteroidal anti-inflammatory drugs, pain control, and blood transfusions. Carrier status is protective against active disease and reinfection; therefore, elimination of the parasite is typically not performed. In nonendemic areas due to high transmission risk to other animals, options are enrollment in an approved treatment program, lifelong quarantine, or euthanasia.

Treatment of choice for clearance of the parasite is imidocarb dipropionate (2.2–4.4 mg/kg IM once q 72 h for four doses). Premedication with n-butylscopolamine (0.3 mg/kg IV) can counteract the anticholinesterase activity of imidocarb. Injection site reactions occur commonly and increased sensitivity to side effects along with fatal reactions has been seen in donkeys and mules. Clearance of the parasite is confirmed by serial negative qPCR or serology results.

Control of Piroplasmosis

Control tactics for piroplasmosis vary greatly among the endemic and nonendemic areas, but typically involve negative serology results. Federal and state regulatory officials must be notified within the United States if a horse tests positive. Nonendemic countries that border endemic countries cannot prevent introduction of ticks completely, therefore, measures must be taken to reduce horses tick exposure. At this time, no vaccination is available to prevent infection.[8]

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CHAPTER 38

Precursor-Targeted Immune-Mediated Anemia and Pure Red Cell Aplasia in Dogs and Cats

CYNTHIA A. LUCIDI

Precursor-Targeted Immune-Mediated Anemia in Dogs
- Pathogenesis
- Diagnosis
- Treatment and Outcome

Precursor-Targeted Immune-Mediated Anemia in Cats
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Acronyms and Abbreviations

AIHA, autoimmune hemolytic anemia; BFU-E, burst-forming unit-erythroid; CFU-E, colony-forming unit-erythroid; DAT, direct antiglobulin test; FeLV, feline leukemia virus; Hct, hematocrit; IMHA, immune-mediated hemolytic anemia; M:E, myeloid to erythroid; nRBC, nucleated red blood cell; PIMA, precursor-targeted immune-mediated anemia; PRCA, pure red cell aplasia; rhEPO, recombinant human erythropoietin.

Immune-mediated nonregenerative anemias are caused by a spectrum of disorders that result in persistent anemia with lack of reticulocytosis and variable bone marrow findings depending on the stage of erythroid cell that is either inhibited or targeted for destruction (Figure 38.1). When erythroid progenitors (burst-forming unit-erythroid (BFU-E), colony-forming unit-erythroid (CFU-E)) or early-stage erythroid precursors are affected (i.e., rubriblasts), the condition results in pure red cell aplasia (PRCA), which is characterized by complete or almost complete lack of erythroid precursors in the bone marrow. Targeting of erythroid precursors results in precursor-targeted immune-mediated anemia (PIMA), which is characterized by ineffective erythropoiesis; the pattern of ineffective erythropoiesis varies among early-, mid-, and late-stage PIMA (see "Diagnosis" in the following text). Targeting of both erythroid precursors and RBCs (or polychromatophils), possibly via a common surface antigen or distinct surface antigens, results in concurrent PIMA and nonregenerative immune-mediated hemolytic anemia (nonregenerative IMHA), which is characterized by evidence of peripheral RBC destruction (e.g., spherocytosis, Coombs positivity, and/or red blood cell agglutination) and bone marrow findings of ineffective erythropoiesis. By contrast, targeting of RBCs results in IMHA, which is characterized by a strong regenerative erythroid response (i.e., reticulocytosis) and effective erythropoiesis with erythroid hyperplasia. Precursor-targeted immune-mediated anemia has historically been called nonregenerative immune-mediated anemia (NRIMA, NIMA) or nonregenerative immune-mediated hemolytic anemia (NRIMHA); the latter term may be considered inappropriate given the lack of evidence of peripheral RBC destruction, and therefore lack of concurrent IMHA, in most PIMA cases, and the former term does not differentiate PIMA from PRCA or preregenerative IMHA.

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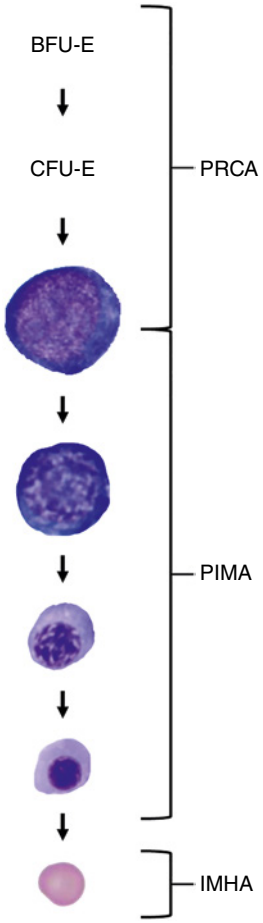


FIGURE 38.1 Schematics of the spectrum of immune-mediated anemias. Orderly erythropoiesis (left column) and resulting hematologic disorders (right column), depending on the stage of erythroid cell that is inhibited or targeted for destruction. Pure red cell aplasia and PIMA affect erythroid progenitors/early erythroid precursors and rubriblasts through metarubricytes, respectively, and are characterized by persistent nonregenerative anemias; by contrast, IMHA affects RBCs and is typically characterized by a strong regenerative erythroid response.

PRECURSOR-TARGETED IMMUNE-MEDIATED ANEMIA IN DOGS

Pathogenesis

Canine PIMA is defined by persistent nonregenerative anemia accompanied by ineffective erythropoiesis. Over the last decades, canine PIMA has been proposed to be caused by immune targeting and destruction of erythroid precursors. This mechanism has been recently demonstrated in a study that showed that a subset of mid- and late-stage PIMA dogs (five of 17 dogs) had increased surface-associated IgG on bone-marrow-derived erythroid precursors when compared to healthy dogs and to sick control dogs without PIMA.[8] In the same study, nRBC phosphatidylserine exposure was significantly higher in PIMA dogs than in healthy dogs, suggesting that apoptosis may also play a role in erythroid precursor targeting and destruction and in the pathogenesis of PIMA. The aforementioned study did not assess early-stage PIMA dogs; the definitive mechanism of the disease in this subset of dogs remains unclear but is presumed to be immune-mediated based on apparent responses to immunosuppressive therapy and relapses occurring after treatment tapering or discontinuation. Currently, canine PIMA is thought to be a primary (i.e., idiopathic) disorder.

Diagnosis

Dogs with PIMA are presented with signs of chronic anemia, and the main clinical complaint is lethargy.[2,7] Diagnosis of PIMA in dogs is based on three criteria, including (1) persistent nonregenerative anemia with duration of at least 5 days, (2) ineffective erythropoiesis, and (3) absence of dysplasia (i.e., dysplastic features in no more than rare cells of any lineage). Anemia is typically severe (Hct median 13%, range 4–28%), and the most common RBC morphologic abnormalities are macrocytosis[6] or elliptocytosis (i.e., ovalocytosis).[2] Mild microcytosis is an uncommon to rare finding.[2] Findings associated with IMHA (i.e., RBC agglutination, spherocytosis, ghost cells) are uncommon to rare in most PIMA dogs, and the vast majority of tested dogs are negative for the Coombs test.[2,7] Rubriphagocytosis (i.e., phagocytosis of intact erythroid precursors by macrophages) is present in most PIMA cases (92% of 66 cases) but is not specific of PIMA. Rubriphagocytosis in PIMA is usually relatively stage-selective (see "morphologic appearance of ineffective erythropoiesis" in the following text).[2]

Some dogs with PIMA have a history of other immune-mediated cytopenias (i.e., immune-mediated thrombocytopenia, immune-mediated neutropenia, or IMHA) or are presented with these cytopenias at the time of PIMA diagnosis or after resolving from PIMA. This supports the concept of multiple[12,14] or shifting [11,13,16,22,23] targets in immune-mediated diseases and hematologic disorders due to dysregulation of the immune system.

The morphologic appearance of ineffective erythropoiesis and bone marrow findings vary with the stage of erythroid precursor being targeted, but typically, ineffective erythropoiesis is characterized by expanded erythropoiesis up to the targeted nRBC stage.[2,6] In early-stage PIMA there is increased M:E ratio, erythroid hypocellularity, and maturation arrest/left shift at the stage of rubriblasts or prorubricytes, cells that may be found within macrophages. Other changes such as myeloid and megakaryocytic hyperplasia may also be present, and mild lymphocytosis or plasmacytosis may be seen in early-stage PIMA. Mid-stage PIMA is typically characterized by an M:E ratio within limits of health, erythroid normocellularity or hypercellularity, and an erythroid maturation arrest/left shift at the rubricyte stage with decreased metarubricytes; rubriphagocytosis of prorubricytes and early/mid-rubricytes is typically present. Late-stage PIMA is most common and characterized by a decreased M:E ratio, erythroid hyperplasia, and erythroid maturation arrest/left shift with decreased metarubricytes and/or polychromatophils; rubriphagocytosis of mid/late-rubricytes and metarubricytes is usually present.

Mild to marked collagen myelofibrosis, as evidenced by H&E and/or trichrome staining of bone marrow cores, is common in PIMA, affecting approximately 50% of PIMA dogs.[2] The cause of myelofibrosis in PIMA is unclear, but may be associated with a chronic inflammatory stimulus.[24,30]

In one study the constellation of moderate-severe nonregenerative anemia, anisocytosis, macrocytosis, and ovalocytosis was predictive of myelofibrosis in dogs, most of which had a diagnosis of PIMA.[6]

Due to collagen myelofibrosis in many PIMA dogs, poorly cellular or marginally adequate bone marrow aspirate samples (or "dry taps") may be a common occurrence, as has been described in a study of dogs with nonregenerative immune-mediated anemia.[20] In these cases, the diagnosis of PIMA relies on observation of left-shifted or arrested erythroid maturation with or without rubriphagocytosis on marginally adequate bone marrow aspirate samples and on the presence of myelofibrosis and ineffective erythropoiesis with expanded erythroid islands in bone marrow core specimens.

Differentials for PIMA include other conditions that can cause ineffective erythropoiesis and persistent nonregenerative anemia, such as phenobarbital-induced cytopenias.[17] Additional differentials include nutrient deficiency (e.g., iron or cobalamin) and familial dyserythropoiesis in English Springer Spaniels; these conditions are uncommon or display characteristic RBC morphologic abnormalities not typically seen in PIMA (e.g., microcytic hypochromic anemia in iron deficiency anemia, multilineage dysplastic changes in cobalamin deficiency). Primary myelodysplastic syndrome can also cause ineffective erythropoiesis and nonregenerative anemia, but dysplastic features should be prominent, while they are minimal or absent in PIMA.

The main differential diagnosis for early-stage PIMA is PRCA, since both can display marked erythroid hypocellularity. However, there is an absence or marked decrease in all erythroid precursor stages in PRCA, whereas early erythroid precursors may be prominent in early-stage PIMA, and early-stage rubriphagocytosis is typically present. These features are best seen cytologically, but expanded rubriblast islands may be seen in bone marrow core samples of early-stage PIMA dogs.

Treatment and Outcome

Glucocorticoids are the drug of choice for treatment of canine PIMA, including prednisone, prednisolone, or dexamethasone, the latter often being used during hospitalization. In one study, all PIMA dogs received prednisone at the median dosage of 2 mg/kg/d (range, 0.7–3.7 mg/kg/d), and 37 of 66 dogs received additional immune-modulating therapy in association with prednisone, including azathioprine (median dosage, 2 mg/kg/d; range, 1.3–2.6 mg/kg/d) and/or cyclosporine (median dosage, 8.4 mg/kg/q12h; range, 6.7–20.8 mg/kg/q12h).[2] Mycophenolate mofetil was used alone or in association with prednisone in a few PIMA cases, at the median dosage of 36 mg/kg/q12h (range, 25.9–46.1 mg/kg/q12h).[2] Cyclophosphamide has also been used in addition to steroids at the dosage of 0.5–2 mg/kg/d for 4 consecutive days a week.[18] Most dogs receive one or multiple blood transfusions during hospitalization.[2,20]

Most dogs respond to immunosuppressive treatment with some degree of reticulocytosis or improvement in Hct (i.e., 29 of 40[20] and 55 of 66 dogs, respectively).[2] Most dogs also achieve remission with a stable Hct ≥ 35% (i.e., 40 of 66 dogs)[2] or complete response with a Hct within or just below the reference interval (i.e., 22 of 40 dogs).[20] In one study, the median times for response and remission were approximately 1 month and 2 months, respectively.[2] Approximately 20% of dogs that achieved remission had a relapse at a median of 302 days after initial diagnosis of PIMA; in the dogs for which follow-up information was available, relapse was documented during weaning from immunosuppressive therapy or after therapy had been discontinued.[2]

Presence of collagen myelofibrosis was not significantly associated with time for regenerative response or median survival time; however, the median survival time for dogs without myelofibrosis (1429 days) was greater than two times that of dogs with collagen myelofibrosis (578 days).[2] One study reports two dogs with myelofibrosis that achieved complete response to treatment and suggests that myelofibrosis does not indicate a poor clinical outcome. This latter study reports resolution of myelofibrosis, as determined by serial bone marrow core biopsy specimens after treatment and resolution of anemia, but it is unclear if these dogs had reticulin or collagen myelofibrosis.[20]

Complications in management of dogs with PIMA include potential transfusion reactions from multiple blood transfusions, side effects of long-term treatment with immunosuppressive therapy (e.g., systemic infections, gastrointestinal bleeding, iatrogenic hyperadrenocorticism), and thromboembolism. Thromboembolism was diagnosed in two of 41 dogs with PIMA in one study (one before and one after treatment).[20] In a different study, nine of 66 and five of 66 PIMA dogs had confirmed or suspected thromboembolism, respectively, and thromboembolism was significantly associated with shorter survival time (54 days in dogs with a thromboembolic event vs. 1365 days in dogs without a thromboembolic event).[2] In the latter study, most dogs (29 of 66 dogs) received antithrombotic drugs at the time of PIMA diagnosis, including two of the nine dogs with confirmed thromboembolism. Antithrombotic therapy included unfractionated heparin, aspirin, and/or clopidogrel; heparin being used during hospitalization. The mechanism of thromboembolism and the optimal antithrombotic treatment in canine PIMA are unknown.

PRECURSOR-TARGETED IMMUNE-MEDIATED ANEMIA IN CATS

Pathogenesis

Precursor-targeted immune-mediated anemia seems to be less common in cats than in dogs, and there are few to rare studies describing this condition distinct from IMHA. The pathogenesis of feline PIMA is unclear, but

an immune-mediated mechanism is presumed based on favorable hematologic responses to immunosuppressive therapy. Its relationship with feline leukemia virus (FeLV) infections is unknown.

Diagnosis

Cats present with severe nonregenerative anemia, and anemia is normocytic normochromic.[3] Lack of response to anemia is determined by aggregate reticulocyte concentration below or within the reference interval. Abnormalities of platelet and WBC concentrations are not expected unless there is a comorbidity. The Coombs test is expected to be negative, but occasional animals may have a positive result.[3] Erythrophagocytosis was reported in the bone marrow of three cats with PIMA, but it is unclear if that involved phagocytosis of RBCs or erythroid precursors (i.e., rubriphagocytosis).[3] Bone marrow lymphocytosis or mild myelofibrosis may be seen in some cats.[3]

Treatment and Outcome

Glucocorticoids have been used to treat cats with PIMA, including oral prednisolone (median 3 mg/kg/d, range 2–4 mg/kg/d), oral methylprednisolone (2 mg/kg/d), and intravenous dexamethasone (0.6 mg/kg).[3] Adjuvant therapy has been used initially in addition to glucocorticoids or following a lack of response to glucocorticoids alone: cyclosporine (10 mg/kg/d) or chlorambucil (2 mg every other day). In one study, most cats achieved remission (defined as Hct > 25% with no clinical signs) in approximately 1 month, including all (2) cats with myelofibrosis.[3] In this study, relapses were common when tapering treatment, but anemia resolved with increasing dosages of glucocorticoids.

PURE RED CELL APLASIA IN DOGS

Pathogenesis

Pure red cell aplasia is an uncommon disorder and causes severe chronic nonregenerative anemia and clinical signs similar to PIMA. Most reported cases of PRCA in dogs are acquired and likely primary.[20,26] Secondary PRCA in dogs has been associated with recombinant human erythropoietin (rhEPO) treatment.[5,18] The mechanism of immune-mediated (primary or secondary) PRCA in people is unclear and is thought to involve (1) production of antibodies against membrane proteins expressed by erythroid progenitors or precursors, (2) cytotoxic T-cell-mediated destruction of erythroid progenitors or precursors, and/or (3) production of molecules (e.g., inhibitory or apoptotic cytokines) by cytotoxic T cells that affect the erythroid lineage and suppress erythropoiesis, among other mechanisms.[9,10,15]

A study investigating the mechanism of primary PRCA in dogs demonstrated that the IgG fraction of serum from a subset of dogs with PRCA inhibited formation of colony-forming units in vitro.[27] Results of the aforementioned study and response to immunosuppressive therapy in most cases[20,26] suggest that primary PRCA in dogs is immune-mediated.

Diagnosis

The main hematologic findings in PRCA are severe nonregenerative anemia with normocytic normochromic RBCs. Evidence of IMHA (i.e., Coombs positivity, spherocytosis, RBC agglutination) is not expected, unless in rare cases when there is concurrent targeting of erythroid progenitors or early-stage erythroid precursors and RBCs.

The bone marrow is characterized by complete lack of or presence of only rare erythroid precursors (i.e., marked erythroid hypoplasia or aplasia)[20,26] and lack of dysplasia in any cell lineage. The granulocytic and megakaryocytic lineages are unaffected, which differentiates this condition from aplastic anemia. Reporting a specific numeric M:E ratio in cases of PRCA may be inappropriate and inaccurate, since the very low numbers of nRBCs and their potentially uneven distribution on the glass slides would require several thousands of hematopoietic cells to be counted in order to achieve an accurate number. Therefore, the symbol > is usually used to convey an approximate estimate (e.g., M:E >99:1).

Bone marrow findings of PRCA and early-stage PIMA may be similar, but focally expanded erythroid islands composed of early-stage erythroid precursors is not expected in marrow cores of PRCA dogs. Also, in PRCA, all stages of erythroid precursors are expected to be decreased or absent.

Treatment and Outcome

The treatment for PRCA in dogs involves immunosuppression and is similar to that described in the earlier text for canine PIMA.[20,26] Combination of corticosteroids and adjuvant immunosuppressive or immunomodulatory drugs, such as azathioprine or cyclophosphamide, may be needed in some cases. One or more blood transfusions may be needed, depending on the time it takes for response to treatment to occur. It is unknown if outcomes differ between early-stage PIMA and PRCA.

PURE RED CELL APLASIA IN CATS

Pathogenesis

Pure red cell aplasia in cats, similarly to people and dogs, can be primary or secondary. The pathogenesis of primary PRCA in cats remains unclear, but an immune-mediated nature is presumed based on responses to immunosuppressive therapy.[19] Pure red cell aplasia secondary to treatment with rhEPO in cats is likely caused by the production of anti-rhEPO antibodies that may cross-react with native EPO.[5] Feline PRCA secondary to FeLV is caused by the virus subtype C (FeLV-C), which impairs differentiation of BFU-E into CFU-E and more mature stages of erythroid precursors.[1]

Diagnosis

Cats with PRCA have severe nonregenerative anemia (range 5–15%), which is normocytic and normochromic or hypochromic.[19,28,31] Cats with PRCA range from 6 months to 3 years old, with most cats being younger than 2 years old.[19,28,31] Clinical signs are often similar to PRCA in dogs and include lethargy, weakness, and pallor.[19,28,31] Abnormalities in white blood cell and platelet concentrations are not expected unless there is a concurrent disorder. The Coombs test is expected to be negative, but occasional positive results have been reported.[19] Bone marrow samples lack or contain only rare erythroid precursors, and the granulocytic and megakaryocytic lineages are expected to display no abnormalities,[19] although concurrent megakaryocytic aplasia has been reported in one case.[31] No dysplasia is seen in any cell lineage. Bone marrow lymphocytosis, represented by small lymphocytes, is not an uncommon finding.[19,28,31] In one study, these lymphocytes were typically arranged in small lymphoid aggregates as seen by examination of bone marrow cores, and in the two tested cats most were polyclonal B cells.[28]

Infections with FeLV-C are uncommon, and feline PRCA secondary to FeLV appears to be much less common than primary PRCA. Testing for FeLV is indicated in the workup of feline PRCA; however, FeLV positivity does not definitively rule out primary PRCA. Diagnostic sensitivity of PCR-based assays for FeLV is higher than ELISA-based assays,[29] so the former method is indicated to properly assess FeLV status in cats. Blood or bone marrow samples may be used for PCR testing, but positive results have been reported when using bone marrow but not blood samples in a few cats with latent FeLV infections and chronic nonregenerative anemias.[21] Some older studies report negative FeLV status in cats with PRCA based on serologic or ELISA testing only; it is possible that some of these cats were infected by FeLV, and the ELISA test yielded a false-negative result.

Treatment and Outcome

Cats with PRCA have been treated with corticosteroids in combination with or without other immunosuppressive or immunomodulatory drugs. In one study, most cats were treated with prednisolone at the dosage of 3.5–5.5 mg/kg/d and cyclophosphamide at the dosage of 2.5 mg/kg/d for 4 days, repeated every 3 days. Most cats responded with an increase in Hct or aggregate reticulocyte concentration within 1.5–4 weeks of treatment and had a Hct within the reference interval within 3–5 weeks.[17] Cyclosporine has also been used with success as an adjunctive drug to glucocorticoids in 10 cats with marked erythroid hypoplasia or PRCA.[25] A different study reports successful treatments in cats with PRCA using a combination of prednisone at the dosage of 4–8 mg/kg/d and chlorambucil at the dosage of 20 mg/m^2 every 2 weeks.[4] Similarly to PIMA and PRCA in dogs, many cats with PRCA require treatment with immunosuppressive drugs for months to years and some relapse after treatment is discontinued.[19,31] Most cats respond to a second course of immunosuppressive treatment.[17] Cats with PRCA often need one or multiple blood transfusions after diagnosis.[17,29]

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CHAPTER 39

Anemia of Inflammatory, Neoplastic, Renal, and Endocrine Diseases

AGATA K. GRZELAK and MICHAEL M. FRY

Inflammation
Neoplasia
Renal Disease
Endocrine Disease

Acronyms and Abbreviations

AI, anemia of inflammation; CKD, chronic kidney disease; DIC, disseminated intravascular coagulopathy; DM, diabetes mellitus; EPO, erythropoietin; IDA, iron deficiency anemia; IMHA, immune-mediated hemolytic anemia.

Many pathologic conditions may cause secondary anemia. This chapter focuses on inflammation, neoplasia, renal disease, and endocrine disease as causes of anemia. These categories are not mutually exclusive—e.g., cancer and chronic kidney disease (CKD) often have an inflammatory component.[27,42,46] Much information is based on studies in people or in rodent research models; however, in general, the pathophysiologic principles are not species-specific. Anemia in individuals with these conditions is usually, but not necessarily, nonregenerative.

INFLAMMATION

For many years physicians and veterinarians have recognized that patients with infectious or inflammatory disease frequently develop anemia, and that this phenomenon is associated with altered iron homeostasis.[13,68] The condition is now known as anemia of inflammation (AI) and occasionally still referred to as anemia of chronic disease. Noninfectious conditions linked to AI include neoplasia, CKD, autoimmune disease, congestive heart failure, chronic pulmonary disease, and obesity.[68] Ultimately, any condition leading to prolonged immune activation can result in anemia. Affected individuals have low serum iron concentrations, but increased iron stores in the mononuclear phagocyte system—seemingly paradoxical findings that are consistent with iron-restricted erythropoiesis caused by iron sequestration. In contrast to patients with classical iron deficiency anemia (IDA), which can be regenerative or nonregenerative, and in advanced cases is microcytic and hypochromic (see Chapter 27), patients with AI typically have normocytic, normochromic, nonregenerative anemia. This pattern is recognized in domestic animals in both research and clinical contexts,[13,64,67] but exceptions can occur. A study that included both AI (n = 12) and IDA (n = 11) groups found microcytosis or hypochromasia in some dogs with AI (those abnormalities tended to be less severe than in dogs with IDA), but no reticulocytosis in dogs with AI.[61] Because a highly specific test is lacking, diagnosis of AI typically involves laboratory data, clinical findings, and exclusion of other causes of anemia.

A proposed evolutionary rationale for AI is that it results from an adaptation to limit microbial access to iron.[19] To this day, studies continue to unravel new molecular mechanisms involved in the development of AI. Many of the insights involve hepcidin, a hormone now generally accepted as the key mediator of AI (see Chapters 20 and 27).[68] Initially referred to as liver-expressed antimicrobial peptide (LEAP-1), hepcidin was subsequently identified as a type II acute phase protein and a key regulator of iron availability.[35,53] It is synthesized mainly by hepatocytes—hence the name hepcidin, intimating both its tissue of origin and its

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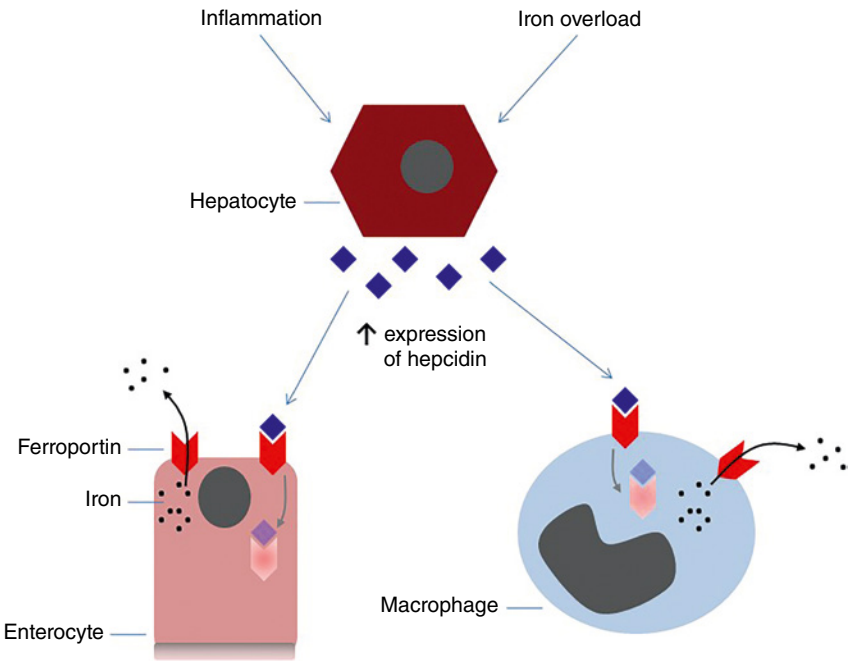


FIGURE 39.1 Hepcidin expression is increased by inflammation and iron overload. Hepcidin binds to ferroportin and causes its internalization and degradation, thus inhibiting efflux of iron into the plasma.

direct antimicrobial effects.[56] The bioactive form is a 25-amino acid peptide that exerts its effects by binding to the cell surface iron efflux protein, ferroportin, and inducing its internalization and degradation.[52] The effect of this interaction is to inhibit both absorption of dietary iron from intestinal epithelium and export of iron from macrophages and hepatocytes.[52]

Hepcidin expression increases in response to acute inflammation or iron overload (Figure 39.1) and decreases in response to hypoxia or iron deficiency.[21,51,53] Interleukin-6 plays a central role in increasing hepcidin production; however, other cytokines and multiple signaling pathways are involved.[51,68] AI also involves mechanisms other than hepcidin-mediated altered iron homeostasis.[68] This may be especially relevant in chronic inflammation, as some research has found persistent suppression of erythropoiesis despite normalization of hepcidin mRNA expression or serum iron concentration.[14,58] Inflammatory cytokines have been implicated directly or indirectly in inhibiting erythropoiesis—via decreased erythropoietin (EPO) production, decreased responsiveness to EPO, or direct effects on erythroid precursors—as well as decreasing erythrocyte survival.[26,31,36]

NEOPLASIA

Recognized causes of anemia in people and veterinary patients with neoplasia include disease- and treatment-related factors. Frequently, the cause of anemia is multifactorial. Anemia in people with cancer is recognized to have negative impacts on quality of life, prognosis, and response to therapy in some cancer types.[27] While neoplasia is one of the most frequently identified causes of anemia in dogs,[12] its deleterious effects have received relatively little attention in the veterinary literature. One study found anemia to be a negative prognostic factor in canine lymphoma patients compared to osteosarcoma patients.[47]

In principle, anemia can occur as a complication of virtually any type of neoplasia, but the risk is higher in some types of malignancy. Potential disease-related factors include inflammation (the link between cancer and inflammation goes at least as far back as Rudolf Virchow[29]), hemorrhage, immunologic dysregulation, nutritional deficiencies, space occupation or alteration of bone marrow microenvironment by neoplastic cells or fibrous tissue, and elaboration of myelotoxic factors by neoplastic cells (such as hyperestrogenism-induced bone marrow hypoplasia secondary to Sertoli cell tumor).[9,27,50,63] The most commonly recognized treatment-related factor contributing to anemia is myelosuppressive chemotherapy or radiotherapy;[27] hemolytic anemia has also been reported secondary to anti-neoplastic therapy.[27]

Anemia due to hemorrhage is a frequent finding in some types of neoplasia. Bleeding may occur directly from a neoplastic lesion, or from distant sites due to defective hemostasis. Hemoperitoneum is a frequent complication of splenic hemangiosarcoma in dogs.[28] Bleeding may be caused by rupture of the primary tumor, but exacerbated by other tumor-related pathology. Thrombocytopenia occurs in the majority of these cases and disseminated intravascular coagulopathy (DIC) is a frequent complication.[28] Anemia and clinical evidence of bleeding are frequent in dogs with gastrointestinal sarcomas and renal neoplasia.[10,17]

Destruction of erythrocytes or their precursors may occur via secondary immune-mediated disease, phagocytosis by neoplastic cells, oxidative insult, mechanical

damage (e.g., microangiopathic hemolysis), or increased apoptosis. Although proof of a causative relationship in animals is elusive, underlying neoplasia can be a differential diagnosis for immune-mediated hemolytic anemia (IMHA).[38] There are several documented cases of IMHA associated with lymphoma in the veterinary literature.[33,40,44,49] In a retrospective study on equine lymphoma, the majority of affected animals were anemic, and autoagglutination was a frequent finding.[44] Canine hemophagocytic histiocytic sarcoma (see Chapter 74) is characterized by malignant histiocytes that are erythrophagic; the large majority of affected dogs are Coombs-negative.[48] A similar condition has been reported in a cat and a cow.[20,39] An association between lymphoma and oxidative damage to erythrocytes, with or without anemia, has been documented in dogs and cats. A recent study found that anemia and eccentrocytosis (see Chapter 24) were more common in dogs with lymphoma than those with inflammatory bowel disease.[55] Another study of canine eccentrocytosis found the prevalence of the condition was only 1.4% (60 of 4251 dogs), but every animal with T-cell lymphoma (five of five) had eccentrocytosis; however, information about the prevalence of anemia in these dogs was not provided.[11] A study in cats found Heinz bodies to occur significantly more often in animals with lymphoma (15 of 17 cases) than in a healthy control group; however, anemia was not a consistent finding in affected individuals.[16] Myelodysplastic syndrome causes cytopenias in part via increased apoptosis of developing blood cells.[3] Eryptosis, an apoptosis-like mechanism of erythrocyte cell death, has been implicated as a contributing factor to anemia in people with many types of disease, including lung cancer.[8,59]

Many anti-neoplastic therapies are known to cause myelosuppression in humans and animals, and such treatment is likely to be a contributing factor to anemia in affected individuals. However, monitoring of hematologic toxicity of chemotherapy or radiotherapy typically focuses on neutrophils and platelets; the impact of myelosuppression on erythrocytes is less severe than on other blood cell types because of the long erythrocyte life span.[38] In the opinion of at least some veterinary oncologists, chemotherapy is more likely to cause treatment-limiting anemia as a result of gastrointestinal hemorrhage than as a result of myelosuppression (A LeBlanc, personal communication).

RENAL DISEASE

Nonregenerative anemia is an expected complication of CKD in humans and domestic animals, and is recognized to contribute to overall morbidity.[5,6,34] Decreased production of EPO is generally regarded as the predominant mechanism of anemia in patients with CKD; other potential contributing factors include inflammation, hemorrhage, iron deficiency, decreased erythrocyte survival, and antiproliferative effects of uremic toxins.[5] Many treatment-related factors are recognized as potential contributors to anemia in patients with renal disease, including repeated phlebotomy and side effects of medications (erythropoiesis-stimulating agents, iron supplementation, phosphate binders, and antacids); detailed discussion of those factors is beyond the scope of this chapter.[6,24,71]

Animals with CKD often have inappropriately low plasma EPO concentrations. In one study of dogs and cats, anemic animals without CKD had significantly increased plasma EPO concentration compared to healthy controls, while a similar increase was not present in animals with CKD.[57] In cats there was no overlap in EPO concentration between anemic animals with and without CKD; in dogs there was considerable overlap between those groups. Another study found no significant difference in EPO concentration between cats with CKD and healthy controls, but significantly lower EPO concentration in anemic cats with CKD than in nonanemic cats with CKD.[30]

It is not clear how frequently or to what degree factors besides decreased EPO production contribute to development of anemia in animals with renal disease. CKD is recognized to be a proinflammatory state in people,[46] and some evidence in the veterinary literature supports the hypothesis that CKD elicits AI. In one study, serum amyloid A concentration was higher in cats with CKD than in healthy controls, and higher in anemic cats with CKD than in nonanemic cats with CKD.[30] Other mechanisms by which CKD may promote anemia, but for which direct evidence in veterinary medicine may be lacking, include potentiation of AI due to decreased renal clearance of hepcidin;[22] decreased erythrocyte survival due to effects of uremic toxins and reactive oxygen species;[1,23,60] and hemorrhage due to uremic ulceration and impaired platelet function.[45]

ENDOCRINE DISEASE

Endocrinopathies are recognized to cause anemia in humans and animals. This section focuses on the relationship between anemia and several endocrine diseases that are well characterized in veterinary medicine: diabetes mellitus (DM), hypothyroidism, hyperthyroidism, and hypoadrenocorticism. Some of these conditions are associated with impaired renal concentrating ability, and decreased plasma volume may mask the severity of anemia.[42,62]

Anemia is common in people with DM and closely linked with CKD.[65] Conversely, DM does not seem to be widely recognized in veterinary medicine as a cause of anemia, and a study in cats found DM to be a negative risk factor for development of CKD.[25] A study in dogs, however, identified a potential mechanistic link between DM-related lipidopathy and anemia: erythrocytes from hyperlipidemic and dyslipidemic dogs with DM were more osmotically fragile than erythrocytes from controls; moreover, incubation in plasma from hyperlipidemic dogs with DM rendered control erythrocytes more fragile, whereas incubation in lipid-depleted plasma from the same diabetic dogs did not.[7] In the

same study, erythrocytes from dogs treated with glucocorticoids were significantly more fragile than controls, and erythrocytes from dogs with hyperadrenocorticism tended to be more fragile than controls (the difference did not reach statistical significance)—suggesting a possible similar mechanistic link between other endocrinopathies and anemia.

Cats with DM have a predisposition to Heinz body formation, but how this relates to development of anemia is not clear. One study found Heinz bodies to occur significantly more often in cats with DM (19 of 21 cases) than in a healthy control group, and the evidence presented suggested a causal relationship to anemia.[15] A subsequent study found that cats with diabetic ketoacidosis had more Heinz bodies than non-ketotic diabetic cats, but did not find a significant association between percentage of Heinz bodies and degree of anemia.[16] Heinz bodies were also detected in five cats with DM and hemolytic anemia associated with hypophosphatemia, but the hemolysis in these cats was presumed to be caused by hypophosphatemia.[2]

Nonregenerative anemia is a recognized complication of hypothyroidism in humans and animals, occurring in approximately one-third of cases in dogs.[32,54] Anemia develops primarily as a result of decreased erythropoiesis, with possible mechanisms including decreased EPO production, decreased responsiveness to EPO, and direct effects on hematopoietic stem cells and erythroid progenitor cells.[32,43]

Despite thyroid hormone effects in promoting erythrocyte production and the fact that hyperthyroidism is recognized as a cause of erythrocytosis,[37,69] anemia occurs in approximately 10–25% of people with hyperthyroidism.[32,69] Decreased erythrocyte survival and ineffective erythropoiesis have been suggested to play a role in development of anemia, but there is conflicting information in the scientific literature. Experimentally induced hyperthyroidism in rats resulted in increased erythrocyte osmotic fragility, suggesting decreased erythrocyte survival as a potential mechanism of anemia;[70] however, a study in people found decreased osmotic fragility in erythrocytes of hyperthyroid patients compared to controls.[4] Another study in people found evidence of both increased proliferation and increased apoptosis in hematopoietic stem cells from individuals with hyperthyroidism.[32] A study in cats found Heinz bodies to occur significantly more frequently in animals with hyperthyroidism (15 of 21 cases) than in a healthy control group, but not all affected animals were anemic and the study did not demonstrate a causal relationship between Heinz bodies and increased erythrocyte destruction.[15]

Dogs with hypoadrenocorticism often develop nonregenerative anemia, but the anemia may be masked by decreased plasma volume from dehydration.[18,66] The pathophysiology of anemia in hypoadrenocorticism is primarily attributed to decreased erythrocyte production, supported by the fact that glucocorticoids interact with EPO and stem cell factor to promote erythropoiesis.[62,66] Additionally, blood loss, especially from gastrointestinal hemorrhage, may also contribute to development of anemia in dogs with hypoadrenocorticism.[41,66]

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CHAPTER 40

Aplastic Anemia

JENNIFER L. BRAZZELL and DOUGLAS J. WEISS

Pathogenesis
Diagnosis
Models of Aplastic Anemia
Aplastic Anemia in Dogs
  Infectious Causes
  Drug-Associated Causes
  Idiopathic Aplastic Anemia
Aplastic Anemia in Cats
Aplastic Anemia in Horses
Aplastic Anemia in Ruminants
  Drug-Associated Causes
  Bracken Fern Ingestion
  Trichloroethylene-Extracted Soybean Oil Ingestion
  Mycotoxin Ingestion
  Idiopathic Aplastic Anemia in Calves
Aplastic Anemia in Chickens

Acronyms and Abbreviations

DNA, deoxyribonucleic acid; FeLV, feline leukemia virus.

Aplastic anemia (also termed aplastic pancytopenia) is a clinical syndrome characterized by bicytopenia or pancytopenia in the blood and replacement of bone marrow by adipose tissue (Figure 40.1).[3,25,33] Excluded from this are pancytopenias associated with myelophthisic disorders, hypoplastic myelodysplastic syndromes, pure red cell aplasia, hemophagocytic syndrome, myelonecrosis, and infectious/inflammatory disorders of bone marrow.[3]

Aplastic anemia can occur in acute and chronic forms. If the bone marrow suppression occurs acutely, as is seen in some types of drug- or virus-induced aplasia, neutropenia typically develops within 1 week followed by thrombocytopenia at 9–14 days.[28] Anemia does not develop for an extended period of time, because of the long life span of RBCs. Therefore, acute aplastic anemia is typically characterized by a neutropenia and thrombocytopenia. Acute aplastic anemia is most frequently associated with destruction of rapidly dividing progenitor or proliferative cells in bone marrow. In chronic aplastic anemia, the hematologic dyscrasia develops more slowly and is characterized by moderate to severe normocytic normochromic nonregenerative anemia, neutropenia, and thrombocytopenia.[16] Chronic aplastic anemia is most frequently associated with stem cell destruction.

PATHOGENESIS

Many etiologic agents have been incriminated in aplastic anemia. These include infectious agents, chemical/physical agents, drugs, toxins, genetic mutations, and immune-mediated causes. The end result of each of these insults is decreased blood cell formation.[29,35] Although several mechanisms could lead to decreased hematopoiesis, direct injury to stem cells or progenitor cells appears to be the primary mechanism involved in clinical aplastic anemia.[25,35] In human patients, immune-mediated destruction of hematopoietic stem cells appears to play a central role in the pathogenesis of aplastic anemia.[23,33,35] The process involves a cell-mediated immune response including CD4 cells, CD8 cytotoxic T cells, regulatory T cells, Th17 cells, and natural killer (NK) cells.[33,35] Cytokines involved in inducing apoptosis of hematopoietic stem cells include interferon-gamma, tumor necrosis factor-alpha, and transforming growth factor-beta.[33,35] Although there is no direct evidence of an immune etiology in animals, clinical responses of dogs and cats with aplastic anemia to immunosuppressive therapy is suggestive of an immune-mediated etiology.[10,18,34]

Beyond the causative immune-mediated etiology, human aplastic anemia has been linked to clonal

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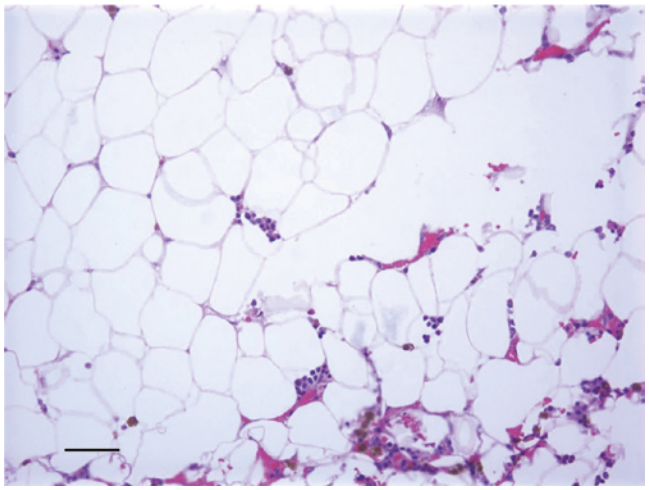


FIGURE 40.1 Bone marrow core biopsy section from a dog with idiopathic aplastic anemia. Cells present are mostly small lymphocytes and plasma cells. H&E stain; bar = 200 µm.

hematopoietic disorders including progression to myelodysplastic syndromes (MDS) and acute myeloid leukemias.[25,33] A variety of clonal changes have been associated with aplastic anemia including gross cytogenic alterations and various clonal somatic mutations. These mutations tend to be similar to those associated with acute myeloid leukemias and congenital bone marrow failure disorders (see Chapter 31). The present theory is that immune-mediated depletion of stem cells and progenitor cells may permit clonal selection of pre-existing stem cells containing genetic mutations that were acquired as part of the normal aging process. These genetic mutations provide a selective growth advantage because they are less immunogenic or relatively resistant to cytotoxicity. Mutations in aplastic anemia patients that result in the loss of class 1 human leukocyte antigen alleles (i.e., involved in immune recognition) support a role of altered immunogenicity of hematopoietic clones in aplastic anemia.[25,33]

The finding of clonal hematopoiesis in aplastic anemia and frequent progression of aplastic anemia to MDS complicate the differentiation of aplastic anemia from hypoplastic MDS.[33] Further, both patients with aplastic anemia and MDS can respond to immunosuppressive therapy. Therefore, a subset of human patients are thought to have a combination of aplastic anemia and hypoplastic MDS. The presence of immune-mediated disease coexisting with MDS also complicates the diagnosis of immune-mediated hemolytic anemia with secondary dysmyelopoiesis (see Chapter 67).

DIAGNOSIS

Inclusion criteria for a diagnosis of aplastic anemia in humans are well-defined. They include: hemoglobin concentration <10 g/dL, reticulocytopenia, platelet count $<50 \times 10^3/\mu L$, a neutrophil count $<1.5 \times 10^3/\mu L$, and hypocellular bone marrow aspirates and core biopsies, as well a battery of ancillary diagnostic tests including cytogenetic analysis of the peripheral blood and bone marrow, fetal hemoglobin percentage in children, flow cytometry for phosphatidylinositol glycan-anchored proteins, liver function tests, vitamin B_{12} and folate concentrations, viral studies, testing for immune-mediated diseases, and diagnostic imaging.[16] Diagnostic recommendations for aplastic anemia in veterinary patients are not well-defined and currently minimally include evaluation of a complete blood count, bone marrow aspiration smear and core biopsy specimen, and ancillary diagnostic tests.[3,29] Fat content of bone marrow depends on location and depth of core biopsy. The bone marrow collection sites should be carefully chosen as fatty marrow samples can be obtained in healthy animals if an improper collection site is selected.[31] Magnetic resonance imaging has been recommended as a supportive diagnostic method for diagnosis of hypocellular fatty bone marrows.[10] Ancillary diagnostic tests should be chosen based on the species and case history. Animals should be evaluated for drug, chemical, and toxin exposure, exposure to specific infectious agents, and the presence of associated diseases such as chronic renal failure.[3,27]

MODELS OF APLASTIC ANEMIA

A variety of in vivo and ex vivo animal models of aplastic anemia have been described.[4] A mouse model has been used to study benzene-induced aplastic anemia. Use of this model has enabled investigators to identify a benzene-induced defect in the capacity of bone marrow stromal cells to process cytokines. A mouse model of busulfan-induced aplastic anemia has been extensively studied.[4] Busulfan treatment results in hematopoietic stem cell depletion with pancytopenia and bone marrow aplasia developing up to a year after treatment. Other studies suggest the presence of a qualitative defect in hematopoietic stem cells or hematopoietic stromal cells. Radiation injury has also been studied in several species.[5] Exposure of mice to 4 Gy ionizing radiation resulted in significant reduction in hematopoietic stem cells.

Immune-mediated aplastic anemia has been reproduced in mice by sublethally irradiating them and injecting them with heterologous lymphocytes.[4] Total body irradiation with 6 Gy and injection of 10 million lymph node cells produced a fatal pancytopenia within 2–3 weeks.

APLASTIC ANEMIA IN DOGS

Aplastic anemia was identified in 2.4% of clinical bone marrow specimens evaluated at a veterinary teaching hospital in the Northern USA.[28] Of these, the majority (1.7%) were idiopathic.[3] Other causes of aplastic anemia included infectious causes, drugs, toxins, and radiation. Alternatively, in Brazil, a retrospective study of bone

marrow aspirates from 65 dogs with pancytopenia revealed a 26% incidence of aplastic anemia.[7] This discrepancy is probably due to the high incidence of *Ehrlichia canis*, *Babesia* spp., and *Leishmania chagasi* and failure to perform bone marrow core biopsies that would have identified bone marrow stromal disorders as a cause of the pancytopenia.

Infectious Causes

Infectious agents that cause aplastic anemia include parvovirus, *E. canis*, and *L. chagasi* (see Chapters 18 and 33).[7] Puppies with parvovirus infection develop erythroid and myeloid hypoplasia. This is not a true aplastic anemia because the hypoplasia is accompanied by degenerative changes including myelonecrosis, edema, and hemorrhage (see Chapter 16). These changes could be caused by the cytotoxic effects of the virus or more likely the effects of secondary bacterial sepsis or endotoxemia on the marrow microvasculature. Parvovirus invades rapidly dividing myeloid and erythroid progenitor cells. Hematopoietic stem cells are not infected. Therefore, bone marrow rapidly regenerates if puppies survive the acute stage of the disease. Dogs with chronic *E. canis* infections and with *L. chagasi* infections develop hypoplastic bone marrows.[7] Appropriate diagnostic testing should be performed in endemic areas.

Drug-Associated Causes

Drugs that induce hematologic dyscrasias are discussed in Chapter 15. In this chapter, some general comments about drug-induced aplastic anemia are presented. The mechanism by which the drug induces suppression of hematopoiesis is important to clinical finding and outcome. Drugs that target progenitor or proliferative cells in marrow, such as anti-neoplastic drugs and sulfadiazine, result in acute aplastic anemia.[30] In some cases, these drugs are directly cytotoxic to rapidly proliferating hematopoietic cells, whereas other drugs act indirectly by inducing immune-mediated hematopoietic cell destruction. Affected dogs are typically neutropenic and thrombocytopenic but not anemic, and frequently undergo prompt hematologic recovery when the drug is withdrawn.[30] Drugs that target stem cells, such as estradiol and phenylbutazone, frequently cause chronic aplastic anemia that is characterized by pancytopenia.[30] Recovery is uncertain and when it occurs it may be delayed for months.

Idiopathic Aplastic Anemia

Idiopathic aplastic anemia was observed in 1.7% of clinical bone marrow specimens examined at a veterinary teaching hospital in the Northern USA and in 9% of pancytopenic dogs in Brazil.[3,7] Affected dogs tend to be young adult with no sex or breed predisposition. The history usually indicates a chronic onset of illness with nonspecific signs including lethargy, anorexia, and exercise intolerance. Fever, petechiae, and epistaxis may also be observed. The anemia varies from mild to severe and is normocytic and normochromic. Neutropenia is usually severe with leukocytes consisting mostly of small lymphocytes. Thrombocytopenia varies from moderate to severe. Bone marrow aspirates and core biopsy specimens are markedly hypocellular (Figure 40.1). In some dogs, small lymphocytes or plasma cells are increased in number.

Treatment of canine idiopathic aplastic anemia is not well-defined. Whole blood, packed RBC, or platelet transfusions are frequently needed. Broad-spectrum antibiotics are indicated to prevent opportunistic infections. Because of the clear connection with the human disease, a primary cell-mediated immune destruction of hematopoietic precursor cells has been suspected in canine idiopathic aplastic anemia. Although administration of immunosuppressive doses of prednisone alone appears to be ineffective as a treatment, administration of mycophenolate mofetil or cyclosporine or azathioprine with or without prednisone has been associated with recovery from idiopathic aplastic anemia.[10,18,34]

APLASTIC ANEMIA IN CATS

Aplastic anemia has been observed in 10% of feline clinical bone marrow specimens at a veterinary teaching hospital.[28] Conditions associated with aplastic anemia include chronic renal disease, feline leukemia virus (FeLV) infection, prolonged anorexia/starvation, cobalamin deficiency, *Leishmania infantum* infection, and adverse drug reactions including griseofulvin, chemotherapeutic agents, propylthiouracil, and methimazole.[15,24,28] Chronic renal disease is typically associated with nonregenerative anemia, and aplastic anemia is an infrequent finding. Some cats with aplastic anemia associated with chronic renal disease have serous atrophy of fat in bone marrow (see Chapter 17).[28] Serous atrophy of fat is typically associated with starvation. Starvation has been implicated in the pathogenesis of the marrow failure in people and other species.

FeLV infection has been associated with a variety of bone marrow disorders including aplastic anemia.[6,28] Cats usually have a severe normocytic normochromic anemia (hematocrit <10%), severe leukopenia, and thrombocytopenia. Affected cats frequently have secondary bacterial infections as a result of the neutropenia. Bone marrow is aplastic; however, the pathogenesis of the aplasia is unclear. Survival time for FeLV-associated aplastic anemia is usually short.

In one study of aplastic anemia in cats, 30% were classified as idiopathic.[28] Many affected cats had a history of prolonged partial or complete anorexia with associated cachexia.[28] One case had serous atrophy of fat and gelatinous transformation in bone marrow. Therefore, malnutrition/starvation may have played a role in the development of the aplasia in these cats.

Although not recognized as a clinical disorder, cat bone marrow appears to be uniquely sensitive to mycotoxins. Mycotoxin exposure results from ingestion of grain that becomes damp during storage permitting

growth of fungi. In a search for an experimental model for T-2 mycotoxicosis, sesquiterpene T-2 mycotoxin was administered to rats, mice, guinea pigs, rabbits, dogs, pigs, sheep, poultry, cattle, horses, and cats.[14] Only cats developed signs of hematotoxicity. This was characterized by pancytopenia and bone marrow was characterized by hypocellularity, hemorrhage, inflammation, and multinucleated giant cells. Although this is consistent with acute toxic injury rather than aplastic anemia, the bone marrow may become aplastic at a later stage of the disease.

APLASTIC ANEMIA IN HORSES

Several reports of equine aplastic anemia have been published. These reports appear to present distinct types of bone marrow aplasia including idiopathic, drug-associated, possible toxin-associated, genetic, and hypoplastic myelodysplastic syndrome. A case of idiopathic aplastic anemia was reported in a 2-year-old Quarter Horse.[2] This horse had severe pancytopenia and bone marrow aplasia typical of aplastic anemia and had no history of exposure to drugs, toxins, or infectious agents. The horse died from hemorrhage as a result of the bone marrow biopsy procedure.

Several suspected drug-induced aplastic anemia cases have been reported. Phenylbutazone has been incriminated as a cause of aplastic anemia. One horse developed aplastic anemia after treatment with penicillin, trimethoprim–sulfadiazine, oxytetracycline, phenylbutazone, dipyrone, flunixin meglumine, and isoxsuprine.[12] The horse was direct Coombs test positive suggesting an immune-mediated pathogenesis.

Aplastic anemia in a 9-week-old Clydesdale foal was associated with biliary hyperplasia and portal fibrosis.[17] Because of this combination of findings, an in utero or neonatal toxicity was suspected. A panhypoplasia of bone marrow has been reported in eight Standardbred horses sired by the same stallion suggesting genetic mutation as a cause.[11]

Finally, a severe nonregenerative anemia and leukopenia that initially had an aplastic bone marrow were reported in a 17-year-old Thoroughbred gelding.[26] Unusual features of this case were marked anisocytosis in the blood, normal numbers of megakaryocytes in bone marrow, and an increase in blast cells in bone marrow. Reevaluation of bone marrow several months later revealed a hypercellular marrow with an increase in lymphocytes and blast cells. This is suggestive of hypoplastic myelodysplastic syndrome (see Chapter 67).

APLASTIC ANEMIA IN RUMINANTS

Drug-Associated Causes

A case of aplastic anemia in a 14-day-old Holstein heifer was associated with prior administration of sulfamethazine, trimethoprim, and sulfadoxine for treatment of bloody diarrhea.[1] Detection of aplastic bone marrow 8 days after initiation of sulfa therapy fits with a drug-induced aplastic anemia.

Bracken Fern Ingestion

Bracken fern (*Pteridium aquilinum*) is typically eaten by ruminants when pastures are overgrazed. After repeatedly ingesting bracken fern, cattle, horses, sheep, and pigs develop aplastic anemia.[19] The major toxin is a thiaminase called ptaquiloside.[31,32] Thiamine depletion in cattle is thought to be involved in the pathogenesis of aplastic anemia. Species other than cattle are more likely to develop neurological signs related to polioencephalomalacia.[31] Development of urinary bladder neoplasia (i.e., chronic enzootic hematuria) is also linked to prolonged ingestion of bracken fern.[19]

Trichloroethylene-Extracted Soybean Oil Ingestion

Many cases of fatal aplastic anemia and renal injury in cattle in the 1950s were traced to feeding of trichloroethylene-extracted soybean oil meal.[13,22] Although trichloroethylene is no longer used to extract soybean oil, it and related compounds are widely used as solvents. The toxic factor was identified as *S*-(1-2-dichlorovinyl)-L-cysteine. Administration of a single dose of this compound intravenously at 4 mg/kg or 0.4 mg/kg per day for 10 days reproduces the aplastic anemia and renal injury.

Mycotoxin Ingestion

A variety of mycotoxins produce aplastic anemia in ruminants. Mycotoxin exposure occurs with ingestion of grain that is contaminated with fungi as a result of becoming damp during storage. Aplastic anemia has been documented in cattle and sheep ingesting feed contaminated with *Stachybotrys* spp.[21] Many other mycotoxins, including T-2 mycotoxin, trichothecene mycotoxin, trichothecene vomitoxin, diacetoxyscirpenol, ochratoxin, and zearalenone, appear to be directly cytotoxic to a variety of cells including hematopoietic precursor cells and lymphocytes.[5] As a result, many mycotoxins cause bone marrow hypoplasia/aplasia and lymphocyte depletion.[6] Initial changes in bone marrow consist of individual cell necrosis or apoptosis; however, by the time affected animals are evaluated clinically, the bone marrow frequently appears aplastic.[6] Depletion of primary and secondary lymphoid tissue may also be seen.

Idiopathic Aplastic Anemia in Calves

An idiopathic aplastic anemia has been identified in calves in Germany.[8] A search for inciting causes has been inconclusive. Circovirus DNA, similar chicken circovirus (chicken infectious anemia), and porcine circovirus were identified in five of 25 affected calves.

APLASTIC ANEMIA IN CHICKENS

Chicken infectious anemia is an emerging infectious disease of young chickens worldwide.[9,20] The disease is caused by a single-stranded DNA circovirus. Affected birds have aplastic anemia, generalized lymphoid atrophy, immunosuppression, and hemorrhages. The diagnosis is straightforward and is based on clinical and histological signs and testing for circovirus infection.[20]

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SECTION IV
Leukocytes

CHAPTER 41

Granulopoiesis

M. JUDITH RADIN and MAXEY L. WELLMAN

Role of the Microenvironment
Lineage Commitment
Transcription Factor Regulation of Granulopoiesis
Cytokine Regulation of Granulopoiesis
  Stem Cell Factor
  Interleukin-3
  Granulocyte-Macrophage Colony-Stimulating Factor
  Granulocyte Colony-Stimulating Factor
  Interleukin-6
Role of MicroRNAs in Granulopoiesis
Developmental Stages of Granulopoiesis
Neutrophils
Eosinophils
Basophils
Mast Cells

Acronyms and Abbreviations

AML-1, acute myeloid leukemia-1; BaP, basophil progenitor; βc, common beta chain for GP140-type cytokine receptors; BMCP, basophil/mast cell progenitor; C/EBP, CCAAT/enhancer-binding protein; CD, cluster of differentiation; c-kit, receptor for SCF; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; CSF, colony-stimulating factor; CXCL12, CXC-chemokine ligand 12; CXCR4, CXCL12 receptor; EoP, eosinophil progenitor; G-CSF, granulocyte colony-stimulating factor; GATA, GATA-binding protein; G-CSFR, granulocyte colony-stimulating factor receptor; GFI-1, growth factor independent-1; GM-CSF, granulocyte-macrophage colony-stimulating factor; GM-CSFR, granulocyte-macrophage colony-stimulating factor receptor; GMP, granulocyte/monocyte progenitor; HSC, hematopoietic stem cell; ICAM-1, intercellular adhesion molecule-1; Id2, inhibitor of DNA binding 2; IL, interleukin; IL-3R, interleukin-3 receptor; IL-5R, interleukin-5 receptor; IL-6R, interleukin-6 receptor; IRF-8, interferon regulatory factor-8; Lin, lineage-specific antigen; miR, microRNA; MCP, mast cell progenitor; MDP, macrophage/dendritic cell progenitor; MEP, megakaryocyte/erythroid progenitor; MITF, melanocyte-inducing or microphthalmia-associated transcription factor; MPP, multipotential progenitor; mSCF, membrane-bound stem cell factor; NeuP, neutrophil progenitor; NGF, nerve growth factor; NK, natural killer cells; PAMP, pathogen-associated molecular pattern; PECAM-1, platelet cell adhesion molecule; pre-BMP, pre-basophil/mast cell progenitor; PRR, pattern recognition receptor; Sca-1, stem cell antigen-1; SCF, stem cell factor; TGF-β, transforming growth factor-β; TLR, toll-like receptor; TNF-α, tumor necrosis factor-alpha; TSLP, thymic stromal lymphopoietin; VCAM, vascular cell adhesion molecule.

In adult mammals, granulopoiesis occurs primarily in the bone marrow, with expansion to extramedullary sites in times of increased demand or with bone marrow disease. Most information about development and regulation of the granulocytic lineages has been obtained from human and rodent models. However, bone marrow cultured from common domestic species indicates that granulocyte development is similar.[6,17,38,41,46,48,50,52]

ROLE OF THE MICROENVIRONMENT

The hematopoietic microenvironment provides the physical structure and chemical milieu permissive for granulopoiesis. The microenvironment comprises the extracellular matrix and stromal cells that include endothelial cells, leptin-receptor-bearing adipocyte

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lineage cells, osteoblasts, perivascular mesenchymal cells, Schwann cells, megakaryocytes, regulatory T cells, and macrophages. Granulopoiesis is regulated by growth factors and cytokines produced by cells in the microenvironment, interactions with the extracellular matrix, and growth factors and cytokines delivered from the blood. Commitment and differentiation are coordinated through changes in expression of cell surface markers, receptors, transcription factors, and signal transduction pathways in the hematopoietic precursors.

In adult mammals, granulocytic lineages originate from pluripotential hematopoietic stem cells (HSCs) within the bone marrow (see Chapter 2).[47] Proliferation, mobilization, and commitment of HSCs to granulocytic differentiation occur within niches along vascular sinusoids.[47] Homing and maintenance of HSCs and committed hematopoietic progenitor cells to the perisinusoidal niches require binding of the CXCL12 receptor (CXCR4) to the CXC-chemokine ligand 12 (CXCL12) on endothelial and stromal cells. Ultimately, as neutrophils mature, there is decreased expression of CXCR4, allowing release of neutrophils into the circulation.

LINEAGE COMMITMENT

Identification of HSCs and other early committed progenitors is based on detection of surface markers. In mice, HSCs are characterized by expression of stem-cell-associated antigen (Sca-1) and c-kit (stem cell factor receptor), but lack of expression of lineage-specific antigens (Lin); however, similar homologs have not yet been clearly identified in other species.[7] CXCL12 signaling appears necessary for maintenance and differentiation of HSCs to CD34+ multipotential progenitors (MPPs) that have multilineage potential but have lost the capability of self-renewal.[47] In the classic hierarchical model of hematopoiesis, MPPs commit to either common lymphoid progenitors (CLPs) or common myeloid progenitors (CMPs) (Figure 41.1). Murine CLPs are distinguished by expression of Lin−Sca-1+c-kit^{lo}, whereas CMPs are Lin−Sca-1−c-kit+CD34+.

CMPs give rise to several lineages, including granulocyte/monocyte progenitors (GMPs), megakaryocyte/erythroid progenitors (MEPs), and macrophage/dendritic cell progenitors (MDPs).[25] In mice, granulocytic lineages differentiate from GMPs to produce neutrophil progenitors (NeuPs), eosinophil progenitors (EoPs), and basophil/mast cell progenitors (BMCPs).[1,25,27] BMCPs subsequently give rise to basophil progenitors (BaPs), which complete their maturation in the bone marrow, and mast cell progenitors (MCPs), which circulate and undergo maturation in gastrointestinal mucosa, skin, lung, and heart.

Although it is helpful to envision progenitor development in the classical hierarchical model, recent alternative models suggest that there may be more plasticity in progenitor commitment. There also may be species differences in generation of eosinophil, basophil, and mast cell progenitors. Some evidence suggests that lineage commitment can occur within the MPPs or as early as some HSCs that have lost self-renewal capability.[8,37] This is evident in leukemic cell lines that express aberrant lineage markers and the ability to modify differentiation of cultured cells by varying culture conditions and transcription factor expression.

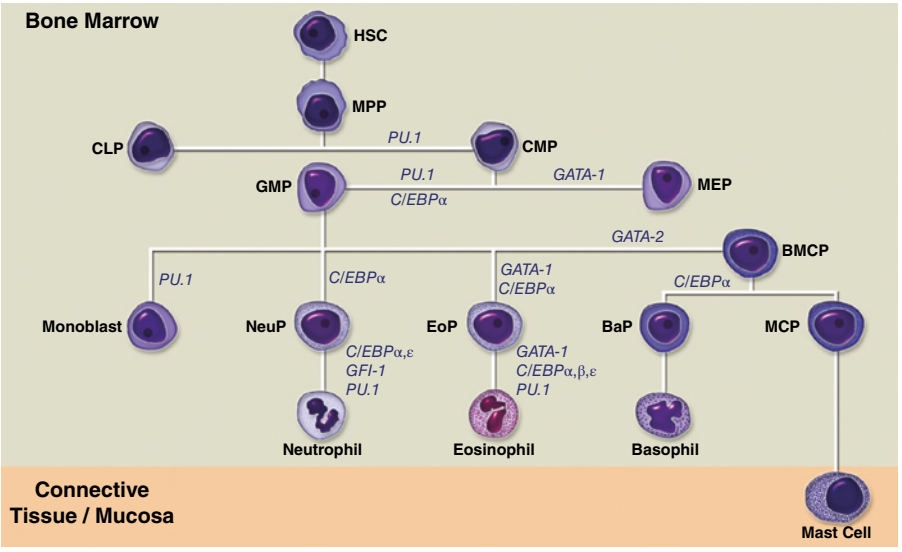


FIGURE 41.1 Granulopoiesis with transcriptional regulation indicated. MCP also may arise directly from MPP, depending on the species or mouse strain (BaP, basophil progenitor; BMCP, basophil/mast cell progenitor; C/EBP, CCAAT/enhancer-binding protein; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; EoP, eosinophil progenitor; GFI-1, growth factor independent-1; GMP, granulocyte/monocyte progenitor; HSC, hematopoietic stem cell; Id2, inhibitor of DNA binding 2; MCP, mast cell progenitor; MEP, megakaryocyte/erythroid progenitor; MPP, multipotential progenitor; NeuP, neutrophil progenitor). (**Source:** Illustrated by Tim Vojt, Biomedical Media, College of Veterinary Medicine, Ohio State University)

TRANSCRIPTION FACTOR REGULATION OF GRANULOPOIESIS

Commitment, differentiation, and maturation of granulocytic progenitors result from a coordinated program of transcription factor expression and gene activation and suppression (Figure 41.1).[25,34,35] This coordinated hierarchy impacts expression of receptors for colony-stimulating factors (CSFs) on hematopoietic progenitors, timing of adhesion molecule expression, and genetic programing that allow transition to committed lineages and functional maturation within a lineage.

Differentiation of MPPs to CMPs requires activation of PU.1, an Ets transcription factor family member.[25,34] PU.1, in conjunction with expression of CCAAT/enhancer-binding protein-α (C/EBP-α), a basic-region leucine zipper transcription factor, promotes transition to GMPs. Continued presence of C/EBP-α and diminished expression of PU.1 result in formation of early neutrophil precursors. This is in contrast to GMPs that maintain high levels of PU.1 but no longer express C/EBP-α, which results in commitment to monocyte production. Maturation of neutrophils beyond the promyelocyte stage and production of specific and gelatinase granules is dependent on increasing expression of PU.1 and C/EBP-ε. C/EBP-ε protein expression is highest in myelocytes and metamyelocytes and is key to stopping proliferation through cell cycle arrest and allowing terminal maturation. Growth factor independent-1 (GFI-1) also is required for transition to promyelocytes and for gelatinase and secretory vesicle formation. Increased production of neutrophils in response to infection and increased demand (termed "emergency" granulopoiesis) requires C/EBP-β to coordinate cytokine-induced proliferation and differentiation.[25] Unlike other C/EBPs, C/EBP-β is not downregulated by inflammatory cytokines and does not inhibit the cell cycle to the same degree, thus allowing amplification of granulopoiesis above steady state.

Production of EoP results from expression of GATA-1 (zinc finger family transcription factors) and continued high levels of C/EBP-α.[44,49] Further, differentiation of EoP to mature eosinophils requires GATA-1, C/EBP-β, PU.1, and inhibitor of DNA binding 2 (Id2). BMCP formation requires GATA-2 expression but inactivation of C/EBP-α.[1,44,49] For BMCPs to commit to production of basophils, there must be reactivation of C/EBP-α to produce BaPs, whereas continued downregulation of C/EBP-α and upregulation of melanocyte-inducing transcription factor (MITF, also called microphthalmia-associated transcription factor) is required for differentiation to MCPs.[12]

CYTOKINE REGULATION OF GRANULOPOIESIS

Granulopoiesis is regulated by cytokines that include stem cell factor (SCF) and CSFs.[3,5,27] Expression of surface receptors for these cytokines on hematopoietic cells is controlled by transcription factors so that sensitivity to individual cytokines coordinates with and facilitates proliferation and differentiation of the various lineages. Most hematopoietic cytokines are constitutively produced at low levels by a variety of cell types to maintain steady-state granulopoiesis, but levels increase in response to inflammation. Increased circulating levels of these cytokines in response to inflammatory mediators stimulate increased granulocyte production in times of high demand.

Stem Cell Factor

SCF (also termed kit ligand and mast cell growth factor) acts on some of the earliest hematopoietic progenitors and is required for survival and self-renewal of HSCs (see Chapter 2).[5,42] SCF is produced as either a membrane-bound (mSCF) or soluble form. mSCF appears to be critical for effective hematopoiesis because soluble SCF is unable to compensate for lack of mSCF in genetically engineered mice.[5] mSCF is found on osteoblasts, endothelial cells, and fibroblasts in the bone marrow, and plays a role in establishing progenitors in their appropriate bone marrow niches. SCF inhibits apoptosis and promotes entry of cells into the S phase of the cell cycle. SCF also potentiates the effects of other cytokines and CSFs to promote proliferation, especially of early committed progenitors. c-kit (CD117), a member of the type III receptor tyrosine kinase family, is the SCF receptor. Binding of SCF to c-kit results in homodimerization and autophosphorylation of the receptor. Expression of c-kit varies depending on the stage of hematopoietic cell development.[22] c-kit expression is present at low levels on quiescent HSCs and increases to its highest levels on early, actively proliferating granulocytic progenitors. With the exception of mast cells, c-kit expression declines with further maturation of granulocytic lineages.

Interleukin-3

Interleukin (IL)-3, a member of the short-chain α-helical bundle type 1 cytokine family, acts on some of the earliest hematopoietic progenitors.[3] It is primarily produced by activated T cells; however, other cell types, such as bone marrow stromal cells, natural killer (NK) cells, eosinophils, and mast cells, also can produce IL-3. IL-3 binds to a specific receptor (IL-3R), which is a heterodimer consisting of α- and β-chains. The α-chain (IL-3Rα or CD123) conveys ligand specificity. The common β-chain (βc or CD131) dimerizes with the α-chain–ligand complex to initiate signal transduction. Transcription of the IL-3Rα is controlled by transcription factors PU.1, GATA-1, and C/EBP-α, whereas constitutive expression of the βc appears to be controlled by PU.1. IL-3R is found on early progenitors such as CD34+ HSCs, MPPs, and CMPs. The primary effects of IL-3 are to promote proliferation, differentiation, and survival of the most primitive precursors by shortening the time in the G_0 stage of the cell cycle. Although IL-3R can be found on more mature granulocyte precursors, the growth-promoting effects of IL-3 continue to be important for eosinophils and basophils.

Granulocyte-Macrophage Colony-Stimulating Factor

Granulocyte-macrophage CSF (GM-CSF), a member of the short-chain α-helical bundle type 1 cytokine family, is

produced by a variety of cells including macrophages, T and B cells, bone marrow stromal cells, osteoblasts, fibroblasts, endothelial cells, neutrophils, eosinophils, and mast cells.[3,27] The receptor for GM-CSF (GM-CSFR) is a member of the GP140 cytokine receptor superfamily. GM-CSFR is a heterodimer that shares the same βc with the IL-3R, but has a unique α-chain (GM-CSFRα or CD116) that confers ligand specificity. Like IL-3R, transcription of the GM-CSFRα is controlled by PU.1, GATA-1, and C/EBP. GM-CSFR is expressed as progenitor cells commit to granulocytic and monocytic differentiation. Binding of GM-CSF to its receptor promotes progenitor survival by inhibiting apoptosis. GM-CSF promotes proliferation and maturation of granulocytes and macrophages.

Granulocyte Colony-Stimulating Factor

Granulocyte CSF (G-CSF) is a primary growth factor for neutrophil production. G-CSF is produced by a variety of cells including macrophages, monocytes, fibroblasts, and endothelial cells of the peripheral tissues and bone marrow.[3,27,30,35] The receptor for G-CSF (G-CSFR) is a member of the GP130 cytokine receptor superfamily and forms a homodimer or homo-oligomer upon ligand binding. G-CSFRs are expressed in highest concentrations by neutrophilic progenitors through mature neutrophils. Sensitivity to G-CSF increases with neutrophil maturation and is related to increasing numbers of G-CSFR.[35] Effects of G-CSF include stimulation of survival, proliferation, and terminal differentiation of neutrophils.

One control of G-CSF production is a feedback loop among G-CSF, IL-23, and IL-17. Under homeostatic conditions, macrophages produce IL-23, a proinflammatory cytokine. IL-23 stimulates IL-17 synthesis by Th17 lymphocytes and NK cells. IL-17 subsequently stimulates production of G-CSF and GM-CSF by endothelial and stromal cells both peripherally and within the bone marrow microenvironment. Phagocytosis of apoptotic and aging neutrophils by macrophages in peripheral tissues and bone marrow results in decreased production of IL-23, subsequent decreased IL-17 secretion, and ultimately decreased secretion of G-CSF. This feedback loop links production of G-CSF to steady-state turnover of aging neutrophils and keeps G-CSF at levels appropriate to support basal neutrophil production. Increased expression of CXCR4 may direct aging neutrophils back to the bone marrow for clearance by resident macrophages, abetting regulation of IL-23 production to maintain steady-state granulopoiesis.[34]

Products released by bacteria of the gut microbiome are implicated as a stimulus of steady-state granulopoiesis through toll-like receptor (TLR) signaling.[2,51] Low-level activation of the TLR-4/MyD88 pathway at the level of the gut and possibly the bone marrow results in a tonic production of inflammatory cytokines such as IL-17 and subsequently G-CSF to help set the size of the bone marrow granulopoietic pool in health.

Concentrations of G-CSF increase markedly in response to infection and help stimulate emergency granulopoiesis. Endotoxin and inflammatory mediators, such as tumor necrosis factor-α (TNF-α), IL-1β, and IL-17, induce G-CSF, thus driving increased neutrophil production and release during an inflammatory response.[35] Increased production of G-CSF by bone marrow stromal cells also may be mediated through direct sensing of pathogen products (pathogen-associated molecular patterns or PAMPs) via TLRs or other pattern recognition receptors (PRRs). Bone marrow stromal cells, HSCs, and progenitors cells may be capable of directly responding to PAMPs through activation of PRRs.[26] Elevated G-CSF shortens maturation time of neutrophilic precursors, decreases marrow transit time, stimulates release of neutrophils from the bone marrow, and prevents apoptosis.

High concentrations of G-CSF result in release of HSCs into the circulation. This technique has been used to harvest circulating HSCs for bone marrow transplantation in people. Release of HSC in response to G-CSF appears to be mediated in part by elaboration of neutrophil enzymes, such as neutrophil elastase, cathepsin G, and matrix metalloproteinase-9 within the bone marrow microenvironment.[27] These enzymes cleave adhesion molecules on HSCs and osteoblasts, thereby releasing HSCs.

Interleukin-6

IL-6 is a member of the GP130 cytokine family, and receptors for IL-6 (IL-6R) are found on some CD34+ hematopoietic precursors.[21] IL-6 can stimulate granulopoiesis via binding to membrane-bound IL-6R. GP130 signaling can also be initiated through "trans-signaling" wherein IL-6 is bound to a soluble form of its receptor and this complex heterodimerizes with GP130 to activate signaling. This latter mechanism appears to be important in granulopoiesis.[20,36] IL-6 can act synergistically with SCF to enhance granulopoiesis in culture and can partially compensate for lack of G-CSF in knockout mice.[27,36] IL-6 can act alone or synergistically with G-CSF to mediate bone marrow granulocytic hyperplasia during times of increased demand for neutrophils.

ROLE OF MICRORNAS IN GRANULOPOIESIS

MicroRNAs (miR) regulate HSC quiescence and proliferation of committed granulocyte precursors.[8] miR-130a confers resistance to the cell cycle inhibitory effects of transforming growth factor-β (TGF-β) in HSCs, myeloblasts, and promyelocytes. Concentrations of different miRs vary as development proceeds from myeloblast to metamyelocyte. For example, with increase in C/EBP-α, production of miR-223 increases, which inhibits translation of proteins that promote cell cycle arrest, thus allowing proliferation of neutrophils. miR-223 inhibits proliferation of eosinophil precursors, while miR-21 promotes eosinophil precursor proliferative responsiveness to IL-5.[49] miRs have also been implicated in the development of myeloid leukemias in people.

DEVELOPMENTAL STAGES OF GRANULOPOIESIS

Committed neutrophil, eosinophil, and basophil precursors undergo a similar maturation sequence (Figure 41.2). The myeloblast is the earliest member of

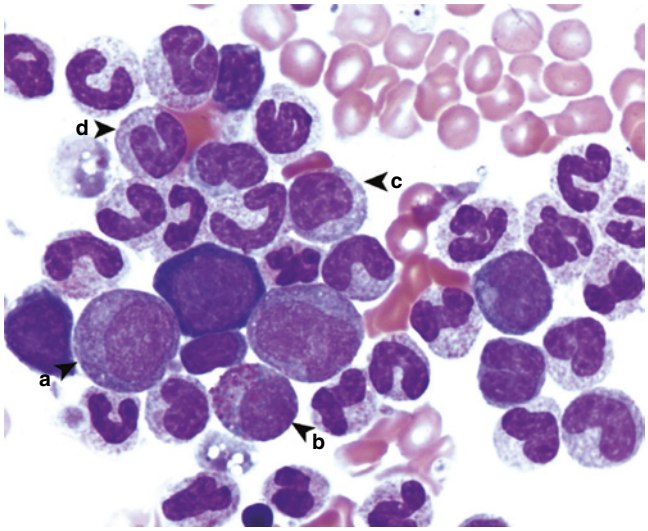


FIGURE 41.2 Granulocytic precursors in canine bone marrow aspirate. (a) promyelocyte; (b) eosinophilic myelocyte; (c) neutrophilic metamyelocyte; (d) band neutrophil. Wright–Giemsa stain; original magnification, 1000×.

granulocytic lineage recognizable by light microscopy. Myeloblasts are characterized by finely stippled chromatin, one to multiple nucleoli, and moderately basophilic cytoplasm. Initially, the cytoplasm lacks granules (type I myeloblast), but small numbers of primary or azurophilic granules begin to appear as myeloblasts mature (type II myeloblast). Promyelocytes (progranulocytes) are larger than myeloblasts and are characterized by increasing numbers of primary granules and decreasing cytoplasmic basophilia. A Golgi area may be apparent and is free of granules. The chromatin pattern remains finely stippled and nucleoli may or may not be present. As promyelocytes mature, primary granules become less apparent by light microscopy and secondary granules appear. Myelocytes are smaller than promyelocytes and are characterized by round to slightly oval nuclei with partially condensed chromatin and lightly basophilic cytoplasm containing secondary granules. Myelocytes are the earliest stage that commitment to a specific granulocytic lineage can most easily be discerned by light microscopy due to the presence of lineage-specific secondary granules. Myelocytes are the last stage at which cellular division occurs. For each myeloblast, there is the potential to produce 16–32 myelocytes.

Metamyelocytes and band granulocytes are no longer capable of cell division, but undergo nuclear maturation characterized by increasing condensation of chromatin and progressive nuclear segmentation. Metamyelocytes have bean-shaped to elongated nuclei, whereas bands have U- or an S-shaped nuclei with smooth, parallel sides. As these cells mature from metamyelocytes to bands, the cytoplasm becomes less basophilic and granule contents mature. Mature granulocytes have condensed chromatin and segmented nuclei with multiple lobes. The degree of segmentation varies with granulocyte type and species (see Section IX). In general, segmented neutrophils have three to five lobes, whereas eosinophils may be bilobed. Mature basophil nuclei may appear monocytoid to bilobed.

NEUTROPHILS

Differentiation from CMPs to GMPs is stimulated by IL-3, GM-CSF, and IL-6. Commitment of GMPs to neutrophil production and proliferation and maturation of myeloblasts to mature segmented neutrophils is primarily driven by G-CSF. Although IL-6 can stimulate granulopoiesis and partially compensate for a lack of G-CSF in knockout mice, G-CSF is required for functional maturation of neutrophils by increasing adhesion factors, chemotactic activity, phagocytosis, and production of bactericidal factors.[35]

Development of myeloblasts to segmented neutrophils is associated with changes in cell surface expression of adhesion molecules and receptors.[15] In people, maturation from myeloblasts to segmented neutrophils is accompanied by decreased surface expression of adhesion molecules that regulate retention in bone marrow such as CD29, CD31 (platelet cell adhesion molecule-1, PECAM-1), CD33, CD49d (binds to vascular cell adhesion molecule, VCAM), and CD49e (binds to fibronectin). There is increased expression of other adhesion molecules associated with neutrophil rolling and adherence (e.g., CD11b, CD11c) as cells mature (see Chapter 45). There is decreased expression of CD54 (intercellular adhesion molecule-1, ICAM-1) during neutrophilic maturation, but expression can be reactivated when neutrophils are exposed to high concentrations of G-CSF during inflammation. Receptors for immunoglobulin (CD16 and CD32) and receptors for modifiers of complement activity (CD11b, CD11c, CD35, and CD55) increase with maturation.

The formation of neutrophil granules is sequential, and the production of granule contents is in step with maturation of the cells (see Chapter 42). A "targeting by timing" model suggests that formation of vesicles is continuous, but protein production and thus packaging of granule contents are sequential and coordinated by transcription factors and cytokines.[8,25] Azurophilic or primary granules contain myeloperoxidase and are the first granules to form as myeloblasts transition to progranulocytes. Synthesis of azurophilic granule proteins is under control of GATA-1, C/EBP-α, C/EBP-γ, c-Myc, and acute myeloid leukemia-1 (AML-1). As c-Myc and AML-1 decrease, protein synthesis transitions to those proteins associated with specific granules. Specific or secondary granules are characteristic of myelocytes and have a strict requirement for C/EBP-ε. C/EBP-ε RNA and protein peak at the myelocyte stage and halt entry into the cell cycle and proliferation. This allows neutrophils to move from the proliferative into the maturation pool. The proteins found in tertiary or gelatinase granules are produced during the metamyelocyte stage as C/EBP-ε falls and C/EBP-β, C/EBP-ζ, and C/EBP-δ increase. Secretory granules form in band and segmented neutrophils during final maturation. Because

of the continuous nature of vesicle formation, overlap in granule contents or subsets of granule types can occur.

Under basal conditions, bone marrow transit time for neutrophils (time to go from myeloblast to segmented neutrophil) is approximately 6 days in dogs, cats, and horses.[4,6,13,38] Production time for neutrophils in calves is 6 days, but neutrophils are held in the bone marrow for one additional day before release.[45,46] Delivery of neutrophils to blood is related to the rapidity with which neutrophils are depleted from the circulation.[45] Although the steady state bone marrow reserve of neutrophils is similar in dogs and calves, the proliferative capacity of bone marrow in calves appears to be less than dogs. If neutrophils are depleted from the circulation, dogs react with leukocytosis and bone marrow hyperplasia characterized by a shortened production time from 6 to 2–3 days.[4,45] There is no apparent shortening of transit time in calves.[45,49] The mechanism underlying the failure of cattle to increase granulopoiesis and shorten bone marrow transit time is uncertain.

Increased demand for neutrophils results in release of inflammatory cytokines and increased production of G-CSF.[26,35] G-CSF is a key mediator of neutrophil mobilization from bone marrow reserves, stimulator of granulopoiesis, and initiator of functional maturation of neutrophil precursors. Dogs, cats, horses, and cattle all show a robust response to administration of G-CSF characterized by neutrophilia and granulocytic hyperplasia in bone marrow.[9,10,16,29,32,41]

EOSINOPHILS

There may be species differences in eosinophilopoiesis. In mice, EoPs originate from GMPs, whereas EoPs appear to arise directly from CMP in human cell lines.[44] EoPs are distinguished by expression of c-kit^{lo} and the IL-5 receptor (IL-5Rα^{hi}). GATA-1 is critical for development of EoPs from GMPs.[44,49] In addition, C/EBP-α, C/EBP-β, PU.1, and interferon regulatory factor-8 (IRF-8) support commitment to EoP. Differentiation and maturation from EoP to eosinophils require C/EBP-β, GATA-1, PU.1, and Id2. miRs also modulate eosinophilopoiesis, with miR-21 enhancing and miR-223 suppressing production of eosinophils.

The main cytokines driving eosinophil production are IL-3, GM-CSF, and IL-5. Eosinophils appear to lack receptors for G-CSF.[31] IL-3 and GM-CSF play a role in the early steps of proliferation and commitment of EoPs, whereas IL-5 is required for proliferation, terminal differentiation, and functional maturation of eosinophils.[44] The IL-5R shares the same signal transducing βc with IL-3 and GM-CSF, but has a unique α-chain (IL-5Rα or CD125) to permit specific binding of IL-5. Thus, eosinophils differ from neutrophils in that βc signal transduction is required through all stages of eosinophil production and maturation. Th2 T helper cells are the primary source of IL-5. IL-5 also can be produced by mast cells, eosinophils, Tc2 cells, γδ T cells, NK cells, and epithelial cells.[43] In addition to its role in proliferation and differentiation of eosinophils, IL-5 prevents apoptosis, stimulates release of eosinophils from the bone marrow, promotes adhesion of eosinophils to endothelial cells, and activates eosinophil effector function. Overproduction of IL-5 is associated with peripheral eosinophilia in transgenic mice and is observed in various naturally occurring and experimentally induced hypereosinophilic conditions. Interestingly, IL-5 knockout mice have normal basal numbers of eosinophils in their bone marrow, but fail to develop tissue or blood eosinophilia when stimulated by a parasite infection or inhalant allergen.[14,25] This suggests that IL-5 is necessary for an expanded eosinophilic response to inflammatory stimuli.

Bone marrow production time from myeloblast to mature eosinophils takes 2–6 days.[19] Secondary granules continue to mature through the segmented stage. Production of specific granule proteins occurs in response to transcription factors. For example, production of major basic protein is controlled by combinations of GATA-1 or PU.1 with various isoforms of C/EBP.[44]

BASOPHILS

Committed BaPs originate from CD34+ myeloid progenitors in the bone marrow. Although the sequence of intermediaries between MPPs and BaPs remains unclear, studies using murine bone marrow cells suggest that basophils share a common progenitor with mast cells (BMCP) and originate from GMPs.[12,18] A second common progenitor, the pre-basophil/mast cell progenitor (pre-BMP), has been described in mice and expresses different surface markers from BMCP.[18] Subsequent differentiation to either BaPs or MCPs is regulated by sequential expression of transcription factors.[12] Upregulation of GATA-2 in conjunction with downregulation of C/EBP-α induces differentiation to BMCPs capable of differentiating into either basophils or mast cells. Subsequent reactivation of C/EBP-α results in committed BaPs and production of basophils.[1,12,18] Maturation of BaPs occurs in the bone marrow, and transit time from myeloblasts to mature basophils takes 2–3 days.[14] Basophil precursors require βc signal transduction initiated by IL-3 and GM-CSF for proliferation and differentiation. IL-5, nerve growth factor (NGF), and TGF-β also act as growth factors. Unlike other granulocytes, a lineage-specific factor has not been identified. Although IL-3 appears to be the main growth factor promoting differentiation and maturation of basophil precursors, it is not required for steady-state conditions in IL-3-deficient mice.[33] However, IL-3 is needed for accelerated production in response to parasites.[33] Both IL-3 and GM-CSF increase effector function of basophils by increasing histamine production through induction of histidine decarboxylase, increasing IL-4 and IL-13 production, and enhancing migration of mature basophils into tissues.

An apparent separate population of basophils can be stimulated by thymic stromal lymphopoietin (TSLP) produced by epithelial cells.[33] Progenitors for this basophil subset in mice develop in either the bone marrow or spleen and do not depend on IL-3. These TSLP-dependent basophils appear functionally distinct from IL-3-dependent basophils in that they express a different pattern of cytokines and inflammatory mediators. TSLP-dependent basophils do not require IgE cross-linking for activation, which may explain why anti-IgE therapies do not work in all basophil-mediated diseases.

MAST CELLS

Mast cell progenitors originate from CD34+ hematopoietic progenitors in the bone marrow. Early development remains unclear and studies suggest various pathways, including common BMCPs descended from GMPs or direct derivation from MPPs or CMPs.[1,12,40] In addition to species differences in derivation of MCP, there may be differences among mouse strains.[40] Commitment to mast cell production from GMPs or CMPs requires high expression of GATA-2. Subsequent suppression of C/EBP-α and increased expression of MITF distinguishes MCP from BaP.[33,40] Committed MCPs exit the bone marrow and undergo proliferation, differentiation, and maturation in mucosal and connective tissues.[12] At the time of migration, these MCPs lack mature granules and the high-affinity receptors for the Fc portion of IgE.[39]

IL-3 and SCF are the primary growth factors for mast cell development.[23,24] Unlike other granulocytic lineages, MCPs maintain high levels of c-kit, the receptor for SCF.[12,28] Stimulation of c-kit promotes proliferation and differentiation of mast cells, and its effects may be potentiated by coincubation with IL-3. In addition, SCF binding to c-kit inhibits apoptosis and prolongs survival of mast cells. SCF is chemotactic for immature and mature mast cells and helps localize mast cells to tissues.[11]

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CHAPTER 42

Neutrophil Structure and Biochemistry

CLAIRE B. ANDREASEN

Neutrophil Structure
Neutrophil Granules and Species Comparisons
  Neutrophil Granule Formation
  Neutrophil Granule Release
  Primary (Azurophilic) Granules
  Secondary (Specific) Granules
  Tertiary (Gelatinase) Granules
Secretory Vesicles
Neutrophil Plasma Membrane
Neutrophil Cytoplasm

Acronyms and Abbreviations

BMAP, bovine myeloid antimicrobial peptide; BPI, bactericidal/permeability-increasing protein; eCATH, equine cathelicidin; e-NAP, equine neutrophil antimicrobial peptide; FMLP, formyl-methionyl-leucyl-phenylalanine; hCAP, human cathelicidin protein; K9CATH, canine cathelicidin; LAMP, lysosome-associated membrane protein; MIF, migration inhibitory factor-related proteins; MRP, migration-related proteins; MPO, myeloperoxidase; NADPH, nicotinamide adenine dinucleotide phosphate; Nramp1, natural resistance-associated macrophage protein 1; PMAP, porcine myeloid antimicrobial peptide.

Neutrophils provide the first line of defense against pathogens by phagocytosis and microbial killing via membrane receptor and cell signaling regulation of cytoplasmic content release. Neutrophil constituents also can result in tissue damage if not adequately regulated. Neutrophils have additional functions (see Chapter 43) and species differences (see Section IX). This chapter will provide an overview of neutrophil cellular contents, including granules, subcellular organelles, cytosol components, and membrane-associated receptors.

NEUTROPHIL STRUCTURE

Mature, segmented neutrophils are approximately 10–12 μm in diameter and contain granules and subcellular organelles. Studies have used electron microscopy, including immunologic techniques, and cell disruption with cavitation followed by density-gradient centrifugation to yield cell fractions for analysis. Scanning electron microscopy has identified numerous small pseudopodia that increase the surface area for receptor contact and phagocytic function (Figure 42.1).

Mature neutrophils have a lobulated nucleus, primarily composed of heterochromatin with a thin central strand of euchromatin, few nuclear pores, and rarely an observed nucleolus[2] (Figure 42.1). Mature neutrophil nuclei can synthesize both RNA and protein.[20] This is important when neutrophils migrate to sites of inflammation and synthesize proteins and chemokines that regulate activation and resolution of the innate immune response.[20]

With the exception of numerous granules, neutrophil cytoplasm contains few organelles and a variable number of glycogen particles. A thin rim at the periphery of neutrophils contains a small area with numerous cytoskeletal proteins.[2]

NEUTROPHIL GRANULES AND SPECIES COMPARISONS

Neutrophil granules contribute to the host defense against pathogens via their matrix content, membrane receptors, and enzymatic modification of tissues. Neutrophil granules contain (1) antimicrobial proteins directed against bacteria, fungi, protozoa, and some

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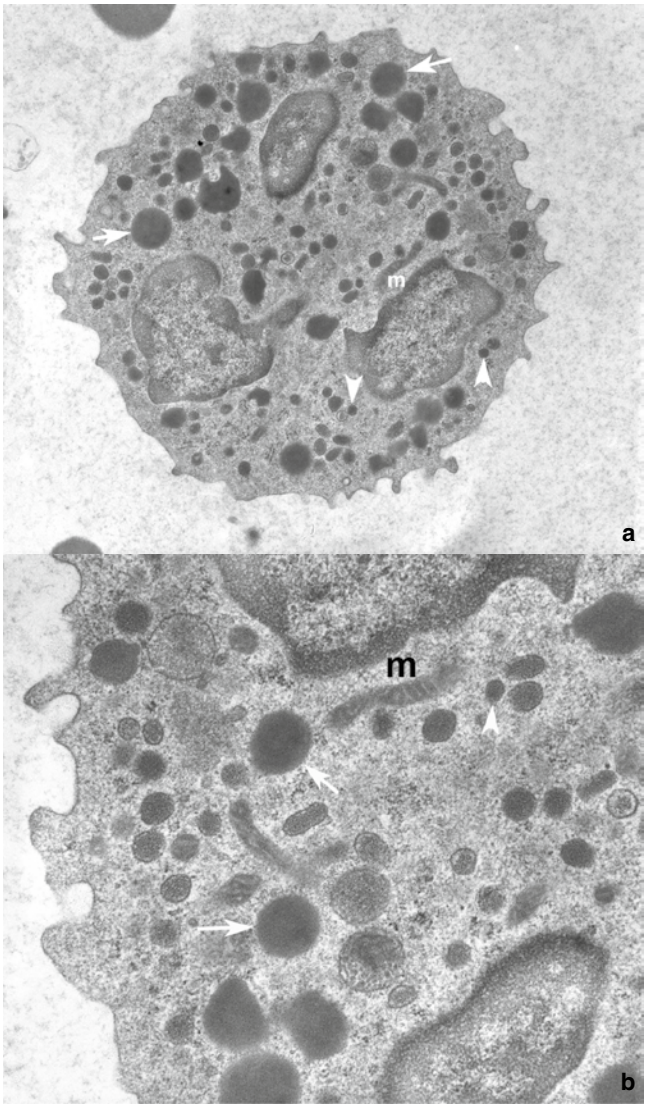


FIGURE 42.1 (a) and (b) Transmission electron micrographs of a segmented canine neutrophil with numerous variably sized round to oval primary (arrows) and secondary (arrowheads) granules. Nuclear lobes contain marginated heterochromatin and central euchromatin. There are few organelles, including rare mitochondria (m), in a cytosol replete with electron-dense glycogen granules. Stained with uranyl acetate and lead citrate. (a) 8400× and (b) 20 000×. (**Source:** Courtesy of Dr. Craig Thompson, Purdue University, College of Veterinary Medicine)

viruses, (2) proteases, (3) components of the respiratory burst, and (4) receptors for endothelial adhesion molecules, extracellular matrix proteins, bacterial products, and soluble mediators of inflammation.[6] Neutrophil granules have a phospholipid bilayer membrane surrounding an intragranular matrix that contains proteins for either exocytosis into the surrounding tissue environment or fusion with a phagosome. These proteins must be properly packaged and exocytosed in order to prevent widespread host tissue damage.[17]

Due to the potential for granule-induced tissue damage, feedback mechanisms occur whereby neutrophils can modulate their local responses and limit host tissue damage.[16] Human neutrophil primary granules contain anti-inflammatory proteins, including alpha$_1$-acid glycoprotein, an acute phase protein that modulates the expression of anti-inflammatory cytokines by macrophages, decreases neutrophil chemotaxis, and has other anti-inflammatory functions.[16] Alpha$_1$-acid glycoprotein recently was confirmed in bovine neutrophil granules.[16]

The granule types and contents have been best characterized in human neutrophils, from which much of the information is derived. In human neutrophils, well-defined granule types are primary (azurophil or myeloperoxidase-positive), secondary (specific), and tertiary (gelatinase) granules; the latter two are both peroxidase-negative. Secretory vesicles will be discussed separately from granules (Table 42.1).[1,3,6,24]

Species-specific distribution of granules and contents can be difficult to compare due to the use of different techniques for neutrophil analysis, such as fraction separation by centrifugation versus whole cell analysis.[24] Most species have neutrophil primary and secondary granules that retain the major enzymes. Some domestic animal species appear to have a third granule type that differs from tertiary granules of human neutrophils.[2] Granule differences in species may impact host response to the pathogenicity of microorganisms; however, this is not well-defined or correlated. This section will address neutrophil granule formation, release, and contents, and the known variations for species.

Neutrophil Granule Formation

Neutrophil granules are a continuous spectrum with differential production of granule contents during neutrophil development (Table 42.1). Primary granules are the first to be formed in the promyelocyte stage, while secondary granules are formed during the myelocyte and metamyelocyte stages.[3] Tertiary granules and secretory vesicles form in the references differ on including metamyelocyte, band and segmented neutrophil stages.[6]

Subsets of primary and secondary granules have been described based on variation in granule contents. Heterogeneity of granule proteins occurs due to segregation of proteins into their respective granules by timing of biosynthesis rather than sorting.[6,9] In this process, the proteins are produced by the endoplasmic reticulum in response to transcription factor expression at specific stages of neutrophil development. For example, primary granules are formed during the promyelocyte stage, when myeloperoxidase is expressed.[6]

Neutrophil Granule Release

The separation of proteins into different granules serves to (1) separate proteins to decrease the risk of untoward enzyme activation, protein degradation, or cellular damage, and (2) allow differential exocytosis of proteins based on varying neutrophil stimulation. This allows the availability of proteins for multiple neutrophil functions (Table 42.1).

Granule release is dependent on receptor-mediated neutrophil stimulation by a variety of molecules. Formyl-methionyl-leucyl-phenylalanine (FMLP), a

TABLE 42.1 Neutrophil granules, stage of granule formation, function, and a partial listing of matrix contents.*

	Primary Granules	Secondary Granules	Tertiary Granules	Secretory Vesicles
Formation	Promyelocyte	Myelocyte/ Metamyelocyte	Band/Segmented	Band/ Segmented
Function	Antimicrobial	Antimicrobial/ Migration	Migration	Adhesion
	Matrix Contents	Matrix Contents	Matrix Contents	Matrix Contents
	Myeloperoxidase	Lysozyme	Lysozyme	Alkaline phosphatase
	Lysozyme	Apolactoferrin	Lactoferrin	Azurocidin
	Defensins	Lactoferrin	Gelatinase	Plasma proteins
	BPI	Alkaline phosphatase	Acetyltransferase	Cytochrome b_{558}
	Elastase	Gelatinase	Cathelicidins	LAMP-3
	Cathepsin G	Collagenase	Defensins (bovine)	
	Proteinase 3	Cathelicidins		
	Beta-Glucuronidase	Cytochrome b_{558}	Migration-related proteins	
	Azurocidin	Migration-related proteins		
	Granzyme B			
	Alpha$_1$-acid glycoprotein			

*Extensive listings of granule and secretory vesicle matrix and membrane contents are further referenced.
Sources: Bainton[1]; Borregaard and Cowland[3]; Faurschou and Borregaard[6]; Styrt[24]

bacterial peptide used in vitro as a neutrophil activator, has species variation in neutrophil activation responses. FMLP activates neutrophils of humans, nonhuman primates, rabbits, guinea pigs, and mice; however, neutrophils of cattle, pigs, dogs, and cats have no FMLP response possibly due to a lack of FMLP receptors.[24]

When neutrophil granules are released, their primary and secondary granule contents are released into the phagocytic vacuole (phagosome), and secondary and tertiary granule contents are released into the extracellular environment.[3] During exocytosis, the granule or vesicle membrane becomes incorporated into the phagosome or neutrophil membrane, respectively. Membrane fusion is crucial, since the granule and secretory vesicle membranes contain many different receptors necessary for neutrophil extravasation, chemotaxis, migration, and immunomodulation.

There is significant overlap in the protein content and function of neutrophil granules; however, there is a general order of their exocytosis inversely related to the order of the granule formation.[6,14] First, secretory vesicles, that facilitate adhesion, are rapidly and completely mobilized during early neutrophil–endothelial interactions while L-selectin is simultaneously shed from the neutrophil surface.[6,19] The vesicles incorporate the vesicle adhesion proteins (e.g., CD11b/CD18), chemoattractant receptors, and complement receptors into the neutrophil cell membrane to permit firm adhesion.[19] This resulting firm adhesion to the endothelium aids in subsequent diapedesis and chemotaxis.[6] Second in order of release are tertiary granules that aid in neutrophil extravasation and migration via matrix-degrading enzymes, including gelatinase and membrane receptors.[6] Third to release are secondary granules that contain antimicrobial substances for microbial destruction and matrix-degrading metalloproteinases that aid in neutrophil tissue infiltration. Last to release are primary granules for microbial destruction that require the greatest stimulus to enter the phagosome for pathogen killing.[6]

Primary (Azurophilic) Granules

Primary granules form during the promyelocytic neutrophil stage and contain many antimicrobial compounds. In most species, except the bovine, primary granules are the largest and most dense granules,[2] and have heterogeneity in size and shape.[6] The major function of primary granules is the secretion of proteolytic enzymes and antimicrobial peptides into the phagocytic vacuole for pathogen killing. Many proteins in the primary granules are synthesized as preforms, but the propeptides are removed during granule maturation and are stored as an active conformation.[6] Additionally, the granule membrane contains a large amount of CD66c and CD63 antigens that have interactions in signaling and membrane adhesion.[20]

Primary granules contain myeloperoxidase (MPO), a potent microbicidal hemoprotein, that contributes to oxygen-dependent pathogen destruction by catalyzing the production of hypochlorite (OCl^-) from chloride and hydrogen peroxide produced by the oxidative burst.[6,14] MPO activity is typically lower in animals compared to humans, except for dogs and nonhuman primates.[18,24] The majority of studies have not detected MPO activity in chicken heterophils, but one study detected a partial DNA sequence and weak MPO activity.[12]

Other proteins within primary granules include cationic alpha-defensins that are powerful pore-forming antimicrobial peptides produced during the promyelocyte and myelocyte stages. Early forming primary granules are defensin-poor and later forming primary granules are defensin-rich.[6] Defensins kill a variety of bacteria, fungi, and viruses (see Chapter 43).

Alpha-defensins also have been identified in neutrophils of rabbits, guinea pigs, and hamsters, but not in mice, cattle, pigs, and birds. Beta-defensins have been identified in neutrophil dense granules of cattle and in avian heterophils, but not in sheep neutrophils.[13] Theta-defensins are in granulocytes of rhesus macaques and other Old World monkeys and orangutans, but not in great apes, humans, and New World monkeys.[13] Horse neutrophils contain only trace amounts of defensins, but do contain significant amounts of equinins (equine neutrophil antimicrobial peptides, e-NAP1 and e-NAP2). These novel proteinase inhibitors have antibacterial and antiviral activities but are structurally different from defensins.[4,15]

Human primary granules contain elastase and beta-glucuronidase that have a role in bacterial killing and endothelial cell and tissue damage during inflammation. A small degree of elastase activity is present in horse and pig neutrophils versus a large amount in rabbits.[24] Beta-glucuronidase has variable concentrations in the neutrophils of many species.[24]

Other human primary granule proteins include bactericidal/permeability-increasing protein (BPI), serprocidins, and granzyme B. BPI binds lipopolysaccharide (LPS) of Gram-negative bacteria, inducing growth inhibition, membrane damage, and mediation of bacterial phagocytosis.[6] Serprocidins, including elastase, cathepsin G, and proteinase 3, degrade extracellular matrix, aid in microbial death, and regulate the immune response.[11] Granzyme B is proposed to act on extracellular targets to enhance neutrophil migration through extracellular matrix and regulate apoptosis or activation in other cell types.[25]

Secondary (Specific) Granules

Secondary granules, formed during myelocyte and metamyelocyte stages, are smaller, less dense, and more numerous than primary granules (Figure 42.1).[2] They can be round, oval, elongated, or dumbbell-shaped.[1,6] Secondary granules have two major functions: (1) antimicrobial activity via peptides and proteases secreted into the phagocytic vacuole to destroy pathogens, and (2) tissue alteration assisting neutrophil migration via matrix metalloprotease release into the extracellular space.[3] Enzymes in secondary granules are stored as inactive latent proforms and are activated upon exocytosis.

The membrane of secondary granules contains membrane-bound molecules that are expressed on the cell surface, including CD11, CD18, CD66a, CD66b, NB-1 (CD177), FMLP receptors, C5a receptors, and cytochrome b_{558}, a component of NADPH oxidase.[6] When cells are stimulated, the surface expression of membrane proteins is increased, and some of the upregulated molecules may be derived from secondary granules. The importance of secondary granules in neutrophil function is apparent in people who lack secondary granules, since these patients are susceptible to repeated skin and respiratory infections, and have defective neutrophil chemotaxis and adhesion.

A major secondary granule component is apolactoferrin, an iron-binding protein that appears to (1) prevent bacteria from obtaining iron that is necessary for growth,[20] (2) modify hydroxyl radical formation, and (3) alter cell adhesion.[20] Haptoglobin released from secondary granules also can inhibit bacterial growth and inflammation.[20]

Secondary granules contain large amounts of antimicrobial substances, especially lactoferrin. Other antimicrobial proteins include hCAP-18 (a cathelicidin), alkaline phosphatase, and lysozyme.[6] Although defensins are stored in primary granules, some human neutrophil defensin-type proteins are stored in secondary granules and then constitutively exocytosed.[7] Collagenase and a small amount of gelatinase act as matrix-degrading enzymes. Release of collagenase augments neutrophil movement through degraded collagen, modifies the inflammatory process, and results in tissue remodeling.[20]

There are limited studies of animal secondary granules. Neutrophil antimicrobial lysozyme was deficient in monkeys, cattle, goats, sheep, cats, and hamsters, and only a small amount of activity was detected in horses, dogs, rats, and guinea pigs.[18,24] Alkaline phosphatase was undetectable in rhesus monkeys, cats, some mouse strains, chickens, and geese, and was low in dogs.[18] Cathelicidins were found in neutrophils of dogs (canine cathelicidin (K9CATH)), horses (equine cathelicidin (eCATH)), cattle (bactenecins, bovine myeloid antimicrobial peptide (BMAPs)), sheep, pigs (protegrins, prophenins, porcine myeloid antimicrobial peptide (PMAPs)), rabbits, guinea pigs, rats, mice, and birds.[13]

Tertiary (Gelatinase) Granules

Tertiary granules, formed during, band and segmented neutrophil stages, are slightly smaller than secondary granules, and fewer in number.[1,10] The major function of tertiary granules is via matrix metalloproteases and receptors required for neutrophil diapedesis and migration.[10] During neutrophil stimulation, both secondary and tertiary granules contain membrane proteins that are upregulated to the cell surface. These granule membranes contain important receptors and proteins, including natural resistance-associated macrophage protein 1 (Nramp1) that may deprive microorganisms of divalent cations.[3,6] The matrix of human neutrophil tertiary granules contains a large amount of gelatinase, released as a major matrix-degrading enzyme,[9] and small amounts of lactoferrin and lysozyme.[1]

Many species, including cattle, sheep, goats, dogs, cats, rabbits, rats, guinea pigs, and horses, have a third granule type that differs from human tertiary granules.[2]

In cattle, these granules are referred to as "large" or "dense" granules, and are the largest (0.4–0.5 μm) and most numerous in mature neutrophils.[8,27] These granules are readily mobilized and responsible for the majority of bovine neutrophil antimicrobial activity, containing lactoferrin, bactenecins (members of the cathelicidin family), and beta-defensins.[27] These are the only neutrophil granules known to contain defensins in bovine neutrophils, in contrast to human neutrophils where defensins are predominantly stored in primary granules.[27]

SECRETORY VESICLES

Secretory vesicles are structures similar to granules that are: 100–200 nm, widely distributed within the cytosol,[19] and identified on electron microscopy as smooth-surfaced vesicles by double-labeling with anti-albumin and either anti-CD11b/CD18 (Mac 1) or anticytochrome b_{558} antibodies.[1] The major function of secretory vesicles is their facilitation of neutrophil adhesion to endothelial cells via their protein and receptor content. In contrast to granules, secretory vesicles lack antimicrobial enzymes. Secretory vesicles contain proteins acquired from endocytosis of plasma (primarily albumin); therefore, they are classified as endocytic vesicles rather than true granules.[1,6]

Secretory vesicles provide an intracellular reservoir from which membrane proteins can be rapidly recruited to the cell surface after weak stimulation.[20] Secretory vesicles can be upregulated to the cell surface in the absence of extracellular calcium, in contrast to secondary and tertiary granules, which require extracellular calcium for release.[20]

Although lysosomes have not been identified in neutrophils, secretory vesicle membranes contain CD63 (lysosome-associated membrane protein-3; LAMP-3) and CD68, but they do not have LAMP-1 or LAMP-2.[1,5] Therefore, secretory vesicles are referred to as regulated secretory granules or lysosome-related organelles.[5] Additionally, secretory vesicle membranes contain many receptors important in extravasation and phagocytosis, including CD11b/CD18, CR1, FMLP-receptor, and CD14. Secretory vesicles also contain alkaline phosphatase, cytochrome b_{558}, and FMLP receptors.[20] Their matrix contains plasma proteins, including albumin.[1,19]

NEUTROPHIL PLASMA MEMBRANE

Constituents of the neutrophil plasma membrane include (1) membrane channels, (2) adhesive proteins, such as CD11/CD18 and CD62L (L-selectin), (3) receptors for various ligands, such as FMLP and C5a receptors that sense stimuli and activate neutrophils, (4) ion pumps, and (5) ectoenzymes. Neutrophil membrane CD13 can inactivate interleukin (IL)-8, eliminating its chemotactic activity, and neutrophil endopeptidase CD10 can inactivate the chemotactic peptide FMLP.[20] CD66 (a, b, c) can activate neutrophils,[20] and some CD45 antibodies inhibit neutrophil chemotaxis.[20] The components of the neutrophil membrane are not uniformly distributed and the membrane can increase the surface area for increased receptor–ligand interactions.

Studies also have indicated the presence of differentiated domains in the membrane called "lipid rafts." These rafts are large noncovalent complexes in cell extracts that contain important signaling molecules, including protein kinases and glycosyl phosphatidylinositol-linked membrane proteins.[20,21] These clusters of molecules may be important in transmembrane signaling by proteins in the complex, and proteins may enter these rafts when they are translocated to the cell surface.[21]

Lipids and carbohydrates also are important to neutrophil structure. Approximately half the neutrophil cellular phospholipid is within neutrophil membranes and secretory vesicles.[20] Neutrophil phospholipids are important sources of inositol 1,4,5-trisphosphate (IP3, a signal-transduction molecule involved in calcium release) and diacylglycerol, which activates protein kinase C. Arachidonic acid in phospholipids, especially phosphatidylcholine, is an important precursor for the production of leukotrienes, prostaglandins, thromboxanes, and lipoxins.[20] Cholesterol and triglycerides constitute most of the non-phospholipid neutrophil lipids.

NEUTROPHIL CYTOPLASM

Neutrophil cytoplasm contains many components common to all cells. A unique aspect is approximately 45% of neutrophil cytosolic proteins consist of migration inhibitory factor (MIF)-related proteins, also known as migration-related proteins (MRP). These cytosol proteins are loosely associated with the neutrophil plasma membrane and within secondary and tertiary granules.[23] MIF/MRP proteins are part of the S100 protein family of calcium-binding proteins expressed by circulating human neutrophils and monocytes. The quantity of MIF/MRP proteins associated with neutrophil plasma membranes increases after stimulation, with a role in neutrophil-mediated inflammation and cytokine production.[23]

Additional cytoplasmic components include annexin I (lipocortin I) that comprises approximately 3% of cytosolic proteins.[20] Annexin I is partially regulated by glucocorticoids and appears to be a mediator of the anti-inflammatory effects of glucocorticoids. Another notable cytoplasmic constituent is glycogen that enables neutrophil energy production via glycolysis and neutrophil function in low extracellular glucose environments.

Cytoplasmic lipid bodies, which are non-membrane-bound cytoplasmic inclusions, provide stores of esterified arachidonate for localized signaling proteins.[26] Their increase during inflammation is stimulated by cis-fatty acids and diglycerides, and is mediated by protein kinase C.

Like many cells, neutrophils contain a complex cytoskeleton that enables neutrophil chemotaxis,

phagocytosis, and exocytosis. Many proteins of this cytoskeleton have been identified, including actin, actin-binding protein, α-actinin, gelsolin, profilin, myosin, tubulin, and tropomyosin. Actin accounts for approximately 10% of neutrophil protein, and is critical for the mobility and migration of neutrophils.[22]

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CHAPTER 43

Neutrophil Function and Response

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Neutrophil Recruitment
- Neutrophil Rolling
- Neutrophil Adhesion and Crawling
- Neutrophil Extravasation

Chemotaxis
Phagocytosis
Antimicrobial Mechanisms
- Respiratory Burst and Generation of Reactive Oxygen Species
- Granular Killing
- Neutrophil Extracellular Traps

Neutrophil Fate
- Apoptosis
- Necroptosis
- NETosis

Immunomodulatory Function of Neutrophils
- Neutrophils in the Adaptive Immune Response
- Neutrophil-Mediated Regulation of Inflammation

Neutrophils in Disease
- Neutrophils in Acute Inflammatory Disease
- Neutrophils in Thromboembolic Disease
- Neutrophils in Immune-Mediated diseases
- Neutrophils in Inflammatory Bowel Disease
- Neutrophils in Cancer

Acronyms and Abbreviations

ANCAs, antineutrophil cytoplasmic antibodies; ARDS, acute respiratory distress syndrome; BAFF, B-cell-activating factor; BPI, bacteria permeability-enhancing factor; C, γ subfamily of chemokines; CC, β subfamily of chemokines; CXC, α subfamily of chemokines; CX_3C, δ subfamily of chemokines; CXCL8, interleukin-8; DAMPs, damage-associated molecular patterns; DC, dendritic cell; ECM, extracellular matrix; fMLP, N-formyl-methionyl-leucyl-phenylalanine; G-CSF, granulocyte colony stimulating factor; IBD, inflammatory bowel disease; ICAM, intercellular adhesion molecule; IFN-γ, interferon-gamma; IL, interleukin; IMHA, immune-mediated hemolytic anemia; LT, leukotriene; LPS, lipopolysaccharide; LFA-1, lymphocyte function-associated antigen-1; MPO, myeloperoxidase; NADPH, nicotinamide adenine dinucleotide phosphate; NETs, neutrophil extracellular traps; NK cells, natural killer cells; PAMPs, pathogen-associated molecular patterns; PRR, pattern-recognition receptors; PSGL-1, P-selectin glycoprotein ligand-1; ROS, reactive oxygen species; SIRS, systemic inflammatory response syndrome; SLE, systemic lupus erythematosus; TNF-α, tumor necrosis factor-alpha; VCAM-1, vascular cell adhesion molecule-1.

Neutrophils are the major pathogen-fighting innate immune cells recruited to sites of infection/inflammation to recognize, phagocytose, and kill pathogens through cytotoxic mechanisms, including production of reactive oxygen species (ROS), release of antimicrobial peptides, and expulsion of nuclear contents to form neutrophil extracellular traps (NETs). The overwhelming infections of cattle, dogs, and cats with either severe neutropenia or neutrophil adhesion deficiencies highlight the importance of functional neutrophils in immunity.[4,18] Neutrophils also play a complex role in the development of the adaptive immune response and in controlling inflammation. When unchecked, neutrophils can also affect significant host tissue damage, making neutrophils central in the development of many inflammatory and immune-mediated diseases.[24] This chapter reviews neutrophil function and the role of neutrophils in adaptive immunity, inflammation, and tissue injury.

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NEUTROPHIL RECRUITMENT

Neutrophils originate in the bone marrow and reach sites of tissue inflammation or infection via the vasculature.[32] Neutrophils are considered to survive in circulation for 8–12 hours and up to 1–2 days in tissues, but based on recent studies they may actually circulate for up to 5 days.[41] The egress of neutrophils from blood occurs primarily in postcapillary venules mediated by the sequential interaction of neutrophil receptors and their ligands that are expressed on activated endothelial cells during inflammation (Figure 43.1).[24] During recruitment, neutrophils (1) attach (tether) to the endothelium, (2) roll along the endothelium, (3) arrest and spread on endothelium, (4) crawl along the endothelium, (5) transmigrate into tissues, and (6) activate and then phagocytose and/or kill pathogens via production of ROS, degranulation, and generation of NETs.[24]

NEUTROPHIL ROLLING

Rolling of neutrophils on the endothelium is mediated by selectins, which are type I integral membrane proteins that recognize specific glycan structures. L-selectin (CD62L) is expressed by leukocytes, E-selectin (CD62E) is expressed by endothelial cells, and P-selectin (CD62P) is expressed by platelets and endothelial cells. P-selectin glycoprotein ligand-1 (PSGL-1) is the main glycoprotein ligand on neutrophils for endothelial and platelet P-selectin, and also is the primary ligand on endothelium for neutrophil L-selectin.[24,50]

Rolling is initiated by inflammatory mediators that induce endothelial surface expression of P-selectin and E-selectin. Endothelial selectins tether or capture free-flowing neutrophils by binding to their respective neutrophil ligands. To reach target tissues, neutrophils must roll along the vascular endothelium during high shear stress which they accomplish with a system of tethers

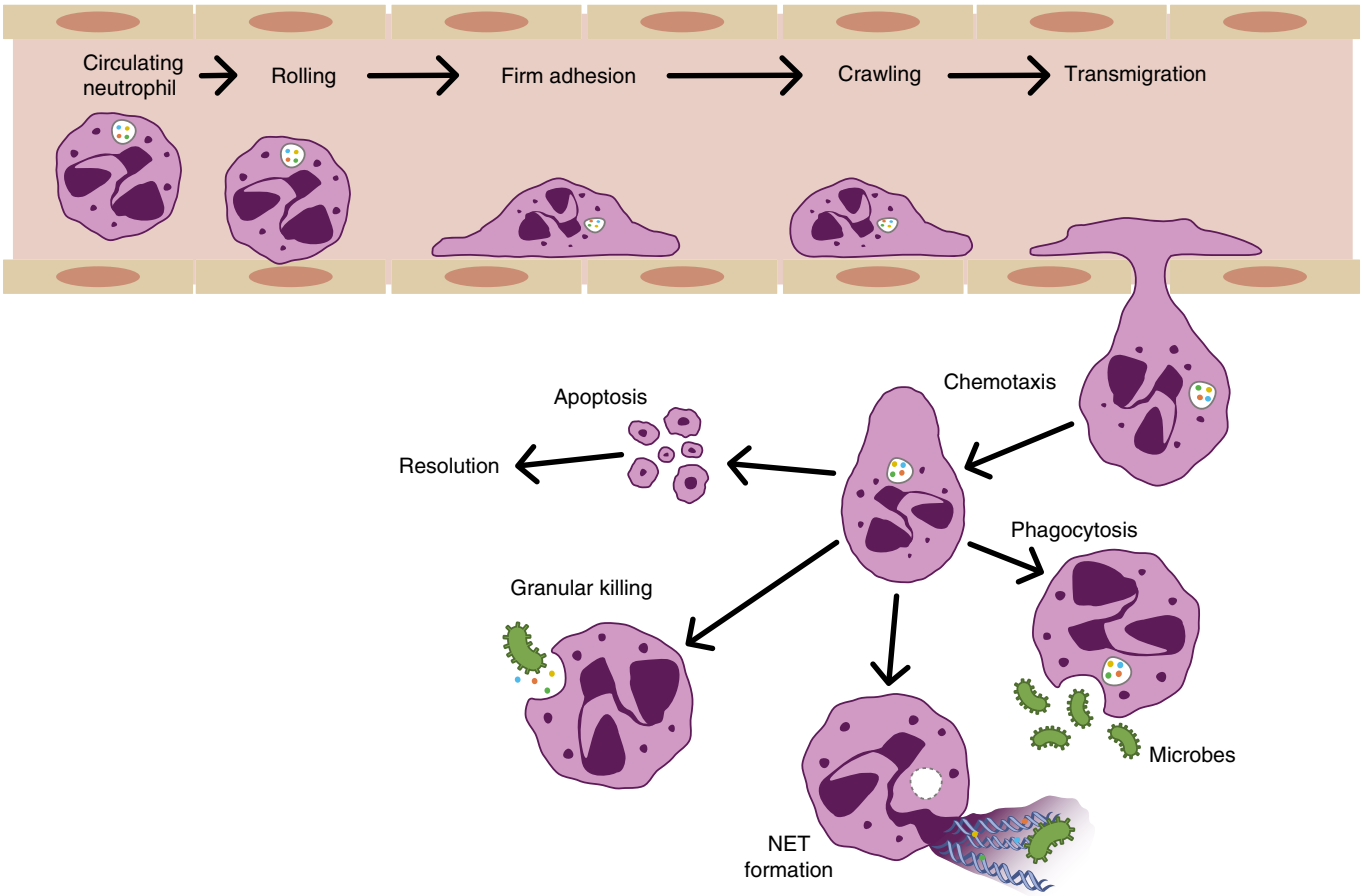


FIGURE 43.1 Antimicrobial activity of neutrophils is a multistep process. Neutrophils in flowing blood accumulate first by direct capture. The tethered neutrophils then roll utilizing slings to slow their rolling and become activated by chemokines. This leads to firm adhesion, crawling, and paracellular or transcellular endothelial migration. Rolling is mostly selectin-dependent, while adhesion, crawling, and transmigration are integrin-mediated. Upon entering tissues, neutrophils migrate toward chemotactic gradients and eliminate pathogens via phagocytosis, release of granular enzymes into phagosomes or extracellularly, or by release of neutrophil extracellular traps (NETs). Pathogen killing inside the phagosome occurs by reactive oxygen species generated by NADPH oxidase and by enzymes released from intracellular granules. Phagocytosis induces neutrophil apoptosis and subsequent clearance by macrophages and ultimately resolution of inflammation. (**Source:** Courtesy of Andrew Kingsbury, Center for Food Security and Public Health, Iowa State University)

and slings. Neutrophils form PSGL-1-mediated temporary tethers behind them that slow rolling, which then pull off the endothelium and sling to the front of the neutrophil, further slowing rolling.[24,47]

Neutrophil Adhesion and Crawling

Following rolling, integrins mediate neutrophil firm adhesion to the endothelium. During rolling, neutrophils are exposed to endothelial chemokines that activate neutrophils and cause integrin conformational changes. These integrin changes enable neutrophils to bind their endothelial ligands.[24] Integrins consist of noncovalent heterodimers (α and β subunits) of transmembrane glycoproteins with subfamilies organized around a particular subunit.[16] β_2 integrins are expressed in an inactive form on neutrophils and other leukocytes. These integrins undergo rapid affinity changes when stimulated by chemoattractants. Four known α (CD11) subunits combine with one common β (CD18) subunit to form unique β_2 integrins; CD11a, b, c, and d. $\alpha_M\beta_2$ (CD11b/CD18, Mac-1, complement receptor 3) is the most abundant integrin on neutrophils, and also is expressed on monocytes, macrophages, natural killer (NK) cells, and lymphocytes.[1] Mac-1 functions include (1) interacting with intercellular adhesion molecules (ICAMs) expressed on resting endothelium and induced on many cell types by inflammatory cytokines, (2) binding to denatured proteins to serve as a scavenger receptor, and (3) binding to iC3b on microbial surfaces with resulting phagocytosis of opsonized pathogens.[1] Following neutrophil activation, intracellular stores of Mac-1 relocalize to the cell surface and Mac-1 changes conformation enabling firm adhesion to endothelial ligands.[24]

The other important neutrophil β2 integrin is $\alpha_L\beta_2$ (CD11a/CD18, lymphocyte function-associated antigen-1 (LFA-1)). Although predominately expressed on lymphocytes, neutrophil LFA-1 binding to endothelial ICAM-1 enables firm adhesion.[24]

Following adhesion, neutrophils elongate and send out pseudopods, probing for endothelial junctions through which they can transmigrate. Neutrophils then crawl toward these junctions, releasing some of their bonds to move forward and form new endothelial adhesions. Active crawling depends on the interaction of neutrophil Mac-1 with endothelial ICAM-1.[24] Crawling also is a shear-driven process involving a complicated interplay of signaling molecules and actin-binding proteins.[24]

Neutrophil Extravasation

Neutrophils leave the vasculature by moving between endothelial cells (paracellular transmigration) and through endothelial cells (transcellular transmigration).[32] During paracellular endothelial transmigration, extensions of the endothelium rich in adhesion molecules (ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1)) form domes around neutrophils (via neutrophil integrins LFA-1 and very late antigen 4) to limit vascular leakage as neutrophils leave the vasculature.[24,32] Although paracellular migration is the prime means of neutrophil egress, transcellular migration may be preferred when endothelial junctions are especially tight, such as at the blood–brain barrier.[32]

CHEMOTAXIS

Once extravasated, neutrophils respond and bind to chemotactic molecules in a hierarchal manner (chemotaxis) to reach inflammatory sites, and find their ultimate target through complex signaling. Neutrophils become less sensitive to initial chemokines, and continue to migrate toward end target chemoattractants, such as bacterial peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) and/or complement components (C5a and C3a), which override other chemotactic signals such as those released by endothelium.[12,24] In addition to fMLP and complement, potent chemoattractants for neutrophils include interleukin (IL)-8 (CXCL8), platelet-activating factor, and leukotrienes (LTs).[18]

The chemokine system involves greater than 50 distinct chemokines, produced constitutively or in response to inflammatory stimuli by a variety of cells, and over 20 G-protein-coupled chemokine receptors.[36] Chemokines have been classified into four subfamilies based on the presence and placement of cysteine (C) residues at the amino terminus: (1) α subfamily (CXC), (2) β subfamily (CC), (3) γ subfamily (C), and (4) δ subfamily (CX_3C) where X represents noncysteine amino acids. Chemokines in subfamilies are named with the letter L (ligand) and given a number (e.g., CXCL8 = IL-8).[43] Each family of chemokines interacts with a reciprocal family of G-protein-coupled receptors, expression of which varies depending on the stage of leukocyte differentiation and stimuli.[17] When chemokines bind to their receptors on leukocytes, they induce conformational changes in cell-surface integrins such as Mac-1 and LFA-1, enabling neutrophil adhesion and locomotion.[24]

PHAGOCYTOSIS

After neutrophil entry into the tissues, neutrophil activation allows for rapid acquisition of phagocytic functions, ROS generation, release of granule proteins, and production of NETs. Neutrophil priming by lipopolysaccharide (LPS), tumor necrosis factor-alpha (TNF-α), and chemokines precedes neutrophil activation. Priming helps limit tissue damage by preventing premature neutrophil activation and granule release. However, misdirected neutrophil activation does occur in autoimmune and chronic inflammatory diseases.[32]

Neutrophils recognize pathogens via cell-surface and intracellular receptors. Neutrophil recognition receptors consist of (1) pattern-recognition receptors (PRR), such as toll-like receptors, engaged by pathogen-associated molecular patterns (PAMPs) or, during sterile inflammation, damage-associated molecular patterns (DAMPs),

(2) opsonic receptors such as complement receptors and FcγRs that detect C3 complement products and IgG, respectively, and (3) G-protein-coupled receptors that detect bacterial fMLP, chemokines, and endogenous inflammatory molecules.[32] The C-type lectin receptor PRRs recognize fungal β-glucan, allowing neutrophils to endocytose fungal pathogens. Although toll-like receptors are nonphagocytic, they prime neutrophil responses to other stimuli and increase their phagocytic capacity.[32] As detailed in the following text, the resulting phagocytic vesicle becomes a site for granular and oxidative microbial killing.

ANTIMICROBIAL MECHANISMS

Intracellular antimicrobial mechanisms within the neutrophil phagosome have generally been divided into oxygen-dependent and oxygen-independent mechanisms, but it is now known that these mechanisms are complexly integrated. Microbial killing is accomplished by lethal reactive oxygen species, granular enzymes, and NETosis.

Respiratory Burst and Generation of Reactive Oxygen Species

Oxygen-dependent mechanisms rely on membrane-bound NADPH oxidase.[2] The NADPH oxidase membrane-associated component, flavocytochrome b_{558}, is found in the cell and granule membranes, and becomes part of the phagosome membrane. Flavocytochrome b_{558}, a heterodimer of $gp91^{phox}$ and $p22^{phox}$, provides the conduit for pumping electrons from the cytosol to oxygen in the vacuole. When stimulated, the cytosolic *phox* components of NADPH oxidase are translocated to the membrane to join the flavocytochrome.[38] The NADPH oxidase complex catalyzes the reduction of molecular oxygen to the superoxide anion ($NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^{\bullet-}$), and the associated rapid consumption of oxygen is the "respiratory burst."

Neutrophils produce large amounts of $O_2^{\bullet-}$ that can combine with H_2O_2 to generate the highly reactive hydroxyl radical $OH^{\bullet}$ via the Haber–Weiss reaction ($O_2^{\bullet-} + H_2O_2 \rightarrow OH^{\bullet} + O_2 + OH^-$), which is preceded by the Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$.[44] Myeloperoxidase (MPO) catalyzes the conversion of H_2O_2 and halide ions to hypohalous acids, primarily hypochlorous acid. Also, H_2O_2 interacts with hypohalous acids to produce high-energy singlet oxygen ($^1O_2$) that attacks double bonds. These ROS intermediates have a critical role in pathogen killing by targeting organism unsaturated lipids, carbon bonds, sulfhydryl and amino groups, nucleic acids, pyrimidine nucleotides, and enzymes.

Products of the respiratory burst also are toxic to host cells. Endogenous antioxidant systems protect cells from harmful oxidants and include cytosolic superoxide dismutase, glutathione peroxidase, glutathione reductase, and catalase, which can convert oxygen radicals to water and oxygen.[39]

Granular Killing

Antibacterial proteins are released from neutrophil granules into phagosomes or extracellularly, thereby enabling neutrophils to kill intracellular or extracellular pathogens.[24] In the phagosome, the NADPH oxidase system provides the ideal environment for granular proteases to achieve enzymatic digestion and microbial killing, which may actually be more important for neutrophil killing than its ROS generation.[44] Electrons pumped into the phagocytic vacuole by NADPH oxidase increase vacuolar pH, making it ideal for protease activity. Influx of potassium ions stimulates release of granular proteins from matrix proteins into a soluble, activated form. That granular enzymes require specific hypertonicity and alkaline pH for maximum activity limits their toxicity and damage to host tissues.[44]

The cationic antimicrobial peptides α-defensin and cathelicidin are major components of neutrophil primary (azurophilic) granules.[10] Defensins (1) mediate killing bacteria, mycobacteria, fungi, parasites, and viruses by forming transmembrane pores, (2) enhance antigen uptake and processing, and (3) stimulate chemotaxis of monocytes, macrophages, and mast cells.[33]

Bacteria permeability-enhancing factor (BPI) is a microbicidal protein in neutrophil primary granules with a high affinity for LPS and neutralizes LPS, thereby limiting inflammatory responses to gram-negative bacteria.[33]

Neutrophil granules contain serine proteases and acid hydrolases with microbicidal activity and different substrate specificities. Some proteases also assist with neutrophil migration by degrading extracellular matrix and cleaving adhesion molecules.[25,40] Serine proteases also modulate chemokine and cytokine activity and thereby inflammatory responses.[40]

Granule lactoferrin inhibits bacterial growth directly by microbicidal activity, indirectly by sequestering iron, an important microbial nutrient, and also reduces inflammation by binding LPS.[45]

Neutrophil Extracellular Traps

During NETosis, neutrophils release their nuclear material in a meshwork consisting of granular-derived antimicrobial proteins, enzymes, and histones.[5] Therefore, NETs are defined by the colocalization of chromatin with histones and granular proteins such as MPO and elastase. Since NETs ensnare pathogens in their meshwork of chromatin and expose them to high concentrations of antimicrobial peptides and enzymes, NETs are recognized as an important mechanism of extracellular microbial killing. Proteins confirmed to be present on NETs include antimicrobials (lactoferrin, defensins, and BPI), proteases (elastase), and enzymes required for ROS generation (MPO).[24] Histones themselves are antimicrobial. NETs have been confirmed to trap bacteria, viruses, fungi, and protozoan parasites.[24] Some bacteria increase their virulence by producing nucleases to escape the NET immune

response by degrading chromatin.[49] Animal models in which NETosis is inhibited have increased susceptibility to pathogens. NETs have been described in dogs, cats, horses, zebrafish, chickens, mice, rabbits, cattle, and humans.[6,8,19,28,37,42,51] NETosis occurs in response to numerous inflammatory stimuli, including combinations of inflammatory cytokines (e.g., IL-8), pathogens (bacteria, fungi, protozoa), and PAMPs.

NEUTROPHIL FATE

Neutrophils are short-lived cells due to constitutive apoptosis of senescent cells shortly after maturation.[35] After stimulation by pathogens or inflammatory mediators, neutrophils undergo apoptosis, necroptosis, or NET release. Extravasated neutrophils also may survive and undergo reverse migration, re-entering the vasculature, where they return to the bone marrow and likely die of apoptosis.[7,24]

Apoptosis

Following phagocytosis and intracellular killing of pathogens, neutrophils often die by apoptosis. During apoptosis, the cell forms apoptotic bodies which are cleared by phagocytic cells. Because intracellular contents are not released, the process is noninflammatory and is associated with wound healing and tissue repair.[32,35]

Necroptosis

Necroptosis is programed neutrophil necrosis that results in chromatin condensation and fragmentation, cell and organelle swelling, and plasma membrane rupture. Extracellular spillage of cell contents occurs inducing inflammation.[35]

NETosis

NETosis is a distinct form of cell death, and the process varies depending on the stimulus.[35] "Suicidal" or "lytic" NETosis induced by phorbol myristate acetate is a slow, NADH-oxidase-dependent process that ruptures the cell membrane leading to cell death.[7,35] "Vital" or "nonlytic" NET release, a rapid, ROS-independent process, often induced by bacteria or fungi, occurs by vesicular trafficking without membrane rupture leaving neutrophils viable.[35] Following vital NETosis, neutrophils can still perform chemotaxis and phagocytosis.[7] Both vital and suicidal NETosis depend on citrullination of histones to enable chromatin decondensation and NET formation. NET release is prevented by phagocytosis, so neutrophils containing phagocytosed bacteria do not subsequently release the bacteria during NETosis.[7] Details of NET clearance are unknown. Endogenous DNAse is speculated to degrade NETs, but macrophages may also phagocytose NETs.[7]

IMMUNOMODULATORY FUNCTION OF NEUTROPHILS

Neutrophils in the Adaptive Immune Response

In addition to their role in innate immunity, neutrophils also modulate the adaptive immune response by signaling other immune cells via soluble or surface molecules. Additionally, neutrophil microparticles can activate platelets and endothelial cells and suppress macrophage function.[32]

In inflammation, neutrophils recruit dendritic cells (DCs) via release of chemokines, lactoferrin, and defensins.[32] When DCs directly bind neutrophils, the neutrophils provide DCs access to their captured pathogens and stimulate DCs to become more effective antigen-presenting cells.[32] Stimulated neutrophils can serve as antigen-presenting cells by expressing low levels of major histocompatibility complex class II and costimulatory molecules.[32] Neutrophils can transport antigens to lymph nodes via lymphatics and present antigen to T cells or DCs. Neutrophils can activate splenic B cells via B-cell-activating factor (BAFF). Neutrophils also have immunosuppressive effects by releasing arginase which depletes L-arginine needed for T-cell proliferation.[32] Neutrophils regulate NK maturation in steady-state conditions, and neutrophil cytokine release during inflammation directly activates NK cells. Many NK-cell-derived cytokines prolong the life of neutrophils to further enhance the inflammatory process.

Neutrophil-Mediated Regulation of Inflammation

Neutrophils are active partners in the overall amplification and resolution of inflammation. Indeed, both pro- and anti-inflammatory subsets of neutrophils exist, but it is unknown if these subsets have different lineages or have adapted different capacities.[24] For example, only about a quarter of neutrophils produce NETs in response to *Staphylococcus aureus* stimulation.[7]

Neutrophils propagate inflammation by secreting inflammatory mediators such as LTs, prostaglandins, cytokines, and chemokines.[24] Neutrophils synthesize LTB4 that (1) primes and directly activates neutrophils, (2) is chemotactic for neutrophils, monocytes, and eosinophils, and (3) stimulates neutrophil aggregation, superoxide production, and integrin expression. Activated neutrophils secrete cytokines that amplify inflammation, including TNF-α, IL-1β, and IL-6 and produce interferon-gamma (IFN-γ), IL-10, and transforming growth factor-β, known to affect adaptive immunity.[14,18,48] Also, neutrophils secrete CC and CXC chemokines.[48]

In later stages of inflammation, neutrophils can be anti-inflammatory and promote tissue healing via growth factors that promote angiogenesis. The neutrophil lipid profile changes so that neutrophils produce lipoxin A4, which inhibits neutrophil migration and recruits monocytes to phagocytose neutrophils.[24] Neutrophils also recruit monocytes to inflamed tissues via release of cytokines.[24] Macrophage phagocytosis of apoptotic neutrophils yields decreased macrophage

IL-23 production, which ultimately leads to decreased granulocyte colony stimulating factor (G-CSF) and decreased marrow neutrophil production.[32] Macrophage phagocytosis of apoptotic neutrophils also results in macrophage production of IL-10, which promotes tissue repair.[32]

NEUTROPHILS IN DISEASE

Not only do they participate in infection control, but neutrophils play a central role in many inflammatory and autoimmune diseases due to their capacity to cause host tissue damage and promote thrombosis. Uncontrolled release of neutrophil granules or oxidative products during phagocytosis is a major cause of tissue damage. Neutrophil granules may fuse with the phagosome before it is completely sealed, releasing cytosolic contents and oxidative products into tissues.[32] Neutrophil phagocytic receptors may detect immune complexes or complement along large surfaces, such as endothelial cells, and subsequently fail to completely engulf these complexes.[32] The subsequent release of neutrophil granules and oxidative products can lead to endothelial damage, vasculitis, and thrombosis. While NETs serve important antimicrobial functions, they have the potential to cause significant host tissue damage, autoimmunity, and thrombosis.

Neutrophils in Acute Inflammatory Disease

In veterinary medicine, localized neutrophil-mediated lung injury occurs due to *Mannheimia haemolytica*. Cattle studies have documented rapid neutrophil recruitment into the lungs within 2 hours, and associated fibrinopurulent pneumonia, endothelial disruption, and alveolar epithelial swelling.[46] Neutrophil depletion before inoculation of *M. haemolytica* into the lungs markedly attenuates alveolar capillary endothelial injury, supporting the role of neutrophil-mediated tissue damage.[46]

Similarly, activated neutrophils create much of the microvascular injury in acute respiratory distress syndrome (ARDS).[26,53] Neutrophils in the pulmonary microvasculature exceed those in circulation by up to fivefold making them ideally poised to respond to insult, but also to cause bystander damage.[53] Neutrophils lodge in extensive pulmonary capillary beds, becoming activated and releasing oxygen radicals and granule contents onto the endothelial surface. Subsequent endothelial injury results in (1) massive leakage of plasma into alveoli, (2) maldistribution of blood flow, and (3) disturbances of oxygen transport and utilization, leading to dyspnea and hypoxemia.[53] Increased production and decreased clearance of NETs likely contribute to pathogenesis of ARDS in people as well.[15]

In mice during early sepsis, NETs provide protective antimicrobial effects by preventing bacterial dissemination; however, later NET-induced host tissue damage outweighs their protective effects.[30,49] Canine and equine neutrophils release NETs in response to LPS, and NETs have been documented in the lungs and in abdominal and pleural effusion samples from septic dogs.[27]

Neutrophils in Thromboembolic Disease

Neutrophils play a central role in immunothrombosis, which can be protective by trapping bacteria, but can result in excessive clot formation. Neutrophilia is associated with increased thrombotic risk and thrombosis causes significant morbidity and mortality in human and veterinary patients.[35] Local and disseminated activation of coagulation frequently accompanies sepsis.[52] Neutrophils can trigger microvascular thrombosis by causing endothelial injury via degranulation, which converts the endothelial surface to a procoagulant surface that binds and activates platelets. Activated neutrophils and activated endothelial cells express a potent platelet agonist, platelet-activating factor.[34] Activated platelets expose their surface P-selectin that interacts with neutrophil surface PSGL-1 forming platelet–neutrophil aggregates. Platelet–neutrophil aggregates are rigid, lodge in capillary beds, and initiate microvascular thrombotic events.[35] Platelet–neutrophil interactions and activated platelet mediator release stimulate nearby neutrophils to generate NETs, which are prothrombotic.[32,35]

NET histones activate platelets and increase thrombin generation. Also, NETs bind factor XII and activate the contact coagulation pathway, increasing the rate of clot formation and stabilizing clots by inhibiting fibrinolysis.[20,31] NET-associated elastase further promotes clot formation by degrading anticoagulants such as tissue factor pathway inhibitor.[31] Additionally, NETs directly inhibit intravascular blood flow and provide a binding site for circulating hemostatic cells and factors.[35] Many animal models of thrombosis depend on NET formation, and NETs are implicated in thrombosis in a multitude of diseases including thrombotic microangiopathies, disseminated intravascular coagulation, and deep vein thrombosis in people.[22,23,31] NETs are present in dogs with immune-mediated hemolytic anemia (IMHA) and may play a role in thrombotic outcomes in this disease.[21]

Neutrophils in Immune-Mediated Diseases

Neutrophils are implicated in autoimmune diseases as direct effector cells that induce tissue injury and as immune-modulating cells. Autoantibodies such as antineutrophil cytoplasmic antibodies (ANCAs) can directly activate neutrophils.[32] ANCA-activated neutrophils can initiate a cascade of increasing inflammation and autoantibody production by releasing NETs, damaging endothelium, and promoting B-cell differentiation via release of BAFF.[32] In systemic lupus erythematosus (SLE) and rheumatoid arthritis, neutrophil recognition of immune-complexes can result in neutrophil activation to establish a self-amplifying loop of inflammation.[32] Neutrophils also promote autoimmunity via NETosis, since NETs expose autoantigen like proteins and nucleic acids in an inflammatory environment.[3] In SLE, there is increased NET formation and decreased NET clearance

due to reduced DNase activity and inhibition of DNase access to NETs by antibodies.[3]

Neutrophils in Inflammatory Bowel Disease

Neutrophils have a role in inflammatory bowel disease (IBD) including Crohn's disease and ulcerative colitis in people and in enteropathies in dogs.[32] In IBD, the degree of intestinal neutrophil infiltration correlates with disease severity. Based on animal models, the IBD pathogenesis appears due to neutrophil transepithelial migration resulting in mucosal barrier breakdown and ROS damage to epithelial integrity.[32] ANCAs are found in human IBD patients and likely contribute to disease pathogenesis. ANCAs are reported in canine chronic enteropathies and correlate with a positive response to strict elimination diets.[11,29]

Neutrophils in Cancer

The relationship between neutrophils and cancer cells is complex with both neutrophil protumorigenic and antitumorigenic effects. Neutrophil chemoattractant chemokines are produced by tumor cells, and increased tissue neutrophils have been associated with increased tumor growth and a poor prognosis.[13,32] Neutrophil tumor-promoting aspects include (1) neutrophil proteinase basement membrane degradation enabling tumor growth and metastasis and 2) neutrophil suppression of other immune effector cells.[32] By contrast, neutrophil antitumor effects include (1) direct killing of tumor cells via ROS, (2) neutrophil-induced apoptosis of tumor cells, and (3) neutrophil presentation of tumor antigens to T cells.[32] The balance between these opposing functions varies in individual patients and cancers. Animal cancer models and studies in human patients suggest a role for NETs in cancer progression. NET-associated elastase may directly induce tumor cell proliferation, NETs may provide a scaffold for metastasizing tumors, and NETs are involved in tumor-associated thrombosis.[9]

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CHAPTER 44

Neutrophil Function Disorders

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Inherited Disorders
- Leukocyte Adhesion Deficiency
  - Leukocyte Adhesion Deficiency, Type I
  - Leukocyte Adhesion Deficiency, Type II
  - Leukocyte Adhesion Deficiency, Type III
- Leukocyte Granule Abnormalities
  - Defects in Oxygen-Independent Killing
  - Lysosomal Storage Diseases
  - Birman Cat Neutrophil Granulation Anomaly
  - Defects in Oxygen-Dependent Killing
  - Myeloperoxidase Deficiency
  - Other Defects
    - Cyclic Hematopoiesis of Gray Collies
    - Autosomal Recessive Hereditary Neutropenia
    - Pelger–Huët (P–H) Anomaly

Acquired Disorders
- Stress
- Neonatal Animal
- Transition Period
- Metabolic Diseases
- Infectious Diseases

Acronyms and abbreviations

ACTH, adrenocorticotropic hormone; BLAD, bovine leukocyte adhesion deficiency; BVDV, bovine viral diarrhea virus; CGD, chronic granulomatous disease; CLAD, canine leukocyte adhesion deficiency; FeLV, feline leukemia virus; G-CSF, granulocyte colony stimulating factor; LAD, leukocyte adhesion deficiency; FLAD, feline leukocyte adhesion deficiency; IBRV, infectious bovine rhinotracheitis virus; MPS, mucopolysaccharidosis; NEFA, non-esterified fatty acid; P–H, Pelger–Huët.

Because neutrophilic phagocytosis is a complex multistep mechanism, several steps may be influenced by inherited or acquired defects. This ultimately leads to an overall decrease of neutrophilic phagocytic activity with increased susceptibility to bacterial infections. For some inherited diseases, the precise molecular mechanisms have been clearly identified. In others, multiple steps of the phagocytic process are influenced and tendency toward infection may be uncommon or less severe.

INHERITED DISORDERS

Inherited diseases impairing steps of phagocytosis are summarized in Figure 44.1.

Leukocyte Adhesion Deficiency

Leukocyte adhesion deficiency (LAD) syndrome is a group of inherited diseases characterized by a failure of extravasation due to disfunction in selectin- or integrin-mediated adhesion to endothelial cells. This frequently leads to persistent neutrophilia, beginning at an early age that is associated with granulocytic hyperplasia in bone marrow. The syndrome is rare, but symptoms may be severe including recurrent bacterial infections, sepsis, and impaired wound repair.

In humans, three monogenic diseases have been identified and designated as type I to III. These mutations have been identified in animals, thus providing possible models of the human conditions.[34]

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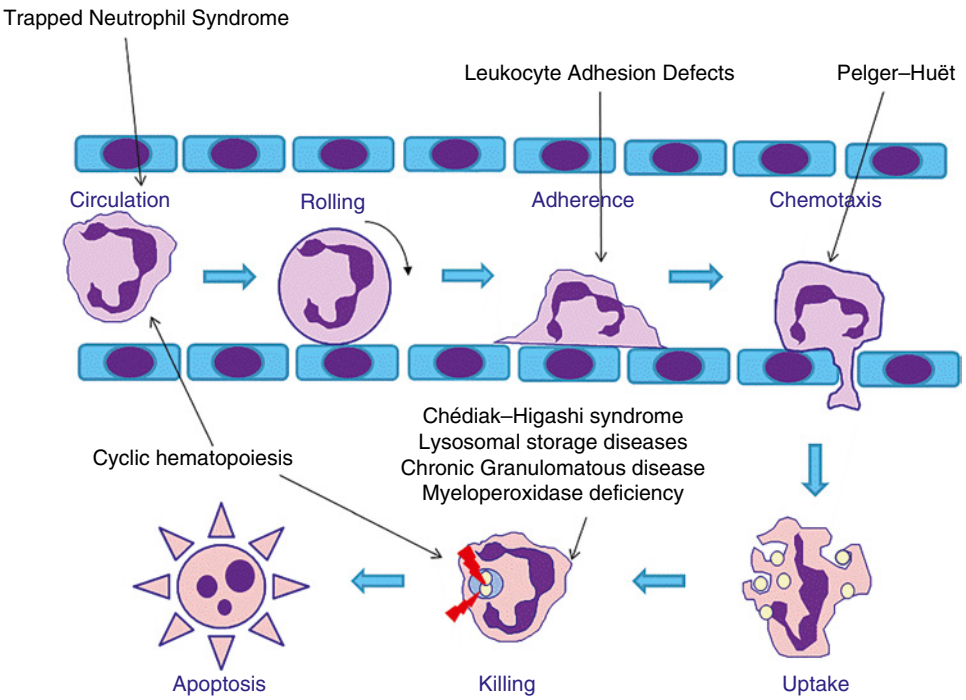


FIGURE 44.1 Summary of most relevant inherited disorders affecting different steps of neutrophil phagocytosis.

Leukocyte Adhesion Deficiency, Type I

Leukocyte adhesion deficiency, type I is the most common in humans and animals and it is linked to a biallelic mutation in the gene ITGB2, coding for the integrin protein CD18. Disease transmission is autosomal recessive, and, in humans, a germline point mutation has been identified leading to an aspartic acid to glycine substitution at amino acid 128 of CD18 polypeptide. All CD18 subfamilies are affected preventing the formation of functional CD11/CD18 complexes. As a consequence, neither the CD18 integrin subunit nor its three subunit partners CD11 come to the cell surface adversely affecting neutrophil adhesion and chemotaxis.

Spontaneous LAD type I has been identified in cows, dogs, and cats, and several mouse models have been generated.[34]

Canine leukocyte adhesion deficiency (CLAD) has been identified in Irish Setter dogs and cross-bred dogs and in one unrelated mixed-breed dog.[16,40] Affected dogs fail to express CD11/CD18 molecules on their surface similar to humans, but the point mutation responsible for CLAD causes a cysteine at amino acid 36 to be substituted for a serine.

Dogs with CLAD have persistent neutrophilia, myeloid hyperplasia in bone marrow, and suffer from recurrent bacterial or mycotic infections without pus formation. Oral cavity and lungs are most frequently affected, and dogs also suffer from fever, poor wound healing, and poor growth. A PCR test for CLAD has been developed and successfully applied in Irish Setters and mixed-breed dogs.

Gene therapy using foamy viruses or lentiviral vectors has been tested in vitro for CLAD resulting in a reversal of CLAD phenotype with long-term correction of the symptoms and neutrophil adhesion defects. This opens interesting perspectives for possible use of gene therapy to treat children with LAD-1.

Bovine leukocyte adhesion deficiency (BLAD), first reported in 1983 in a Holstein heifer with recurrent infections, periodontitis, oral ulcers, and marked, persistent neutrophilia, was initially termed bovine granulocytopathy syndrome. The term BLAD was introduced in 1990 when the lack of CD18 was demonstrated.[22] BLAD has been identified worldwide as an autosomal recessive trait in Holstein cows, thanks to the development of a rapid and economical PCR test useful in identifying carriers. Carriers are reported to range from 5% to 20% of the Holstein population in the USA, Japan, and Europe.

The molecular basis of BLAD is a single point mutation identical to that identified in humans leading to a 56–90% decrease in expression of CD18 in heterozygous calves. Affected cows show a severe decrease of both adherence and chemotaxis, but carriers show no detectable decrease in neutrophil function despite decreased expression of the CD18 molecule.[36] Currently, the most used approach to control in clinical practice is identification and elimination of carrier bulls from mating.

Feline leukocyte adhesion deficiency (FLAD) has been reported in a domestic longhair adult cat with recurrent infections and neutrophilia.[3] Laboratory tests revealed a lack of CD18 expression in all leukocytes and a severe decrease of unstimulated neutrophil adhesion. Genetic analysis showed a mutation in the ITGB2 gene, due to a 24 bp deletion causing a truncated gene product. In contrast with dogs and cattle, FLAD mutation appears extremely rare, although associated with a less severe phenotype probably associated with a CD18-independent T-cell activation pattern.

Leukocyte Adhesion Deficiency, Type II

Leukocyte adhesion deficiency, type II is a severe disease also associated with poor growth. In humans, a biallelic mutation of SLC35C1 gene, encoding for a fucose-transporter protein, has been reported. In spite of the lack of association between fucose metabolism and adhesion molecules, the disease is associated with a severe decrease in neutrophil rolling. Engineered mice with an analogous mutation have no spontaneous diseases consistent with the LAD II phenotype.

Leukocyte Adhesion Deficiency, Type III

Leukocyte adhesion deficiency, type III is a severe deficiency associated with reduced neutrophil adhesion and chemotaxis and platelets dysfunction (Glanzmann-like) causing severe bleeding. A biallelic mutation in FERMET3 gene, encoding for the protein kindlin 3, has been identified. Kindlin 3 is an intracellular protein linked to the cytoskeleton and binds CD18.

FERMET knockout mice show a similar phenotype to human LAD III. In addition, spontaneous diseases mimicking LAD III have been reported in one German Shepherd dog and in a mixed-breed dog.[4,19]

Leukocyte Granule Abnormalities

Leukocyte granule abnormalities may involve either oxygen-independent or oxygen-dependent mechanisms, affecting killing ability and, in some cases, are accompanied by alterations in steps in the phagocytic process or other cell types. The appearance of granules in the cytoplasm of neutrophils in blood smears may be helpful in differentiating some types of leukocyte granule abnormalities.

Defects in Oxygen-Independent Killing

Chédiak–Higashi Syndrome Chédiak–Higashi Syndrome is a rare inherited disorder reported in humans, cattle, Persian cats, Aleutian mink, Arctic foxes, killer whales and some strains of mice. In humans, the disorder is inherited in an autosomal recessive manner. Abnormal lysosome granule formation leads to giant lysosomes in leukocytes, platelets, and melanocytes (Figure 44.2). Decreased neutrophil chemotaxis, and oxygen-independent killing, mild to moderate bleeding, and oculocutaneous partial albinism are frequently seen in affected animals, but the severity of symptoms is quite variable among species, being generally more severe in cattle and mice than in cats. In humans and mice, a defect in LYST (CHS1) gene has been identified encoding for a protein involved in regulating vesicular size and trafficking. In Japanese Black cattle, a similar mutation has been identified and a PCR test to identify carriers has been developed. Stained blood smears commonly show pink to magenta staining granules due to the fusion of primary and secondary granules, which may stain positive with myeloperoxidase stain.

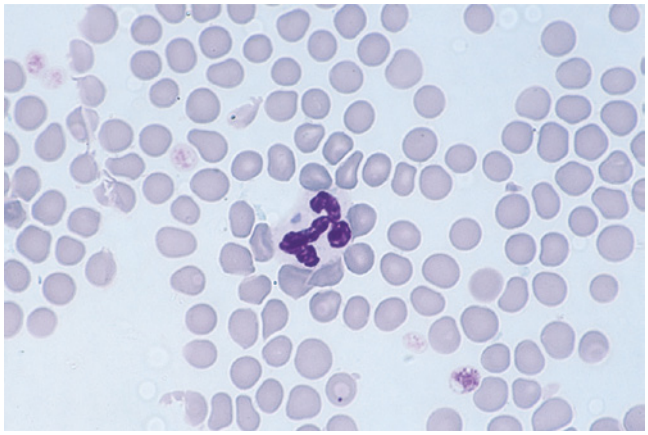


FIGURE 44.2 Giant lysosomes in the neutrophil cytoplasm of a cat that has Chédiak–Higashi.

Lysosomal Storage Diseases

Lysosomal storage diseases are a group of rare inherited enzyme deficiencies leading to accumulation of metabolic by-products within leukocyte granules and variable impairment of killing ability of neutrophils (see Chapter 49). Among these, mucopolysaccharidosis (MPS) is associated with altered mucopolysaccharide (glycosaminoglycan) catabolism and classified into different types according to genes involved and consequent enzymes affected. Several MPS have been described in humans, with recognized counterparts in dogs and cats: MPS type I (Hurler's syndrome), due to deficiency of alpha-L-hyaluronidase; MPS type II (Hunter syndrome), due to deficiency of hyaluronate sulfatase; MPS type III (Sanfilippo syndrome), due to various enzyme deficiencies leading to heparan sulfate accumulation; MPS IV (Morquio syndrome), due to deficiency of galactose-6-sulfatase and beta-galactosidase; MPS VI (Maroteaux–Lamy syndrome), due to deficiency of arylsulfatase; and MPS VII (Sly syndrome), due to deficiency in beta-glucuronidase. Other lysosomal storage diseases involving leukocytes include GM1 gangliosidosis (reported in Friesian cattle, Siamese and Korat cats, English Springer Spaniels, Beagles, Portuguese Water dogs, and Shiba Inus), GM2 gangliosidosis (in Yorkshire piglets, German Shorthaired Pointers, Golden Retrievers, and cats), and fucosidosis (in English Springer Spaniels). Pink to purple small granules are often visible in leukocytes stained with Wright's or Toluidine blue dyes, but the overall neutrophil function may be minimally affected. Type and severity of clinical signs are variable and more related to other tissues affected.

Birman Cat Neutrophil Granulation Anomaly

Birman cat neutrophil granulation anomaly is a rare hereditary trait affecting purebred Birman cats characterized by red azurophilic granules in the cytoplasm of neutrophils stained with Wright's stains. The color of these granules resembles that of promyelocytes, and it is

probably attributable to a different affinity for dyes. The trait is not associated with clinical signs or recurrent infections, and neutrophil functions do not appear impaired. The granules have normal electron microscopic morphology and do not stain for acid mucopolysaccharide, thus differentiating this condition from mucopolysaccharidosis types VI and VII.[17]

Defects in Oxygen-Dependent Killing

Chronic Granulomatous Disease Chronic granulomatous disease (CGD) is a group of heterogeneous inherited defects caused in humans by more than 40 different mutations in genes encoding components of NADPH oxidase. The majority of cases are transmitted as a mutation on the X-chromosome (X-linked trait), but some autosomal recessive diseases have been described. Affected children have recurrent, but rarely fatal, infections due to bacterial (mainly catalase-positive) or fungal microorganisms. Neutrophil inability to activate a respiratory burst is demonstrable by the nitroblue tetrazolium test, dihydrorhodamine (DHR) test, or cytochrome C reduction assays (see Chapter 45).

Five phenotypically different CGDs have been described, and knockout mouse models but no spontaneous animal models of human CGD have been demonstrated. Impairment of oxygen-dependent killing, partly resembling human CGD, has been reported in eight closely related Doberman Pinscher dogs with chronic recurrent bronchopneumonia and rhinitis that tended to relapse when antibiotic therapy was discontinued. Neutrophil chemotaxis and ingestion were normal, but the ability to kill *Staphylococcus aureus* in vitro, reduce of nitroblue tetrazolium, and produce superoxide was impaired, thus supporting decreased oxygen radical generation.[6]

A group of Weimaraner puppies with recurrent fevers, diarrhea, pneumonia, pyoderma, lymphadenopathy, stomatitis, and osteomyelitis have been described.[11] A defect in chemiluminescence response to phorbol ester, leading to impaired oxygen-dependent killing, was the major neutrophil alteration. Serum IgG and IgM concentrations also were decreased, but this finding was not associated with any decrease in humoral or cellular immune responses.

Myeloperoxidase Deficiency

Myeloperoxidase deficiency is the most common inherited defect of neutrophils in humans, generally leading to mild clinical signs. Myeloperoxidase deficiency is rare in animals, but has been reported in cyclic hematopoiesis of Gray Collies and is associated with a decrease in oxygen-dependent killing.[18]

Other Defects

Although not directly correlated with a severe decrease of neutrophil function, some inherited disorders may be considered among neutrophil function abnormalities.

Cyclic Hematopoiesis of Gray Collies

Cyclic hematopoiesis of Gray Collies is an inherited autosomal recessive disease due to a mutation in the beta subunit of adaptor protein complex-3 (AP3B1).[13] The mutation results from an insertion of an extra adenine residue in exon 21 of the canine AP3B1 gene leading to a frameshift with premature termination. Paradoxically, heterozygous carrier puppies show a normal AP3B1 transcript and protein, while homozygous puppies have a heterogeneous population of AP3B1 mRNA containing both mutant and wild-type transcripts. This paradox is probably attributable to transcription errors. AP3B1 protein is a membrane cargo protein involved in transport of several proteins, including neutrophil elastase, from Golgi to lysosomes. Therefore, cyclic hematopoiesis of Gray Collie is considered analogous to a hereditary mutation of the human elastase gene (ELANE). Accumulation of elastase on neutrophil membranes likely induces an increased degradation of important signaling protein promoting hematopoiesis leading to decreased hematopoiesis. Decreased neutrophil elastase allows a temporary boost in hematopoiesis. This hypothesis is supported by the asynchronous fluctuations of platelet and reticulocytes that tend to increase during the neutropenic phase.

Affected puppies die in the first weeks or show stunted growth, recurrent bacterial infections (enteric, respiratory, buccal, cutaneous), bleeding, and shortened life span. Affected dogs often have dilution of skin pigmentation and ocular lesions. Diagnosis is generally confirmed by recurrent neutropenia, every 11–14 days, lasting for about 3–4 days, followed by neutrophilia, thrombocytopenia, and reticulocytosis. Neutrophils have mildly reduced myeloperoxidase activity and platelet function may also be impaired.

Several treatment options have been proposed including antibiotics to manage bacterial infections, endotoxin, and lithium carbonate to stimulate hematopoiesis, granulocyte colony stimulating factor (G-CSF) subcutaneously or delivered using lentiviral vectors. Bone marrow transplantation may also be effective.

Autosomal Recessive Hereditary Neutropenia

Autosomal recessive hereditary neutropenia, also termed trapped neutrophil syndrome, has been described in Border Collies accompanied by recurrent infections, fever, and lameness due to lytic bone lesions.[1] The causative mutation has been identified in the canine VPS13B gene and is similar to human Cohen syndrome.[35] Neutropenia is associated with accumulation of mature and immature neutrophils in bone marrow which are unable to escape. A PCR test is available to reveal carriers and affected animals.

Pelger–Huët (P–H) Anomaly

Pelger–Huët (P–H) anomaly is a hereditary disorder described in several breeds of dogs, domestic shorthair cats, rabbits, and Arabian horses. The anomaly is an

autosomal dominant trait and heterozygous animals express the typical phenotype, although generally do not show clinical symptoms. Neutrophils with P–H anomaly may show decreased chemotaxis due to a more rigid nucleus, but other studies contradicted this hypothesis. Homozygous cats and rabbits have been associated with skeletal deformities and recurrent infections leading to death.

Pelger–Huët anomaly is characterized by failure of neutrophil nuclei to segment (Figure 44.3). They show round or hypolobulated nuclei, chromatin pattern is more clumped than those of band neutrophils and no toxic changes are identified (Figure 44.4). The features of persistent left shift without any clinical signs of infection are highly suggested of P–H anomaly. Other leukocytes and megakaryocytes in bone marrow may also show hypolobulation.

The underlying mutation likely involves the gene encoding for lamin B receptor, which is an inner nuclear membrane protein participating in nuclear segmentation and the trafficking of heterochromatin to the nuclear membrane. The mutation has been identified in human and mice, but not in domestic animals. The anomaly should be differentiated from transient acquired conditions due to inflammation or myelodysplasia (pseudo-Pelger–Huët) with asynchronous neutrophil maturation.

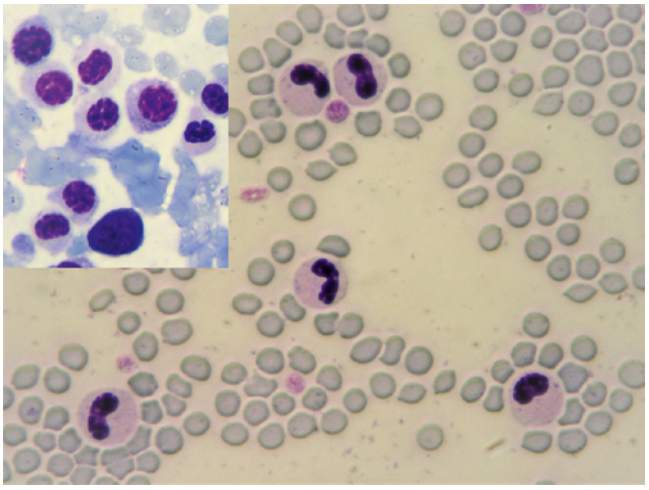


FIGURE 44.3 Peripheral blood and bone marrow (inset) from a cat with Pelger–Huët anomaly. Note hyposegmented neutrophils with a clumped chromatin pattern.

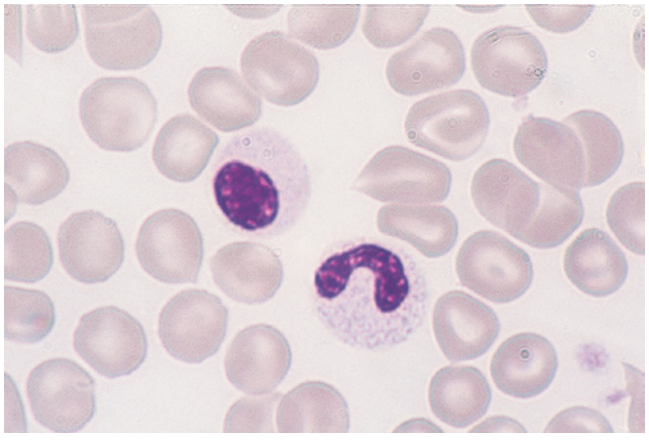


FIGURE 44.4 Band and myelocyte neutrophils from a Border Collie that has Pelger–Huët anomaly. (**Source:** Courtesy of Dr. K. S. Latimer, University of Georgia, Athens, Georgia)

ACQUIRED DISORDERS

Several physiological and pathological conditions have been reported to potentially influence neutrophil function. In contrast with congenital disorders, these conditions are quite frequent, but phagocytosis impairment is generally mild. A majority of reports are inconsistent and focused on in vitro effects on neutrophil function that do not necessarily reflect an in vivo impairment of the phagocytic process.

Stress

Immunosuppression due to prolonged stress is a well-recognized factor potentially increasing susceptibility to infections and both innate and specific immunity may be affected. Endogenous cortisol, released in stressful conditions, increases neutrophil counts by recruiting neutrophils from the marginal pool, following decreased selectin expression on endothelial cell and neutrophil membranes. However, data on the direct effects of cortisol on single steps in the phagocytic process are inconsistent among studies. Some authors reported increased adherence of sheep neutrophils 2 hours after mimicking acute stress by administration of adrenocorticotropic hormone (ACTH). Bovine neutrophils exposed to multiple corticoid treatments, mimicking chronic stress, showed decreased oxidative burst. Bovine neutrophils exposed to glucocorticoids have been described to show differential expression of genes involved in immune function, apoptosis, and tissue remodeling, and a downregulation of L-selectin which impaired neutrophil rolling.[8]

After 30 minutes of transportation, 4–6-month-old Friesian calves showed an increase in neutrophil superoxide production and decreased neutrophil apoptosis associated to increased cortisol, suggesting a possible pro-inflammatory effect. Other authors reported that transport stress may induce decreased expression of surface selectins and integrins, but increased chemotaxis. Transport stress may modulate peripheral blood neutrophil function, enhancing migration into the udder, thus contributing to increased milk somatic cell counts.

Neutrophils from neonatal calves exposed to cold stress showed no effects on killing ability. In addition, cold exposure seemed to restore ACTH-induced reduction in neutrophil chemiluminescence in Japanese Black steers.

Stress due to weaning was associated with decreased particle uptake and expression of L-selectin but not bactericidal activity in beef calves. This was associated

with circulating neutrophilia and impaired rolling and adherence.

Effect of stress due to exercise on circulating neutrophils has been investigated in horses. Endurance rides reduced oxidative burst in equine neutrophils.[12] Similarly, chemotaxis and chemiluminescence were decreased by a single bout of intense exercise. Untrained horses subjected to moderately intense exercise showed increased oxidative burst and phagocytosis; by contrast, high-intensity training caused an impairment of oxidative burst and phagocytosis.[29] These reports suggest a suppressive effect on equine neutrophil function of high-intensity or long-duration exercise; however, these effects are mild.[14]

Neonatal Animal

Impaired neutrophil activity in neonatal animals may predispose to a greater susceptibility to infectious diseases; however, studies in foals and calves are inconsistent. In studying neonatal calves, some researchers reported decreased phagocytosis, killing, and respiratory burst, while others reported increased superoxide production and increased chemotaxis and shape change.

Transition Period

Transition period is defined as the interval from 3 weeks before to 3 weeks after calving. Transition period is characterized by several concurrent conditions potentially predisposing to infectious diseases including reduced leukocyte function, negative energy balance, hypocalcemia, systemic inflammatory response, and oxidative stress.[38] Decreased neutrophil function, particularly in the ability of neutrophils to migrate into udder, has been recognized as an important predisposing factor for bovine mastitis.[21,28] A few weeks after calving, neutrophils have been reported to show reduced myeloperoxidase activity, antibody-dependent bacterial killing, diapedesis, chemotaxis, and phagocytosis. This impairment has been partly attributed to both direct alterations in neutrophils and to soluble mediators in serum.[2] Although stress due to parturition may be involved in depressing neutrophil function, proteomic studies showed that more than 70 genes are differentially expressed in neutrophils from transition cows and from cows inoculated with steroids. This implies that stress-related modifications are not the only factors impairing neutrophil function.[25] Other authors suggest that impairment of neutrophil function in the dry period might anticipate the onset of infectious diseases including mastitis and metritis. Myeloperoxidase activity is decreased before calving in cows that developed metritis, and myeloperoxidase and chemotaxis are affected in cows that developed retained placenta. Neutrophil chemotaxis is reduced in cows that subsequently developed mastitis.[9] The hypothesis is that pre-parturition impairment of neutrophil phagocytosis contributes to the onset of infectious disease after calving, even if other factors (ketosis, hypocalcemia, selenium concentration, and lypomobilization) might play a determinant role at the onset of infections.[38]

Metabolic Diseases

Metabolic diseases associated with the peripartum period in dairy cattle have been widely investigated to better elucidate the role of ketosis, increased non-esterified fatty acid (NEFA), and hypocalcemia on increased susceptibility to infections in the first week after calving.

Ketone bodies have been reported to impair bovine neutrophil function in vitro with decreases in chemotaxis, phagocytosis, and respiratory burst while bacterial killing capacity appears unaffected. In sheep, phagocytosis, chemotaxis, and bactericidal activity have been reported to be decreased in vitro by a concentration of ketone bodies similar to those seen in moderate to severe ketosis, while adherence appears unaffected.

The direct effect of NEFA due to lypomobilization on neutrophil activities is more controversial. Some authors reported a dramatic increase of respiratory burst, and necrosis of bovine neutrophils incubated with NEFA while phagocytosis was not affected.[33] Other authors have variously showed a reduced myeloperoxidase activity ex vivo, decreased respiratory burst activity, or increased chemotaxis and phagocytosis.

Cows with experimentally induced postpartum hypocalcemia exhibited severe impairment of immune cell function. Additionally, neutrophils from hypocalcemic cows had reduced phagocytosis and oxidative burst.[20,26]

All these data describe multifactorial effect of metabolic imbalances occurring after calving in dairy cows. This imbalance may increase susceptibility to infections mainly mastitis and endometritis.

Effects of diabetes mellitus and hyperglycemia have been investigated in cats and dogs. Neutrophil adherence, chemotaxis, and chemokinesis were reported to be decreased in poorly controlled diabetic dogs, but not in well-controlled ones.[24] Neutrophil adherence is decreased in whole blood with increased blood glucose, but no difference was observed when isolated cells were evaluated.[37] This implies that plasma factors may be responsible for decreasing phagocytic activities, thus predisposing to infections. Neutrophils from diabetic cats were reported to show an exaggerated oxidative burst that may contribute to diabetes-associated microvascular damages.[15] However, other authors found no evidence of increased oxidative stress or decreased neutrophil function in feline diabetes mellitus.[39]

Among nutritional factors, malnutrition has been associated with impaired neutrophil chemotaxis, respiratory burst, the release of lysosomal enzymes, and impaired microbicidal activity. Selenium and vitamin E are known to potentially improve neutrophil function, while copper deficiency has been reported to decrease the neutrophil's ability to kill yeast.[5,10] In cats, taurine deficiency has been associated with decreased neutrophil respiratory burst.

Infectious Diseases

Impairments of neutrophil function, possibly increasing susceptibility to bacterial infections (such as respiratory diseases) or comorbidities, have been described for some viral and parasitic diseases.

In cattle, the bovine viral diarrhea virus (BVDV) may induce impaired iodination capacity, random migration, phagocytosis, killing, and respiratory burst activity.[7] Similarly, altered neutrophil function was observed after vaccination with modified live BVDV vaccines.[32] Infectious bovine rhinotracheitis virus (IBRV), parainfluenza 3 virus, bovine herpes virus-1, and bovine immunodeficiency virus-1 may induce decreased neutrophil random migration and chemotaxis predisposing to bacterial pneumonia.[27] Neutrophils from cats affected by feline leukemia virus (FeLV) showed decreased chemotaxis and chemiluminescence.[23] Among parasites, decreased neutrophil functions were described in a dog infected by *Prototheca zopfi* and in cattle infected with *Eimeria spp*.[30,31]

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CHAPTER 45

Clinical Evaluation of Neutrophil Function

STEFANO COMAZZI

Specimen, Storage, and Neutrophil Isolation
  Neutrophil Isolation by Lysis
  Sedimentation Techniques
  Gradient Centrifugation Techniques
  Immunological Separation Techniques
Classical Techniques for Evaluation of Phagocytic Process
  Cellular Movement and Chemotaxis
  Adherence
  Particle Uptake
  Killing
Flow Cytometric Tests for Neutrophil Function
  Cytoskeletal Activity
  Surface Adhesion Molecules
  Phagocytosis
  Reactive Oxygen Species Production
Evaluation of Neutrophil Death
Evaluation of Neutrophil Extracellular Traps

Acronyms and abbreviations

BAL, bronchoalveolar lavage; CV, coefficient of variation; DCF, 2,7-dichlorofluorescein; DHE, dihydroethidium; DNA, deoxyribonucleic acid; DPI, diphenyleneiodonium chloride; FMLP, formylmethionine-leucyl-phenylalanine; H2-DCFDA, dichlorodihydrofluorescein diacetate; LPS, Lipopolysaccharide; MCLA, methyl cypridina luciferin analog; NBD, N-(7-nitrobenzofurazan-4-yl); NBT, nitroblue tetrazolium salt; NET, neutrophil extracellular traps; PAF, platelet-activating factor; PMA, phorbol myristate acetate; ROS, reactive oxygen species; TdT, terminal deoxyribonucleotidyl transferase; TUNEL, terminal-deoxynucleotidyl-transferase-mediated dUTP nick-end-labeling; ZAS, zymosan-activated serum.

Neutrophils play an important role in innate immunity as the first defense against microbes, mainly bacteria and fungi.

In vivo, innate immunity response is the result of neutrophil function and localized or systemic conditions including vessel permeabilization, priming, and opsonization, cytokines, complement fractions, and soluble mediator secretion, the interaction between polymorphonuclear leukocytes (PMN) and extracellular matrix, or other inflammatory and endothelial cells. However, testing neutrophil function in vivo is complex and does not permit discrimination between direct and indirect alterations. Thus, in vitro assays, which can be standardized, are more useful in pathological conditions and allow testing the effects of different substances, drugs, and activators on neutrophils.

SPECIMEN, STORAGE, AND NEUTROPHIL ISOLATION

Theoretically, a purified population of cells supplies more correct information about functional abnormalities. However, isolation techniques may activate cells; thus, using techniques requiring whole blood (such as flow cytometry or chemiluminescence) may be preferable in order to limit activation of cells.

Neutrophils from peripheral blood, milk, exudates, and bronchoalveolar lavage (BAL) have been tested. Peripheral blood or fluids must be collected in anticoagulated tubes. Definitive studies on the influence of anticoagulants on neutrophil function in animals are not available, but heparin is generally preferred, since

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ionized calcium, which is necessary for phagocytic activity and cell movement, is not chelated. However, EDTA and acid–citrate–dextrose are often used as good alternative choices in dog, pig, and ruminants. The samples should ideally be processed within 4 hours, but, in some studies, analysis has been delayed up to 24 hours after collection. Samples should be stored at room temperature. In order to avoid neutrophil activation, plastic instead of glass tubes should be used and sterility of all solutions should be carefully maintained.

Neutrophil Isolation by Lysis

The simplest method of preparation of blood leukocytes is osmotic lysis of RBCs, by adding distilled water to an aliquot of whole blood followed by restoring isotonicity with a hypertonic solution. Red blood cells are ruptured while leukocytes are more resistant, and RBC membranes debris is removed by washing steps. This technique is very simple, but it does not allow isolation of leukocyte subpopulations and purity of neutrophils is similar to that of whole blood. On the other hand, the method induces only minimal activation of neutrophils. A modification of this technique is the method described by Carlson and Kaneko[1] and widely used in ruminants. It involves osmotic lysis after the removal of buffy coat that contains most of the mononuclear cells. The method yields a high percentage of granulocytes, but eosinophils cannot be separated from neutrophils. The method does not provide good purity and recovery in non-ruminant species.

Red blood cell lysis can also be performed by means of chemical solutions such as tris-buffered ammonium chloride. Unfortunately, this technique frequently interferes with some neutrophil functions or induces apoptosis; therefore, it should be limited to flow cytometric evaluation of membrane antigens.

Sedimentation Techniques

Neutrophils can be enriched by adding an equal volume of dextran solution to whole blood. Dextran is a polysaccharide solution that induces rouleaux formation by increasing sedimentation of erythrocytes and mononuclear cells. After 30 min, the granulocyte-rich supernatant is collected, centrifuged, and washed. This method has been described in humans and dogs as a single step or followed by gradient centrifugation, and it requires a minimal amount of blood.[21] However, isolated cells are characterized by low purity and dextran can partially influence neutrophil function.

Gradient Centrifugation Techniques

Several isolation techniques use colloidal suspension at different densities to concentrate cells according to their specific gravity. Discontinuous gradients are more feasible and adaptable in routine isolation of cell populations than continuous gradients. However, continuous gradient may help to set up isolation conditions for specific cell subpopulations compared with density-labeled beads. The most frequently used technique in veterinary medicine is separation using a discontinuous gradient of Percoll®, silica particles coated with polyvinylpyrrolidone. For canine neutrophils, two different densities (1.092 and 1.071 by using 74% and 55% of Percoll, respectively, in saline solution) are layered and leukocyte suspensions are put in top of the tube. After centrifugation, a neutrophil-rich band is easily recognizable at the interface between two densities (Figure 45.1). This layer can be removed and after washing provides a very pure population of neutrophils. The method can be adapted to different species by adjusting densities of Percoll (swine 65% and 55%; bovine 70% and 50%; horse 75% and 60%). The main advantage of this technique is the high purity and yield of neutrophils, mainly in healthy animals. However, cell densities often vary with physiological and disease conditions, which could lead to failure to isolate atypical or activated cells.

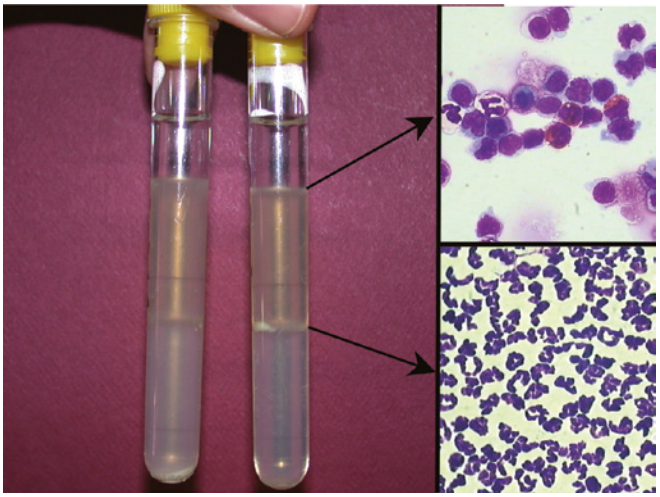


FIGURE 45.1 Isolation of blood neutrophils using a discontinuous Percoll gradient. After centrifugation, a neutrophil-rich band is located at the interface between the two Percoll densities. (**Source:** Courtesy of V. Spagnolo)

A similar technique uses another commercially available polymer, Ficoll-Paque®, which is able to separate mononuclear cells and platelets (top of the tube) from granulocytes and erythrocytes (bottom of the tube). This method is generally more reserved to concentrate peripheral blood mononuclear cells.

Finally, an interesting method using discontinuous metrizamide gradient has been developed to isolate bovine neutrophils.[3] This technique is reported to provide neutrophils with high purity and yield and minimal interference with function tests, but it has not been widely used.

Immunological Separation Techniques

Increase in availability of lineage-specific monoclonal antibodies has permitted separation of leukocyte subpopulation with a high percentage of purity. Fluorescence-activated cell sorting (FACS) is widely used for isolating lymphocyte subpopulations, but is of

limited use for myeloid cells, due to the paucity of specific antigens, the difficulty in maintaining sterility, and possible activation due to manipulation. Separation methods using magnetic beads have been developed for cattle.[17] Briefly, magnetic beads are labeled with anti-neutrophil-specific monoclonal antibodies and then incubated with leukocyte suspensions. Leukocytes bound to the beads are then captured using the magnetic system, while unbound cells (mononuclear and other granulocytes) are eluted and discarded. After removal of the magnetic field, collected neutrophils are collected in high percentage, purity, and yield. However, this technique requires neutrophil-specific antibodies that do not recognize other polymorphonuclear leukocytes. Moreover, incubation with antibodies may induce activation of neutrophils, thus influencing function tests. For these reasons, antibodies should be chosen carefully. A possible solution is the enrichment of neutrophil by depleting other cell populations, using a mixture of lymphocyte- and monocyte-specific antibodies labeled with magnetic beads. Magnetic negative methods may provide neutrophils with high yield, but purity may be low.

CLASSICAL TECHNIQUES FOR EVALUATION OF PHAGOCYTIC PROCESS

Different techniques are described, some of which are able to evaluate the whole phagocytic process, while others are used to discriminate single steps of phagocytosis. Tests for specific steps of phagocytosis are useful because some diseases or conditions lead to alterations in a single function without perturbing the whole phagocytic process. In this case, a panel of tests for simultaneous evaluation of different steps of phagocytosis is strongly recommended in order to correctly assess neutrophil function. Alternatively, tests for the whole phagocytic process are easier to perform and provide a complete picture of interactions with nonspecific immunity, but are less informative regarding disease pathogenesis.

Cellular Movement and Chemotaxis

The simplest technique to evaluate increased cellular movement is microscopic detection of conformational changes of neutrophils after activation. Activated neutrophils show a bipolar configuration within a few minutes after exposure to activating agents with a gradual return to spheric shape. However, this method is difficult to standardize and does not distinguish spontaneous motility, random migration (chemokinesis), and directional migration of the cell toward an activator substance (i.e., chemotaxis). Many chemoattractant substances have been recognized, but some of them (interleukin-8, zymosan-activated serum (ZAS), casein, LTB4, C5a, TNF-α, platelet-activating factor (PAF), and formylated peptides such as N-formylmethionine-leucyl-phenylalanine (FMLP) are more commonly used in chemotactic tests. However, some species differences have been recognized. Neutrophils from cows, pigs, and cats do not respond to formylated peptides and those from dogs require a priming agent in order to express enough receptors for such substances.

A frequently used method to test chemokinesis and chemotaxis is evaluation of neutrophil migration using a modified Boyden chamber.[23] Briefly, suspensions of isolated neutrophils are put into the upper wells of the migration chamber, while chemoattractant is added in the lower well. For evaluating random migration, chemoattractant is omitted from the lower well.

Two different methods have been described according to the type of filter used between the two wells:[20]

Method 1: In the leading front method (Figure 45.2), a 150 μm-thick cellulose nitrate filter is used. The chamber is incubated for 30 min at 37 °C in humidified, 5% CO_2 atmosphere, and then the filter is removed, fixed, stained, dehydrated, clarified in xylene, and mounted on a glass slide. Cellular movement is evaluated using the fine adjustment and micrometer of a microscope. The distance between the top of the filter and the leading front, where the two last cells are in focus, is determined.

Method 2: In the lower surface count method, a thin (12 μm) polycarbonate filter with a pore size of 2 μm diameter is used. After incubation, the filter is removed and the number of cells that pass through the filter is enumerated in the lower well, using a cell counter or a spectrophotometric method.[5] Cells attached to the lower surface of the filter are counted after wiping the upper surface of the filter and staining with the Wright method.

These two techniques provide different data on neutrophil chemotaxis, but, according to some investigators, both methods are strongly influenced by the degree of adherence and gravity.

Thus, assays to evaluate chemotaxis in a collagen gel matrix or under-agarose have been developed.[11] The collagen gel matrix assay consists of a visual assay obtained by two capillary tubes with their ends covered by a dialysis membrane and connected by a polyethylene tubing sleeve. The compartment formed between the ends of the two tubes is filled with a solution of monomeric collagen and put under a microscope. Cell movements are recorded microscopically after the addition of cell suspension and chemoattractant. This technique is quite complex, but it allows the study of neutrophil movement step-by-step in a microenvironment similar to an "in vivo" system.

The under-agarose technique is a simple, inexpensive, and widely used method based on the preparation of a gel matrix in a Teflon chamber. Wells are cut in the gel, and cell suspensions are added to the wells. Different chemoattractants can be put in separate wells, and in some cases chemoattractant is omitted in order to evaluate spontaneous motility. After incubation, agarose is flooded with methanol, followed by 35% formaldehyde in order to harden the gel, and, after staining, the migration distance of neutrophils toward chemoattractant wells is evaluated by microscopy.

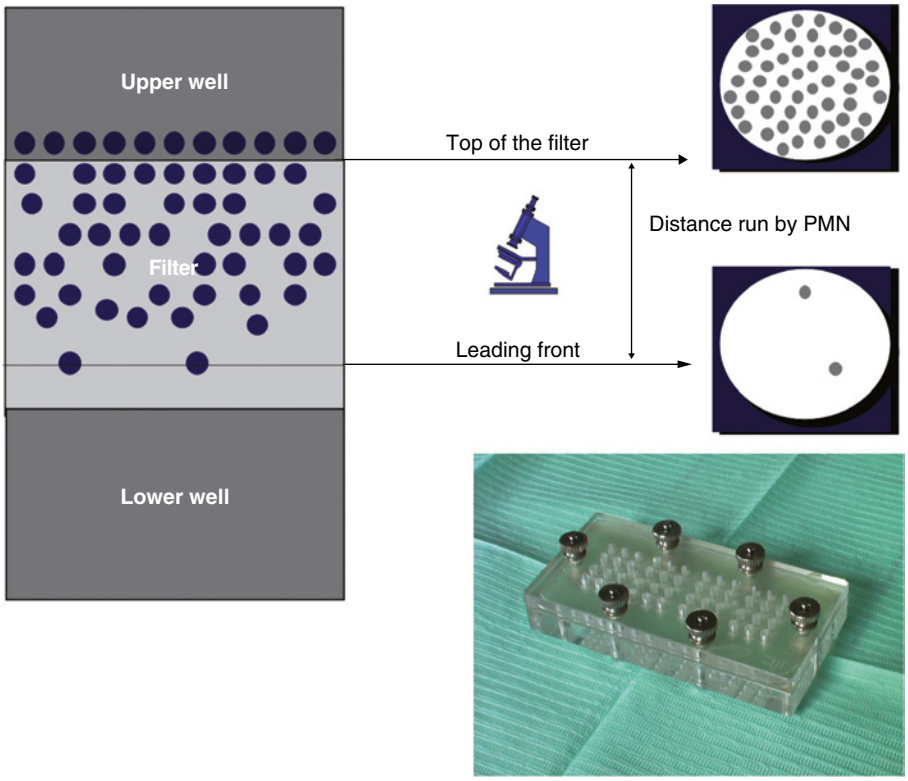


FIGURE 45.2 Evaluation of chemotaxis in a Boyden chamber, using the leading front method. Neutrophils migrate from the upper to the lower well (toward a chemoattractant) through a cellulose nitrate filter. The distance traveled by the cells is evaluated after staining the filter, using the fine adjustment on the microscope.

Recently, some authors developed a real-time cell migration assay based on a microfluidic device and a dedicated application for smartphone. To date, no applications to veterinary medicine have been described.[22]

Finally, some authors suggested a more complex method that requires a culture system made of successive layers of collagen, fibroblasts, and a confluent monolayer of epithelial cells coated on a porous membrane.[16] Cells are put at the basal side of the membrane, and cells passing over the epithelial layer toward a chemoattractant stimuli are counted. This technique is particularly useful for studying chemotaxis and diapedesis of neutrophils during infection in the mammary gland, but it is quite difficult to perform and standardize.

Adherence

Assays for evaluation of adhesive capability of neutrophils could be schematically divided into two groups: (1) those evaluating adherence to inert substances, such as polysaccharides, nylon fibers, glass, or plastic, and (2) those that study adhesiveness to biological structures, such as endothelial cells, monolayers, or cell culture systems. The latter assays reproduce interactions between neutrophils and biological barriers in a more complex way, similar to that which occurs in vivo. However, they are strongly influenced by extragranulocytic factors; thus, most of these techniques are difficult to standardize and lack reproducibility.

Techniques that evaluate adherence to inorganic substances have been developed using column or microtiter plates. Briefly, borosilicate glass column can be filled with packed Sephadex or nylon fibers and neutrophils suspension is percolated through the column. The percentage of adhered cells is then calculated by counting the percentage of recovered cells. Different stimulators or substances can be used.

All these methods lack precision; thus, it is strongly recommended that these tests be run in triplicate. Assays in microtiter plates are performed by adding a suspension of PMN in flat bottom wells together with different activators or inhibitors and they may also be useful to test the influence of different substances on neutrophil adhesiveness. Nonspecific adherence to plastic wells or adherence after activation with an activation agent (e.g., phorbol myristate acetate (PMA) or ZAS) can be evaluated. In order to remove nonspecific activation due to contact with plastic wells, some wells can be precoating with gelatin. After incubation and washing to remove nonadherent cells, the percentage of adhered cells is evaluated by microscopic count using an inverted microscope or neutrophils can be lysed and indirectly evaluated by means of chemical or fluorometric methods. Fluorometric labeling with calcein[10] or colorimetric methods to quantify myeloperoxidase or acid phosphatase is commonly used.[15] Results should be reported as the number of adhered cells compared to a standard curve obtained by different dilutions of neutrophils from the same animal. Assays in microtiter plates are more reproducible in comparison with those in columns and require a smaller amount of blood; thus, they are more applicable for use in small animals.

Many assays have been reported to test adhesive capability of neutrophils to cell layers or tissues. The simplest methods use cultured endothelial cells packed into an adherence column instead of nylon fibers or

Sephadex. Other techniques using monolayer of cultured cells of different origin (endothelial, fibroblasts, myocytes) have been reported. Target cells may be isolated from fresh tissues and cultured on a glass coverslip until confluent. An adhesion assay chamber is obtained by adding a second coverslip separated from the monolayer by a rubber O-ring. Neutrophils are added in this incubation chamber and adhesion is evaluated by a visual method, using an inverted microscope equipped with phase-contrast optics. The number of neutrophils remaining on the slide after washing off the nonadherent cells is enumerated. This technique reproduces the complex interactions between neutrophils and tissue cells and is very useful for studying the production of cytokines by endothelial or mesenchymal cells or the mechanism of interaction between neutrophil adhesion molecules and their ligands. However, the technique is difficult to perform, lacks reproducibility, and is very influenced by extraneutrophilic factors.

Particle Uptake

Although there are several methods to evaluate neutrophil capability to uptake particles, the basic concept is measuring the amount of labeled foreign material engulfed after incubation with or without activating substances. Particles can be evaluated by visual, spectrophotometric, or flow cytometric assays. Various particles can be used including latex beads, yeasts (e.g., *Saccharomyces* or *Candida*), or bacteria (e.g., *Escherichia coli*, *Staphylococcus aureus*, or *Streptococcus spp*.). Particles may be incubated directly on a glass slide with a suspension of isolated neutrophils, then ingestion is stopped adding an ice-cold isotonic solution, and phagocytosis is evaluated by fluorescence microscopy. The simplest method is microscopic evaluation of the percentage of cells that have phagocytized particles, after staining adherent cells. In other assays, particles can be prelabeled with a fluorescent dye (fluorescein isothiocyanate or acridine orange) and the mean number of ingested particles per neutrophil is determined using a fluorescence microscope. Fluorescence of noningested particles is inactivated by treating cellular suspension with trypan blue solution.[21] The main issue with this technique is that results are strongly affected by adherence to the glass slide; therefore, assays using neutrophil suspensions in tubes or flasks have been developed.[18]

Another interesting technique uses polystyrene latex beads (1.09 μm diameter).[14] After incubation, noningested particles are removed by centrifugation and the supernatant is discarded; then neutrophils are ruptured and ingested latex particles are solubilized with 1,4-dioxan. The amount of latex is indirectly determined in a spectrophotometer at 255 nm. This method is very simple, inexpensive, and rapid, but it does not permit determination of the percentage of neutrophils phagocytizing particles.

Finally, ingestion can be evaluated after labeling bacteria with radioactive materials followed by measuring the quantity of radioactivity in cell suspensions.[13] However, this technique is complex and expensive and requires handling of radioactive materials.

Killing

Many assays have been developed to evaluate bactericidal activity of neutrophils. These may be divided in three groups:

Group 1: This group includes techniques that evaluate residual viability of bacteria incubated with neutrophil suspensions. These assays are easy to perform and can be applied to whole blood providing an overall evaluation of innate immunity. The method consists of incubation of a suspension of live bacteria with neutrophil suspensions and serum in order to allow opsonization. After incubation, bactericidal activity is determined by counting colony-forming units, after cultivation on blood agar plates, or by enumerating live bacteria using a spectrophotometric assay (at 560 nm) after addition of tetrazolium compound.[19] Live bacteria reduce tetrazolium to red-purple formazan. Results are compared with a standard curve obtained with a known number of live bacteria.

Group 2: This group includes techniques that quantify neutrophil lysosomal enzymes. Many assays have been developed to quantify the activity of lysosomal enzymes (myeloperoxidase, lysozyme, lactoferrin, cationic proteins, neutral proteases, acid hydrolases, and proteases) after neutrophil lysis. Most of them utilize colorimetric or ELISA methods that can be performed using a spectrophotometer or a microtiter plate reader. In horses, microtiter ELISA tests were used to evaluate myeloperoxidase and elastase activities and an automated laser hematology system (ADVIA 2120) has been proposed to evaluate changes in myeloperoxidase content of neutrophils associated with experimental endotoxemia.[8]

Group 3: This group includes techniques that quantify the production of oxygen compounds produced by the respiratory burst. Neutrophils generate reactive oxygen species (ROS) that play an important role in bactericidal activity. Many ROS are involved, but superoxide anion is the precursor. Several assays have been developed to test the oxidative burst of neutrophil. Most of them are performed in multiwell microplates. Neutrophil suspensions are incubated in the presence of activators and, after incubation, several methods to measure ROS production can be used. The differential between intracellular and extracellular ROS production can be quantified by pretreating cell suspension with a NADPH-oxidase inhibitor (diphenyleneiodonium chloride or DPI) or with superoxide dismutase or catalase, which are superoxide and hydrogen peroxide scavengers, respectively. These substances do not cross cell membranes; therefore, they inhibit only extracellular ROS.

A rapid assay to detect respiratory burst activity evaluates the capability of neutrophils to reduce nitroblue tetrazolium salt (NBT) to formazan with the production of a red-purple color that can be evaluated using an ELISA reader.[9] Also, chemiluminescence assays have been described.[12] In the cytochrome C reduction assay,

ferricytochrome C is added to wells and it is reduced by superoxide. Changes in absorbance at 550 nm are spectrophotometrically evaluated at 10-min intervals. This assay is reported to be a specific indicator of extracellular superoxide production. Chemiluminescence can be evaluated by adding methyl cypridina luciferin analog (MCLA). After addition of MCLA, the decrease in chemiluminescence is measured at 10-min intervals using a microplate luminometer. A commercially available test (Amplex-Red-dependent fluorescence) consists of a reaction mixture (resorufin and horseradish peroxidase) that is added to neutrophils. Samples are evaluated in a fluorimeter at an excitation wavelength of 530 nm and an emission wavelength of 590 nm every 15 min. The test detects extracellular hydrogen peroxide. Other fluorescent assays include the dihydroethidium (DHE)/dichlorodihydrofluorescein diacetate (H2-DCFDA) fluorescence assay and the luminol/isoluminol chemiluminescence assay.

All these assays are suitable for detection of neutrophil burst in bovine. Intra-assay repeatability is good; however, interassay variability is high. The luminol assay has the lowest interassay coefficient of variation (CV), while other tests such as cytochrome C reduction, Amplex Red, and DHE have high interassay variability (CV > 30%). In dogs, studies using evaluation of ROS production using cytochrome C reduction, luminol chemiluminescence, H2-DCFDA, and NBT reduction have been reported.

FLOW CYTOMETRIC TESTS FOR NEUTROPHIL FUNCTION

The previously described measurement of neutrophil function, using physical, microscopic, or chemical methods, remains time-consuming. Moreover, because phagocytosis is due to sequential activation of multiple steps (rearrangement of cytoskeleton, chemotaxis and adherence, degranulation and production of oxygen radicals) and alteration in any single step can influence the whole process, a panel of assays is usually applied. This requires large numbers of neutrophils that are difficult to obtain from small or diseased animals. Flow cytometric assays for testing neutrophil function have significant advantages compared to classical ones: they are very rapid to perform, require small amounts of cells (at least 50–100 times less than conventional assays), and can be applied on heterogeneous populations of cells such as whole blood, avoiding neutrophil isolation that could induce significant activation of cells. Flow cytometry measures fluorescence and light scatter signals through a laser source (see Chapter 142). Using the gating technique, the neutrophil population can be isolated from a mixed population of cells. Resolution may be further increased using a multicolor approach and lineage-specific monoclonal antibodies labeled with different fluorochromes. In some cases, subsets of neutrophils (e.g., immature neutrophils or activated neutrophils) may be distinguished and their activities separately interrogated and compared. Many different flow cytometric assays have been described in human medicine, but only a limited number have been validated and used in veterinary medicine.

Cytoskeletal Activity

Actin is a major cytoskeletal protein of neutrophils and it can be found in a globular monomeric form (G-actin) and in a filamentous polymeric form (F-actin). During activation, G-actin changes into F-actin and accumulates at the periphery of cells. This process is involved in adhesion, deformability, and cell movement. Dysfunction in actin dynamic polymerization has been linked to susceptibility to infectious diseases. Flow cytometric detection of F-actin can be assessed using the fluorochrome N-(7-nitrobenzofurazan-4-yl) (NBD)-phallacidin. Whole blood cells are activated, then cells are fixed with 3% paraformaldehyde and RBCs are lysed. Leukocytes are permeabilized and stained with fluorochromes. In the flow cytometer, fluorochromes within neutrophils are excited with standard (488 nm) laser light and the amount of emitted green fluorescence is correlated with F-actin content. The technique has been used in bovine in which great increases of F-actin were found after neutrophil activation.

Recently, an interesting method to quantify receptor clustering via flow cytometry has been described. Receptor clustering is mediated by cytoskeletal protein and is highly suggestive of cell activation and polarization. In flow cytometry, receptor clustering may be evaluated measuring in real time the decrease in the ratio between fluorescence/forward scatter time of flights and the increase in the ratio between height/area fluorescence signals.[2] No application of such technique is available at the moment in veterinary medicine.

Surface Adhesion Molecules

Flow cytometric assays for neutrophil adhesion are mainly based on the detection of adhesion molecules on the cellular surface. Adhesion molecules may be divided into two groups: the selectin family, which establishes a weak association of neutrophils with endothelial cells, and the integrin family, which interacts with the immunoglobulin superfamily on endothelial cells, resulting in a firm bound that allows neutrophil diapedesis. The expression of adhesion molecules can be easily quantified using specific monoclonal antibodies. Whole blood is preferable to avoid neutrophil activation during the isolation procedure. After labeling cells with fluorochrome-conjugated antibodies, the expression of membrane surface molecules is evaluated on gated neutrophils and expressed as mean (or median) fluorescence channel units. Integrins increase on neutrophil cell membranes after activation due to the externalization of adhesion molecules from cytoplasmic granules (Figure 45.3). Expression of selectins on the membrane of activated cells is controversial.

This assay is very simple and rapid, but it requires careful standardization of procedures, number of cells, and antibody titration in order to work with a large excess of antibody.

In veterinary medicine, several studies using this technique have been reported with particular attention given to canine and bovine adhesion molecules. Some studies in dogs investigated the expression of integrins

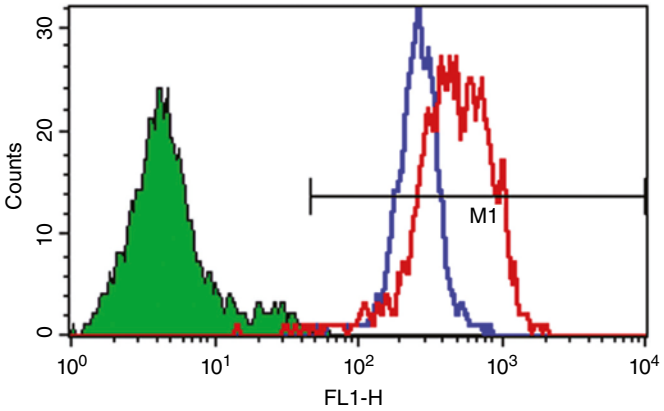


FIGURE 45.3 Flow cytometric evaluation of membrane integrin CD11b in bovine neutrophils. Lipopolysaccharide (LPS)-activated neutrophils (red line) express increased fluorescence compared to nonactivated neutrophils (blue line). Shaded line indicates isotypic control.

(CD35, CD18, CD11b, CD11c) and Fc receptors (CD16). In cattle, CD11b, CD11a, CD11c, CD18, L-selectin, CD47, and CD44 have been studied on neutrophils from peripheral blood and milk.

Phagocytosis

Many different assays to quantify neutrophil phagocytic capabilities have been described. These methods entail incubating whole blood with fluorescent-conjugated particles of different origin (inorganic beads, bacteria, yeast). The amount of fluorescence is taken as an indicator of phagocytic activity (Figure 45.4).

Heparinized whole blood is the preferred sample because other anticoagulants chelate Ca^{2+} that is necessary for phagocytosis. A major limitation is that nonspecific adherence to the neutrophil membrane can induce an overestimation of phagocytosis. This aspect could be partially limited by quenching the fluorescence of nonphagocytized particles using trypan blue or crystal violet solution. Alternatively, a control sample can be incubated at 4 °C, thus, inhibiting phagocytosis and allowing quantification of nonspecific adherence.

An interesting variant of this method allowing simultaneous evaluation of phagocytosis and oxidative burst has been described in cows, dogs, cats, and horses. The technique couples evaluation of ROS production using fluorescent dyes (dihydrorhodamine 123 or 2,7-dichlorofluorescein (DCF)) with ingestion of propidium-conjugated bacteria that emit a red fluorescence. Two-color analysis allows easy measurement of particle uptake and oxidative burst activity simultaneously. Recently, commercially available flow cytometric assays have been validated for the bovine species, and show a good agreement with other classic methods.[6]

Reactive Oxygen Species Production

Many flow cytometric tests to measure ROS production have been developed and applied in cows, dogs, and horses. One of the most frequently used is the DCF assay.

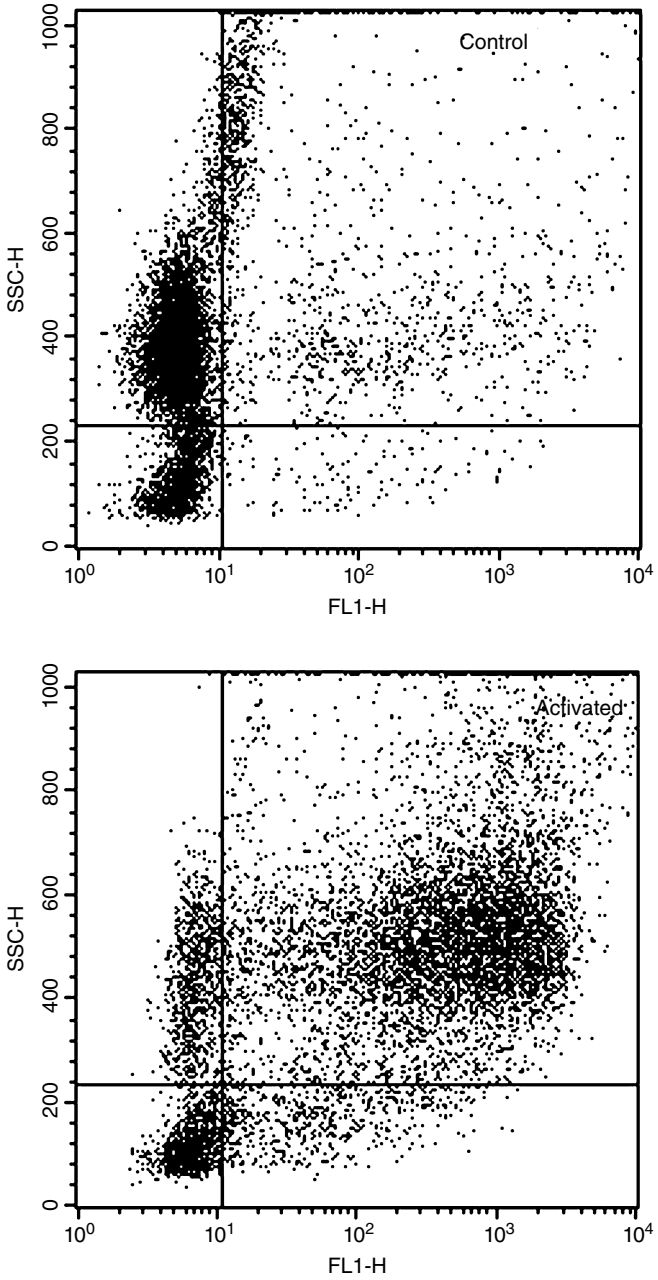


FIGURE 45.4 Flow cytometric evaluation of phagocytosis of fluorescein-labeled *Escherichia coli*. A high percentage of neutrophils shows fluorescence due to bacterial ingestion. In the control sample, phagocytosis is inhibited by incubating cells at 4 °C.

The acetate form enters the cells and is oxidized mainly by hydrogen peroxide resulting in green fluorescence. Similar techniques use dihydrorhodamine 123, which is converted to the fluorescent rhodamine 123, or hydroethidine, which is converted to the fluorescent ethidium bromide. These three techniques are useful to assess neutrophil phagocytosis, but they react differently with different oxygen compound produced during the respiratory burst. The choice of probe depends on the ROS of interest, while a combination of techniques can provide a more accurate and specific evaluation.

Eosinophils, which are not differentiated based on scatter properties, are capable of an oxidative burst. Thus, results in whole blood must be carefully evaluated in animals with hypereosinophilia.

EVALUATION OF NEUTROPHIL DEATH

The study of neutrophil death, occurring by apoptosis (programed cell death) or necrosis, is another challenging area of neutrophil function. Neutrophils are normally characterized by a very short life span that can vary in physiological or pathological conditions. Evaluation of neutrophil death by apoptosis and/or necrosis is interesting because many different cytokines and substances produced by pathogens can influence the rate at which neutrophils undergo apoptosis and/or necrosis.

Many assays using conventional and flow cytometric techniques have been developed to test cell death. Conventional techniques use mainly cytochemical stains (terminal-deoxynucleotidyl-transferase-mediated dUTP nick-end-labeling (TUNEL)) or colorimetric evaluation of proteases involved in the apoptosis process (e.g., caspase-3).

Flow cytometric techniques have the advantage of differentiating apoptosis versus necrosis in a large number of cells and are rapid and easy to perform. Many different assays have been developed that can evaluate the following:

1. *Scatter properties:* Apoptotic cells lose water and decrease granularity. This aspect can be easily identified by decreases in both forward and side scatter. However, this method cannot distinguish between apoptotic and necrotic cells.
2. *DNA content:* The content of DNA can be evaluated using fluorescent dye propidium iodide (or other dyes such as acridine orange, ethidium bromide, Hoechst 33342, or actinomycin D) after cell permeabilization. Apoptosis results in DNA fragmentation; thus, the detection of cells with less DNA content than normal diploid neutrophils is suggestive of apoptosis or necrotic cells.
3. *Combined test for evaluating cell death:* Many tests to evaluate cell viability are commercially available. Each of them focuses on a specific stage on the death process; Annexin V is a polypeptide that exhibits a strong affinity for phosphatidylserine molecules that are present only on the inner layer of the cell membrane in viable cells. During early apoptosis, phosphatidylserine residues are exposed on the cell membrane. Early apoptotic neutrophils and necrotic cells can be easily detected by double labeling cells with fluorescent-conjugated annexin V and propidium iodide that enters only necrotic cells. Modifications of this technique use other fluorescent dyes instead of annexin V to evaluate membrane integrity (SYBR-14) or mitochondrial function (rhodamine 123).
4. *TUNEL:* The principle of this assay is that, during apoptosis, terminal deoxynucleotidyl transferase (TdT) adds nucleosides to free 3′-ends produced from DNA cleavage. Neutrophils are permeabilized and incubated with biotinylated nucleosides and TdT; after washing, fluorescent-conjugated streptavidin, which binds to the biotin, is added and the number of cells in apoptosis is detected using a fluorescent microscope or flow cytometer. This assay is one of the more widely used methods to detect apoptosis. However, nonspecific positivity in necrotic cells is reported; thus, it should be combined with one or more other techniques.

EVALUATION OF NEUTROPHIL EXTRACELLULAR TRAPS

Neutrophil extracellular traps (NETs) are networks of DNA, nuclear material, and proteins released in response to many inflammatory or infectious stimuli (see Chapter 43). NETs are components of innate immunity that entraps and kills pathogens. However, excessive NET formation may enhance tissue damage, thrombosis, and autoimmunity. The evaluation of NET formation has recently been reported in several species, mainly in dogs. Isolated cells are incubated in multiwell plates with activator substances. DNA release is quantified by adding a cell-impermeable nucleic-acid dye and reading absorbance using a spectrophotometer.[4] Alternatively, two nucleic-acid dyes, one that stains cells with intact membranes (SYTOX green) and one that stains cells with permeable membranes (SYTOX orange), may be used and NET formation is evaluated using a fluorescence microscope equipped with an image analyzer.[7]

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CHAPTER 46

Eosinophils and Their Disorders

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Regulation, Production, and Release
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Acronyms and Abbreviations

CLCP, Charcot–Leyden crystal protein; EBP, eosinophilic bronchopneumopathy; ECF-A, eosinophil chemotactic factor of anaphylaxis; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; EoSV, eosinophil sombrero vesicle; EPO, eosinophil peroxidase; Gal-10, galectin-10; HES, hypereosinophilic syndrome; HETE, hydroxyeicosatetraenoic acid; HHT, hydroxyheptadecatrienoic acid; GI, gastrointestinal; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; MBP, major basic protein; MCP-3, monocyte chemotactic peptide-3; MIP-1α, macrophage inflammatory protein-1α; PAF, platelet-activating factor; PGE, prostaglandin E; RER, rough endoplasmic reticulum; Siglec, sialic-acid-binding Ig-like lectin; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment receptor; TGF, transforming growth factor; TNF-α, tumor necrosis factor-α.

Eosinophils, named by Ehrlich based on their affinity for anionic dyes such as eosin,[34] defend against helminthic parasites, are bidirectional partners in basophil- or mast-cell-mediated inflammation, and may damage host tissues. Eosinophils participate in innate, acquired, and adaptive immunity, tissue remodeling, and developmental biology, and are pleotrophic multifunctional cells that serve complex physiologic roles. This chapter emphasizes structure–function relationships to elucidate the varying roles of eosinophils in many complex processes. The reader is referred to several recent comprehensive reviews.[5,29,43,46,61,66]

REGULATION, PRODUCTION, AND RELEASE

Eosinophils develop in bone marrow and to a lesser extent in thymus, spleen, lung, and lymph nodes in some laboratory species.[21,36,78] Type-2 helper T (TH_2) cells secrete interleukin (IL)-5 and IL-13 and are important regulators of all aspects of eosinophil development and function, including increased production by bone marrow, mediated by IL-5, and recruitment to tissues by eotaxins, regulated by IL-13. IL-3 and granulocyte macrophage colony-stimulating factor (GM-CSF) also play roles in production but are less lineage-specific (see Chapter 41).

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Produced in bone marrow over 2–6 days, eosinophils comprise <10% of marrow nucleated cells; the marrow-to-blood eosinophil ratio varies between 300:1 in guinea pigs and 3.4:1 in humans. The half-life of circulating eosinophils varies from <1 hour in dogs to 18–24 hours in humans. Eosinophils migrate into tissues, especially the gastrointestinal (GI) tract and lungs, and live for about 2 days unless anti-apoptotic factors, such as IL-5, prolong their survival for up to 2 weeks. Under pathologic conditions, generation and emergence times can be altered, and eosinophils may re-enter circulation.[17,76]

TISSUE DISTRIBUTION AND METABOLISM

Eosinophils reside mainly in the skin, respiratory tract, and GI tract. Their location and number vary with species, stage of estrous cycle, diet, and histamine content of the tissue; most eosinophils are found in the GI tract where they are recruited by the chemokine eotaxin-1 (CCL11).[50,53]

STRUCTURE AND MORPHOLOGY

Eosinophils are polymorphonuclear leukocytes that become recognizable with the appearance of specific (secondary) granules at the progranulocyte/myelocyte stage (Figure 46.1). Myelocytes have numerous immature specific granules, an extensive Golgi complex, and abundant rough endoplasmic reticulum (RER).[29] The nucleus undergoes segmentation, and granules continue to mature in band and segmented forms. Eosinophils contain three types of granules—specific granules, primary granules, and small dense granules—as well as eosinophil sombrero vesicles (EoSVs),[52] lipid bodies, mitochondria, free ribosomes, sparse RER, a small Golgi, and glycogen.[2,19]

Specific granules containing potent cytotoxic proteins and comprising the majority of the granule population have a bicompartmental structure in many species, including humans, rhesus monkeys, cats, goats, rabbits, opossums, guinea pigs, rats, and mice,[2,40,51] with a distinctive electron-dense core (crystalloid) characterized by both longitudinal and cross-sectional periodicity and surrounded by more lucent matrix (Figure 46.2a–d). Cores vary in density, size, and shape among species; variation is especially wide in cats. Approximately 10% of canine granules have distinct cores[37]; approximately 50% of sighthounds and individuals of other canine breeds can have eosinophils with poorly staining granules, termed gray eosinophils. This staining difference appears due to eosinophil peroxidase (EPO) deficiency; other deficiencies may also be present. Using Romanowsky stains, gray eosinophils from Greyhounds are similar to murine eosinophils deficient in both EPO and major basic protein (MBP).[4,27,38,39] Equine eosinophil granules contain dense cores that lack crystalline patterns, are often located eccentrically, and are surrounded by less-dense matrix.[65]

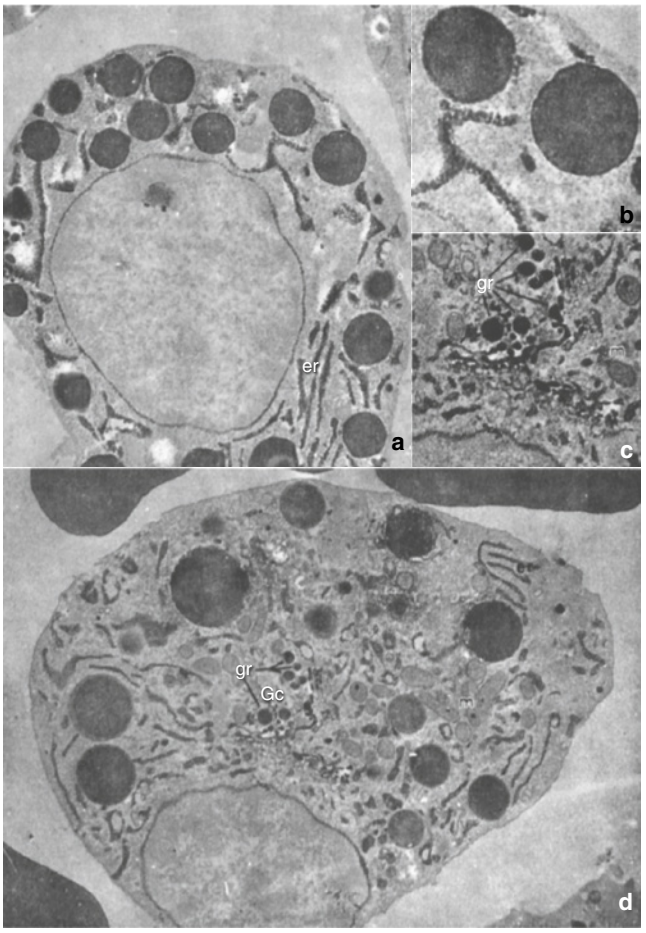


FIGURE 46.1 Eosinophil progranulocyte and myelocyte from canine bone marrow stained for eosinophil peroxidase (EPO) activity. (a) A progranulocyte contains dense, homogeneous granules and cisternae of rough endoplasmic reticulum (er), highlighted in (b). The myelocyte shown in (d) contains condensing granules of varying morphology, an active Golgi complex (Gc) producing granules (gr), profiles of rough endoplasmic reticulum, and numerous mitochondria. (c) An enlargement of the Golgi area from the cell in (d). Eosinophil peroxidase activity is characterized by the electron-dense material in the perinuclear cisterna, rough endoplasmic reticulum, Golgi complex, and specific granules. (a) 10 080×. (b) 18 900×. (c) 14 280×. (d) 10 080×.

Homogeneous granules are found in eosinophils from cattle, mink, and gorillas.[40,51]

Features of the four major proteins are described in Table 48.1. Major basic protein, located in the core, comprises >50% of the granule protein content. Deposits of MBP can be found at sites of eosinophilic inflammation. The other proteins are found in the matrix. Eosinophil peroxidase is found in progranulocytes and myelocytes in the RER, perinuclear cisterna, Golgi, and specific granules. Thereafter, EPO is located solely in specific granules.[2,19] Eosinophil peroxidase is distinct from and more potent than myeloperoxidase.[2] Different sources conclude that EPO is involved in bacterial killing and has activities against parasites and viruses.[59,72] This enzyme has been widely reported to be absent from feline

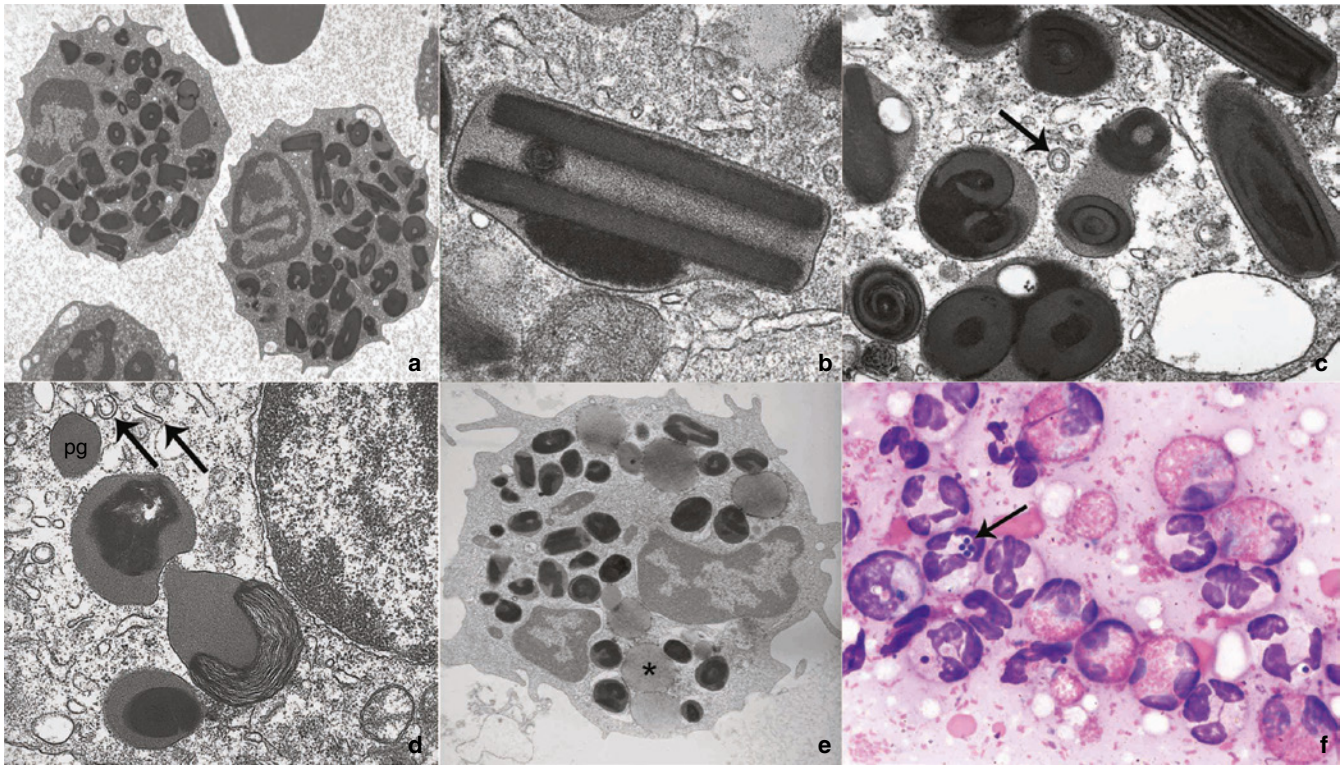


FIGURE 46.2 (a)–(e) Transmission electron micrographs and (f) cytologic preparation of feline eosinophils in blood and tissue. (a) Two blood eosinophils containing numerous heterogeneous bicompartmental granules; 34 500×; (b) A rod-shaped specific granule with electron-dense cores (which may be disassembling) and lucent matrix; 340 000×; (c) Multiple specific granules with disassembling electron-dense cores surrounded by more lucent matrix. The arrow indicates an eosinophil sombrero vesicle (EoSV); 340 000×; (d) Specific granules, some of which are undergoing piecemeal degranulation. Arrows indicate C-shaped and elongate EoSV that transport and store granule proteins. A primary granule (pg) is present; 340 000×; (e) Transmission electron micrograph of an activated eosinophil from a transtracheal wash of a cat with allergic bronchitis. Lipid bodies (asterisk) are increased; 39 100×; (f) Eosinophilic inflammation in the skin of a cat with staphylococcal hypersensitivity. A neutrophil contains bacterial organisms (arrow). Wright's stain; 1000×.

eosinophils; however, protein peaks separated from feline eosinophil granules are most compatible with EPO, eosinophil cationic protein (ECP), and MBP.[25] ECP constitutes approximately 30% of the granule protein and damages membranes by a colloidal osmotic process, causing formation of transmembrane non-ion-selective pores; this protein promotes mast cell degranulation and is bactericidal. Two isoforms have been identified by chromatography.[29] Eosinophil-derived neurotoxin (EDN) is also expressed in mononuclear cells and possibly neutrophils; it damages myelinated nerve fibers and is implicated in activity against respiratory viruses.[59] Other granule constituents include catalase, peroxisomal lipid β-oxidation enzymes (enoyl-CoA hydratase, 3-ketoacyl-CoA thiolase), flavoprotein (acyl-CoA oxidase), β-glucuronidase, cathepsin D, serine:pyruvate aminotransferase, and zinc.[19,40] Acid phosphatase and arylsulfatase activities may be unmasked in the matrix in activated eosinophils.[19] Four basic proteins have been isolated from equine eosinophils, but could not be correlated definitively to human proteins, although one is likely the analog of human MBP.[56]

Membrane-bound primary granules are most numerous in progranulocytes and can be found in all stages of eosinophils in some species (Figure 46.2d). Their lysophospholipase activity has been reported to reside in Charcot–Leyden crystal protein (CLCP), but is now believed to be due to other eosinophil lysophospholipases.[11,35,45] CLCP, renamed galectin-10 (Gal-10) based on homology with other members of that protein family and its presence in regulatory T cells,[11,45] comprises 7–10% of total eosinophil protein in humans and other primates.[11,19,20,45] This protein polymerizes to form hexagonal crystals deposited in tissues with eosinophilic inflammation even when intact eosinophils are no longer visible; its function in humans remains obscure, and CLCP/Gal-10 does not appear to be present in species of veterinary importance.[11,35,45] Lysophospholipase dampens inflammation by impeding generation of arachidonic acid metabolites and may inactivate cell-damaging lysophosphatides produced during degranulation.[29] A third type of eosinophil granule, the small dense granule, contains acid phosphatase, arylsulfatase, and perhaps ECP, catalase, and peroxidase.

Lipid bodies store arachidonic acid used in the generation of lipid mediators such as leukotrienes and prostaglandins.[19] Eosinophils also synthesize cytokines, proteoglycans, vitamin-B-binding proteins, and numerous enzymes, including collagenase, histaminase, phospholipase D, and nonspecific esterases. At least

TABLE 46.1 Summary of major preformed proteins released from specific granules of eosinophils.

Protein	Location	Characteristics	Actions
Major basic protein (MBP)	Specific granule core (also found in basophils)	Cationic protein, 14 kDa, pI 10.9, 117 amino acid (aa) single polypeptide rich in arginine; premolecule (207 aa) has a pI of 6.2 owing to a 90 aa acidic portion thought to protect cell from toxic effects of MBP during transport	Cytotoxic to parasites, protozoa, bacteria, normal mammalian epithelial cells, murine tumor cells; stimulates signaling pathways and mediator release from mast cells, neutrophils, and basophils; neutralizes heparin; activates platelets, basophils, mast cells, and neutrophils; induces bronchospasm; activates complement via classical and alternative pathways; activates remodeling factors from epithelial cells; increases cutaneous vasopermeability; adversely affects smooth muscle contraction responses; implicated in regulating peripheral nerve plasticity by inhibiting apoptosis; no enzymatic activity
Eosinophil peroxidase (EPO)	Specific granule matrix	Heavy (58 kDa) and light (14 kDa) subunits; pI 10.8; genetically and biochemically distinct from myeloperoxidase	In presence of H_2O_2 and halide, generates oxygen radicals toxic to helminths, bacteria, mycoplasmas, fungi, protozoa, viruses, and tumor cells; in absence of H_2O_2 acts as cationic toxin; toxic to host respiratory epithelium; induces release of granules, histamine from mast cells; inactivates leukotrienes
Eosinophil cationic protein (ECP)	Specific granule matrix	Cationic protein, 18–21 kDa, pI 10.8, 133 aa; secreted and stored forms differ; ribonuclease gene superfamily; 66% sequence homology with EDN	Toxic to helminths, protozoa, bacteria, and tracheal epithelium; neurotoxic; neutralizes heparin; promotes mast cell degranulation
Eosinophil-derived neurotoxin (EDN, also called eosinophil protein X [EPX])	Specific granule matrix	Cationic protein, 18–19 kDa, pI 8.9, 134 aa; ribonuclease gene superfamily; 66% sequence homology with ECP	Toxic to myelinated nerve fibers; significant ribonuclease activity; antiviral activity in respiratory infection

35 cytokines, chemokines, and growth factors are produced by eosinophils.[35] Eosinophils are the chief producers of cytokines such as transforming growth factor (TGF)-β in a variety of eosinophil-associated diseases such as asthma.

The plasma membrane contains NADPH oxidase to generate an oxidative burst and alkaline phosphatase in some species (cattle, horses, dogs, and cats).[40] Eosinophils lack bactericidal lysozyme, lactoferrin, and phagocytin.[18]

SURFACE MOLECULES

By scanning electron microscopy, surfaces of eosinophils have short blunt processes and sparse microvilli. Their many cell surface receptors include receptors for cytokines, chemokines, complement proteins, immunoglobulins (Ig), adhesion molecules, lipid mediators, enzymes, and signaling and apoptotic factors.[81] Adhesion receptors link eosinophils with endothelial cells or extracellular matrix, allowing their emigration and directing their movement in tissues and extracellular spaces. Chemotactic factors and chemokines recruit eosinophils to sites of inflammation. Many factors, including lipid mediators, Igs, and complement components, act through receptors to activate eosinophils to perform effector functions and connect them with their targets.[40,46]

Other surface molecules have been identified on eosinophils and include toll-like receptors and inhibitory receptors, such as sialic-acid-binding Ig-like lectin (Siglec-8).[46,60] Activation of inhibitory receptors suppresses eosinophil activation and induces negative signaling in murine eosinophils; cross-binding Siglec-8 induces apoptosis of eosinophils.[35]

SIGNS OF ACTIVATION

Eosinophil activation results in changes in morphology, surface characteristics, and functional activities.[7,19,46] These changes are acquired after leaving the circulation, but may be found in circulating eosinophils of patients with allergic disease and hypereosinophilic syndrome (HES). Activated eosinophils are typically hypodense and have fewer specific granules and increased numbers of lipid bodies (Figure 46.2e) and vesiculotubular structures. Lipid bodies are repositories of arachidonate-containing lipids for oxidative metabolism and synthesis of lipid mediators; vesiculotubular structures, now termed EoSVs, store and transport granule products.

Upon activation, EoSVs move to the plasma membrane and fuse with it to rapidly release mediators. In a canine model of asthma, eosinophils in bronchoalveolar lavage fluid had either a typical polymorphonuclear appearance or the appearance of a globule leukocyte. Other changes noted in human activated eosinophils are increased primary granules and small dense granules. Functionally, cytotoxicity to parasites and mammalian cells is increased, generation of reactive oxygen species is enhanced, and enzymes, such as acid phosphatase, are activated.

INVOLVEMENT IN HOMEOSTASIS AND THE IMMUNE SYSTEM

Eosinophils participate in homeostatic functions and mucosal immunity in the GI tract. Under homeostatic conditions, eosinophils traffic into the thymus, mammary gland, uterus, and the GI tract.[60] They are recruited by eotaxin-1 to the thymus during the neonatal period and are associated with thymocyte deletion.[68] They participate in T-cell-mediated immunity through recognition and presentation of microbial, viral, and parasitic antigens, as well as superantigens. Their immunologic roles may be related to their location at surfaces in contact with the outside environment, their capacity to respond to particulate antigens (inhaled, ingested, or granuloma-encapsulated antigens), and their expression of Fc receptors for IgG, IgE, and IgA.[75] Eosinophils can prime B cells for IgM production and regulate T-cell polarization and proliferation of effector T cells.[60,71] Eosinophils appear to play regulatory roles in development of the postpubertal mammary gland, onset of estrous cycling, and blastocyst implantation.[30,58,60]

TRAFFICKING IN EOSINOPHILIC INFLAMMATION

Trafficking of eosinophils into tissues is directed by cytokines produced by TH_2 cells and endothelial cells; these include IL-4, IL-5, and IL-13, adhesion molecules (β1-, β2-, and β7-integrins), chemokines (CCL5 and the eotaxins), and many other molecules, including acidic mammalian chitinase.[79] Eotaxins-1, -2, and -3 (CCL11, CCL24, and CCL26, respectively)[42,64] are produced by respiratory epithelium and skin fibroblasts in human hypersensitivity disorders; CCL11 appears at the earliest stages, followed by CCL24 and then CCL26.[35]

Circulating eosinophils participate in inflammation through the following sequence of events:

1. Quiescent circulating eosinophils express L-selectin, permitting the eosinophil to roll along the vessel wall.[46]

2. Eosinophils meet a priming stimulus, such as IL-3, GM-CSF, IL-5, IgG, and IgA, released from perivascular immune cells, endothelial cells, and local inflammatory sites.[44] IL-4 mediates ε isotype switching and brisk production of IgE.[26] IL-5 initiates signal transduction in eosinophils resulting in expression of genes for surface receptors, structural and secreted proteins, and transcription factors.[44,69] Priming leads to adhesion, chemotaxis, degranulation, release of lipid mediators, and oxidative burst.

3. One priming function of IL-5 is to increase the number of responsive eosinophils.[14,55] Primed eosinophils express β1- (very late activation [VLA]-4), β2- (Mac-1 and leukocyte-function-associated antigen [LFA]-1), and β7-integrins that mediate secure adhesion to endothelial ligands (vascular-cell-adhesion molecule [VCAM]-1 and intercellular adhesion molecule [ICAM]-1) induced by IL-1.[6,7] The VLA-4/VCAM-1 pathway is selective for eosinophils and basophils.[6] Expression of tissue factor by eosinophils may play a role in migration from blood.[54] Adhesion further primes eosinophils to respond to chemoattractants and lipid mediators.

4. Recruited by various chemotactic factors, primed eosinophils migrate between endothelial cells into interstitial spaces. Depending on the target organ, eosinophils migrate into tissues by a regulated process involving coordinated interaction between networks involving CCL11, eosinophil adhesion molecules (α4β1, α4β7, αmβ2, αLβ2), and adhesion receptors on the endothelium (MAdCAM-1, VCAM-1, and ICAM-1).

5. Eosinophils approach their final destination guided by interactions with adhesion molecules in the extracellular matrix (fibronectin, laminin, and fibrinogen) and a concentration gradient of chemoattractants, including C5a, *N*-formyl-methionyl peptides, leukotrienes, platelet-activating factor (PAF), hydroxyeicosatetraenoicacids(HETEs),hydroxyheptadecatrienoic acids (HHTs), monocyte chemotactic peptide-3 (MCP-3), macrophage inflammatory protein-1α (MIP-1α), eosinophil chemotactic factor of anaphylaxis (ECF-A), histamine, parasite-derived factors, and chemokines such as CCL5.[15,46]

6. Eosinophils are activated by cytokines, lipid mediators, chemoattractants, and Igs to release granule proteins, lipid mediators, and oxygen radicals.

7. Eosinophil survival is enhanced through exposure to inflammatory cytokines, such as IL-5.[33]

8. Eosinophils contribute to wound healing, or sclerosis, by secretion of TGF-β, procoagulation through activation of factor XII by ECP, and fibrinolysis by activation of plasminogen.[9,16,70,73]

Eosinophilic predominance in certain disorders, such as parasitic disease and asthma, is likely mediated by several eosinophil-selective molecules, including

adhesion molecules, primers, chemoattractants, and anti-apoptotic agents. Finally, a major consequence of chronic eosinophilic inflammation is tissue damage. Injury to airway epithelium mediated by MBP appears to be reversible, but myocardial fibrosis, perhaps induced by granule proteins or TGF, is permanent in hypereosinophilic syndrome.[7,29,46]

EFFECTOR FUNCTIONS

Defense Against Helminthic Parasites

Eosinophils are capable of phagocytosis, but helminths are too large, and eosinophils secrete mediators directly onto the target rather than engulfing them and, working in concert with T-cell-derived perforins, bind to larvae opsonized with IgG, IgE, or complement, flatten against the integument, release basic toxins and hydrolytic enzymes directly onto the target, and migrate under the damaged larval skin.[8,10,46,51] Oxidative burst may contribute to anti-helminthic action, but the cytotoxic effect of MBP to schistosomula of *Schistosoma mansoni* occurs independently of oxygen metabolites.[46] Eosinophils contribute to host defense by dampening immediate pathologic responses and participating in tissue remodeling and immunomodulation. IL-5 may play a role in the effectiveness of parasite vaccines in mice.[49]

The role of eosinophils in helminthic cytotoxicity in vivo remains uncertain. Some human studies suggest that eosinophils may prevent reinfection rather than clear an initial infection.[31] Studies of transgenic mice that overproduce IL-5 or mice deficient in IL-5, eotaxin, or the eotaxin receptor yield conflicting results about the role of eosinophils and their mediators in helminthic infections. Parasitized mice in which eosinophil and IgE responses were ablated by anti-IL-5 antibody had no changes in parasite burden or resistance to infection.[32,63] Interpretation of findings is confounded by use of parasites for which the mouse is not the natural host, equating murine and human eosinophils, which have functional differences, and lack of ability to ascertain that eosinophils are degranulating in response to parasitic infection. Horses with insect-bite hypersensitivity treated with an anti-IL-5 vaccine that reduced tissue, but not peripheral blood, eosinophils did not have greater intestinal parasite counts compared with placebo-treated horses.[24] After many years, the beneficial role of eosinophils in control of parasite infection remains poorly understood.

Effector Cells in Asthma and Allergic Disease

The definitive contributions of eosinophils in hypersensitivity disorders are still being determined. Asthma is characterized by inflammation, hyperresponsiveness, mucus secretion, and remodeling with smooth muscle hyperplasia, collagen deposition, neovascularization, and metaplasia of goblet cells.[3,9] Studies in animals and people suggest an important role for eosinophils in airway remodeling and in acute exacerbations in asthma, but uncertainties remain.[57]

Eosinophils promote damage and dysfunction through elaboration of proinflammatory mediators, such as leukotriene C4, PAF, and eicosanoids, and release of granule proteins.[80] Eosinophils recruited into tissues by cytokines then recruit CD4+ cells into tissues by releasing IL-16. IgE-mediated mast cell degranulation, induced upon re-exposure to allergens and parasite antigens, plays a role in eosinophil recruitment in hypersensitivity disorders (Figure 46.2f).[46] Eosinophil granule products, reactive oxygen species, and lipid mediators cause damage to airway mucosa and nerves and bronchoconstriction. Major basic protein is toxic to airway epithelium and triggers degranulation of mast cells and basophils.[60] Eosinophils from asthmatic patients are hyperadhesive and have increased collagen receptor expression. TGF-β from eosinophils may play a role in airway remodeling. Leukotrienes secreted by eosinophils mediate vascular permeability, secretion of mucus, and constriction of smooth muscle. Cytokines involved in recruitment enhance effector functions and prolong survival of eosinophils by delaying apoptosis.

Modulation of Inflammation

Eosinophils suppress immediate hypersensitivity reactions.[46] They phagocytose immune complexes, peroxidize mast cell granule debris, and release histaminase to neutralize histamine, phospholipase B to inactivate mast cell PAF, MBP to inactivate mast cell heparin, plasminogen to reduce local thrombin activity, arylsulfatase B and hypochlorous acid to inactivate leukotrienes, prostaglandin E1 (PGE1) and PGE2 to inhibit degranulation of basophils and mast cells, and lysophospholipase to prevent generation of arachidonic acid metabolites.

Phagocytosis

Eosinophils have the capacity to phagocytose immune complexes, antibody-coated RBCs, mast cell granules, inert particles, yeast, and bacteria, including *Mycoplasma*. They are less efficient at killing bacteria than neutrophils despite having higher levels of peroxidase activity, oxidative responses, and hydrogen peroxide (H_2O_2) production.[18,40] Eosinophils lack several bactericidal substances and have a lower density of complement receptors than neutrophils.

Antitumor Effect

Eosinophil products are cytotoxic to or induce apoptosis of tumor cells in vitro,[41] and antitumor roles for IL-5 and eosinophils are suggested in murine models of increased expression of IL-5 and eotaxin deficiency, respectively. In some neoplasms, local infiltration by eosinophils in the absence of peripheral eosinophilia improves prognosis.[48] In human colorectal carcinomas, improved prognosis associated with eosinophil infiltration was independent of staging, vascularization, p53 expression, and histologic grade.[23] However, in other studies, eosinophilic infiltrates in tumors have been associated with a poorer prognosis or had no impact on prognosis.[1,47]

MECHANISMS OF EOSINOPHIL EFFECTOR FUNCTION

Release of Granule Proteins

Granule products are released through exocytosis, piecemeal degranulation, or cell lysis.[52,62] In piecemeal degranulation, transport vesicles carry granule products to the plasma membrane. Unique to eosinophils is a dynamic system of vesicular compartments, termed EoSVs, generated by specific granules. Using electron tomography, EoSVs are demonstrated to be highly pleomorphic curved tubular structures that are round, C-shaped, or elongated (Figure 46.2c,d). They change shape to accommodate transport of membrane-bound proteins over long distances in the cell and store certain proteins for quick release upon stimulation.[52] EoSV "dock" and then fuse with the plasma membrane, releasing proteins. The docking complex consists of soluble *N*-ethylmaleimide-sensitive factor attachment receptors (SNARE) located on the plasma membrane and the vesicle. Piecemeal degranulation is selective and tightly regulated so that only specific proteins are released after receiving a distinctive stimulus: IgE induces release of EPO and IgA binding can cause release of ECP and EPO, whereas IgG binding results in release of ECP and EDN.[43,46,51] Equine eosinophils degranulate through a complex process of compound exocytosis and cumulative fusion of multiple granules, resulting in a degranulation sac and focal release of granule proteins.[62]

Release of Lipid Mediators

Eosinophils respond to PAF and also produce and secrete it on activation. Platelet-activating factor enhances many eosinophil activities, including adherence to endothelium, chemotaxis, binding of IgE, production and release of oxygen radicals, release of granule proteins, and synthesis of prostanoids. Other lipid mediators released by eosinophils include leukotrienes (e.g., LTB4 and LTC4), thromboxane A2, and prostaglandins.[46] With the exception of PGE, which may downregulate eosinophil effector functions, lipid mediators contribute to host dysfunction, such as bronchial hyperreactivity.[46]

Cytokine Release

Eosinophils exhibit paracrine regulation of other inflammatory cells, endothelial cells, and fibroblasts through release of IL-1, IL-2, tumor necrosis factor-α (TNF-α), TNF-β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-16, IL-18, TGF-α/-β, eotaxin, and CCL5. Autocrine control results from elaboration of IL-3, IL-5, and GM-CSF by eosinophils.[7,75]

MECHANISMS OF EOSINOPHILIA AND EOSINOPENIA

The number of eosinophils in circulation is influenced by diurnal variation related to cortisol levels, age, exercise, and exposure to environmental stimuli. The least accurate method to quantify blood eosinophils is the differential leukocyte count performed on a stained blood film, but this method is useful in most situations. Eosinophil counting chambers can be used, but automated methods that stain peroxidase provide the most accurate counts. Eosinophil peroxidase is not detected by this method in all animal species, especially cats. Reference intervals for eosinophils vary among species and geographic regions. Eosinophilia is not always present in the face of eosinophilic inflammation in tissues where eosinophil survival is increased.

Eosinophilia

Eosinophilia results from elaboration of eosinophilopoietic factors, principally IL-5, by T cells sensitized by parasite antigens or allergens.[28,32] IL-5 primes eosinophils, enhances their function, and prolongs their survival.[77] Eosinophilia associated with parasitism occurs when parasites are located in tissues rather than in the intestinal lumen. In eosinophilic bronchopneumopathy (EBP), previously termed pulmonary infiltrates with eosinophilia, eosinophilic infiltrates are found in the bronchial mucosa and interstitium[12,13]; hypersensitivity may be initiated by heartworm infection, migration of larval parasites, and antigens associated with chronic infections. Chronic eosinophilia is associated with inflammatory disorders of mast cell-rich organs, namely, skin, lung, GI tract, and uterus. Other conditions include eosinophilic dermatitis, previously termed eosinophilic plaque/granuloma/indolent ulcer, and eosinophilic inflammatory bowel disease in cats and eosinophilic myositis, eosinophilic panosteitis, and eosinophilic gastroenteritis in dogs. Paraneoplastic eosinophilia is caused by tumors, such as lymphoma, mast cell tumor, and solid tumors, that produce IL-5 and other cytokines.[67] Eosinophilic leukemia is a rare disease, and the diagnosis depends on ruling out other causes (see Chapter 66). In HES, characterized by persistent eosinophilia of unknown origin, increased survival of eosinophils in circulation, tissue infiltrates, and organ dysfunction, one proposed mechanism is clonal expansion of T cells generating eosinophilopoietic factors.[74] Increased levels of IL-5 in some patients may override the apoptotic effects of corticosteroids. In a subset of people with HES, constitutive expression of tyrosine kinases leads to production of eosinophils independent of growth factors.[60] Eosinophilia is sometimes recognized in animals with hypoadrenocorticism owing to decreased or absent cortisol.

Eosinopenia

Detection of low numbers of eosinophils has limited significance, and underlying mechanisms of eosinopenia are unclear. Corticosteroids cause eosinopenia, perhaps by neutralization of circulating histamine and inhibition of mast cell degranulation. The lympholytic effect of corticosteroids may attract eosinophils into lymphoid tissues after release of cytokines; alternatively, production of cytokines promoting growth and function of

eosinophils may be diminished. Corticosteroids inhibit cytokine-dependent eosinophil survival. Thus, eosinopenia may occur in hyperadrenocorticism or after exogenous administration of corticosteroids. Catecholamines may induce eosinopenia through a β-adrenergic effect, as the effect is eliminated by β-blockers. Eosinopenia present in acute inflammation or infection may result from release of corticosteroids and catecholamines.

THERAPEUTIC APPROACHES

Novel therapeutic interventions based on molecular and cellular mechanisms of eosinophilic inflammation may transform medical management of patients with eosinophilic inflammation.[7,22,24,35,46,60] Glucocorticoids have been the therapeutic mainstay and act by destabilizing eotaxin mRNA, suppressing transcription of IL-4, IL-5, and eotaxin genes, and inhibiting survival of eosinophils.[61] Not all patients respond or maintain a response to glucocorticoids. Other approaches include reduction of eosinophil production by myelosuppressive drugs (e.g., hydroxyurea), inhibition of eosinophil degranulation and effector functions by interferon (IFN)-α, and interference with transcription of cytokines, such as IL-5 and GM-CSF, by cyclosporin A. Novel approaches include anti-IL-5 antibodies such as mepolizumab, used in eosinophilic asthma; therapeutic recombinant vaccination against IL-5; antagonists to leukotrienes or their receptors; tyrosine kinase inhibitors, such as imatinib, that may be effective in patients with HES caused by constitutive production of tyrosine kinases; lidocaine, demonstrated to shorten eosinophil survival; third-generation antihistamines; blockers of eosinophil adhesion; inhibitors of the eotaxin/CCR3 pathway; anti-human IL-13 antibody; and induction of eosinophil apoptosis by antibody cross-linking of Siglec-8.

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CHAPTER 47

Basophils, Mast Cells, and Their Disorders

BRANDY C. KASTL and LISA M. POHLMAN

Origin, Development, and Tissue Distribution
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Acronyms and Abbreviations

AP-1, activator protein-1; BaP(s), basophil progenitor(s); bFGF, basic fibroblast growth factor; BMCP, basophil–mast cell progenitor; C/EBPα, CCAAT-enhancer-binding protein alpha; CCL; C-C motif chemokine ligand; CCR, C-C chemokine receptor; CD, cluster of differentiation; COX, cyclooxygenase; CXCR, C-X-C chemokine receptor; ELAM, endothelial-cell–leukocyte adhesion molecule; ERK, extracellular receptor-activated kinase; Fc region, fragment crystallizable region of an antibody; FcεRI, Fc region of IgE receptor I; FcγRIIb, Fc region of the IgG receptor IIb; FcγRIIIa, Fc region of the IgG receptor IIIa; GATA1, GATA-binding protein 1; GATA2, GATA-binding protein 2; GM-CSF, granulocyte macrophage colony-stimulating factor; GMP(s), granulocyte–monocyte precursor(s); ICAM, intercellular adhesion molecule; IFN, interferon; IFN-γ, interferon-gamma; Ig, immunoglobulin; IgG1, immunoglobulin G 1; IL, interleukin; JAK2, janus kinase 2; KIT(c-KIT), receptor tyrosine kinase KIT; LFA, lymphocyte-function-associated antigen; Lin^-, lineage negative; LT, leukotriene; LTB_4, leukotriene B_4; LTC_4, leukotriene C_4; LTD_4, leukotriene D_4; LTE_4, leukotriene E_4; MAdCAM-1, mucosal vascular addressin cell adhesion molecule-1; MAPK, mitogen-activated protein kinase; MCP(s), mast cell progenitor(s); MC_T, mast cell subset with tryptase; MC_{TC}, mast cell subset with tryptase and chymase; MCP(s), mast cell progenitors; MITF, melanogenesis-associated transcription factor; NFAT, nuclear factor of activated T cells; NF-κβ, nuclear factor κβ; PGD_2, prostaglandin D_2; PGE_2, prostaglandin E_2; PLA_2, phospholipase A_2; SCF, stem cell factor; pre-BMP(s), pre-basophil and mast cell progenitors; STAT5, signal transducer and activator of transcription 5; TGF-β, transforming growth factor-beta; Th1, T-helper lymphocyte type 1; Th2, T-helper lymphocyte type 2; TNF-α, tumor necrosis factor-alpha; TLR, toll-like receptor; TSLP, thymic stromal lymphopoietin; TXB_2, thromboxane B_2; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor.

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Basophils and mast cells are often referred to as sister cells due to similarities in their chemical compositions and biologic functions. Both cells are well known for their involvement in T-helper lymphocyte type 2 (Th2)-mediated immunity and hypersensitivity reactions. However, these cells are distinct with unique maturation pathways, tissue localizations, and chemokine-signaling responses. Additionally, basophils and mast cells function independently in their protective and immunologic roles. Therefore, laboratory abnormalities of basophils and mast cells have distinctive differential diagnoses.

ORIGIN, DEVELOPMENT, AND TISSUE DISTRIBUTION

Basophils and mast cells arise from shared early progenitor cells in bone marrow and spleen via separate maturation pathways.[5] In the few reported cases of defective basophilopoiesis or basophil function, patients lacked concurrent mast cell defects providing clinical evidence for separate maturation pathways.[9]

Common Origin of Basophils and Mast Cells

Within the bone marrow, hematopoietic stem cells develop into common myeloid progenitor cells which, in turn, differentiate into granulocyte–monocyte precursors (GMPs).[5] GMPs are agranular, lineage negative (Lin^-), cluster of differentiation (CD)34^+, receptor tyrosine kinase KIT (c-KIT^+), CD16/32^+ cells, which have the potential to differentiate into mast cells, basophils, eosinophils, neutrophils, or macrophages.[5] The final maturation pathway selected by these multipotent bone marrow GMPs is influenced by specific chemokines, interleukins (ILs), and activated transcription factors. IL-3 signaling via the janus kinase (JAK)2/signal transducer and activator of transcription 5 (STAT5) pathway is crucial for development of both basophils and mast cells.[29]

The majority of GMPs within bone marrow express FcεRI, the high-affinity receptor for the fragment crystallizable (Fc) region of IgE, which is important for basophil and mast cell function.[5] These $FcεRI^+$ GMPs are also referred to as pre-basophil and mast cell progenitors (pre-BMPs).[5] Both pre-BMPs and $FcεRI^-$ GMPs are capable of differentiating into either basophil progenitors (BaP) or mast cell progenitors (MCP).[5] In murine bone marrow, the differentiation of pre-BMPs into either BaPs or MCPs depends upon the relative expression of the transcription factors GATA-binding protein 2 (GATA2), CCAAT-enhancer-binding protein alpha (C/EBPα), and melanogenesis-associated transcription factor (MITF).[25] MITF expression is necessary for mast cell development and is disrupted by high concentrations of C/EBPα expression.[29] Thus, MCPs have high MITF and low C/EBPα gene expression; BaPs have low MITF and high C/EBPα gene expression.[29]

Using the murine animal model, the common origin of basophils and mast cells within the spleen follows a slightly different pathway than that described in bone marrow. The murine spleen contains Lin^-, $CD34^+$, c-KIT^+, $FcεRI^-$ multipotent GMP-like cells analogous to bone marrow $FcεRI^-$ GMPs.[25] However, splenic GMP-like cells are capable of promoting enhanced type-2 cytokine responses (i.e., IL-4, IL-5, IL-9, and IL-13 production).[25] GMP-like cells develop into a bipotent basophil–mast cell progenitor (BMCP), which is immunophenotypically distinct from the murine bone marrow pre-BMP.[4,25] Splenic BMCPs are believed to be capable of further differentiation into both BaPs and MCPs.[4,25]

Basophil Development and Tissue Distribution

Under the influences of IL-3 and thymic stromal lymphopoietin (TSLP), BaPs within bone marrow or spleen develop into mature basophils which are released into circulation.[25] In health, basophil maturation within bone marrow is completed in about 2.5 days.[27] Once in blood, basophils have a brief circulating half-life of approximately 6 hours and constitute about 0.5% of blood leukocytes.[27] From the blood, basophils are recruited into tissues in response to inflammatory stimuli where they may live as long as 2 weeks.[27] Within tissue, basophils retain their unique morphologic features, but are not routinely identified in H&E-stained tissue sections.

IL-3 is the major basophil growth factor and promoter of basophil production, differentiation, and survival;[25] it upregulates essentially all basophil immune responses. IL-3-induced basophils arise primarily from bone marrow as part of immunoglobulin E (IgE)-dependent, Th2-mediated immunologic responses.[25] IL-3 activates GATA1, GATA2, and STAT5 transcription factor pathways in multipotent GMPs resulting in amplified basophiloiesis, bone marrow basophil hyperplasia, and increased peripheral basophil concentration.[25] Additional growth factors that contribute to basophil production and differentiation include TSLP, granulocyte macrophage colony-stimulating factor (GM-CSF), IL-4, IL-5, and stem cell factor (SCF).[25,27]

Basophils emigrate from blood to tissues along chemotactic gradients in order to participate in inflammatory responses. After stimulation, basophils release cytokines, including tumor necrosis factor-alpha (TNF-α) and IL-4, which increase expression of adhesion molecules on endothelial cells, thereby initiating emigration. Rolling of basophils along endothelium is mediated by basophil selectins, which act to tether the cells loosely to their carbohydrate counter ligands on endothelial cells. Adherence of basophils to the vascular endothelium involves intercellular adhesion molecule (ICAM)-1, endothelial-cell–leukocyte adhesion molecule (ELAM)-1, and vascular cell adhesion molecule (VCAM)-1, which are expressed on the surface of activated endothelial cells. Basophil integrins act to establish firm adhesion. Transendothelial migration and movement through the extracellular matrix are mediated by similar mechanisms. In addition to directing basophils to inflammatory sites, chemotactic cytokines enhance basophil degranulation.[27]

Mast Cell Development and Tissue Distribution

Unlike other leukocyte lineages, most mast cells do not complete maturation within the bone marrow or spleen. Instead, immature poorly granulated MCPs are released into circulation and migrate into perivascular, subendothelial, and perineuronal areas within connective and mucosal tissues.[5] There, they proliferate, differentiate, and mature into well-granulated mast cells under the influence of a variety of interleukins including IL-3, IL-4, IL-9, and IL-10.[27] Mature mast cells are resident tissue cells capable of surviving for weeks to months, even after degranulation and subsequent cytoplasmic granule regeneration.[5]

Mast cell survival, growth, and differentiation are dependent upon normal interactions between SCF and its cell-surface receptor c-KIT (CD117).[39] SCF is produced by endothelial cells, epithelial cells, fibroblasts, and other cell lines in culture. Mast cells deprived of SCF undergo apoptosis. KIT is a transmembrane receptor tyrosine kinase which uses the mitogen-activated protein kinase (MAPK) signaling cascade and is encoded by the c-KIT proto-oncogene.[39] Mutations in the c-KIT proto-oncogene can result in mastocytosis and mast cell neoplasia (see Chapter 73).[39] The effects of SCF on tissue mast cells are antagonized by GM-CSF, interferon-gamma (IFN)-γ, and transforming growth factor-beta (TGF)-β. Mast cell differentiation and proliferation are also supported by IgE, IL-6, IL-9, C-C motif chemokine ligand (CCL)11, CCL24, CCL26, and neuronal growth factor.[5,7]

Mast cells are distributed within tissues commonly associated with clinical symptoms of allergic disease: gastrointestinal tract, lungs, and skin. Rodents, and potentially other mammals, have a large pool of MCPs and mast cells within the peritoneum and associated adipose tissues.[5] Migration of MCPs into the gastrointestinal tract is primarily mediated by C-X-C chemokine receptor (CXCR)2 and α4β7 integrin, which binds to mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1) and VCAM-1 on endothelial surfaces.[8,12] Similar MCP homing mechanisms have been described in the lungs utilizing CXCR2, C-C chemokine receptor (CCR)2, and CCL2.[5] The MCP integrins, α4β7 and α4β1, interface with VCAM-1 on pulmonary endothelial cells and thereby facilitate MCP migration into the pulmonary parenchyma.[5] The primary chemotactants for mast cells in the skin are leukotriene (LT)B_4, IgE, and CCL2/CCR2.[5] Once in tissue, mast cells participate in protective and pathologic processes.

OVERVIEW OF BASOPHIL AND MAST CELL BIOLOGIC FUNCTIONS

In addition to participating in allergic responses, mast cells and basophils also perform protective roles in innate immunity, host defense, and inflammation.[36] In response to microbial invasion, microbial products, and tissue damage, basophils and mast cells release potent inflammatory mediators, such as histamine, proteases, chemotactic factors, cytokines, and arachidonic acid metabolites.[31] Both cells may also be activated by other inflammatory molecules, such as defensins, neuropeptides, adenosine, and endothelins.[4]

Basophils

Basophils participate in type 2 immunity and allergic inflammation. Type 2 immunity defends the host from helminths and ticks and is achieved by the stimulation of a Th2-mediated response. Basophils are critical in the development of IgE-mediated chronic allergic inflammation and recruit neutrophils and eosinophils to inflammatory sites.[36] Circulating basophils enter inflamed tissues during the late phase of IgE-mediated immediate type I hypersensitivity reactions where, like mast cells, they have deleterious effects. The importance of basophils in type I hypersensitivity responses is demonstrated by the ability of genetically engineered mast-cell-deficient mice to mount an IgE-mediated anaphylactic response.[13] Basophils are also crucial players in many other allergic conditions such as allergic rhinitis, hives (e.g., urticaria), asthma, allergic conjunctivitis, allergic gastritis, drug-induced or insect-bite-induced anaphylaxis, and inflammatory bowel disease.[25] Basophils may also antagonize or promote hemostasis by means of the anticoagulant actions of secreted heparin and the procoagulant effects of kallikrein.[27]

Mast Cells

Mast cells have critical roles in type 1 hypersensitivity reactions, but their other immunologic functions are vast. Mast cells protect hosts against a wide range of parasitic and bacterial infections, degrade venom toxins, mediate tolerance to skin transplants, contribute to tumor rejection, and exacerbate autoimmunity.[36]

Mast cells promote hypersensitivity reactions characterized by vasodilation, increased vascular permeability, bronchoconstriction, mucus secretion, and nerve stimulation. They modulate the immune response by stimulating T cells; play a role in host defense against tissue parasites; and promote acute and chronic inflammatory responses by stimulating leukocyte migration, endothelial–leukocyte adhesion, angiogenesis, fibrin deposition, fibroblast proliferation, and fibrosis.[11,36] Mast cells also play a protective role in innate immune responses to invading bacteria by releasing mediators that promote clearing infection.[36]

Mast cells are central mediators of wound healing and fibrosis; they release fibroblast-activating factors and cytokines, such as basic fibroblast growth factor, vascular endothelial growth factor, macrophage inflammatory protein-1, TGF-β, and IL-4.[11] Mast cells contribute to pathologic fibrotic disorders. For instance, after cardiac infarcts, mast-cell-mediated fibrosis and angiogenesis result in the replacement of injured cardiomyocytes with granulation tissue, thus, perpetuating cardiac insufficiency.[8]

MAJOR PRODUCTS OF BASOPHILS AND MAST CELLS

Many of the immediate effectors of basophils and mast cells are preformed and stored within their cytoplasmic granules. Basophils and mast cells also synthesize de novo products following activation. After degranulation, basophils and mast cells are capable of regenerating both their granules and granular contents.[5]

Stored Products

The primary substances stored in mast cell and basophil granules include proteoglycans, biogenic amines, enzymes, and, in basophils, major basic protein.[37] Stored products enhance localized innate immune responses following activation and degranulation.

The chemical composition of stored proteoglycans, such as heparin, serglycin, and chondroitin sulfate, is responsible for the characteristic metachromatic staining of basophil and mast cell cytoplasmic granules.[37] Among stored biogenic amines, histamine is arguably the most important due to its effects on local blood flow, vascular permeability, and smooth muscle contractility in inflammatory reactions. Other stored amines, which vary between species, include serotonin, adenosine, and dopamine.[37]

The collections of stored enzymes within mast cell and basophil granules are notably different. In addition, the enzymes within mast cell granules vary somewhat between mucosal (i.e., contain tryptase) and connective tissue (i.e., contain tryptase and chymase) mast cell populations.[37] Mast cell granules typically contain tryptase and sometimes chymase along with β-hexosaminidase, carboxypeptidase, cathepsin G, phospholipase A_2 (PLA_2), acid hydrolases, and chitinase.[37] By contrast, basophil granules contain bradykinin-generating neutral peptidases, elastase, β-glucuronidase, tryptase, and cathepsin-G-like enzyme.[27] Mast cells also store small quantities of various growth factors and cytokines, particularly TNF-α.[31]

De Novo Products

Because of their active roles in immediate inflammatory responses, basophils and mast cells are capable of de novo synthesis of various lipid-derived inflammatory mediators, cytokines, and interleukins. Basophils and mast cells preferentially utilize different arachidonic acid metabolism pathways in the production of their respective repertoires of lipid-derived products. Basophils tend to favor the lipoxygenase pathway with production of leukotrienes such as LTC_4, LTD_4, and LTE_4.[31] Mast cells utilize both the cyclooxygenase (COX) and lipoxygenase pathways to synthesize prostaglandin D_2 (PGD_2), PGE_2, LTC_4, LTB_4, LTE_4, and thromboxane B_2 (TXB_2).[31] Basophils and mast cells produce lipid-derived platelet-activating factor.[31] The dominant interleukins and cytokines produced by these cells are those important in type 2 immune responses, specifically IL-4, IL-3, IL-8, IL-13, TNF-α, and CCL3. Basophils, in particular, produce abundant IL-4, which recruits eosinophils and helps drive immunoglobulin class switching to IgE.[31] Mast cells also produce IL-1, IL-6, and GM-CSF, known for their roles in systemic acute inflammatory responses, as well as IL-5, a potent mediator of eosinophil proliferation.[31] Additionally, mast cells synthesize several growth factors and chemokines involved in mast cell proliferation, wound healing, fibrosis, and vascular dynamics including SCF, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), nerve growth factor, and endothelin.[31,37]

ROLE OF BASOPHILS AND MAST CELLS IN TYPE 2 IMMUNE RESPONSES

Basophils and mast cells participate in type 2 immune responses.[36] Adaptive type 2 immunity protects the host from large multicellular invaders, such as helminths and ticks, and is characterized by activation of Th2 lymphocytes and antibody class switching to IgE.[36] The ultimate goal of a type 2 immune response is to create an inhospitable environment culminating in the expulsion of invading pathogens from the host's body. If pathogen expulsion cannot be achieved, type 2 immune responses will encapsulate the invader within a granuloma which shields local tissues from further damage.[36]

Activation of Basophils and Mast Cells

There are numerous initiators of basophil and mast cell activation and degranulation including IgE-mediated FcεRI cross-linking, cytokines, chemokines, chemical agents (e.g., radiocontrast solutions, narcotics, muscle relaxants, calcium ionophores), physical stimuli (e.g., cold), peptides (e.g., neutrophil lysosomal proteins, venoms, bacteria, and bacterial products), and viruses.[27] Basophils and mast cells also express their own unique sets of toll-like receptors (TLR), which facilitate activation during innate immune responses. Basophils of various mammalian species may express TLR1, TLR2, TLR4, and TLR6.[17] The TLR complement of mast cells is much broader, potentially due to their role as tissue sentinel cells, and includes TLR1, TLR2, TLR3, TLR4, TLR6, TLR7, and TLR9.[17]

Mast cell degranulation is also controlled by surface catecholamine receptors (α- and β-adrenoceptors) with opposing effects. Stimulation of α-receptors (e.g., norepinephrine and phenylephrine) or blockade of β-receptors (e.g., propranolol) induces mast cell degranulation. β-blockade, resulting in increased mast cell degranulation and tissue inflammation, can be caused by some respiratory pathogens such as *Bordetella pertussis* or *Haemophilus influenza*.[27] Animals with these infections are also predisposed to development of respiratory allergies.[27] Conversely, mast cell degranulation is inhibited by molecules that block α-receptors or stimulate β-receptors such as isoproterenol, epinephrine, and salbutamol. Thus, these medications may be employed in treatment of type I hypersensitivity reactions.[27]

Additional pharmacologic inhibitors of mast cell and basophil activation include corticosteroids, cyclosporine A, and theophylline.[15]

Degranulation and Production of Mediators by Basophils and Mast Cells

Following activation, basophils and mast cells release preformed inflammatory mediators stored within their cytoplasmic granules. The preformed contents have immediate local and, in some cases, systemic effects. Histamine, a major constituent of basophil and mast cell granules, is a short-lived vasoactive amine that causes an immediate increase in local blood flow and vessel permeability. Enzymes, such as chymase, tryptase, and serine esterases, activate matrix metalloproteases. Matrix metalloproteases, in turn, cause tissue destruction by breaking down tissue matrix proteins.

In addition to releasing preformed mediators, activated basophils and mast cells synthesize additional effector molecules. Synthesis and secretion of lipid mediators are induced via the activation of the cytoplasmic enzyme PLA_2. Activation of PLA_2 results in hydrolysis of mast cell membrane phospholipids and the production of arachidonic acid and subsequent prostaglandins and leukotrienes by the actions of cyclooxygenase or lipoxygenase.[27] The major arachidonic-acid-derived mediators produced by mast cells are PGD_2, LTC_4, LTD_4, and LTE_4. Basophils produce predominantly LTC_4.[27] Platelet-activating factor causes bronchoconstriction, retraction of endothelial cells, and relaxation of vascular smooth muscle.

Production and Action of IgE

After antigen exposure, B cells within lymph nodes undergo antibody class-switching to IgE via IL-4- and IL-13-mediated interactions with Th2 lymphocytes.[36] Upon subsequent exposures, preformed IgE is readily available to rapidly activate basophils and mast cells via IgE-mediated FcεRI cross-linking.[36]

IgE-mediated mast cell and basophil responses are critical in defense against parasitic infections. IgE-dependent mast cell activation in the gastrointestinal tract and lungs promotes the physical expulsion of parasites by initiating mucus release and triggering gastrointestinal peristalsis. Mast cells may also reduce the reproductive capability of gastrointestinal parasites, thereby reducing parasitic load.[14]

IgE-mediated mast cell and basophil responses are also protective against blood-sucking ticks. Upon initial exposure, skin at the site of a tick bite contains degranulated mast cells, basophils, and eosinophils. With subsequent tick-feedings, basophil-dependent resistance to the tick bite develops.[36] Basophils may confer this resistance via activation of the basophil cell-surface receptors, FcεRI, and Fc region of the IgG receptor IIIa (FcγRIIIa).[36] This resistance to subsequent tick feedings reduces transmission of *Borrelia burgdorferi* in mice.[36,42]

Interactions of Basophils and Mast Cells with Eosinophils

Mast cells, basophils, and eosinophils interact with each other at sites of inflammation in protective and pathologic roles. Mast cells produce IL-5 and CCL11, CCL24, and CCL26 that stimulate the release of eosinophils from the bone marrow.[27] Molecules such as CCL11, CCL24, and CCL26, histamine, LTB_4, 5-hydroxytryptamine, and platelet-activating factor attract eosinophils to a site of inflammation.[23] Basophil influx occurs in the late-phase of an allergic response. Eosinophil degranulation releases major basic protein and platelet-activating factor causing additional mast cells and basophils to degranulate. This effect is augmented by mast cells, which further synthesize and secrete IL-3, IL-5, and GM-CSF, thereby enhancing eosinophil and basophil growth, differentiation, and activation.[27]

ROLE OF BASOPHILS AND MAST CELLS IN TYPE I HYPERSENSITIVITY REACTIONS

Most of our knowledge of mast cell- and basophil-mediated responses stems from the investigation of hypersensitivity diseases. Type 1 hypersensitivities are characterized by: (1) Th2 cell activation, (2) high concentrations of IgE, and (3) eosinophilia. Mast cells, basophils, and their granular contents are the central effectors of IgE-mediated immediate type I hypersensitivity reactions and allergic diseases.[27] The clinical and pathologic manifestations of type I hypersensitivity disorders depend upon (1) the tissue location, (2) the degree of previous antigen sensitization, (3) the quantity of antigen to which the animal is exposed, (4) the route of antigen administration, and (5) the chronicity of the resulting inflammatory process.[27]

Exposure to environmental antigens occurs routinely. Yet, most animals show no obvious clinical consequences because they produce primarily IgG or IgA antibodies. However, some animals develop an exaggerated Th2 response to such commonplace antigens. Basophils are believed to be key players in this exaggerated Th2 response by releasing the IL-4 required to initially activate CD4+ T cells and induce immunoglobulin class-switching to IgE production.[31]

IgE antibodies attach to the $\alpha\beta\gamma_2$ form of the FcεRI, the high-affinity surface Fc receptor specific for IgE ε-heavy-chains, on basophils and mast cells. Upon subsequent antigen exposure, the cell-surface-bound IgE molecules immediately join with their complimentary antigen. When antigen concurrently binds to two adjacent cell-surface-bound IgE molecules, it induces aggregation of the IgE molecules and associated FcεRI receptors. This cross-linking of FcεRI by IgE–antigen complexes initiates a signaling cascade resulting in three types of biological responses: (1) secretion of the preformed granule contents, (2) synthesis and secretion of lipid mediators, and (3) synthesis and secretion of cytokines, all of which work together to initiate a local inflammatory response.[27]

Degranulation and release of stored inflammatory mediators, such as histamine, are responsible for the clinical signs of immediate hypersensitivity reactions observed within minutes of allergen exposure. IgE-mediated cross-linking of FcεRI activates protein kinases to induce gene transcription factors, such as nuclear factor of activated T cells (NFAT), nuclear factor κβ (NF-κβ), and activator protein-1 (AP-1). These transcription factors initiate the production of IL-4, IL-5, IL-6, IL-13, and TNF among others. Newly synthesized inflammatory mediators are responsible for the clinically observed late-phase of type I hypersensitivity reactions 2–12 hours after allergen exposure.[27]

LABORATORY EVALUATION OF BASOPHILS

Staining Characteristics

For a description of morphologic features, see section IX.

Basophil Activation Tests

The majority of histamine within peripheral blood is contained within basophil granules. In an attempt to assess hypersensitivity states in several species, including horses and sheep, basophil histamine release assays have been developed. In people, flow cytometric assays are available to assess basophil activation.[20]

Basophil Concentrations in Blood

Due to the low concentration of circulating basophils in most mammals, manual 100-cell differentials are imprecise estimates of the true basophil concentration. Regardless, manual differential counts are often considered the most reliable method of basophil enumeration.

Further complicating basophil enumeration, automated hematology analyzers are fraught with error in basophil identification in veterinary species. Current automated methods are based upon the unique ability of human basophils to resist lysis by a strong surfactant known to lyse nonbasophil leukocytes. Thus, in theory, cells that remain intact after exposure to the surfactant are either basophils or atypical lysis-resistant circulating cells. However, this method does not appear to work well for dogs and cats. For example, the Technicon H1®, Advia 2120®, Sysmex XT-2000iV®, CELL-DYN 3500®, and likely the LaserCyte® do not accurately detect canine basophils even in the presence of pronounced basophilia.[18,19,27,33–35,41] Feline basophils have been identified on the Sysmex XT-2000iV WBC/DIFF scattergram via fluorescence-activated flow cytometry as a distinct population of cells between the neutrophil, eosinophil, and lymphocyte clusters.[32] However, these cells were not detected in the Sysmex XT-2000iv® basophil channel, further confirming the inadequacy of basophil detection via the basophil channel in feline patients.[32] Although the Sysmex XT-2000iV® basophil channel fails to detect canine and feline basophils, mast cells of both species appear to be lysis-resistant and have been detected within the Sysmex XT-2000iV® basophil channel.[18] Interestingly, the Advia 2120® can accurately enumerate lysis-resistant rabbit basophils.[19] The Technicon H1®, Advia 2120®, and Sysmex XT-2000iV® have indicated the presence of basophilia when basophilia was not detected by blood smear inspection.[27,34] Therefore, an increased automated concentration of basophils should be confirmed via blood film evaluation.

Basophilia

Basophilia in mammals is relatively rare. For domestic mammals, only marked or persistent increases in basophil concentrations >200–300 basophils/μL should be considered abnormal.[27] When observed, basophilia is typically associated with an IgE-mediated disorder and often accompanied by eosinophilia. Therefore, primary considerations should be given to allergic diseases and parasitism. Parasites documented to cause basophilia include ectoparasites, gastrointestinal parasites, and vascular parasites.[27]

If parasites and allergic inflammation are ruled out, neoplastic and other nonallergic inflammatory conditions should be considered. Basophilic leukemia is rare, but has been reported in dogs.[22] Basophilia may also occur secondary to other neoplastic disorders such as mast cell neoplasia, lymphomatoid granulomatosis, myeloid leukemias, polycythemia vera, essential thrombocytosis, or idiopathic canine myelofibrosis.[6,7,28,40]

Basopenia

Circulating basophils are in very low concentrations in healthy animals, often resulting in the inclusion of 0 cells/μL within basophil reference intervals. Therefore, basopenia is not recognized in most species. Nevertheless, decreased concentrations of circulating basophils have been reported in people with disorders such as urticaria and anaphylaxis, some inflammatory and immunologic conditions, corticosteroid administration, neoplasia, and hemorrhage.[10]

LABORATORY EVALUATION OF MAST CELLS

Structure, Morphology, and Staining Characteristics

Mast cells are round cells with a moderate volume of pale cytoplasm that contains numerous coarse, metachromatic granules which may stain various shades of red to deep purple. Mast cell nuclei are frequently obscured or faintly stained amid their abundant mass of deeply staining, purple, cytoplasmic granules. When visible, nuclei are usually round and centrally located. Chromatin patterns can range from coarsely granular to finely stippled, depending on cellular maturity. Prominent nucleoli are not typically seen in normal mast cells, but may be seen in mast cell neoplasia. In some species, highly granulated basophils can be confused with mast cells.

When stained with methanolic Romanowsky stains, mast cell granules are easily recognizable. However, mast cell granules may stain poorly or not at all when aqueous-based Romanowsky-type quick stains are used.[1] The exact reason for this phenomenon is unknown. Prolonging the fixation time in order to prevent the theorized dissolution of mast cell granules within the aqueous stain solutions was recently proven ineffective at improving mast cell granule-staining intensity.[16]

Mastocythemia and Marrow Mastocytosis

Because mast cells are normal tissue residents, occasional mast cells (i.e., 0–1/1000 nucleated cells) are present in healthy bone marrow.[2] Bone marrow mastocytosis has been reported in dogs with myelofibrosis, lymphoma, myelosuppressive chronic monocytic ehrlichiosis, aplastic anemia, systemic mastocytosis, and mast cell leukemia.[24,38] Mast cell leukemia is rare in dogs.[6]

Mastocythemia (aka mastocytemia) is the presence of circulating mast cells within peripheral blood. In the past, mastocythemia in dogs was considered highly suggestive of mast cell neoplasia; however, in one study, 95.5% of canine patients with circulating mast cells had no concurrent evidence of mast cell neoplasia.[21] Furthermore, mastocythemia may be observed in animals with inflammatory disease, regenerative anemia, tissue injury, neoplasia other than mast cell tumors, and necrosis.[3,21,30] In addition, mastocythemia of high magnitude is not always indicative of underlying mast cell neoplasia and may occur with inflammatory conditions.[21,30] If other concurrent disease states have been ruled out and/or circulating mast cells have atypical cytologic features, evaluation for visceral, cutaneous, or bone marrow mast cell neoplasia should be performed. In contrast to canines, feline mastocythemia, although rare, is most commonly associated with visceral or cutaneous mast cell neoplasia.[26] Feline mastocythemia has also been reported in feline patients with lymphoid neoplasia, hemangiosarcoma, and chronic kidney disease.[26]

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CHAPTER 48

Monocytes, Macrophages, and Dendritic Cell Production

CLEVERSON D. SOUZA and DOUGLAS J. WEISS

Monocyte Production
Macrophages Production
Dendritic Cell Production
Summary

Acronyms and Abbreviations

AP-1, activator protein-1; BATF3, basic leucine zipper ATF-like transcription factor 3; C/EBP-α, CCAAT/enhancer-binding protein-alpha; CCL2, C-C motif chemokine ligand 2; cDCs, conventional DCs; c-KIT, tyrosine-protein kinase kit; cMoPs, common monocyte progenitors; CMP, common myeloid progenitor; DCs, dendritic cells; FLT3, fms-related tyrosine kinase 3; EMPs, erythro-myeloid progenitors; GMP, granulocyte/monocyte progenitors; HSC, hematopoietic stem cells; IL, interleukin; IRF8, interferon-γ-responsive transcription factor; M-CSF, macrophage colony-stimulating factor; MDP, macrophage/dendritic cell progenitors; miRNAs, microRNA; MPS, mononuclear phagocyte system; pDCs, plasmacytoid DCs; PU.1, hematopoietic transcription factor; SCF, stem cell factor; TLR, toll-like receptor.

The mononuclear phagocyte system (MPS) is organized as a complex structure of ontogenetically and functionally distinct cells, that have been best described in mouse and human and to a lesser extent in other species.[8,15,25] These cells consist of multiple subsets of monocytes, macrophages, and dendritic cells (DCs) traditionally defined by their expression of various surface markers, morphologic characteristics, and biological function.[8] Recent studies have challenged previous models of MPS ontogenesis.[7,8] Monocytes and macrophages protect the body against bacterial infections through production of microbicidal compounds including reactive nitrogen intermediates and proinflammatory cytokines. These cells also function to preserve tissue homeostasis through scavenger activity and production of molecules to aid tissue repair.[12] Dendritic cells are highly efficient antigen-presenting cells with key roles in regulation of tolerance and immune defense against pathogens and tumors.[17] This chapter aims to describe the ontogenesis, transcriptome profiles, and features of the MPS, particularly in mice and human. This area is incompletely understood and will undoubtedly continue to undergo redefinition and modification.

MONOCYTE PRODUCTION

Monocytes represent approximately 3–10% of all nucleated blood cells with a marginal pool of considerable size in the spleen and lungs that can be mobilized on demand.[2,4] These cells are defined morphologically as well as by their characteristic cell-surface markers, and gene and microRNA expression signatures. Recent studies indicated that monocytes initiate inflammation, patrol the vasculature, transport antigen to lymphoid tissue, and participate in tumor cell destruction.[3,4]

Monocyte development in adult mice, and likely most species, depends on macrophage colony-stimulating factor (M-CSF). Monocytes arise from myeloid precursor cells in the fetal liver and bone marrow, during both embryonic and adult hematopoiesis (Figure 48.1).[3,4] In contrast to adult monocytes, fetal liver monocytes proliferate independent of M-CSF.[3,4] However, in-depth information on these cells is lacking. Monocyte ontogeny gained considerable attention in the last decade with the identification of dedicated clonotypic precursor cells, termed *macrophage/dendritic cell progenitors* (MDPs), in mouse bone marrow.[5,7,10] MDPs

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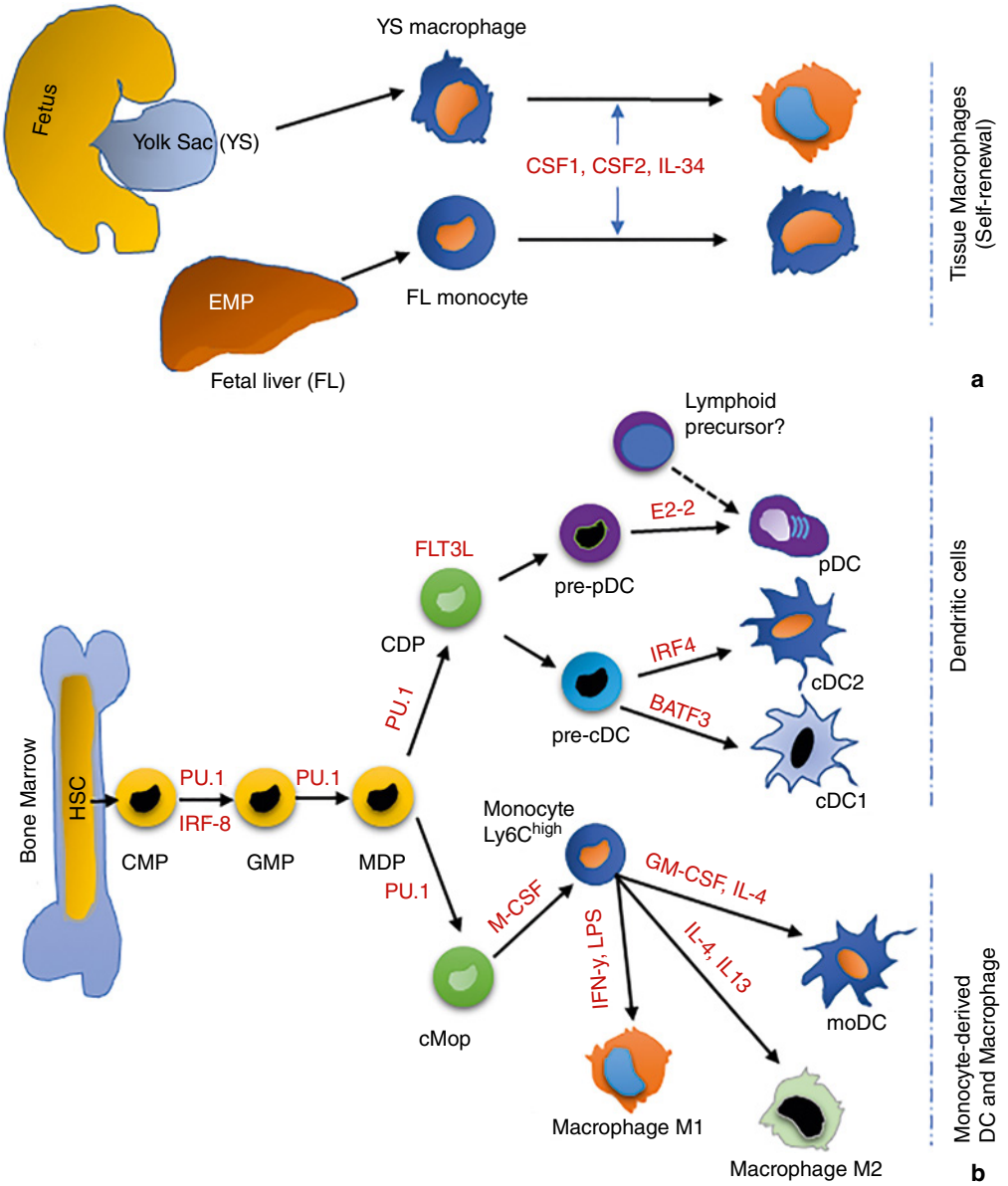


FIGURE 48.1 Current understanding of embryonic and hematopoietic development of mononuclear phagocytes (MNPs) in mice. (a) Some tissue macrophages have an embryonic origin, from yolk sac macrophages and fetal liver monocytes; (b) In bone marrow, monocytes, macrophages, and dendritic cells (DCs) originate from distinctive cell lines. DCs and monocytes originate from bone marrow committed DC progenitors (CDP) and common monocyte progenitors (cMoPs), respectively. Dendritic cells express diverse transcription factors important for its development. Examples include basic leucine zipper ATF-like transcription factor 3 (BATF3) for cDC1s, interferon regulatory factor 4 (IRF4) for cDC2s, and E2-2 for plasmacytoid DCs (pDCs). These cells are also dependent on various growth factors such as fms-related tyrosine kinase 3 (FLT3) ligand for DCs, M-CSF for monocytes, and immunomodulatory cytokines for macrophages and monocyte-derived dendritic cells.

are defined as lineage-negative cells that express tyrosine-protein kinase kit (c-KIT), fms-related tyrosine kinase 3 (FLT3), and M-CSF receptor.[3,18] MDPs lack the potential to produce neutrophils, but can give rise to monocytes, plasmacytoid dendritic cells (pDCs), and conventional dendritic cells (cDCs).[3,5,10,11,16] MDP differentiation toward monocytes includes a defined transient intermediate, the common monocyte progenitor (cMoP).[3,11,16] cMoPs differ from MDPs by their lack of FLT3 expression, as well as by inability to differentiate in cDC or pDC. cMoPs have been shown to give rise to both Ly6C+ and Ly6C− monocyte subsets. In mice, the spleen is a major source of Ly6C+ and Ly6C− monocytes that are mobilized to the peripheral blood in response to tissue damage.[18] This response often results in an acute monocytosis that facilitates tissue repair.

Variable phenotypic changes and expression of transcription factors are involved in defining monocyte lineage commitment.[2,11,28] A multitude of factors, including chronic stress, inflammation, and infection, have been shown to effect myeloid differentiation. For example, diabetic mice have increased proliferation of both

common myeloid progenitor (CMP) and granulocyte/monocyte progenitors (GMP), resulting in increased levels of peripheral blood monocytes and neutrophils.[28] In addition, bacterial infections increase monopoiesis, through toll-like receptor (TLR) signaling, which increases C-C motif chemokine ligand 2 (CCL2) signaling in bone marrow and causes rapid monocyte emigration. Most of these signals have been shown to modify peripheral blood monocytes by stimulating the proliferation of progenitor cells and regulating the release of cytokines.[28] Moreover, it is likely that epigenetic changes occur within myeloid progenitors during disease, which can trigger transcriptional reprograming of monocytes.

Monocyte differentiation is regulated by lineage-specific transcription factors that are turned on or off during different phases of hematopoiesis.[2,11] Hematopoietic transcription factor (PU.1) is a key transcription factor important in the early phases of myeloid and lymphoid development. PU.1-deficient mice lack monocytes as well as granulocytes, and some subsets of lymphocytes.[2,28] Proteins of the CCAAT/enhancer-binding protein (C/EBP) family are crucially important in the development and maintenance of myeloid cell subsets. Analysis of C/EBPβ knockout mice shows a selective reduction in peripheral blood monocytes, but not of other lineages. How C/EBP regulates commitment of monopoiesis is poorly understood.[2,11,28]

Interferon-γ-responsive transcription factor (IRF8) is critical for monocyte development in bone marrow.[2,11,28] IRF8 promotes monocyte differentiation and inhibits granulocyte production. IRF8 expression persists in differentiated monocytes and macrophages, but declines in neutrophils. Other transcription factors thought to be involved in the production of monocytes have been reviewed.[28] MicroRNAs (miRNAs) can suppress target protein production by inducing RNA degradation and by hindering protein translation. How miRNAs regulate myelopoiesis is poorly understood.[28] miR-146a is differentially regulated in human and murine monocyte subsets and appears to control Ly6C+ monocyte propagation during inflammation, but has no effect on Ly6C– monocytes.[28] Interestingly, miRNAs have been shown to participate in macrophage polarization to M1 or M2 subsets.[23] The intricacies of transcription factor regulation and function during monocyte development are incompletely understood. Understanding how these transcription factors are controlled by noncoding RNAs, miRNAs, and metabolic and epigenetic changes that occur in response to environmental cues may aid the development of novel therapeutic targets to assist in controlling the inflammatory response.

MACROPHAGES PRODUCTION

Macrophages are rarely present in blood. These cells occur as phenotypically heterogeneous forms depending on the tissue of residence. Some examples include alveolar macrophages, Küpffer cells, Langerhans cells, osteoclasts, and microglia. These mononuclear phagocytes (MNPs) play critical roles in many fundamental biological processes such as host defense against pathogens and tissue homeostasis.[6]

For many years, the overall assumption was that macrophage populations were derived from blood monocytes.[15,26] Current murine and human studies challenge this assumption and have shown that the majority of tissue-resident macrophages develop during embryogenesis independently of hematopoietic stem cells (HSC).[7,10,23] These macrophages are characterized by the ability to self-renew and to self-maintain in adult tissues without the need for replenishment by monocytes. The two main sources of tissue-resident macrophages are most likely from yolk-sac macrophages (contributing to microglia production) and yolk-sac endothelium (which produce erythro-myeloid progenitors (EMPs); Figure 48.1). EMPs migrate, proliferate, and differentiate into monocytes or premacrophages in the fetal liver.[10,23] Subsequently, monocytes or premacrophages colonize various fetal tissues where they differentiate into long-lived macrophages. Interestingly, murine EMPs seem to be the source of most tissue-resident macrophages.[7] Hematopoietic-stem-cell-derived monocytes originate from the aorta–gonad mesonephros, proliferate in the fetal liver, and colonize the bone marrow before birth.[7,10,23] Bone marrow hematopoietic stem cells produce Ly6C+ monocytes.[10,23] During tissue damage, Ly6C+ monocytes quickly travel into inflamed tissues and differentiate into inflammatory macrophages and DCs.[1,4,12] Some tissue-resident macrophage populations, including intestinal macrophages and skin dermal macrophages, are derived from Ly6C+ monocytes and not from embryonic, self-renewable macrophages.[1,4,10] Thus, during times of need, there will be variability in the origin of populations of mononuclear phagocytes in tissue.

PU.1 is the most important transcription factor in macrophage development.[2,11,28] Studies have shown the majority of monocytes, macrophages, and mature B cells are absent in PU.1 knockout mice. Moreover, fetal progenitor cells containing macrophage precursors are also decreased in PU.1 knockout mice. PU.1 works in harmony with other transcription factors including C/EBPs, activator protein-1 (AP-1), and IRFs.[28] M-CSF signaling is required for the proliferation, survival, and differentiation of macrophages, as shown in M-CSF receptor (M-CSFR)-knockout mice studies.[2,11,28] Two ligands have been described for the M-CSFR. One is M-CSF and the other interleukin (IL)-34. The interaction of these factors with the M-CSF receptor activates tyrosine signaling pathways, thus, regulating cell proliferation and survival.[28] As discussed in the earlier text, most tissue-resident macrophages are self-renewing and long-lived; however, the molecular mechanisms involved in these processes remain undefined.

DENDRITIC CELL PRODUCTION

One of the most important functions of macrophages and DCs is detection of danger through recognition of damage-associated and pathogen-associated molecular

patterns. In addition, DCs have greater ability to prime and sensitize immune cells compared to macrophages. Hence, DCs play a key role in organized and effective immune responses.[20] Recent studies advanced our understanding on the ontogeny of DCs in the murine system through the recognition of adult, bone-marrow-derived, monocyte-independent DC lineages.[9,17] This is of central importance for DC distinctiveness as highlighted by the discovery of conserved transcription factors involved in the DC development and by the documentation of a unique, well-conserved, transcriptomic profile in DCs that is distinct from monocytes and macrophages.[14,17,19,20,22,24]

Murine ontogenic studies have identified the committed DC progenitors (CDPs) with a select ability to differentiate solely into DC.[14] CDPs differentiated in cDC1, cDC2, and pDC with very specific transcription factor dependency.[7] This pathway is distinct from DC originated from monocyte-derived dendritic cells (MoDCs). MoDCs are ontogenetically and functionally distinct, which represent a steady-state DC in the mucosa and dermis, which is vital for tissue homeostasis.[17,20] In addition, MoDC plays a key role as inflammatory DC.[17,20]

Several recent studies, mostly in the murine model, have identified cell-lineage-defined hematopoietic factors required for differentiation of DC and MoDC.[7,14,17,18] FLT3 represents a key growth factor for development of cDC1, cDC2, and pDC, whereas M-CSF is critical for MoDC development.[14,17,18] DC differentiation and proliferation are driven by a plethora of well-defined transcription factors. In the murine model, the crucial factors for DC production are E2-2 for pDC, basic leucine zipper ATF-like transcription factor 3 (BATF3) for cDC1, and IRF4 for cDC2.[7,14,17] Given the degree of conservation, it can be expected that similar transcription factors will control DC development in veterinary species.

The analysis of DC uniqueness through transcriptomic profiling in murine and human DCs has shown that this approach is powerful for differentiating macrophages from DC.[27] In addition, transcriptomic analysis represents a novel and elegant tool to determine the phylogenetic relationship between immune system cell types, including monocytes, macrophages, DCs, and lymphocytes.[13,27] In this regard, studies have shown that many differentially expressed genes on DC subsets are conserved between human and mice.[14,21] A similar approach should be productive in veterinary medicine.

SUMMARY

Until recently, the MPS provided an effective framework for understanding the development of mononuclear phagocytes. This model, in which monocytes were the common precursor to macrophages and DC, has given way to a system where long-lived macrophages and pure DC lineages contribute to tissue cell populations. Mouse models have been crucial for the analysis of hematopoiesis and continue to provide insights into the pathways of monocytes, macrophages, and DC development in humans and veterinary species. While in vivo and in vitro studies of monocyte-derived macrophages and DCs have provided many stimulating experimental results, the real physiological context of this body of work remains incompletely understood. In the future, single-cell-RNA-sequencing analysis technologies, immunophenotypic assessment using mass cytometry, epigenetic programing, and more classical immunophenotype techniques will be instrumental to assessment of functional aspects of these cells, particularly at sites of inflammation. It will be possible to identify previously unknown cell subsets and to identify interactions with other cells of the immune system. Understanding the ontogeny of the monocytes, macrophages, and DC more fully offers the potential for the discovery of novel cell-mediated therapies. In this regard, complementary research in veterinary species is essential.

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CHAPTER 49

Monocytes and Macrophages and Their Disorders

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Monocytes
  Subsets and Functions
  Phenotypic Appearance and Ultrastructure
Macrophages
  Subsets and Functions
  Phenotypic Appearance and Ultrastructure
Dendritic Cells
  Subsets and Functions
  Phenotypic Appearance and Ultrastructure
Monocyte Disorders
  Monocytopenia
  Monocytosis
  Monocyte Dysfunction
Macrophage Disorders
  Acquired Defects in Macrophage Activation and Function
  Hemophagocytic Syndrome
  Lysosomal Storage Diseases
  Idiopathic Systemic Granulomatous Disease in Horses and Cattle

Acronyms and Abbreviations

ADCC, antibody-dependent cellular cytotoxicity; AIDS, acquired immunodeficiency syndrome; APC, antigen-presenting cells; CD, cluster of differentiation; CDP, common dendritic cell precursor; CLEC 9A, C-type lectin domain containing 9A; CPV, canine parvovirus; CSF, colony-stimulating factor; DAMPs, danger-associated molecular patterns; DCs, dendritic cells; DCIR2, dendritic cell inhibitory receptor-2; DTH, delayed-type hypersensitivity; EHV, equine herpesvirus; ETs, extracellular traps; FGF, fibroblast growth factor; FIV, feline immunodeficiency virus; HIV, human immunodeficiency virus; HPS, hemophagocytic syndrome; IFN, interferon; IL, interleukin; IMHA, immune-mediated hemolytic anemia; IRF, interferon regulatory transcription factor; ISGD, idiopathic systemic granulomatous disease; LPS, lipopolysaccharide; LSDs, lysosomal storage diseases; MARCO, macrophage receptor with collagenous structure; M-CSF, macrophage-colony stimulating factor; MCP-1, monocyte chemoattractant protein-1; MDP, macrophage/DC precursor; MDSCs, myeloid-derived suppressor cells; METs, macrophage extracellular traps; MHC, major histocompatibility complex; MIP-1α, macrophage inflammatory protein-1α; mTOR, mammalian target of rapamycin; MMP9, matrix metallopeptidase 9; NADPH, nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor-kappa B; NLRs, nucleotide-binding domains and leucine-rich repeat containing receptors; NO, nitric oxide; PAMPs, pathogen-associated molecular patterns; PAS, periodic acid–Schiff; PRRs, pattern recognition receptors; RANTES, regulated upon activation, normal T cell expressed and presumably secreted; RLRs, retinoic-acid-inducible gene-I-like receptors; ROS, reactive oxygen species; RTX, repeats in toxin; SNPs, single-nucleotide polymorphisms; STAT, signal transducer and activator of transcription; TAMs, tumor-associated macrophages; TEM, transmission electron microscopy; TLRs, toll-like receptors; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; WC1, workshop cluster-1.

Formerly known as the reticuloendothelial system, the mononuclear phagocyte system is composed of blood, bone marrow, and tissue-derived monocytes, macrophages, dendritic cells (DCs), and osteoclasts.[17] The mononuclear phagocyte system plays an important role in innate and adaptive immunity, interacting with

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lymphoid cells to mediate the body's immune response to pathogens, vaccines, tumors, and other sources of self- and non-self-antigens.[15,17] These cells also contribute significantly to tissue repair, including angiogenesis, collagen deposition, and matrix remodeling.[32] Although there are shared phenotypic and functional traits among the cells of the mononuclear phagocyte system, there is also considerable heterogeneity, which derives from their surrounding microenvironment.[6,24]

MONOCYTES

Once released into circulation, monocytes have a half-life of 1–2 days, after which time, they migrate into various tissues, where they differentiate into macrophages and dendritic cells.[51] Similar to neutrophils, they are frequently separated into a circulating pool and a marginating pool to help conceptualize their distribution and kinetics within the vasculature.[49] Although rabbits and mice have large marginating pools of monocytes, their presence has not been documented in other veterinary species.[49] Monocyte reservoirs exist within the bone marrow and spleen, specifically within the subcapsular red pulp.[37]

Subsets and Functions

Two distinct subsets of monocytes have been identified in mice, based on surface expression of the molecule Ly6C.[6,32,37] Using the monoclonal antibody *Gr1*, murine monocytes can be separated into Ly6C^{high} (inflammatory monocytes) and Ly6C^{low} (patrolling or resident monocytes), which correspond with the human subtypes CD16– and CD16+, respectively.[37] Although expression of Ly6C has garnered the most attention, expressions of CD11a, CD11b, CD16, CD62L (L-selectin), CD43, and lymphocyte function-associated antigen-1 (LFA-1) have also been shown to differ significantly between these two populations.[15,37,50] Ly6C^{high} monocytes, due to expression of C-C chemokine receptor type 2 (CCR2), respond to monocyte chemoattractant protein-1 (MCP-1 or CCL2), MCP-3 (CCL7), and MCP-5, and are recruited to sites of tissue injury to participate in inflammatory and phagocytic functions.[22] in vitro experiments in mice have shown that Ly6C^{high} monocytes are the predominant subtype in wound infiltrates up to 6 hours after muscle injury or tissue trauma.[32,37] These inflammatory monocytes ultimately differentiate into classically activated, M1-like macrophages and various DC subtypes, as shown in infectious disease models involving *Listeria monocytogenes* and *Toxoplasma gondii*.[15] In addition, Ly6C^{high} monocytes may contribute to myeloid-derived suppressor cells (MDSCs)—a group of myeloid cells shown to aggregate in the lymphoid tissues of mice with bacterial and parasitic infections, autoimmune diseases, traumatic lesions, neoplasia, and other causes of chronic inflammation.[13,50] MDSCs are capable of suppressing T-cell function, and enhancing tumor growth and metastasis by promoting angiogenesis via production of factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and matrix metallopeptidase 9 (MMP9).[13] Carcinomas (i.e., mammary, colon) demonstrate the most significant expansion in response to MDSCs.[50] MDSCs are phenotypically distinct from tumor-associated macrophages (TAMs), as the former demonstrate relatively increased expression of F4/80 and the macrophage-colony stimulating factor (M-CSF) receptor (CD115).[13]

By contrast, Ly6C^{low} monocytes, which express CX_3CR_1 (the chemokine receptor for fractalkine) and CCR5 (the receptor for macrophage inflammatory protein- 1α (MIP-1α), MIP-1β, and regulated upon activation, normal T cell expressed and presumably secreted (RANTES)), are suspected to play more of an anti-inflammatory role and contribute to reparative functions within damaged tissues.[22] Also termed patrolling or resident monocytes, Ly6C^{low} monocytes have been shown to slowly crawl along the luminal aspect of vascular endothelial cells, surveying for potential damage or infection.[15,37] This stands in stark contrast to the "rolling" demonstrated by inflammatory monocytes and granulocytic cells preparing for diapedesis. Influx of Ly6C^{low} monocytes occurs during the later phase of tissue injury, and they serve as the predominant subset during repair. The method of influx, however, appears to differ depending on the site of injury. While studies involving myocardial infarction reveal recruitment of a distinct population of Ly6C^{low} monocytes 4 days after injury, similar studies involving skeletal muscle are more suggestive of in situ conversion of inflammatory Ly6C^{high} monocytes.[15,32] In addition to promoting angiogenesis and collagen deposition, Ly6C^{low} monocytes differentiate into alternative, M2-like macrophages and CD103^{-} pulmonary DCs.[15,22]

Phenotypic Appearance and Ultrastructure

Monocytes are cytologically similar across species (see Section IX). They are large leukocytes (approximately 15–20 μm in diameter) with a variably shaped (ovoid, reniform, or convoluted) nucleus, granular to lacy chromatin, and abundant amounts of blue-gray cytoplasm that is variably vacuolated, depending on degree of activation or reactivity.[5,36] They have large Golgi complexes and surface filopodia, which can be appreciated using transmission electron microscopy (TEM).[5] After activation with interferon-γ (IFN-γ), cytoplasmic volume increases due to increased numbers of free ribosomes, mitochondria, and lysosomal granules.[5,49]

Monocytes in reptilian species are different in that they may display azurophilic (pink/purple), fine to coarse, intracytoplasmic granules.[45] While azurophils are considered a variation of the monocyte in non-squamate reptiles, they are a distinct type of leukocyte in snakes, and demonstrate functional similarities to the neutrophil.[1,45] Azurophils of snakes have round nuclei, while those in other reptiles have lobulated nuclei.[1,45]

MACROPHAGES

Unlike other myeloid cells, macrophages are long-lived, persisting in tissues for several months to more than a year in some species, except for those responding to inflammatory stimuli, or those derived from the differentiation of monocytes.[42,49]

Subsets and Functions

Although macrophages perform several conserved functions that are crucial to the orchestration of innate and adaptive immune responses, they represent a heterogeneous population of cells whose differences are governed by origin, activating stimuli, and microenvironment. While blood monocytes contribute to resident populations of macrophages within the gastrointestinal tract and dermis, those in the bone marrow, central nervous system (CNS), liver, and lung originate from precursors that derive from the yolk sac and fetal liver.[6,17] Function is intricately linked with tissue distribution. For example, macrophages within the splenic red pulp contribute to heme catabolism, while macrophages in the splenic white pulp contribute to clearance of apoptotic lymphocytes.[17] Shared functions include the phagocytosis of antigens and pathogens, which occurs via phagocytosis, and the recognition of specific moieties and organic ligands by pattern recognition receptors (PRRs). PRRs, which include toll-like receptors (TLRs), nucleotide-binding domains and leucine-rich repeat containing receptors (NLRs), and retinoic-acid-inducible gene-I-like receptors (RLRs), are expressed both intracellularly and extracellularly, and require coreceptors (i.e., CD14, CD36) for binding and signaling.[17] Recognition of pathogen-associated and danger-associated molecular patterns (PAMPs and DAMPs, respectively) results in the activation of an intracellular signaling cascade involving the adaptor protein MyD88. After the translocation of nuclear factor-kappa B (NF-κB) or IRF3/IRF7 into the nucleus, synthesis of specific cytokines (i.e., interleukin (IL)-1β) or interferons ensues, resulting in continued leukocyte recruitment and activation.[24]

Pathogen inactivation can also occur through a programed cell death pathway ("ETosis") that involves the production of extracellular traps (ETs). Similar to granulocytes and mast cells, macrophages are capable of releasing a web-like complex (macrophage extracellular traps, METs) of nuclear material, histones, and cellular proteins, which entrap and eliminate some microorganisms.[8] This process, which can occur in less than 30 minutes, has not been consistently observed during different experimental conditions, and it is therefore suspected that certain macrophage subtypes may be more prone to ETosis. ET release may be intrinsically linked to certain pathogenic proteins and toxins, as ETosis has been shown to occur in a leukotoxin-dependent and repeats-in-toxin (RTX)-dependent manner during in vitro studies involving macrophages and the bacterial pathogens *Mannheimia hemolytica* and *Escherichia coli*, respectively.[3,8] Similar to neutrophils, ET formation by macrophages is also dependent on NADPH-oxidase activity.[3] Further studies are necessary to determine what role METs or their overproduction may play in disease pathology, including thrombosis, coagulation disorders, and metastatic disease.[3,27]

Despite the phenotypic and functional heterogeneity of macrophages, they are commonly subdivided into two distinct classes (M1 and M2), which were derived based on in vitro studies (Table 49.1). Commitment to these subtypes has been shown to occur within an hour of exposure to stimulatory cytokines in vitro and is dependent on mammalian target of rapamycin (mTOR).[37] The macrophages, however, can transition in vivo during stages of inflammation and repair. Although convenient, the subsets fail to capture the heterogeneity of the macrophage population and overlook macrophages that exhibit characteristics from both the M1 and M2 phenotypes, or do not correspond to either.[32,37]

M1 macrophages are considered "classically activated," as their differentiation is triggered after exposure to cytokines (i.e., IFN-γ, tumor necrosis factor (TNF)-α) and TLR agonists (i.e., lipopolysaccharide (LPS)).[6,24] These macrophages are proinflammatory and release high levels of IL-12 and IL-23, contributing to a Th1 response, which further supports microbicidal activity and pathogen killing.[6,39] The high levels of reactive oxygen intermediates and nitric oxide released by M1 macrophages also promote tumoricidal activity.[39] M1 macrophages can be further divided into M1a and M1b subtypes, which primarily differ based on receptor expression. M1a macrophages express high levels of major histocompatibility complex (MHC), costimulatory molecules (CD40, CD80, and CD86), and complement receptors (CD35, CD11a/CD18, CD11b/CD18, and CD11c/CD18), which enhance their phagocytic abilities and function as antigen-presenting cells (APCs).[24,37,49] Alternatively, M1b macrophages express high levels of Fc receptors and scavenger receptors workshop cluster-1(WC1), the latter of which recognizes negatively charged macromolecules, and is concurrently expressed on gamma delta T cells.[37,49] Fc receptors (i.e., FcγR, FcεRI), which are also expressed on the cell surface of monocytes, neutrophils, and mast cells, enhance phagocytosis and antibody-dependent cellular cytotoxicity (ADCC) by allowing for the recognition of immunoglobulin-coated cells or pathogens, and opsonins of innate immunity (i.e., C-reactive protein).[25]

M2 macrophages, which have been broadly classified as anti-inflammatory, or "alternatively activated" macrophages, secrete anti-inflammatory IL-10, chemokine (C-C motif) ligands, support a Th2 response, and play an important role in tissue repair.[24,39,49] They have enhanced endocytic capability due to the increased expression of mannose receptor (CD206) and dectin-1, which allow for the recognition of mannosyl residues, fucosyl residues, and other carbohydrate signatures,

TABLE 49.1 Characterization of macrophage subtypes.

Phenotype	Stimulus	Increased expression/production	Biological function
M1a$^{mTOR\ low}$	IFN-γ + LPS or TNF-α	Complement receptors MHC-I and MHC-II CD80/CD86 IL-1β, IL-12, IL-6, TNF-α CXCL9, CXCL10, CXCL11 CCL2, CCL3, CCL5 CCL2, CCL3, CCL5 Reactive oxygen species (ROS), nitric oxide (NO)	Classically activated Th1 responses; type I inflammation; delayed-type hypersensitivity (DTH); killing of intracellular pathogens; tumor resistance
M1b$^{mTOR\ low}$	LPS	Fcγ receptors Scavenger receptors Co-stimulatory signals IFN-β, IL-1β, IL-6, TNF-α CXCL9, CXCL10, CXCL11 CCL2, CCL3, CCL5 ROS Macrophage receptor with collagenous structure (MARCO)	Innate activated macrophages Share many of the proinflammatory properties with M1a macrophages, but they do not secrete biologically active IL-12, and therefore do not directly drive Th1 responses
M2a$^{mTOR\ high}$	IL-4 and IL-13	Fcγ receptors Scavenger receptors MHC-II CD80/CD86 IL-1RA, IL-10, TGF-β CCL2, CCL13, CCL17 Macrophage fusion Autophagy	Th2 responses; type II inflammation; allergy; killing and encapsulation of parasites
M2b$^{mTOR\ high}$	Immune complexes and TLR/IL-1R ligands	MHC-II CD80/CD86 IL-10 CCL1, CCL20 CXCL3	Th2 activation; immunoregulation
M2c$^{mTOR\ high}$	IL-10, TGF-β, glucocorticoids	Fcγ receptors Scavenger receptors IL-1RA, IL-10, TGF-β CXCL4, CXCL13, CXCL23 CCL18	Immunoregulation; matrix deposition and tissue remodeling

and permit the binding of parasitic organisms, bacteria, and yeast.[24,32,39,49] Due to decreased production of nitric oxide, M2 macrophages have a reduced ability to eliminate intracellular pathogens.[24] M2 macrophages can be divided into three subtypes, which differ based on activating stimuli from the surrounding environment. M2a macrophages are stimulated by IL-4 or IL-13, which interact with the common receptor IL-4Rα, subsequently activating signal transducer and activator of transcription 6 (STAT6).[32,37,39] M2b are activated following exposure to LPS, immune complexes, and CD40 ligand, while M2c macrophages differentiate following exposure to IL-10, transforming growth factor (TGF)-β, and glucocorticoids.[37,39] M2a and M2c macrophages have been shown to increase fibroblast collagen production and myofibroblast differentiation in culture, which likely occurs through the production of TGF-β.[32]

Phenotypic Appearance and Ultrastructure

Macrophages share similar cytologic features and ultrastructural characteristics to monocytes, particularly when unstimulated (Figure 49.1). In addition to large Golgi complexes, they possess many primary lysosomes, which contain acid hydrolases, lysozyme, collagenase, elastase, and other enzymes important for the elimination of intracellular and extracellular pathogens.[5,49] After activation, macrophages generally display enlarged Golgi complexes, along with increased numbers of lysosomes, mitochondria, and pinocytic vesicles.[49] The heterogeneity of their phenotypic appearance, however, is reflected in their ultrastructural appearance, with significant variation noted on TEM. Fusion of macrophages, which can occur secondary to antigenic stimulation or cytokine stimulation from T lymphocytes, results in the

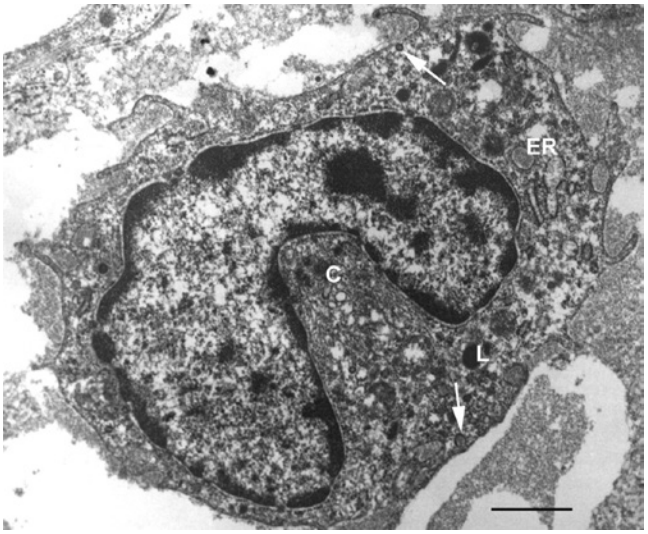


FIGURE 49.1 Porcine tissue macrophage from the skin. Endoplasmic reticulum (ER), primary lysosomes (L), clathrin-coated pits (arrows). Bar, 2 μm. (**Source:** Courtesy of W. L. Steffens III, University of Georgia)

formation of multinucleated giant cells.[5] These cells, which are components of granulomatous inflammation, have extensive, undulating membranes that form from the interdigitation of individual cells. Although cytokinesis is inhibited in these cells, karyokinesis is not.[5]

A specific group of pigmented macrophages has been defined in most fish, amphibians, and reptiles.[7] Melanomacrophages are found in lymphoid and nonlymphoid tissues, including kidney, spleen, and intestines, and are occasionally observed in circulation, particularly during inflammatory conditions.[18,46] The size and number of melanomacrophage centers in amphibians and reptiles have been reported to increase with season and age, respectively.[20,43] Despite their designation, melanomacrophage pigments consist predominantly of lipofuscin and hemosiderin, with smaller amounts of melanin.[18] Functions of these cells include phagocytosis of effete erythrocytes, iron storage, neutralization of free radicals, and phagocytosis of pathogens. It has been suggested that melanomacrophages are the primary defenders against infection during hibernation because they have been shown to retain their effectiveness at near-freezing temperatures in turtles.[18] Due to their role in antigen storage and processing, their close association with plasma cells, and their 5′-nucleotidase profile, melanomacrophages are speculated to function similarly to follicular dendritic cells, which supports the theory that melanomacrophage centers represent primitive germinal centers.[18,46]

DENDRITIC CELLS

Dendritic cells are MHC-II+, CD11C+ APCs that serve as essential conduits between the innate and adaptive immune responses. DCs are the APCs mainly responsible for the cross-presentation of exogenous antigens to the MHC class I pathway, which enables activation of cytotoxic CD8+ T cells in vivo.[19] Although much regarding their origin remains to be elucidated, it has been shown that DC subsets arise from both the MDP, a common progenitor cell for monocytes, macrophages, and DCs, as well as a common dendritic cell precursor (CDP), which does not respond to colony-stimulating factor-1 (CSF-1).[2,28] It is suspected that the MDP pathway is involved in DC generation during inflammation, while the CDP pathway maintains stable populations of DCs during homeostasis.[2]

Subsets and Functions

DCs are generally classified into two categories—plasmacytoid (pDCs) and classical (cDCs), the latter of which constitute all DCs other than pDCs.[28] Present within blood and lymphoid tissues, pDCs have a plasma-cell-like morphology and express a narrow range of PRRs, including TLR7 and TLR9. They produce high levels of type I interferons after exposure to viral antigens or TLR ligation.[6] They can also serve as antigen-presenting cells and mediate T-cell responses, despite their relatively low expression of MHC class II and CD11c.[2,28]

Classical DCs, which reside within most lymphoid and nonlymphoid tissues, are highly phagocytic cells that express high levels of MHC class II and costimulatory molecules (CD80/CD86) following activation.[2,5,28] They can migrate from tissues to the T- and B-cell zones of lymphoid organs via CCR7 in order to regulate lymphocyte responses to antigens during the steady state, as well as during inflammation. When circulating in lymph, DCs are referred to as veiled cells.[5] Within nonlymphoid tissues, DCs comprise between 1% and 5% of tissue cells, and are divided into two major subsets—CD103+CD11b− and CD103+CD11b+, which differ based on origin, expression of FcγRI, FLT3, and responsiveness to FLT3 ligand.[28] Within lymphoid tissues, DCs are divided into two subsets, CD8+ and CD11b+, and are colloquially referred to as interdigitating or follicular DCs based on location within the lymph node, and preference for T-cell activation versus B-cell-activation.[5,28] CD8+ DC function and origin are shared with the previously described CD103+CD11b− DCs. Therefore, this subset expresses high FLT3 levels and lectin receptors, including CD205, C-type lectin domain containing 9A (CLEC9A), and langerin.[28] CD11b+ DCs, which exclusively express dendritic cell inhibitory receptor-2 (DCIR2), are the predominant cDCs in lymphoid tissues, apart from the thymus. This subset can potentially be further divided based on the expression of endothelial cell-specific adhesion molecule (ESAM), particularly within the spleen.[28]

Langerhans cells are a specific type of DC that primarily reside within the suprabasal epidermis, where they serve to process and transport cutaneous antigens to draining lymph nodes for T-cell activation. They also have been documented within the gingiva, epithelium of the gastrointestinal tract, and lymphoid tissues, such

as lymph nodes, thymus, and tonsils.[5] Langerhans cells express lower levels of MHC class II than other DC subsets. They are characterized by expression of E-cadherin and high levels of CD207 (a calcium-dependent C-type lectin).[28]

Phenotypic Appearance and Ultrastructure

Dendritic cells are characterized by the presence of surface dendrites, which transform from filiform to beaded dendrites after activation.[5] Ultrastructural studies have identified nodose enlargements of the beaded dendrites that are responsible for the entrapment of antigen–antibody complexes.[5] Langerhans cells can be identified ultrastructurally by the presence of intracytoplasmic Birbeck granules, which are rod- or racquet-shaped granules, that appear as electron-dense striations. Langerin (CD207) is involved in their formation.[5,28]

MONOCYTE DISORDERS

Monocytopenia

Although monocytopenia is generally not considered to be of clinical significance, it can be observed secondary to a variety of infectious and non-infectious disorders (i.e., estrogen toxicity, chemotherapy). Common infectious causes include parvovirus (canine parvovirus-1 (CPV-1), CPV-2a, CPV-2b) and chronic canine monocytic ehrlichiosis, both of which can result in bone marrow hypoplasia and the destruction of leukocyte precursors.[16] Monocytopenia can occur in up to 53% of cases of dogs with chronic monocytic ehrlichiosis caused by *Ehrlichia canis*.[29] Acute cases, alternatively, can result in a monocytosis. In addition to affecting absolute numbers, infection with CPV-1 has been shown to impair monocyte function, resulting in decreased phagocytic ability and impaired pathogen killing, which likely predisposes to comorbidities.[16] Monitoring the monocyte count may be useful for predicting recovery from bone marrow suppression, since monocytes and neutrophils share a common progenitor cell, and the time taken for monocyte production (3 days) is shorter than the time necessary for neutropoiesis (6 days).[16,49]

Monocytosis

Monocytosis is a common, reactive response to inflammatory disorders that typically reflects a need for macrophages in diseased blood or tissues.[44] It is commonly observed in infectious disorders (e.g., acute and chronic bacterial, fungal infections, rickettsial infections, protozoal infections), immune-mediated diseases (e.g., immune-mediated hemolytic anemia (IMHA)), tissue necrosis (e.g., infarction, trauma), and neoplasia. A reactive monocytosis rarely exceeds 10,000 cells/μL.[49] A neutrophilia often occurs concurrently, since monocytes and neutrophils share a common progenitor cell. A monocytosis can also be observed secondary to the effects of corticosteroids (primarily in dogs and cats), and in obesity. In the latter, adipose tissue macrophages and inflammasome-dependent IL-1β production can directly stimulate the proliferation and expansion of bone marrow myeloid progenitors, thereby resulting in a neutrophilia and monocytosis.[30,41]

Malignant proliferations of monocytes can also occur. Disease processes, including acute and chronic monocytic leukemia and myelomonocytic leukemia, are discussed in Chapters 66 and 68.

Monocyte Dysfunction

Monocyte dysfunction, without a concurrent or consistent alteration in absolute number, can occur secondary to multiple metabolic and systemic diseases, including liver failure, renal failure, diabetes mellitus, and obesity.[12] Monocytes in human patients with end-stage cirrhosis, for example, have lower MHC class II and CCR2 surface expression, decreased phagocytic and oxidative function, and decreased inflammatory cytokine (i.e., TNF) production, thereby resulting in secondary infection and increased mortality.[14] Experimental studies in mice and nonhuman primates involving high-fat and high-cholesterol diets have demonstrated increased MCP-1-mediated chemotaxis, and a skewed expression of Ly6C^{high} monocytes, resulting in a proinflammatory state.[4,41] Continual secretion of proinflammatory cytokines (TNF-α, IL-6) and monocyte "priming" have subsequently been shown to contribute to insulin resistance, diabetes mellitus, kidney disease, and cardiovascular disease.[4]

Several infectious diseases can also result in monocyte dysfunction, particularly those viruses (i.e., lentivirus, herpesvirus, influenza A virus) with a tropism for peripheral blood mononuclear cells. Lentiviruses (of the retrovirus family) are of particular interest, since they affect multiple species, including nonhuman primates (simian immunodeficiency virus), equids (equine infectious anemia virus), bovine (bovine immunodeficiency virus), sheep (maedi–visna virus), goats (caprine arthritis encephalitis virus), and felids (feline immunodeficiency virus). Lentiviruses can serve as translational, comparative models for human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS).[10] Infected monocytes in sheep experimentally infected with maedi–visna virus, for example, demonstrate decreased surface expression of CD80 (B7-1), which is a costimulatory ligand that is crucial for T-cell activation and survival.[38] It is important to note, however, that not all lentiviruses are associated with monocyte dysfunction and disease. Although more virulent strains have been isolated, bovine immunodeficiency virus generally causes mild symptoms, including lymphocytosis, in affected cattle.[10,49] Certain viruses (i.e., porcine reproductive and respiratory syndrome virus) show a restricted tropism for macrophage subsets, as opposed to their circulating counterparts.

MACROPHAGE DISORDERS

Malignant proliferation of macrophages can be difficult to differentiate from reactive processes, particularly if the neoplastic population does not display overt criteria

of malignancy. A thorough review of localized and systemic histiocytic sarcoma and cutaneous and systemic histiocytosis is provided in Chapter 74.

Acquired Defects in Macrophage Activation and Function

Although macrophages play a crucial role in defense against a variety of infectious organisms, including *Mycobacterium*, *Leishmania*, *Rhodococcus*, *Toxoplasma*, *Listeria*, *Brucella*, *Salmonella*, *Yersinia*, and *Rickettsia*, certain pathogens are adept at suppressing macrophage activation or neutralizing their mechanisms of killing. Yeast forms of *Cryptococcus neoformans* and *Cryptococcus gattii*, for example, can prevent phagocytosis and subsequent activation of macrophages by blocking dectin-1 and CD11b.[23] Other pathogens, such as *Rhodococcus*, are able to decrease surface expression of key glycoproteins involved in antigen-presentation (i.e., CD1b), while *Mycobacterium avium* subsp. *paratuberculosis* can interfere with the acidification and fusion of intracellular lysosomes.[33,49] Such interference results in the persistence of the pathogen, resulting in patient morbidity and mortality.

Hemophagocytic Syndrome

Hemophagocytic syndrome (HPS), which is also known as hemophagocytic lymphohistiocytosis, is a reactive proliferation of macrophages that label positive with monoclonal antibodies against CD14, CD18, MHC class II, and Thy-1.[47] Although in humans it can either be inherited as a genetic (primary or familial HPS) or acquired (secondary or reactive HPS) disorder, only acquired cases arising secondary to infectious (*Trypanosoma congolense*, African swine fever virus, simian retrovirus, ehrlichiosis, parvovirus, blastomycosis), IMHA, and neoplastic (lymphoma, multiple myeloma) diseases have been documented in dogs, cats, cattle, swine, and a rhesus macaque.[31] Idiopathic cases have also been reported. In humans, primary or familial HPS is an autosomal recessive disorder that has been linked to reduced perforin function and subsequent defects in cell-mediated cytotoxicity.[31,49] Perforin monomers are calcium-dependent, channel-forming proteins that result in the osmotic lysis of, and allow for, the introduction of apoptosis-inducing granzymes into target cells.[31] Although more investigation is required, preliminary studies in Bernese Mountain Dogs with and without histiocytic diseases concluded that single-nucleotide polymorphisms (SNPs) within the perforin gene were unrelated to the expression of disease, since the SNPs did not result in alteration of amino acid coding sequences.[31]

Due to its similar presentation to other disease processes, including bacterial sepsis and the systemic inflammatory response syndrome, HPS can be difficult to document both clinically and cytologically.[49] Documented cases, which require bicytopenia or pancytopenia in peripheral blood, and >2% hemophagocytic macrophages in a cellular bone marrow, can present with vague clinical signs including pyrexia and diarrhea, as well as hepatosplenomegaly.[31,48,49] Abnormalities that can be observed on serum biochemical panels include hypoalbuminemia and hypocholesterolemia.[9] In canines, a strong breed predilection has been documented in Bernese Mountain Dogs, Flat-Coated Retrievers, Rottweilers, and Tibetan Terriers.[31,48] Mortality rates reportedly range from 22% to 62%, but overall, canines with HPS secondary to infection have better survival rates than cases arising from immune-mediated, neoplastic, or idiopathic causes.[48,49]

When evaluating bone marrow samples, HPS must be distinguished from malignant histiocytosis (also known as disseminated histiocytic sarcoma), and other causes of macrophagic proliferation within the bone marrow (i.e., necrosis, IMHA). Criteria that can be used to differentiate malignant histiocytosis from HPS include highly anaplastic nuclear features (i.e., multinucleated giant cells, karyomegaly, bizarre mitotic figures) and >30% neoplastic cells in a normocellular or hypercellular bone marrow.[9,48] An expansion of well-differentiated macrophages can also be seen on aspirates of the liver and spleen in cases of HPS. However, since cytologically malignant features can be observed in cases of HPS, flow cytometry scatter plots or immunophenotyping may be necessary to differentiate HPS from malignant histiocytosis.[9,47,48]

Lysosomal Storage Diseases

Lysosomal storage diseases (LSDs) are a relatively uncommon, broad category of inherited, autosomal recessive, and X-linked disorders that result in atypical lysosomal function, and subsequent accumulation of incompletely degraded macromolecules, including sphingolipids, cholesterol esters, glycoproteins, and mucopolysaccharides.[11,21] Such accumulation affects the function of cells, tissues, and organs, resulting primarily in central nervous system dysfunction and neurodegenerative disease. Signs associated with LSDs, however, can be multisystemic, resulting in musculoskeletal, cardiovascular, and ocular disorders.[11] Electron microscopy of affected tissues reveals membranous cytoplasmic bodies or zebra bodies, which represent concentric and stacked membranous material, respectively.[21] Natural cases of LSDs, which include GM1 and GM2 gangliosidosis, mucopolysaccharidosis, glucocerebrosidosis (Gaucher disease), sphingomyelinosis (Neimann–Pick disease), β- galactocerebrosidosis (globoid cell leukodystrophy or Krabbe disease), α- and β-mannosidosis, and α-l-fucosidosis, have been documented in dogs, cats, ruminants (cattle, goats, sheep), guinea pigs, rodents (mice and rats), pigs, Japanese quail, emus, and flamingos.[11,21,49] Breed predispositions and specific inherited mutations are documented elsewhere.[21] Genetic testing for canines and felines is currently offered through the University of Pennsylvania's PennGen Laboratories.

Cases of acquired LSDs have also been documented, particularly in herbivores following exposure to toxic agents, such as *Swainsona*, *Oxytropis*, and *Astragalus* spp. Also known as locoweeds, the plants of these genera can cause disease similar to α-mannosidosis in horses, cattle,

and sheep due to the inhibition of α-mannosidase within lysosomes and the Golgi complex by swainsonine.[21] Similar clinical signs and histological lesions have been documented in horses, deer, and goats, following ingestion of *Sida carpinifolia*, a swainsonine-containing wireweed.[26] Ingestion of *Trachyandra divaricata*, *Phalaris* spp., and *Solanum* has also been associated with acquired LSDs.

Idiopathic Systemic Granulomatous Disease in Horses and Cattle

Idiopathic systemic granulomatous disease (ISGD) is an uncommon, multisystemic, non-caseating granulomatous disease that is characterized by the presence of fibrinoid necrosis, perivascular infiltrates composed of lymphocytes, macrophages, plasma cells, and multinucleated giant cells.[34,35,40] Closely resembling human sarcoidosis, this disease process has no age, breed, or sex predilection, and is suspected to originate from a dysregulated immune response. Unlike the human disease, however, ISGD is not associated with hypercalcemic states or soft-tissue mineralization.[35] A similar disease process has been documented in cattle and horses after ingestion of *Vicia villosa* (hairy vetch).[34,35,40] The granulomatous lesions that develop secondary to ingestion of this legume are suspected to represent either a type IV hypersensitivity reaction against the plant constituents or direct activation of T cells by lectin.[34] ISGD is currently a diagnosis of exclusion, and requires that bacterial (i.e., *Mycobacterium spp.*), viral (i.e., equine herpesvirus-2 (EHV-2)), and fungal (i.e., *Coccidioides immitis*) causes of granulomatous disease be ruled out.

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CHAPTER 50

Lymphocyte Ontogeny and Lymphopoiesis

AMY L. WARREN and ROBIN M. YATES

Immunologic Diversity
  Combinatorial Diversity
  Junctional Diversity
  Somatic Hypermutation
B Cell Development
  B-2 B Cells
  B-1 B Cells
  Marginal Zone B Cells
T Cell Development
  TCR Production
  Positive Selection
  Negative Selection
  Natural Killer T Cells
Natural Killer Cell Development

Acronyms and Abbreviations

αβ, alphabeta; AID, activation-induced deaminase; AIRE, autoimmune regulatory gene; BCR, B cell receptor; Blimp-1, B lymphocyte-induced maturation protein-1; C, constant; CCR, chemokine receptor; CD, cluster of differentiation; CDR, complementarity determining region; c-Kit, mast/stem cell growth factor receptor or CD117; CLP, common lymphoid progenitor; D, diversity; DN, double negative; DNA-PK, DNA-dependent protein kinase; DP, double positive; FLT3, FMS-like tyrosine kinase 3 (CD135); G, guanine; γδ, gammadelta; GATA3, GATA-binding protein 3; HSC, hematopoietic stem cell; Ig, immunoglobulin; IFNγ, interferon gamma; IGF1, insulin-like growth factor 1; IL, interleukin; J, junctional; KLRG1, killer cell lectin-like receptor G1; MALT, mucosa-associated lymphoid tissue; MHC, major histocompatibility antigens; MPP, multipotent progenitor; NK, natural killer (cell); NKT, natural killer T (cell); RAG, recombination-activating gene; RSS, recombination signal sequences; sca-1; stem cell antigen-1; SCID, severe combined immunodeficiency; TCR, T cell receptor; TdT, terminal deoxynucleotidyl transferase; TGF-β, transforming growth factor-beta; U, uracil; V, variable.

Lymphocytes develop from bone-marrow-derived pluripotent hematopoietic stem cells (HSCs). Pluripotent stem cells have the capacity either to self-renew or to differentiate into all blood cell types.[11] HSCs initially give rise to multipotent progenitors (MPPs) that retain the ability to differentiate into all cell lineages, but lack the ability to self-renew (see Chapter 41). MPPs differentiate into oligopotent progenitors—the common myeloid progenitors and the common lymphoid progenitors (CLP). CLPs give rise to the three lymphocytic lineages: B cell progenitors, T cell progenitors and natural killer (NK) cell progenitors.[6] As hematopoietic cells mature, they express different cell surface markers, forming the basis of identification of cells by flow cytometry. CLPs are characterized by the expression of interleukin (IL)-7, as well as mast/stem cell growth factor receptor (c-Kit or CD117), stem cell antigen-1 (sca-1), and FMS-like tyrosine kinase 3 (FLT3 or CD135).

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IMMUNOLOGIC DIVERSITY

Lymphocytes are unique in their ability to recognize and bind to an almost unlimited array of antigens through their T-cell receptors (TCR) and B-cell receptors/immunoglobulin (BCR, Ig). The process by which this diversity is achieved is similar in both T and B cells and involves combinatorial diversity and junctional diversity. A third

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process in B cells, somatic hypermutation, further diversifies Ig after T-helper-cell stimulation.

Combinatorial Diversity

Unlike many genes, there is not a single contiguous segment of the genome that encodes TCRs and Igs. Instead, in germline DNA, there are multiple segments, named variable (V), diversity (D), and junctional (J) segments, with multiple copies of each of these. Combinatorial diversity occurs through random joining of different V, D, and J segments, during V(D)J rearrangement to create millions of V(D)J permutations. Specific gene segments called recombination signal sequences (RSS) and recombination-activating gene (RAG) enzyme complexes are crucial for V(D)J rearrangement. RSS flank Ig and TCR V, D, and J gene segments and are composed of a conserved heptamer, a non-conserved spacer of 12 or 23 base pairs and a conserved nonamer. The orientation of RSS and the size of the non-conserved spacer region (12 vs. 23) ensure orderly recombination of V, D, and J segments, and prevent the recombination of similar gene segments (e.g., V–V). RAG-1 and RAG-2 proteins are nuclear recombination enzymes unique to developing lymphocytes that recognize and bind to RSSs adjacent to different V, D, and J gene segments. In general, RAG enzyme production ceases at the end of V(D)J rearrangement. RAG enzymes and other more universally expressed DNA-repair enzymes including DNA-dependent protein kinase (DNA-PK), artemis nuclease, terminal deoxynucleotidyl transferase (TdT), and DNA ligase IV form complexes that bind, excise, and repair V, D, and J segments to form a unique V(D)J sequence. This occurs in a multistep process where first RAG-1 and RAG-2 enzymes recognize and bind to two RSSs on different D and J regions, creating a nick in one strand of the DNA. The intervening DNA forms a hairpin between the selected D and J segments. Nucleotides can then be added or deleted to create blunt-ended double-stranded DNA breaks (see junctional diversity). The cut ends of the DNA are held in place by RAG enzymes that are then reannealed by a complex of artemis nuclease, DNA-PK, and DNA ligase IV. V(D)J rearrangement is initiated by joining of one D region with one J region. A V region is then added and the intervening DNA is deleted, resulting in a single recombined V(D)J genomic sequence (see Figure 50.1).

There are differences in the BCR (Ig) and TCR components as to whether all three V, D, and J segments are incorporated. TCR α-chain and BCR (Ig) light chain use only V and J rearrangement, whereas TCR β-chain and BCR (Ig) heavy chain use V, D, and J rearrangements. There is reduced diversity in the number of V-segments encoding for γδ TCR, accounting for some of the decreased antigenic diversity of this receptor.

V(D)J rearrangement defines specific stages of B- and T-cell development. In B cells, successful heavy-chain rearrangement is required before light-chain rearrangement can begin. In T cells, rearrangement of the TCR loci follows a specific order starting with delta, then proceeding through the gamma, beta, and finally alpha genes. Two distinct subpopulations of TCR expressing cells are formed from these chains: αβ and γδ T cells. To underscore the importance of V(D)J rearrangement, foals that genetically lack DNA-PK die early in life due to severe combined immunodeficiency (see Chapter 55).

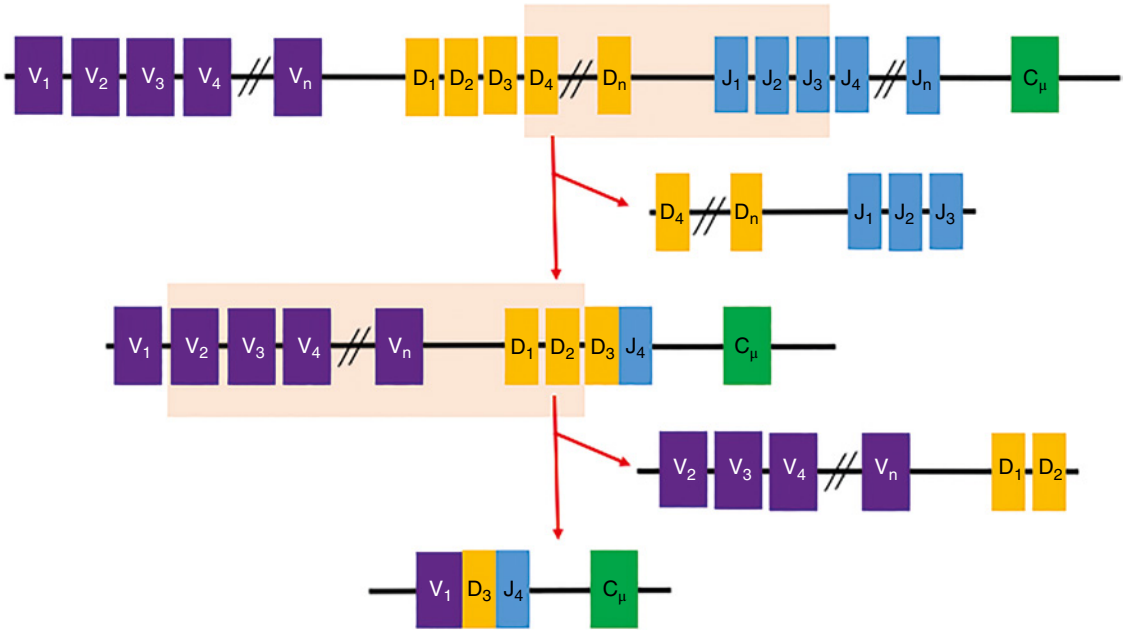


FIGURE 50.1 V(D)J rearrangement. RAG enzyme complexes bind to RSS sequences adjacent to V, D, and J segments, excising the intervening DNA and binding cut ends. D and J segments recombine first, excising the intervening DNA. Thereafter, V segments produce a final rearranged product containing one V, D, and J segment.

Junctional Diversity

Junctional diversity is created by the random addition of nucleotides to DNA ends that are cleaved during recombination. TdT is a DNA polymerase that adds variable numbers of nucleotides to the V, D, and J exons during rearrangement. These additional nucleotides at the V(D)J junctions are called N regions and provide additional diversity of the Ig heavy chain and the TCR. As the addition or subtraction of nucleotides by TdT is random, there is also the possibility that during this process, a frameshift or nonsense mutation is introduced, resulting in nonfunctional TCR or Ig open reading frames. This necessitates a quality-control step in both T- and B-cell maturation to ensure proper formation of TCR and Ig chains. Combinatorial gene rearrangement and junctional diversity are initiated first in one paired chromosome and move to the second chromosome if a functional chain fails to form. Failure of both chromosome rearrangements to form a functional chain results in apoptosis of the cell. TdT expression declines as B cells mature and does not increase light-chain diversity.

Somatic Hypermutation

In activated, proliferating follicular B cells, additional immunoglobulin diversity is achieved through programed somatic hypermutation within the Ig genes. Although this occurs after lymphopoiesis, it is an essential component of B-cell maturation and markedly hones the specificity of the acquired immune response. Somatic hypermutation occurs in proliferating B cells that have been activated by antigen-bearing T-helper cells. It is restricted to the BCR genes predominantly in the complementarity determining regions (CDR), the area of the BCR that binds antigen. Hypermutation in this region occurs at approximately 10^5–10^6 times higher than in other regions and consists mostly of single nucleotide replacements. Activation-induced deaminase (AID) mediates somatic hypermutation by deaminating cytosine residues in CDRs to uracil, resulting in a uracil–guanine (U:G) mismatch. Base-excision-repair enzymes remove these, which are then repaired by error-prone DNA polymerases that create mutations in one to a few nucleotides surrounding the U:G mismatch. As B cells are proliferating at a high rate when somatic hypermutation occurs, this results in thousands of variations of antigen-recognizing B cells, some of which will have enhanced recognition of the antigen. The B cells with the highest affinity for the antigen have a selective advantage to receive signals to clonally expand and eventually to differentiate into plasma cells and memory B cells.

B CELL DEVELOPMENT

B cell lymphopoiesis is a lifelong process in mammals that begins in the fetal liver and transitions to bone marrow after birth. In birds, B-cell maturation occurs in the bursa of Fabricius, in fish it occurs in the kidneys and in amphibians in the spleen, bone marrow, and kidney.[3] Mature B cells form the cornerstone of humoral immunity, producing and secreting highly antigen-specific antibodies. Mammalian BCR are comprised of two heavy chains and two light chains, bound together by disulfide bonds. Each paired N-terminus of the heavy and light chain forms an antigen-binding domain—the location of the highest amount of variability which is divided into three regions of hypervariability, CDR1, CDR2, and CDR3.[5] The paired C-terminuses of the heavy chains are relatively constant and can be attached to a membrane through a membrane-binding domain (BCR) or are free in solution (Ig). A B cell can, throughout its life, express different BCR forms. These forms maintain their antigen specificity, but vary in the C-terminus of the heavy chain, creating different antibody isotypes (e.g., IgG, IgM, IgA, etc.) with the IgM isotype the initial BCR expressed by all developing mammalian B cells.

B-2 B Cells

There are several different types of B cells: B-2, B-1, and marginal zone B cells. B-2 are the more conventional type of B cell that on maturation reside in secondary lymphoid tissue follicles and differentiate after activation into memory B cells and plasma cells. Much of what is understood about B cell lymphopoiesis pertains to B-2 B cells.

IL-7 is important in the initial proliferation of committed B cell progenitors. Later stages of developing B cells require only soluble factors for development. IL-1α, IL-3, IL-4, IL-11, transforming growth factor-beta (TGF-β), interferon gamma (IFNγ), insulin-like growth factor (IGF1), c-Kit ligand, FLT3 ligand, and estrogen are known to play a role in B cell proliferation, differentiation, and regulation.

The sequential V(D)J rearrangement of BCR gene closely follows the sequence of B-cell maturation. Heavy-chain rearrangement occurs first in pre-/pro-B cells where D and J gene segments recombine. This is followed in pro-B cells where variable segments are recombined to the rearranged (D)J segments. This completed V(D)J forms the heavy chain and is the first quality assurance step in B-cell maturation. To test if the recombined heavy chain can bind to a light chain and be expressed on the surface of the cell, the cell binds the heavy chain to a surrogate light chain to produce a pre-BCR. If the cell fails to produce a pre-BCR, genetic recombination of the heavy chain occurs again on the paired chromosome. The second checkpoint in BCR production assesses the pre-BCR for heavy-chain self-reactivity. Expression of a functional pre-BCR inhibits further heavy-chain rearrangement, stimulates cell replication, and is necessary for the survival and positive selection of pre-B cells after which recombination of the light chain commences.

Light-chain rearrangement occurs in late pre-B cells. Kappa rearrangement proceeds first. Lambda rearrangement will occur only if kappa rearrangement is unsuccessful. After successful production of the light chain, the newly formed BCR forms and is expressed on the cell surface. If the light chain produced is unable to bind to the heavy chain, the other parental chromosome is

rearranged, and if the light-chain production fails for a second time, the cell undergoes apoptosis. Light-chain and heavy-chain proteins combine to produce a BCR. The third checkpoint occurs at this stage where the newly formed BCR is exposed to self-antigen and those with high affinity are deleted.[12] As cytoplasmic IgM is exported to the surface membrane, cells transition into immature B cells that express CD21. Surviving immature B-2 B cells migrate to peripheral lymphoid tissues where they begin to express surface IgD as well as IgM. As these cells interact with antigen and T cells, they undergo the fourth checkpoint: the process of class switching, somatic hypermutation, and affinity maturation (see Figure 50.2).

Further, functional diversity in immunoglobulins and BCR comes from varying isotypes (IgM, IgA, IgD, IgG, IgE, etc.). These different isotypes are determined by multiple gene encodings for different constant regions—Cμ, Cδ, Cγ, Cε, and Cα corresponding to IgM, IgD, IgG, IgE, and IgA. During isotype switching, different constant regions are expressed, while the variable region (the antigen-binding region) remains the same. The Cμ gene is adjacent to the V(D)J region in the genome, and as such, B cells initially express IgM isotype and some IgD, switching later after antigen and T-helper activation in the periphery to IgE, IgA, and IgG.

B-1 B Cells

B-1 B cells play more of a role in innate than acquired immunity. It is currently thought that most B-1 B cells are produced in the yolk sac, fetal liver, and bone marrow, with a lesser number produced in the adult bone marrow. Most agree that B-1 B cells are derived from a different, parallel lineage to B-2 B cells, arising from a B-1 CLP.[13] After maturation in the fetal liver, B-1 cells mostly localize to pleural and peritoneal cavities where they establish self-renewing populations. Most B-1 B cells express cell surface CD5 and produce predominantly IgM antibodies. While they are the minority population in humans and rodents, they form the majority of B cells in ruminants and rabbits. There is much less BCR diversity in B-1 B cells as they lack the enzyme TdT required for junctional diversity, they do not undergo somatic hypermutation, and there are many fewer possible DJ and V(D)J permutations. Their antigenic repertoire, while narrower, is honed to bacterial polysaccharides and lipids, the predominant pathogen in peritoneal and pleural cavities. B-1 B cells are capable of activation independent of Th-cells.[9] Their capacity to quickly respond to bacterial antigens gives them unique advantages over B-2 B cells.

Marginal Zone B Cells

A second subset of less common B cells is the marginal zone B cells that are localized to the marginal zone of the spleen–the primary site of blood antigen monitoring in the body. Like B-1 B cells, marginal B cells are able to respond to antigen in a T-helper-independent manner and tend to have higher responsiveness to non-protein

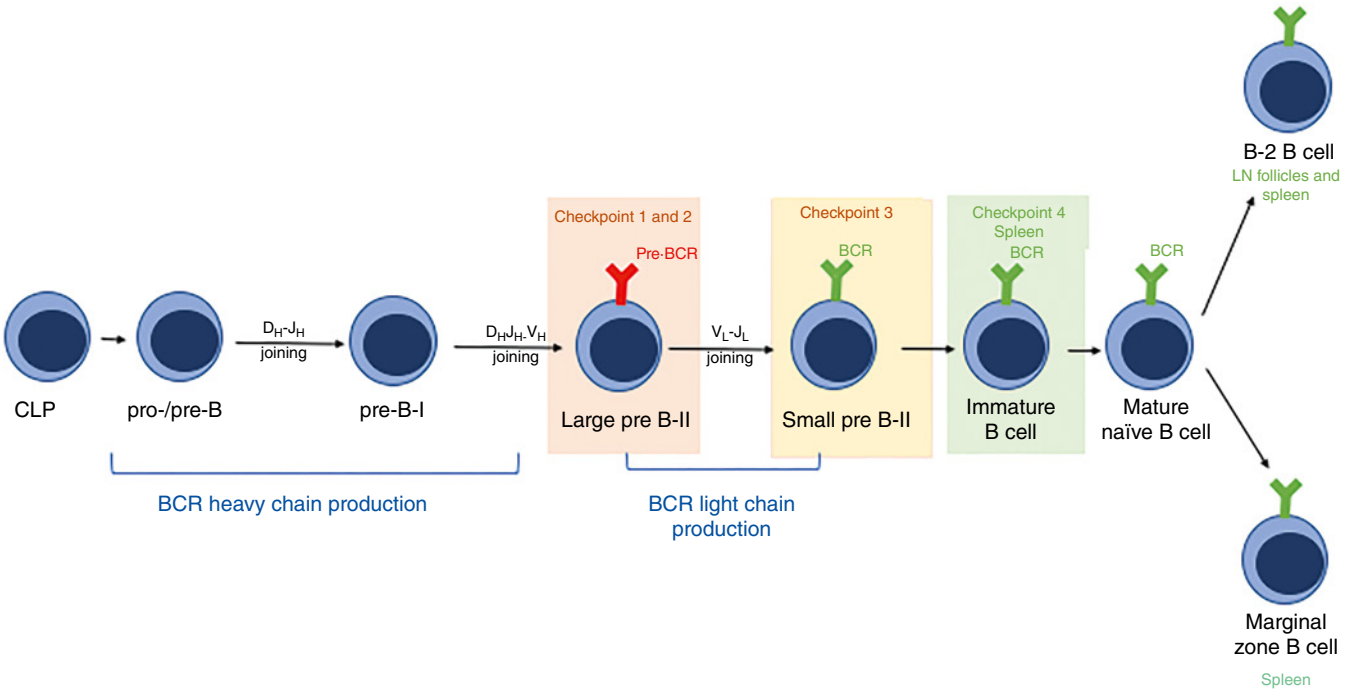


FIGURE 50.2 B cell development. BCR heavy-chain rearrangement begins in pro-/pre-B cells and ends in large pre-B-II cells. After production of a heavy chain, a pre-BCR is produced and the first two checkpoints of the heavy chain's ability to bind to the surrogate light chain and not react with self is interrogated. Light-chain production follows with expression of fully formed BCR and the third checkpoint to eliminate self-reactivity of the formed BCR. Immature B cells are thought then to migrate to the spleen where a fourth checkpoint for self-reactivity is believed to occur. (Source: Modified from Melchers[12])

antigen than B-2 B cells. Similar to B-1 B cells, they are only able to produce IgM antibodies, and compared to B-2 B cells, they have reduced BCR diversity. Our understanding of marginal B cell lymphopoiesis is still emerging. The current belief is that marginal B cells are derived from bone marrow, but develop into marginal B cells in the spleen in response to a combination of BCR antigen affinity strength and Notch2 signaling.[8]

T CELL DEVELOPMENT

Similar to B cells, T cells are characterized by enormous TCR diversity. The derivation of the TCR closely follows T-cell development and maturation. Initial T cell precursors are produced in bone marrow from CLP cells. These cells migrate to the thymus for maturation and are termed "thymocytes." The thymic stroma is a uniquely organized epithelial network on a mesenchymal cell backbone with interspersed dendritic cells and macrophages (see Chapter 51). This cellular network provides the extracellular matrix ligands and growth factors critical for T-cell selection and development. Thymic precursors enter at the corticomedullary junction via molecules such as CD44, CD62P, and chemokine receptor 9 (CCR9), and migrate initially to the outer cortex, then later to the medulla. Initial T-cell development in the thymus is driven by notch expression by thymic stromal cells that interacts with the notch receptors on thymocytes. Notch signaling is essential for T versus B cell fate decision and promotes αβ T-cell development over γδ T cells.[16] Notch1 with GATA3 is important for the transition from the T/NK progenitor to sole T-cell commitment.[15] Notch1 alone directs cells toward γδ TCR development, but HEB (one of the basic helix–loop–helix E-box-binding proteins) directs cells toward αβ TCR.[14] IL-7 is also necessary for T cell development. Defects in the IL-7/IL-7 receptor complex and signaling pathway are responsible for several reported T cell defects. B lymphocyte-induced maturation protein (Blimp-1) has been shown to be essential for terminal differentiation of T cells.[10]

T cell development proceeds down two primary pathways as defined by expression of different TCR types: αβ and γδ. In most species, approximately 90% of immature cells develop into αβ T cells through thymic selection, while the remaining cells develop into γδ T cells. αβ TCRs are only capable of recognizing antigen when it is complexed to either major histocompatibility antigens (MHC)-I or MHC-II molecules and have a much wider range of TCR diversity, compared to γδ TCR. γδ TCRs can recognize unprocessed antigen, not associated with MHC, and the narrow range of antigen they are capable of binding to includes lipid antigens (including those produced by mycobacteria), phosphorylated ligands, and heat shock proteins.[4] The narrow range of variability in γδ T cells relates to a lower number of V-gene segments. αβ T cells are CD3+ and the majority also express either CD4 or CD8.

TCR Production

The earliest progenitors of T cells in the thymus are the double negative (DN) cells, so called because they lack expression of either CD4 or CD8. As DN cells mature, they undergo variable expression of CD44 and CD25. The earliest DN1 cells are CD44+/CD25−. DN2 cells are CD44+/CD25+, followed by decreased CD44 expression in DN3 cells (CD44−/CD25+) to the dropping of both markers in DN4 cells (CD44−/CD25−). TCR production accompanies this maturation. TCR β-chain rearrangement occurs first in DN3 (CD44−/CD25+) cells. DN4 (CD44−/CD25−) cells produce a pre-TCR by combining the newly formed β-chain with a surrogate TCRα chain to express a pre-TCR that complexes with CD3 on the cell surface. This is termed beta-selection, a quality assurance checkpoint. Successful formation of the pre-TCR leads to survival, proliferation, and upregulation of CD4 and CD8. Cells that fail beta-selection undergo apoptosis. TCRα rearrangement and expression then commence in CD4+/CD8+ double positive cells to produce rearranged αβTCRs.

Positive Selection

After a functional TCR is produced, double positive cells undergo positive selection. This is to ensure that the TCR–CD3 complex is able to interact with MHC class I and II molecules. Positive selection occurs in the cortex of the thymus where double positive thymocytes interact with low- to moderate-affinity MHC peptide ligands on cortical epithelial cells to receive a survival signal. Although the basis of positive selection is the TCR–MHC interaction, CD4 and CD8 are also required for selection. Double positive cells that select MHC-II molecules will become CD4 T cells, while those that select MHC-I molecules will become CD8 T cells, forming the basis for lineage commitment. Positive and negative selection occurs during interaction with dendritic cells and other antigen-presenting cells at the corticomedullary junction. Differential downregulation of either CD4 or CD8 occurs in the thymic medulla.[16] CD4+ T cells can become T-helper cells including Th1, Th2, Th17, and likely T-regulatory cells, and CD8+ T cells can become T-cytotoxic cells.

Negative Selection

As a diverse population of TCRs is produced, the next stage removes self-reacting T cells. Thymocytes that survive positive selection upregulate cell-surface expression of the chemokine receptor CCR7 and migrate to the thymic medulla for elimination of autoreactive T cells. Medullary epithelial and dendritic cells are induced to express peripheral antigens (e.g., those expressed by the pancreas, liver, etc.) through the autoimmune regulatory gene called AIRE. In the medulla, thymocytes interact with high-affinity self-ligands on the medullary epithelium. Cells that react strongly receive an apoptotic signal. Surviving cells undergo final maturation and then exit the thymus. Greater than 95% of developing lymphocytes fail one of these selection processes (see Figure 50.3).

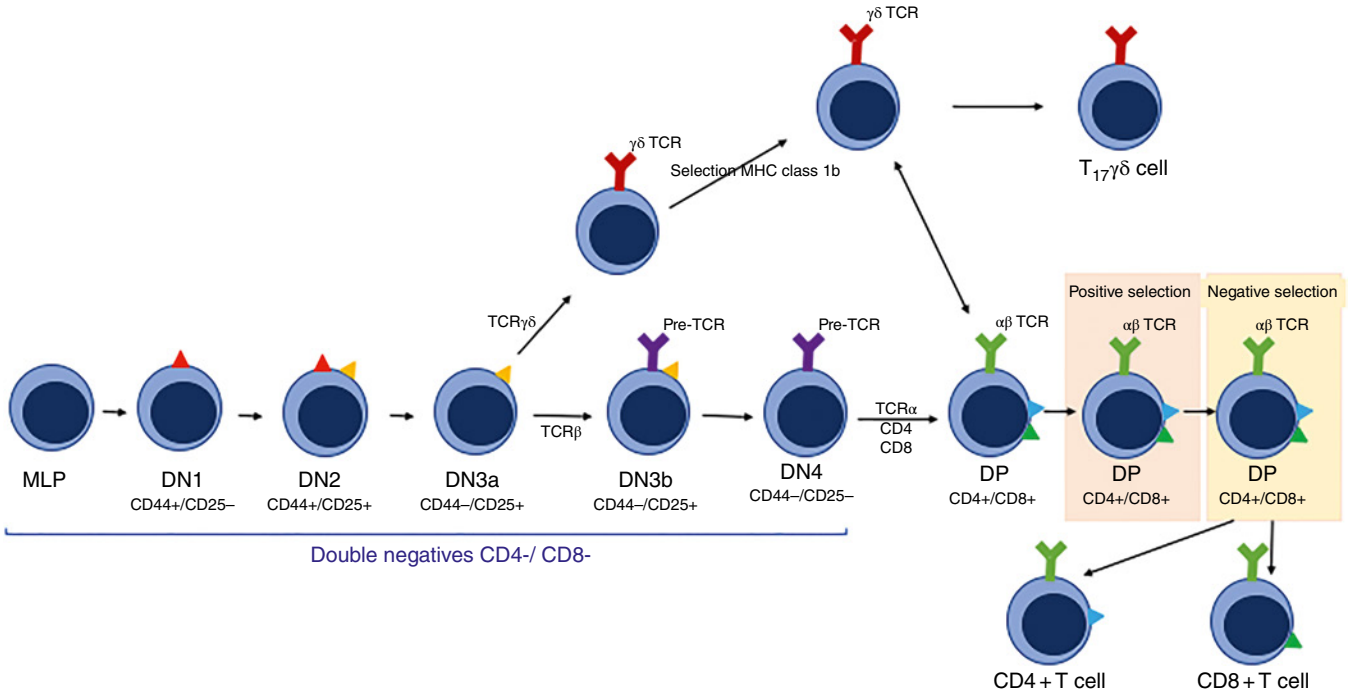


FIGURE 50.3 T cell development. CLPs migrate from bone marrow to the thymus where thymocytes are termed double negative (DN) cells lacking CD4 or CD8 expression. In the thymic cortex, DN cells mature through several stages with differential expression of CD44 and CD25 that results in TCR β-chain rearrangement, production of pre-TCR, and beta selection checkpoint. TCRα chain production and expression of both CD4 and CD8 follow, producing double positive (DP) cells. The functional TCR undergoes both positive and negative selection in the medulla of the thymus after which differential reduction in either CD4 or CD8 expression occurs producing functional CD4+ and CD8+ cells that exit the thymus. (**Source:** Modified from Gerondakis et al.[7])

Natural Killer T Cells

Natural killer T (NKT) or NK1.1 T cells are a unique subpopulation of αβ T cells that functionally bridge the innate and adaptive immune systems. NKT cells express NK1.1, a marker typically found on NK cells, can be CD4 and CD8 positive or negative and express CD16, CD56, and produce granzyme. Unlike conventional T cells, NKT cells recognize antigen presented in CD1d molecules, rather than MHC, a consequence of which is their enhanced ability to recognize lipid and glycolipid antigen (e.g., mycobacteria). NKT cells undergo selection via interactions with CD1 ligands in the cortical region of the thymus after they reach the double positive stage of development. TCR development in NKT cells can progress in a manner similar to conventional T cells; however, a subset of NKT cells (invariant iNKT cells) produces an invariant α-chain and have limited β-chain diversity.[2]

NATURAL KILLER CELL DEVELOPMENT

NK cells are cytotoxic innate lymphocytes that kill altered host cells. NK cells are thought to be activated by alterations in the balance between activating and inhibitory signals expressed on the surface of host cells. Activating signals are often normal cell surface proteins whose structure is modified by increased intracellular stress, as can happen with viral infection. Normal MHC-I expression can inhibit cell killing, and altered MHC-I expression can induce killing. NK cells lack TCR, CD3, and BCR expression and NK-receptors do not undergo recombinatorial events, but instead are constitutively expressed. NK development occurs in the fetal liver and thymus, and after birth in the bone marrow and peripheral lymphoid tissues.[1] Like other lymphocytes, NK cells develop from CLP cells with the expression of IL-15 receptor β-chain (CD122) identifying the earliest committed pre-NK cell progenitors. Three stages of NK development are currently recognized: lineage commitment, NK receptor selection, and functional maturation. NKG2D expression occurs initially, followed by NK1.1 and NCR1 expression. Mature NK cells are characterized by the expression of CD51, CD49, CD43, CD11b (Mac-1), and finally Ly49. Maturing NK cells then migrate to secondary lymphoid organs to complete maturation characterized by the expression of killer cell lectin-like receptor G1 (KLRG1). The transition from CLP to T/NK precursor is mediated, at least in part, by Notch1 and the T-box transcription factors, T-bet and Eomesodermin. IL-1, IL-7, and IL-15 are required for NK growth and maturation. The process by which NK cells are educated to not self-react is not fully understood, but may involve mechanisms similar I to T cell or B cell anergy or binding through self-MHC-I-specific inhibitory receptor interactions.

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CHAPTER 51

Structure, Function, and Disorders of Lymphoid Tissue

AMY L. WARREN and ROBIN M. YATES

Structure, Function, and Disorders of Primary Lymphoid Tissue
- Bone Marrow
- Thymus
- Bursa of Fabricius

Structure, Function, and Disorders of Secondary Lymphoid Tissue
- Lymph Nodes
- Tertiary Lymphoid Centers
- Spleen
- Hemal Nodes
- Mucosa-Associated Lymphoid Tissue

Acronyms and Abbreviations

APC, antigen-presenting cell; BALT, bronchus-associated lymphoid tissue; BLV, bovine leukemia virus; BVDV, bovine viral diarrhea virus; CD, cluster of differentiation; D, diversity; FeLV, feline leukemia virus; GALT, gut-associated lymphoid tissue; H&E, hematoxylin & eosin; HEV, high endothelial venules; HSC, hematopoietic stem cell; Ig, immunoglobulin; J, joining; MALT, mucosa-associated lymphoid tissue; MHC, major histocompatibility complex; NK cell, natural killer cell; PALS, periarteriolar lymphoid sheath; PCB, polychlorinated biphenyls; PCV2, porcine circovirus type 2; RBC, red blood cell; SCID, severe combined immunodeficiency; TCR, T cell receptor; Th2 cell, T-helper 2 cell; V, variable; VALT, vulvovaginal-associated lymphoid tissue.

The lymphoid system consists of primary and secondary lymphoid tissues. Primary tissues, including the bone marrow and thymus, are sites of lymphocyte production, development, and maturation. Secondary lymphoid tissues are the sites where lymphocytes interact with each other as well as antigen-presenting cells (APC) in the orchestration of the adaptive immune response. Subsets of lymphocytes develop and mature in different ways and in different tissues. T cells are formed in the bone marrow but migrate to the thymus for maturation and selection. B cells are formed and primarily mature in the bone marrow, though there is some evidence that B cell maturation and education may also occur in the spleen and peri-intestinal lymphoid tissues. NK cells, the only known innate lymphoid cell, develop and mature in bone marrow and then are released into and spend much of their time circulating in the blood and lymphatic systems.

Other highly specialized structures support the hematopoietic system. These include the epithelial cells of the thymus that sheath the blood vessels and form the saccular structures of the thymic cortex that are important for T cell maturation and selection. The fibrovascular supporting framework of the lymph node and spleen plays crucial roles in antigen-presenting cell and lymphocyte migration, orchestrating their interactions during adaptive immune responses. The vascular endothelium of the hematopoietic system includes the regionally undifferentiated cells lining the lymphatics as well as the regionally differentiated

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endothelial cells of the blood vascular system, which include the high endothelial venules of the lymph node paracortex with specific cell surface markers permitting the adhesion of lymphocytes in transit and their transmural migration to enter the node paracortex. The specialized circulating blood cells and their conducting and supporting structures are uniquely packaged in a series of organs of either separate design like the thymus, lymph nodes, and spleen or are incorporated into other organs like the bone marrow. These also include the free and fixed tissue macrophages and dendritic cells. These cells and organs of the blood vascular system constitute remarkably interrelated and integrated systems.

STRUCTURE, FUNCTION, AND DISORDERS OF PRIMARY LYMPHOID TISSUE

Bone Marrow

In mature animals, the bone marrow is the primary site of lymphocyte production, arising from common lymphoid progenitor cells, which in turn differentiate from common hematopoietic stem cells (HSC) (see Chapter 50). During embryogenesis, hematopoiesis occurs predominantly in the liver and migrates to bone marrow by the time an animal is born.

Lymphocyte production starts in the intersinusoidal spaces with initial differentiation to early lymphoid progenitors that later differentiate into the T cell, B cell, and NK cell lineages.[5,6,10,12,16]

Due to the rapidly dividing nature of hematopoietic cells, including lymphopoietic cells, the marrow is particularly susceptible to toxic and infectious agent injury as well as radiation and chemotherapeutic drugs. Disorders of the bone marrow are wide-ranging and encompass primary and secondary infections, neoplastic, developmental, toxic, and nutritional diseases (see Chapters 12 and 16).

Thymus

The thymus, the primary site of T cell maturation, is located in the cranial mediastinum in most species, as well as the ventral portion of the neck in young calves and pigs, and occasionally in kittens and foals. It is comprised of maturing T cells, termed thymocytes while in the thymus, and epithelial cells arranged in lobules with a distinct cortex and medulla (Figures 51.1 and 51.2). The cortex stains darker than the medulla on H&E stain due to a higher density of smaller, more basophilic lymphocytes in this area. An intricate network of epithelial cells and blood vessels form the structural basis of the thymus. Unlike other lymphoid organs, the normal thymus contains no afferent lymphatics or B cells. The thymus reaches maximal development at puberty and regresses in adulthood, but thymic remnants remain through the animal's life. Thymic function does not necessarily correlate to thymic size.

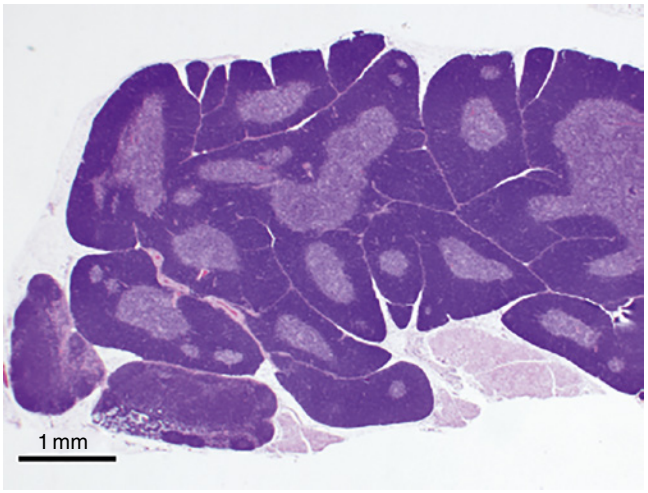


FIGURE 51.1 Thymus from a young adult male rat. The organ is composed of closely faceted lobules with a sharp distinction between the darker cortex and the lighter medullary areas of each lobule. H&E stain; bar = 1 mm.

The epithelial cells of the thymus are derived from the third and fourth pharyngeal pouches. This migration occurs very early in embryologic development and is immediately followed by seeding of the thymus with progenitor cells from the blood islands of the yolk sac. The first stream of embryonal epithelial migration forms the isolated reticular epithelial cells of the cortex and medulla of the adult thymic lobule, which forms the scaffold through which maturing lymphocytes migrate. The second epithelial migration forms the thymic duct epithelium and later the Hassall's corpuscles of the thymic medulla (Figure 51.3). Early reticular epithelial cells form loose cuffs around small vessels that persist in adult life and become obvious in conditions of lymphoid atrophy. Also in early development, the ductular component of epithelium forms the branching system that communicates between the lobules of a single thymic lobe.

The epithelial cells surrounding thymic vessels, as well as continuous endothelium of the capillaries and cuffs of macrophages, form a barrier to blood-borne antigens in the thymic cortex. This provides a sequestered environment for naïve T cell maturation, such that they receive their immunologic training solely from the reticular epithelial cells. Lymphoid germinal centers occurring in the thymus are not necessarily pathologic or an indication of autoimmune disease if they occur near a blood vessel and lie within the vascular epithelial sheath, as in those areas they are immunologically outside of the thymus. Detection of the epithelial sheath can require the application of a cytokeratin stain. The cortical and medullary epithelium produces trophic hormones that assist lymphocytic colonization and are the source of nonpathogenic cysts lined by ciliated epithelium that frequently develops in adult life. An additional cellular component to the thymus is myoid cells. They surround the Hassall's corpuscles and are important in the pathogenesis of myasthenia gravis. In young animals, particularly rabbits,

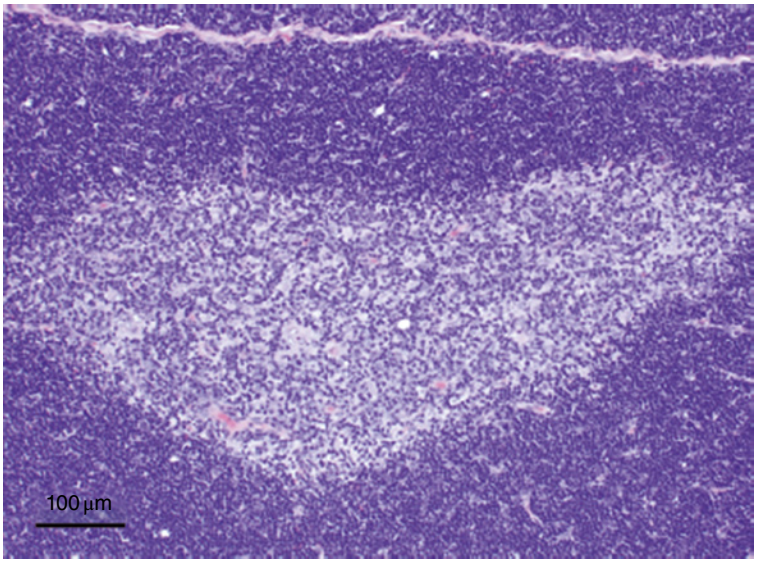


FIGURE 51.2 Mammalian thymus. In mammals, the medulla and cortex are sharply delineated in young healthy animals with the widths of the cortices and medulla approximately equal to one-third the width of the lobe. H&E stain; bar = 100 µm.

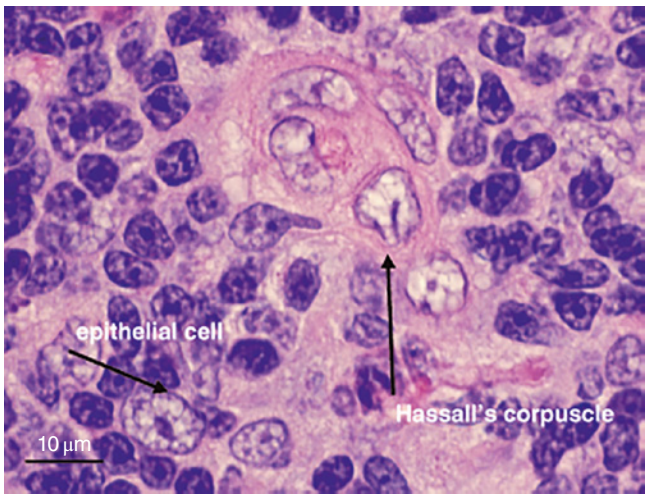


FIGURE 51.3 Hassall's corpuscle in the thymic medullary area consisting of concentric laminations of squamous epithelial cells. The surrounding lymphocytes are characteristically a mixture of small and medium cells and epithelial cells with large pale nuclei (arrow). These cells with moderately abundant cytoplasm separating the nuclei give the medulla a less dense appearance than the cortex on histological examination. H&E stain; bar = 10 µm.

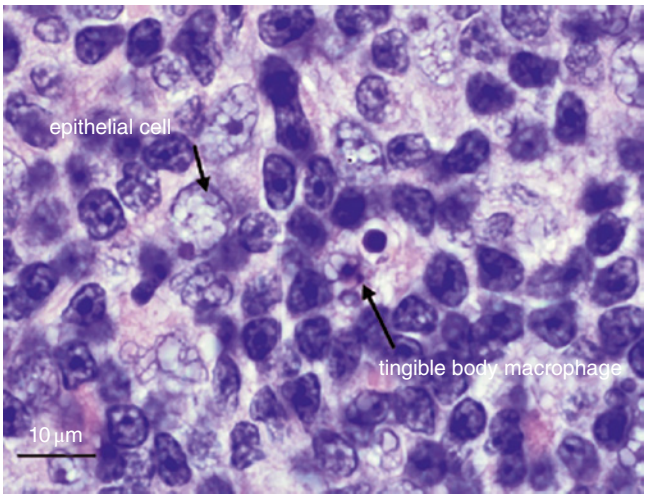


FIGURE 51.4 Thymic cortex composed of densely packed small lymphocytes. A macrophage in the center has cytoplasm that contains several pyknotic nuclear fragments (tingible bodies) of apoptotic cortical thymocytes. The larger vesicular nuclei above and to the left of the macrophage are probably epithelial cells involved in the self-recognition selection process of developing T cells. H&E stain; bar = 10 µm.

heterophils are frequently found in the thymus, particularly in lobular connective tissue. Eosinophils may occasionally be found in the thymic connective tissues and mast cells are present in the thymic capsules in most species and are particularly frequent in rats.

After production in the bone marrow, immature CD4−/CD8− T cells (prothymocytes) home to the cortex of the thymus for T cell receptor (TCR) rearrangement, maturation, and immunological selection for tolerance to self-antigens. Here, they proliferate in niches created by thin-walled pouches (caveolae) consisting of reticular epithelial cells. In caveolae, maturing T cells undergo negative selection by epithelial nurse cells in the outer cortex and dendritic cells in the inner cortex for reactivity to self-antigens, of which only about 1% will survive. Physiologic development continues when lymphocytes enter the medullary areas with exposure to self-antigen expressed by medullary epithelial cells. During this stage, cells also acquire CD3, CD4, and CD8 as they adopt the potential for differentiating into helper T cell, cytotoxic T cell, and suppressor T cell roles (see Chapter 50). The cells that are not selected undergo apoptosis and are removed by thymic macrophages, termed tingible body macrophages owing to the hematoxylin-stained apoptotic cell remnants in their phagolysosomes (Figure 51.4). During times of stress or atrophy, there is an increase in the proportion of tingible body macrophages and epithelial cells, resulting in lighter staining and a punched-out appearance to the cortex. in H&E stained sections.

Thymic involution and atrophy are histologically indistinguishable, characterized by a reduced size in thymic lobules and blurring of the corticomedullary junction. Involution is a natural, physiologic process, whereas atrophy occurring in young animals can be secondary to a variety of disease states. This can include atrophy secondary to poor nutrition and chronic high levels of glucocorticoids (usually due to chronic stress) as well as toxic insult (dioxin, polychlorinated biphenyls (PCB), lead, fumonisin B1 and B2) and viral infection (canine parvovirus, canine distemper virus, feline leukemia virus, feline panleukopenia virus, feline immunodeficiency virus, equine herpesvirus-1, classical swine fever, porcine reproductive and respiratory syndrome virus, and bovine viral diarrhea virus [BVDV]). The thymus can also be severely hypoplastic or absent in developmental diseases that either congenitally or genetically inhibit lymphocyte development, such as seen in severe combined immunodeficiency (SCID) and nude mice. Thymic hyperplasia occurs largely through an increase in number of lobules rather than increased lobule size, and is typically a nonspecific adaptive response to chronic antigenic stimulation. This can include the formation of functional B-cell follicles, termed follicular hyperplasia. Thymic hemorrhage and hematomas are found in young dogs, secondary to trauma, overstretching of the neck, rupture of aneurysms, hemorrhage from a thymic neoplasm, or secondary to anticoagulant (e.g., rodenticide) ingestion. An idiopathic form of spontaneous thymic hemorrhage is thought to occur due to the fragility of thymic blood vessels during thymic involution. Thymitis is seen in epizootic bovine abortion and salmon poisoning (*Neorickettsia helminthoeca*). Thymic cysts, often nonclinical, occur in the remnants of the brachial cyst ducts, and are lined by a single layer of ciliated epithelial cells.

Primary thymic neoplasia occurs in two forms: thymoma, derived from the thymic epithelial cells, and thymic lymphoma, derived from the thymic T cells. Thymomas are often benign, found in older animals as expansile mediastinal masses. A variety of autoimmune diseases have been associated with thymomas including myasthenia gravis and exfoliative dermatitis in cats.[11] Thymic lymphoma is more often seen in young animals, particularly young cats and cattle, and is often a lymphoblastic lymphoma.

Bursa of Fabricius

The bursa of Fabricius is a specialized lymphoid organ unique to birds. It is located in the dorsal diverticulum of the cloaca, and, like the thymus, is comprised of epithelial and lymphoid components. It is most active in young birds, reaching maximal size at around 6 months of age and progressively atrophying during adulthood. The bursa of Fabricius is the site of B cell maturation in birds, which in mammals generally occurs in the bone marrow. The bursal lymphoid follicles are located immediately subjacent to the folded epithelium of the dorsal cloaca in a similar structure to the Peyer's patches. Bursal lymphoid follicles contain lymphocytes, macrophages, dendritic cells, and plasma cells. Immature B cells are thought to migrate to the bursal mesenchyma where they interact with dendritic cells, and undergo V(D)J rearrangements and selection (see Chapter 50). After V(D)J rearrangement, B cells migrate to the bursal epithelium where they proliferate and generate bursal follicles. The epithelium overlying the bursal follicles has phagocytic abilities, sampling cloacal luminal antigens for presentation to dendritic cells and follicular lymphocytes, akin to the function of mammalian mucosa-associated lymphoid tissue (MALT) M cells.

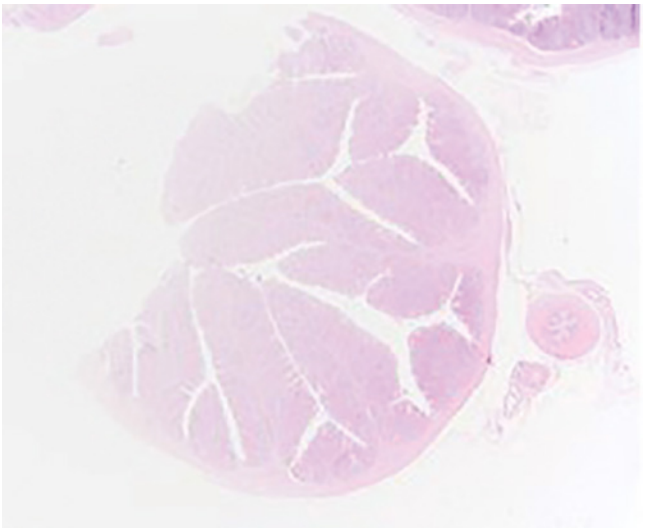


FIGURE 51.5 Bursa of Fabricius from a chicken infected with infectious bursal disease virus. There is severe diffuse atrophy of the bursa secondary to marked depletion of B cells H&E stain.

Infectious bursal disease, caused by infectious bursal disease virus (a birnavirus), specifically targets and kills maturing, IgM-producing, B cells in the bursa of Fabricius, the MALT, and in circulation, resulting in severe immunosuppression (Figure 51.5).

STRUCTURE, FUNCTION, AND DISORDERS OF SECONDARY LYMPHOID TISSUE

Lymph Nodes

The lymph node is the primary site of the adaptive immune response to antigens, bringing together antigen, APC, and lymphocytes. Lymph nodes are situated at multiple points of lymphatic vessel convergence and interface with vascular supply throughout the body. The anatomical location and number of nodes vary depending on the species. The lymph node architecture is critical to its function, as it dictates the spatial organization of lymphocytes and dendritic cells, orchestrating their interactions and performing a key role in which direction an immune response will develop (CD4+ vs. CD8+ vs. TH fate).[7] The lymph node is comprised of multiple repeating subunits—the lymphoid lobules. Lymphoid lobules can be singular in smaller lymph nodes, but in larger lymph nodes are arranged adjacent to each other,

radiating from the hilus and their vascular supply.[18] Lymphoid lobules are divided into the cortex, paracortex, and medulla. This arrangement is consistent in all domestic species, with the exception of the pig, where the organization of the medulla and the cortex are reversed. In the pig, afferent lymphatics enter the center of the node where the germinal centers are located first, such that functional delivery of antigen to the node is similar to other mammals.

The lymph node is surrounded by a peripheral capsule that is thin and taut in conditions in which there has been rapid enlargement of a lymph node. By contrast, lymph node atrophy results in relative thickening of the capsule that on H&E stained slides is thickened and wavy, with widening of the peripheral sinuses. In cattle and likely in other species, the lymph node capsule is contractile and under neural control, thus assisting lymph flow.[8]

The structure of the lymph node is centered on two circulatory systems—the lymphatic and vascular systems—and supported by a fine meshwork of supportive reticular cells. Lymph enters the capsule of the lymph node through afferent vessels that drain into subcapsular sinuses of the cortex (Figure 51.6). In lymph nodes draining areas of inflammation, the subcapsular sinus is a common area for the relevant inflammatory cells (neutrophils, macrophages, and eosinophils) as well as infectious agents to accumulate. Cortical, paracortical, and transverse sinuses percolate through the cortex and paracortex connecting lymph flow to the medullary sinuses in an intricate meshwork. The medullary sinuses converge near the hilus of the lymph node, eventually forming the efferent lymphatic that exits the node. Entering the hilus is the lymph node arteriole. This arborizes in the medulla, following medullary cords, dividing into small arterioles and a capillary network that supplies the cortex and paracortex. Postcapillary high endothelial venules (HEV) in the paracortex are important sites of T cell migration. As the venules enlarge and re-enter the medulla, they converge, leaving the hilus again as a single lymph node vein. The reticular network is comprised of stellate and spindled fibroblastic reticular cells that form a delicate network through which APCs and lymphocytes migrate. The vascular, lymphatic, and reticular network of the lymph node forms the basis of lymphocyte colonization during embryogenesis and later, after colonization, the lymphoid lobule.

The cortex of the lymph node is comprised of follicular and interfollicular regions. Cortical follicles contain mostly B cells and are the location of germinal centers following immune stimulation. Germinal centers are the site of B cell clonal expansion, somatic hypermutation, and affinity maturation (see Chapter 50). In unstimulated lymph nodes, B cells form dense follicles in the superficial cortex. After stimulation, germinal centers develop; displacing naïve B cells that then form the mantle zone. The mantle zone is eccentrically thickened near the capsule and forms a thinner rim closer to the paracortex. The naïve B cells in the mantle zone have small dense nuclei, staining darker on H&E. The proliferating germinal center is divided into two compartments: the dense zone (deep pole), adjacent to the T cell parafollicular zone, and the light zone (superficial pole), nearest the capsule. The dense zone is comprised almost exclusively of B cells with a high nucleus: cytoplasm ratio and dense chromatin, producing a darker basophilic stain on H&E. The light zone contains a mix of cells, which includes B cells, follicular dendritic cells, T cells, and migrating naïve B cells.[17] Tingible body macrophages are found throughout both zones of the germinal center, phagocytozing B cells that become apoptotic during B cell selection (see Figures 51.7 and 51.8). Previous work has suggested two different types of B cells present in dense and light zones: centroblasts and centrocytes, respectively. It was thought that centroblasts in the dense zone underwent clonal expansion and antigen receptor

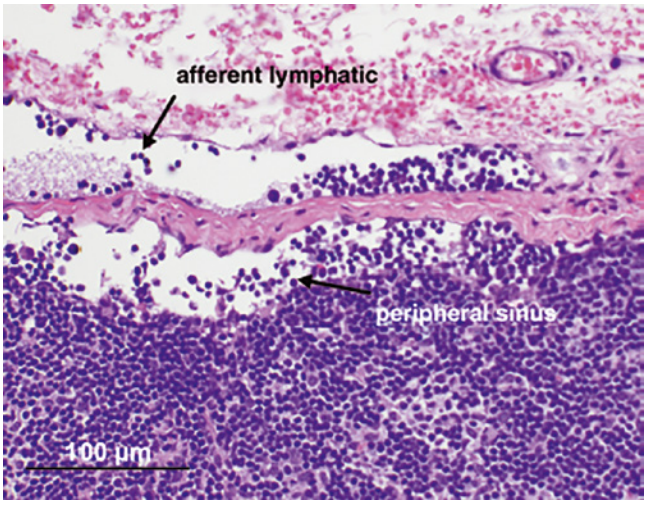


FIGURE 51.6 Lymph node outer cortex from a healthy dog. An afferent lymphatic (arrow) leads into the peripheral sinus beneath the node capsule. The sinus is open and contains a few cells in transit, whereas to the left and right, the sinus is compressed by developing germinal centers. H&E stain; bar = 100 µm.

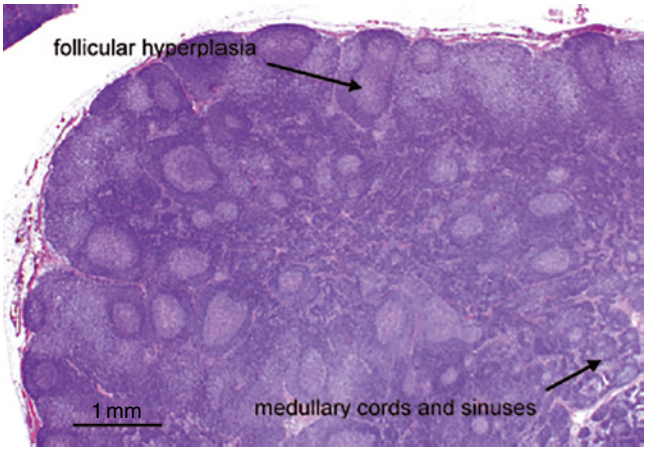


FIGURE 51.7 Architecture of the lymph node in Figure 51.6 showing follicular hyperplasia and densely cellular medullary cords and sinuses (arrows). H&E stain; bar = 1 mm.

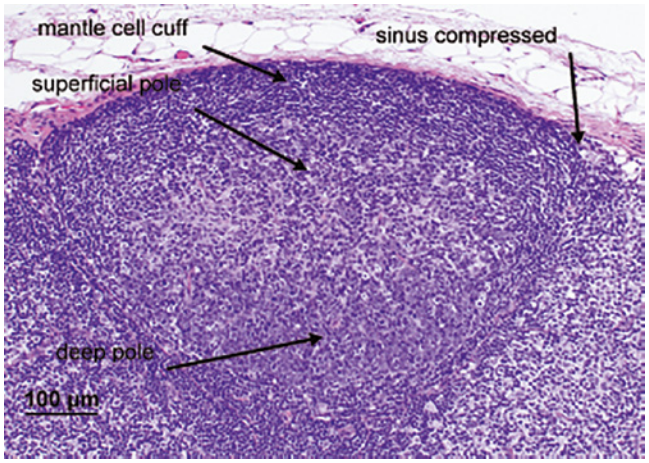


FIGURE 51.8 Lymph node cortex of Figure 51.6 with subcapsular germinal center having a polarity directed toward the antigens being delivered from the peripheral sinus into the superficial pole of small lymphocytes. In the middle of the germinal center, there are small and large lymphocytes with macrophages removing apoptotic lymphocyte nuclei. Near the bottom of the picture, the deep pole area consists of lymphocytes with large nuclei and a narrow rim of highly basophilic cytoplasm. H&E stain; bar = 100 µm.

diversification that matured to centrocytes that unidirectionally migrated to the light zone where B cell selection and antigen binding occurred. Numerous recent studies have challenged the centroblast/centrocyte model with no differences in B cells in the zones with respect to size, morphology, gene expression, or dynamic behavior and B cells seem to move back and forth between the zones during the immune reaction.[1,2,14] Instead, it is thought that B cells cycle between the dense and light zones, with proliferation occurring in the dense zone and selection occurring in the light zone, in repetitive iterations of selection of high-affinity clones, proliferation, mutation, and further selection–leading to affinity maturation of the B cell response.[17] Proliferation, hypermutation, and selection of B cells in germinal centers produce both memory B cells and plasma cells. Plasma cells can remain in the lymph node or can migrate to the medullary cords, or bone marrow. B cells that are stimulated in a T-cell-independent manner (by foreign polysaccharides or CpG DNA) proliferate outside of the follicles and do not produce germinal centers.[7]

T cells home to the deep cortex (also called the *paracortex*) and interfollicular region of the cortex, entering through HEVs where they migrate and survey dendritic cells.[13] HEVs are sophisticated structures with pockets between endothelial cells and the basement membrane that have the capacity to store circulating lymphocytes, prior to entry into the lymph node, regulating lymphocyte entry. A reticular framework supports the interfollicular cortex and paracortex, similar to the rest of the node, within which are dendritic cells. The fibroblastic reticular cells that form the framework are believed to produce cytokines that establish the spatial segregation of T and B cells in the lymph node and induce and aid in T cell migration through these areas. The deep cortex is comprised of semi-ovoid aggregations of T cells that center on lymphatic openings. With stimulation, proliferating T cells form dense nodular aggregates, termed deep cortical units. Unlike germinal centers, the distribution of cells in proliferating deep cortical units is more homogenous (Figure 51.9). Central areas of deep cortical units can have a mottled appearance histologically, due to varying proportions of large and small lymphocytes with some macrophages and HEVs (Figure 51.10). The edges of the deep cortical units gradually meld with the surrounding medullary cords and sinuses.

The medulla of the lymph node is comprised of medullary cords and sinuses. The medullary cords are centered on centrifugally radiating medullary arterioles and medullary veins that are returning to the hilus centripetally. Surrounding the blood vessels is a meshwork

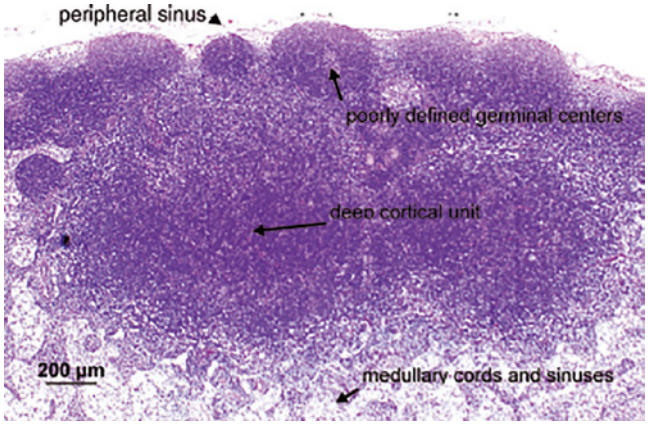


FIGURE 51.9 Cross-section of rat mesenteric lymph node. The upper area of the node is occupied by typically poorly defined germinal centers. The medullary cords and sinuses lie along the bottom of the image and abut a large uniformly dense oval deep cortical unit that lies across the cut surface of the node. A rim of less-dense cellularity lightly outlines the unit. H&E stain; bar = 200 µm.

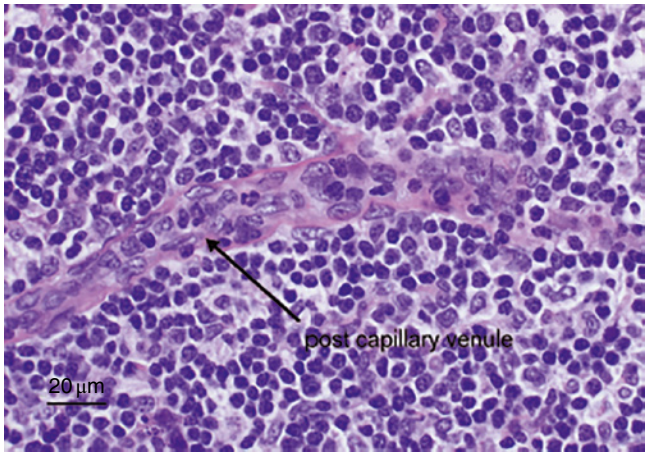


FIGURE 51.10 Paracortex (deep cortex) of rat mesenteric lymph node with a high endothelial postcapillary venule crossing the center. Several lymphocytes within the wall of the venule can be seen to be in transmural transit exiting the blood to enter the node cortex (near arrow). H&E stain; bar = 20 µm.

of pericytic reticular cells within which are migrating, recirculating lymphocytes.[18] Stimulated B cells forming precursor plasma cells migrate to the medullary cords where they mature to plasma cells. Interdigitating between the medullary cords are the medullary sinuses that drain lymph from the cortical and paracortical sinuses to the efferent lymphatic at the lymph node hilus. In the medullary sinuses, macrophages adhere to reticular cells phagocytozing debris, bacteria, RBCs, and infectious agents in the circulating lymph (Figure 51.11). Proliferation of medullary macrophages in response to increased draining debris is termed "sinus histiocytosis." This can be seen in lymph nodes draining areas of tissue necrosis, infection, or hemorrhage, and in the case of hemorrhage often contain large amounts of cytoplasmic hemosiderin, pigmenting the center of the node. In lymph nodes draining areas of chronic dermatitis, melanin pigment can be found phagocytosed in macrophages. The fine reticular network of the medullary cords forms a fertile microenvironment for extramedullary hematopoiesis. In myeloid leukemias, the malignant cells also colonize the medullary cords, and under these circumstances, there may be both tumor and benign extramedullary hematopoiesis coexisting.

Developmental diseases of lymphocytes can result in lymph node atrophy in a manner similar to that seen in the thymus. Foals with SCID have small lymph nodes that lack germinal centers and have poorly populated paracortical zones (see Figure 51.12). Severe T cell deficiencies have developed cortical follicles, but a poorly developed paracortex, and severe B cell deficiencies have the opposite, developed paracortex, but virtually absent cortex. In adulthood, severe cachexia and advanced age can result in lymph node atrophy, commonly associated with relative thickening of the capsule and the medullary trabeculae. Cachectic atrophy is more common in old sheep and goats and senile atrophy is more frequently seen in aged dogs, cats, and primates.

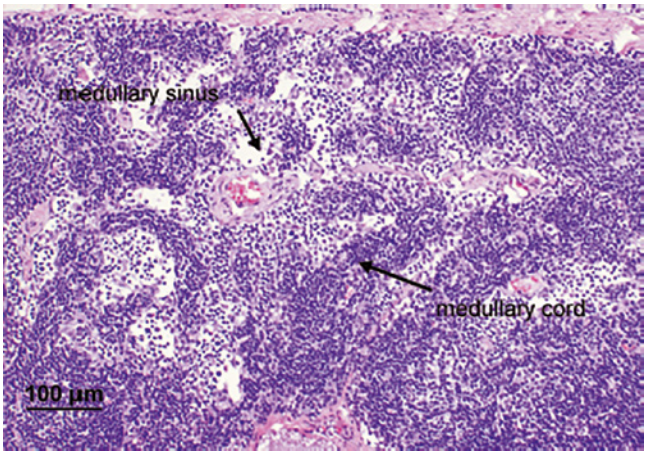


FIGURE 51.11 Medulla of a lymph node from a dog with normal cords and sinuses. The cords are densely cellular and surrounded by a thin endothelial membrane with the sinuses containing a few fixed macrophages and cells in transit in a background of lymph. H&E stain; bar = 100 µm.

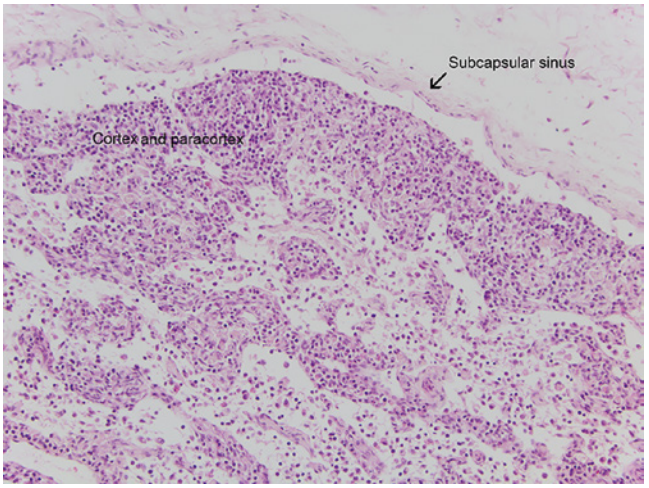


FIGURE 51.12 Hypoplastic lymph node from a 4-month-old Arabian foal with SCID. Marked hypoplasia of the cortex and paracortex, populated mostly with dendritic cells, reticular cells, and macrophages. H&E stain.

Lymph node atrophy can also occur secondary to a chronic infection with a lympholytic virus, e.g., BVDV.

Lymphadenitis occurs when an infectious or inflammatory agent specifically inflames lymphoid tissue, as opposed to benign hyperplasia, a reactive immunologic response to inflammation in the draining field. In acute lymphadenitis, typical features of acute inflammation (vasodilation, hyperemia, and edema) are present in the lymph node, as well as an increased proportion (typically >25% on cytology) of the relevant inflammatory cells. As with most infectious processes, neutrophilic lymphadenitis occurs in response to bacterial agents, including *Streptococcus* species, *Brucella* species, *Trueperella pyogenes*, *Salmonella* species, and *Toxoplasma* species. *Francisella tularensis* and *Yersinia pestis* frequently cause severe liquefactive necrosis of the lymph node, as well as suppuration. Chronic lymphadenitis can produce fibrosis around the node that fixes them in the surrounding connective tissue. Typically, the capsule and trabeculae are also thickened and sclerotic. Chronic inflammation can be mixed or granulomatous. It can be seen nonspecifically with chronic bacterial infections (usually mixed inflammation), as well as with specific agents including *Corynebacterium pseudotuberculosis* (also called caseous lymphadenitis), *Mycobacterial* agents (granulomatous inflammation), *Rhodococcus equi* (granulomatous inflammation), and fungi (granulomatous inflammation) (Figure 51.13). Porcine circovirus type 2 (PCV2)-associated diseases can also cause granulomatous lymphadenitis with characteristic botryoid, intracytoplasmic, viral inclusion bodies in macrophages. Eosinophilic lymphadenitis occurs in response to parasitic infections of the node (e.g., demodex lymphadenitis) or hypersensitivity reactions.

Lymphadenopathy (better termed lymphadenomegaly) is a generalized term used for enlargement of the lymph node due to nonspecific lymphoid hyperplasia. Lymphoid hyperplasia can be skewed to a predominantly

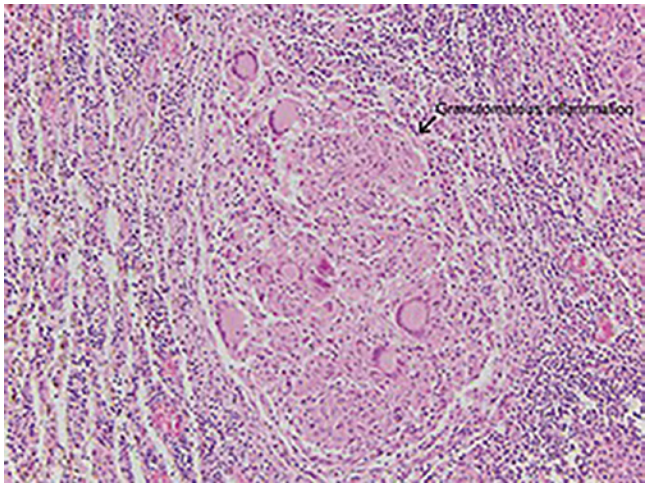


FIGURE 51.13 Granulomatous lymphadenitis in an adult cow infected with *Mycobacterium bovis*. The granuloma is located in the paracortex and consists of epithelioid and multinucleated Langhans-type macrophages. Increased numbers of hemosiderin-laden macrophages are present in the medullary sinuses on the left producing brown pigmentation of the lymph node. This is consistent with the lymph node draining hemorrhage in the local area.

B cell response in which follicular hyperplasia and cortical expansion are prominent, or a predominantly T cell response in which hyperplastic deep cortical units in the parafollicular or deep cortical area are predominant (Figure 51.8). Increased proportions of plasma cells in medullary cords and sinus histiocytosis are other features of a reactive immune-driven hyperplasia. When the lymph node is draining an area of active inflammation, there are increased numbers of often innate immune cells that drain into the subcapsular sinuses.

Differentiation of marked lymphoid hyperplasia from lymphoma can be difficult. Key, commonly used features are the disruption of the normal lymphoid architecture and the homogeneity of the lymphocyte population in lymphoma. Disruption of the outer sinuses and invasion of the capsule are features seen in lymphoma, but not in lymphoid hyperplasia. While some lymphomas can have two types of neoplastic lymphoid cells histologically (e.g., a T-cell-rich B cell lymphoma), there are no known neoplastic processes with three different lymphoid types. Chromatin and cytoplasmic homogeneity is generally expected in lymphoma. Follicular hyperplasia can be differentiated from follicular lymphoma based on the architecture of the follicles, as well as by the preservation of an intact mantle cell corona in a hyperplastic process. The polarity of germinal centers is important to distinguish benign and atypical follicular hyperplasia from follicular lymphomas. In follicular lymphoma, the mantle is often effaced, there is often concurrent paracortical atrophy, and the follicles themselves lack the lymphocytic heterogeneity expected in a reactive follicle.[15] Follicular lymphoma have few or no tingible body macrophages, as the tumor cells, unlike the benign cell selection process, are not undergoing apoptosis. A further distinction between true follicular lesions, whether benign or malignant, and pseudofollicular lesions is that the postcapillary venules are between the follicular or nodular proliferations and never within them.[15]

As lymph nodes are the hub of lymphatic drainage and surveillance, they are also a common site for metastatic disease, particularly from primary neoplasms in their draining area. Following the route of drainage, metastatic neoplastic cells are frequently found at the periphery of the node in subcapsular sinuses and in the medullary sinuses as single or clusters of neoplastic cells (Figure 51.14). In non-lymphoma metastatic disease, the lymph node is generally atrophied. Myeloid leukemias tend to home to medullary cords, which are also sites of extramedullary hematopoiesis. Spread of neoplastic lymphoma cells to lymph nodes tends to reflect the cell type and homing receptors of the neoplastic cells such that B cell lymphoma will tend to spread to B cell areas in the cortex and T cell lymphoma spread to T cell areas in the paracortex.

Tertiary Lymphoid Centers

Where lymph nodes, the spleen, and MALT comprise the secondary lymphoid centers with the anatomic localization of these organs similar between individuals of a species, tertiary lymphoid centers with similar structure to lymph nodes develop ectopically in response to chronic inflammation or neoplasia. Similar to lymph node development, tertiary centers are believed to be induced by chemokine secretion by stromal cells localized to the site of chronic inflammation. A similar structure to a lymph node develops including T cell zones with high endothelial blood vessels, B cell follicles, and germinal centers of proliferating B cells.

Spleen

The spleen is the primary blood-filtering organ of the immune system. Analogous to the role of lymph nodes in surveilling antigen in the lymph, the spleen's primary

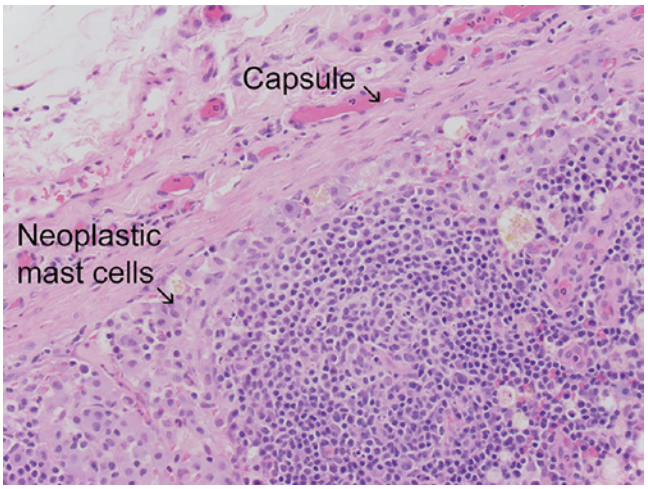


FIGURE 51.14 Lymph node from an adult dog. Neoplastic mast cells infiltrate the subcapsular sinus of a draining lymph node.

immunologic function is surveillance of antigens in the blood, as well as removal of effete or defective erythrocytes or white blood cells. The anatomy of the spleen reflects this blood-filtering role: it is comprised of a complex arborizing network of arteries and arterioles that drain into porous venous sinuses of the red pulp where lymphocytes, dendritic cells, macrophages, and other immune cells can be maximally exposed to circulating cells and pathogens. The venous system similarly arborizes into increasingly sized veins that eventually merge to form the splenic vein. Both the major arterial supply and venous outflow enter through the hilus of the spleen. While the spleen contains efferent lymphatics, no afferent lymphatics are present. The spleen has additional functions in lymphopoiesis and extramedullary hematopoiesis. The spleen forms early in embryonic life and is a site of active hematopoiesis during the fetal period, and recolonization of these sites occurs at times of increased demand during adulthood.

The vascular anatomy of the spleen is critical to its function. It contains arborizing arterioles that, as they become smaller, are surrounded by a lymphoid sheath, termed the periarteriolar lymphoid sheaths (PALS). The PALS is formed by a cuff of densely packed T cells which, at points along the length of the artery, dilate eccentrically with B cell follicles, located to the periphery of and surrounded by the T cell cuff (Figure 51.15). At the periphery of both the PALS and the follicles is a looser arrangement of mixed T and B cells called the marginal zone. Together, the periarteriolar sheath, B cell follicles, and marginal zone lymphocytes form the white pulp.

Similar to the lymph node, the PALS form a distinct T cell zone, and the follicles form distinct B cell zones. The PALS is comprised centrally of dense accumulations of darkly staining T cells, most of which are CD4+ T cells interacting with interdigitating dendritic cells and lesser numbers of CD8+ T cells. B cells in follicles function similarly to the lymph node, where, once activated, B cells migrate to the follicle, proliferate, and undergo clonal expansion, isotype switching, and somatic hypermutation (see Chapter 50). The marginal zone forms a key transit area for lymphocytes leaving the blood stream and entering the white pulp, in contrast to the lymph node, where lymphocytes mostly enter through afferent lymphatics or HEVs. At the periphery of both the PALS and B cell follicles is the marginal zone, a complex, highly organized zone with key functions in cell migration. The marginal cell zone layer is more loosely arranged and is comprised of T cells, B cells, marginal zone macrophages, and marginal metallophilic macrophages whose scavenging properties play a key role in antigen presentation (Figure 51.16). While the B and T cell zones of the white pulp are almost exclusively involved in adaptive immune responses, the marginal zone is involved in both innate and adaptive responses. Marginal zone B cells are specialized to detect blood-borne pathogens, can differentiate into plasma cells producing high levels of IgM, and can function as antigen-presenting cells. They can migrate to the B cell follicles, initiating T-cell-independent humoral immunity. Marginal zone B cells acting as APC can also migrate to the T cell zone where they activate naïve CD4 T cells. Lymphocytes exit the white pulp either through the efferent lymphatics or back into the blood stream through the marginal zone.[9]

B cell follicles are supplied by very small arterioles that branch off the larger sheathed arteriole. In some species, these small arterioles end in the marginal sinus (the area between the white pulp and the marginal zone), whereas in others, the arterioles transverse the marginal zone and directly drain into venous systems of the red pulp.[9] These areas receive some of the more recently arrived cells to the spleen, and in cases of

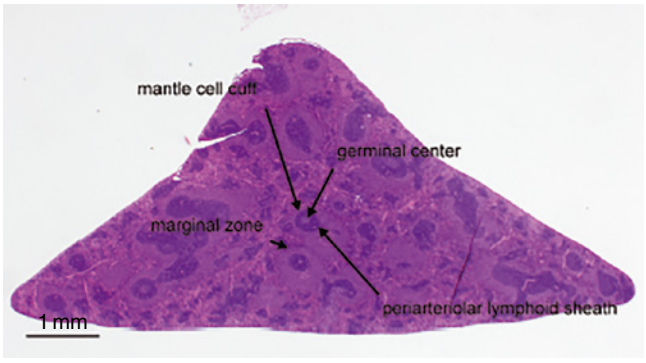


FIGURE 51.15 Cross-section of spleen from a young adult male rat. At the architectural level, the spleen is occupied by multiple, dispersed round dark areas representing lymphoid nodules with the intervening lighter areas consisting of sinuses. The lymphoid or white pulp areas may have a germinal center in the middle of the mantle cell cuff (arrows). The lighter mantle and marginal zone areas surround these areas. H&E stain; bar = 1 mm.

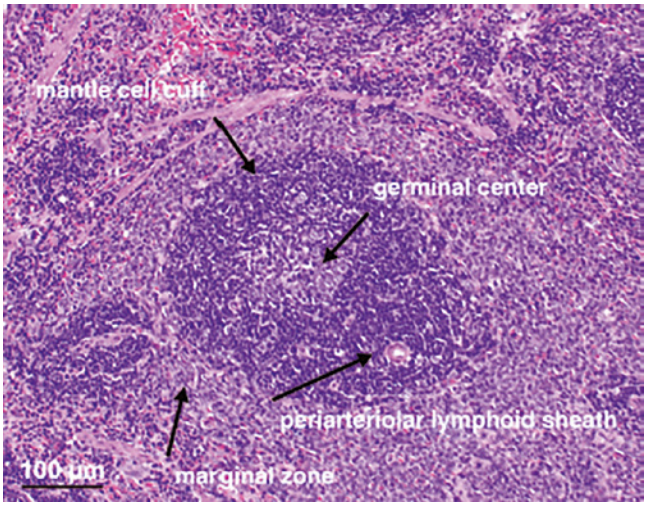


FIGURE 51.16 Details of Figure 51.5. Unlike larger mammals, rats tend not to have well-formed splenic germinal centers. In the middle of the image, a lighter zone constitutes the dendritic cell bed of an ill-defined germinal center eccentric to and abutting a small muscular arteriole at lower right (arrows). The germinal center is surrounded by a cuff of darker mantle lymphocytes that has an outer envelope of lighter-staining marginal zone cells that interface with the surrounding splenic red pulp. H&E stain; bar = 100 µm.

marked neutrophilia, neutrophils can form a cuff around the marginal zone. The splenic arterioles terminate in pencillary vessels that drain into large venous sinuses, which then drain into splenic veins. Ellipsoid reticular cells surround the pencillary vessels in some species, controlling the amount of blood flowing into the venus sinuses.

The red pulp is comprised of splenic cords and sinuses. Approximately 3% of the blood flow in the venous sinuses perfuses into the splenic cords (also called the *Billroth cords*) of the red pulp. The splenic cords are comprised of a network of reticular cells and fibers that are arranged such that cells exiting the macrophage-rich cords into the red pulp sinus have to squeeze through junctions formed by reticular cells. As erythrocytes age, or are damaged, they lose their deformability and their ability to squeeze effectively through reticular junctions. The cords are also rich in macrophages and the slowing of aged, less-deformable erythrocytes through reticular junctions allows for the phagocytosis and removal of senescent or deformed RBCs (including those with inclusion bodies or reticulocytes), senescent leukocytes, circulating foreign material, and iron breakdown products. The large venous sinuses of the red pulp are highly perforated, slowing blood as it flows through the red pulp. Macrophages, lymphocytes, and plasma cells are embedded in the Billroth cords and blood flow through this area is slow, such that there is maximum exposure of erythrocytes to surveilling cells. By this process, the entire blood volume passes through the filtering system of the spleen at least once each day.

Similar to the lymph node, after B cells proliferate and are selected in the germinal centers, they migrate as plasmablasts to the red pulp, often just outside the marginal zone, where they mature to plasma cells and produce antibodies.[9] The position of plasma cells in the red pulp allows rapid entry of antibodies into the blood stream.

The venous sinuses of the spleen constitute the expansile regions of the organ and thus may vary greatly in their content of blood, particularly in species with a high level of smooth muscle in the splenic capsule. In a muscular spleen in the contracted state, all of the blood would pass directly from the arteriole to the venous system in a "closed" circulation, permitting maximal utilization of blood cells in the systemic circulation. By contrast, any influence that causes the splenic capsule to dilate (e.g., anesthesia, portal hypertension due to hepatic fibrosis, or marked increase in extrasinusoidal macrophages) results in a greater proportion of blood passing through the filtering system.

A variety of changes occur in the spleen in response to systemic states, the most notable of which is reactive lymphoid hyperplasia of the PALS in an adaptive immune response to systemic infection. This is mostly in response to infections that involve the blood and can include systemic viremia (e.g., malignant catarrhal fever and equine infectious anemia), bacteremia/endotoxemia, blood cell parasitemia (e.g., malaria and trypanosomiasis), and immune-mediated diseases directed against circulating cells (most commonly, immune-mediated hemolytic anemia). In septicemia or cases of marked neutrophilia, aggregates of neutrophils can be seen in the marginal zone. Additionally, when acute infection is present, the spleen is often hyperemic and enlarged. This is most marked in anthrax, where the septicemic response is almost entirely vascular (Figure 51.17).[16] Acute toxic diseases, overwhelming infection, and certain viremias can result in lysis of lymphocytes in the germinal centers and replacement by residual proteinaceous debris referred to as follicular hyalinosis. Diagnosticians are aided by the fact that nuclear debris after acute lympholysis is cleared within 24 hours, but the hyalin debris may remain for many months. In systemic amyloidosis, the germinal centers may become sites of amyloid deposition, while atrophic changes due to starvation, aging, cancer, or chemotherapy may result in atrophy which impacts one or both of the thymic-dependent arteriolar sheaths or bone-marrow-dependent germinal center systems. In chronic hemolytic anemias, a marked increase in hemosiderin-bearing macrophages in sinus areas and iron deposition can occur in the connective tissue of the spleen, particularly in old dogs. Nodular hyperplasia is common in older dogs and occasionally old bulls. The nodules are comprised predominantly of proliferating large lymphocytes and differentiation from lymphoma can be difficult. Lymphoma and leukemias can localize to the spleen, resulting in splenomegaly. Hemangiosarcoma and hemangiomas are common vascular tumors of the spleen that can rupture, resulting in life-threatening hemoabdomen. Rupture of the spleen can also occur secondary to splenomegaly associated with lymphoma or as a result of abdominal trauma. As a blood-filtering organ, metastatic disease is also common. Splenic infarction occurs in focal areas of the spleen in dogs with hemangiosarcoma and is always of hemorrhagic type. Ischemic infarction can occur in dogs with myeloma due in part to the greater

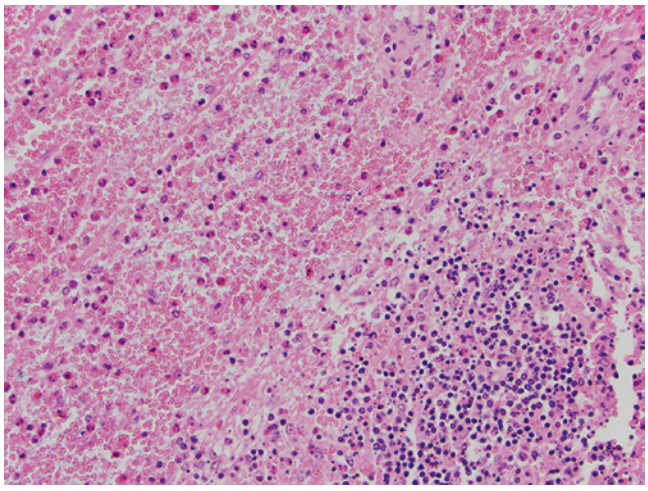


FIGURE 51.17 Spleen from a cow with anthrax. There is severe hyperemia of the red pulp with large number of neutrophils. Lymphocytolysis is present in the white pulp. Large numbers of faintly basophilic rod-shaped bacteria (*Bacillus anthracis*) are present in the background.

viscosity of blood secondary to hyperglobulinemia. Splenic torsion occurs primarily in dogs and rarely in adult pigs. In dogs, this is often secondary to gastric dilation and volvulus.

Hemal Nodes

Hemal nodes, found in ruminants and rats, have a similar structure to lymph nodes, but instead of draining and surveilling antigen in lymph, they drain and survey blood, a function more similar to the spleen. Arteries enter the hilus, similar to the lymph node, but arborize in the cortex, draining to veins. There are germinal centers in the cortex; however, the paracortex (T cell) is much diminished compared to a lymph node. Macrophages are present in trabecular areas and phagocytose efferent erythrocytes or defective erythrocytes.

Mucosa-Associated Lymphoid Tissue

The MALT is a system of small lymphoid aggregates located in the submucosa of most mucosal surfaces in the body including the respiratory, gastrointestinal, and reproductive tracts, as well as the skin, eye, excretory ducts, and mammary and salivary glands. MALT samples surface (mucosal) antigen, stimulating mucosal immunity. Although less familiar than lymph nodes and the spleen as sites of immunity, there are more immune cells in the intestinal mucosa than the any other site in the body.[3] MALT is key in regulating mucosal immunity, most commonly through Th2-driven IgA production and to a lesser degree Th1 and cytotoxic T cell reactions. The components of the MALT can be subdivided anatomically and as such, differentially named, e.g., gut-associated lymphoid tissue (GALT), bronchus-associated lymphoid tissue (BALT), vulvovaginal-associated lymphoid tissue (VALT), etc. (Figure 51.18). There is considerable species variation as to the prominence or even presence of the subsets of MALT. Generally, the anatomy and immunological function of the MALT are similar regardless of location.

Functionally, MALT serves as either inductive or effector sites. Inductive sites are areas where antigen is sampled from the mucosal surface and then presented to and stimulates cognate T and B cells. Follicles are present in inductive sites, forming germinal centers with activation that allows B cell proliferation and class switching, secondary to Th2 cell activation (see Chapter 50; Figure 51.19). The mucosa covering inductive sites contains specialized cells called "M cells" that phagocytose microbes and particles from the mucosal surface, transporting them through the epithelium to the underlying lymphoid tissue, exposing lymphocytes to antigens from the mucosal surface. Ultrastructurally, the subepithelial surface of M cells contains deep invaginations in which B and T cells as well as macrophages are located (Figure 51.20). Follicular areas mostly contain B cells embedded in a network of follicular dendritic cells with fewer CD4+ T cells and macrophages. Interfollicular areas are rich in T cells and recirculating lymphocytes can enter the MALT through HEVs. Efferent lymphatics connect MALT with regional lymph nodes, further inducing and augmenting an adaptive immune response. Effector sites are sites where effector cells, after stimulation, perform their immunologic function. Effector sites have more diffuse anatomy, spread along mucosal surfaces, most commonly in the lamina propriae.[4]

The GALT forms a relatively continuous chain from the oral cavity to the anus. There are generally few lymphocytes in the epithelium and mucosa of the esophagus. The Peyer's patches in the intestine and the tonsils form inductor sites for the GALT and can be the target of disease processes and pathogen portal of entry. *Mycobacteria*, *Salmonella*, *Yersinia*, and *Listeria* often gain entry to the body through M cells. Prion proteins have been isolated from the tonsillar tissue of sheep, cattle, elk, and deer. Aujeszky's disease in pigs targets tonsils causing a necrotizing tonsillitis. Many lymphotropic

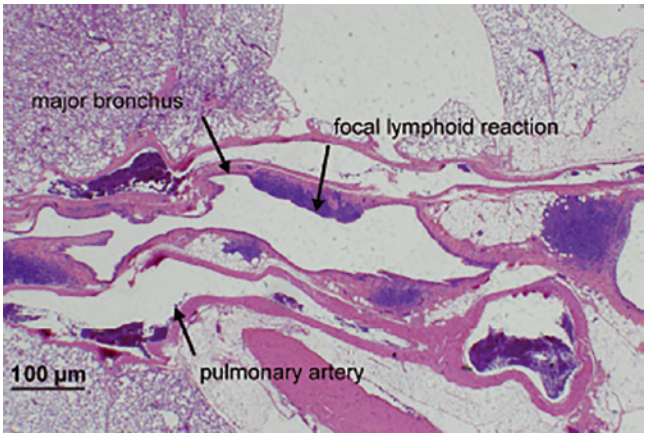


FIGURE 51.18 Lung from a normal rat with longitudinal section of a major bronchus. Note that the focal areas of lymphoid proliferation are both peribronchiolar and subepithelial. H&E stain; bar = 100 µm.

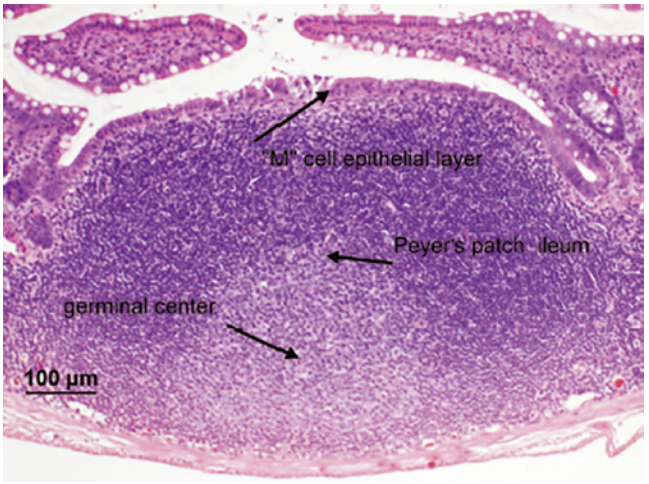


FIGURE 51.19 Lower small intestine of normal rat. A focal submucosal proliferation of lymphocytes in a dome area characterized by absent villi and modified epithelium to assist antigen recognition and sorting. H&E stain; bar = 100 µm.

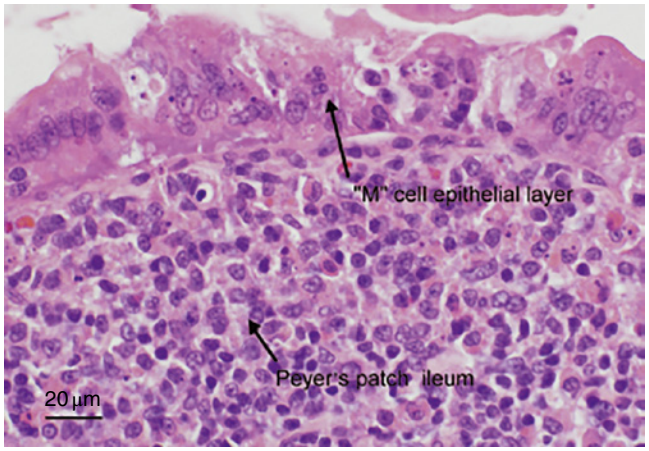


FIGURE 51.20 Details of Figure 51.15. The epithelium above the lymphoid follicle is modified to contain M cells that have deeply folded cellular membranes that enclose intraepithelial lymphocytes. The M cells are capable of uptake and of processing antigen from the intestinal lumen and providing immune instruction to maturing lymphocytes. In this area, there are lymphocytes in the epithelium undergoing apoptosis presumably similar to the selection process in node germinal centers. H&E stain; bar = 20 µm.

viruses, including feline panleukopenia virus, canine parvovirus, canine distemper, bovine viral diarrhea, rinderpest, and swine vesicular disease virus target Peyer's patches and tonsils with Peyer's patch necrosis particularly prominent in BVDV and rinderpest. Like the lymph node, PCV2 can cause granulomatous inflammation in Peyer's patches. The tonsils are also a site of viral persistence with feline calicivirus infection.

Intraepithelial lymphocytes (IEL) interdigitate between mucosal epithelial cells. Their unique expression of $\alpha_E\beta_7$ integrin interacts with E-cadherin on epithelial cells, allowing IELs to enter and remain within the epithelium. Unlike many lymphocytes, IELs do not circulate but remain as residents in the mucosal epithelium. In many species, the majority of intraepithelial lymphocytes express γδ TCR, as opposed to more common αβ TCR, and CD8αα as opposed to the more common CD8αβ. There is also a population of recently identified TCR-negative IELs. IELs play roles in both the innate (in a natural-killer-cell-like function) and adaptive immunity.

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CHAPTER 52

Systemic Lupus Erythematosus

LUC CHABANNE

Pathogenesis
  Autoimmunity
    Autoantibodies
    B and T Cell Response
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    Levamisole
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Acronyms and Abbreviations

ANA, antinuclear antibody; ACR, American College of Rheumatology; CD, cluster of differentiation; CIC, circulating immune complex; DLA, dog leukocyte antigen; dsDNA, double-stranded DNA; ENA, extractable nuclear antigen; FeLV, feline leukemia virus; FIV, feline immunodeficiency virus; Ig, immunoglobulin; LE, lupus erythematosus; mAb, monoclonal antibody; MHC, major histocompatibility complex; MMF, mycophenolate mofetil; NSDTR, Nova Scotia Duck Tolling Retriever; RNP, ribonucleoprotein; SLE, systemic lupus erythematosus; SS-A, Sjögren syndrome antigen A.

Systemic lupus erythematosus (SLE) is a chronic inflammatory, multisystem, autoimmune disease with protean clinical manifestations and laboratory features. It is characterized by an autoantibody response and complement activation, immune complex formation, and immune dysregulation that cause damage to multiple organs and lead to a wide spectrum of clinical manifestations.

In animals, SLE has been described in monkeys, dogs, cats, and horses. A number of inbred strains of mice (e.g., the New Zealand black/white strain) spontaneously develop lupus-like syndromes, and this has allowed intensive study of disease mechanisms. SLE-like disorders have also been reported in poikilothermic species, such as snakes and iguanas. Considered as an infrequent disease in the dog, many studies have been published on canine SLE since its initial description in 1965,[22] while feline and equine SLE are rare. An experimental canine model of SLE has been developed, and could be interesting for a better understanding of this complex disease and evaluation of new therapeutic approaches.[8]

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PATHOGENESIS

Autoimmunity

Systemic lupus erythematosus is the prototypic multisystem autoimmune disease in which immune dysregulation leads to failure of the mechanisms that normally prevent the development of self-response.[32]

Autoantibodies

SLE patients develop a wide variety of autoantibodies (Table 52.1).[26,28,35]

Antinuclear antibodies (ANAs) are the most significant autoantibodies found in SLE. One proposed mechanism for development of ANAs involves a defect in apoptosis that causes increased cell death. During apoptosis, blebs of cellular material form on the surface of dying cells. Antigens that are normally buried within the cell are exposed on the surface of these blebs and they may trigger an immune response (Figure 52.1). These exposed antigens include nucleosomes (DNA and histone proteins), extractable nuclear antigens (ENA, including ribonucleoproteins (RNP)), and anionic phospholipids. The clearance of apoptotic debris is abnormal in patients with lupus. Normally, apoptotic cells are removed by phagocytes without causing inflammation. However, macrophages from SLE patients show defective phagocytosis of apoptotic cells, which, as a result, accumulate in tissues.

Unlike humans, dogs do not develop ANAs against native DNA (double-stranded DNA (dsDNA)), and there is a restricted pattern of reactivity against the various individual histone antigens (H1, H2A, H3, and H4 polypeptides), while ANAs specific to nucleosome or histone–DNA complexes are rare.[26,28] Canine ANAs also appear to have additional reactivities not found with human SLE sera (e.g., for the glycoprotein hnRNP G).[34] By contrast, the pattern of reactivity of other ENAs found in dogs is similar to that found in humans; e.g., anti-Sm antibodies and anti-SS-A/Ro antibodies. Antibodies to the proliferating cell nuclear antigen have also been described in dogs.

ANAs mediate tissue inflammation and injury in SLE either by direct pathogenic effect (e.g., anti-SS-A/Ro activity against skin) or due to their association with antigens to form immune complexes either in situ or in circulation. Moreover, a wide range of abnormalities contribute to an impaired clearance of circulating immune complexes (CIC) in SLE (e.g., abnormal complement receptors, deficiency of complement components), and CICs are not effectively removed from the circulation. Thus, the net result is prolonged high levels of CIC, which become available for deposition in nonlymphoid sites such as kidneys, synovium, or skin. Inappropriate tissue deposition or formation in situ may result in complement activation, granulocyte chemotaxis, and release of inflammatory mediators leading to vascular injury (type III hypersensitivity) and tissue damage.

TABLE 52.1 Autoantibodies in canine SLE.

Autoantibody	Percent positive
Antinuclear (indirect immunofluorescent techniques)	95–100
Anti-dsDNA	<3
Anti-ENA	
Total	40
Sm	16
RNP	8
hnRNP G	20
Canine type 2	9
SS-A/Ro	4
Anti-histones	
Total	65
H1	8
H2A	22
H2B	20
H3	54
H4	54
Anti-nucleosome	7
Anti-phospholipids	ND
Anti-erythrocyte antibodies (Coombs test)	17
Anti-IgG antibodies (rheumatoid factors)	20
Circulating immune complexes	75
IgG deposits on cutaneous biopsies	75

Note: ENA, extractable nuclear antigens; ND, not done; RNP, ribonucleoprotein; SS-A/Ro, Sjögren syndrome antigen A (or its equivalent Ro).
Source: Monestier et al.[26]; Monier et al.[28]; Stone[35]

B and T Cell Response

Although ANAs are characteristics of SLE, many other autoantibodies are produced suggesting that affected patients have abnormalities in B cell function resulting in B cell hyperactivity. Abnormalities in T cell functions play a part in the induction and maintenance of B cell hyperactivity. T cell cytokines affect B cells by stimulating cell division, switching antibody production from IgM to IgG, and promoting a change in the molecular sequence of the secreted antibody so it binds more strongly to the driving antigen. Thus, T cells help make possible the production of high-affinity IgG autoantibodies. Defective suppressor T cell function also can fail to regulate B cells.

Data from canine studies have shown immunologic alterations involving T cells and support defective suppressor T cell function: (1) a rapid decline in the level of a soluble thymic factor in canine SLE sera; (2) resistance to induced tolerance in some cases of canine SLE; (3) deficient concanavalin-A-induced suppressor cell function; and (4) a decrease in the number of circulating CD8+ T cells.[7] The extent of this last abnormality is directly related to disease severity.

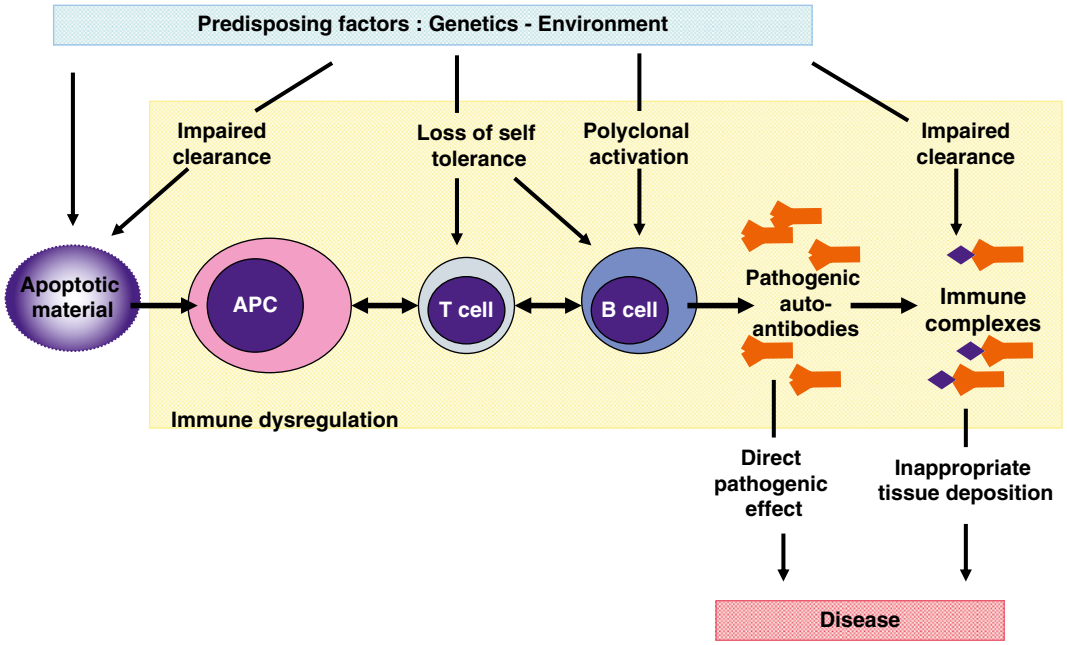


FIGURE 52.1 A simplified diagram of the immunologic events thought to occur in SLE leading to ANAs formation and immune complex deposition. Initiation of the disease involves a combination of predisposing factors that give rise to accumulation of apoptotic debris and immune dysregulation. Antigens that are normally buried within the cells are exposed on the surface of the dying cell, and may trigger an immune response. Loss of tolerance toward self-antigens combined with polyclonal stimulation perpetuates the autoantibody secretion. Autoantibodies may cause direct pathogenic effect or lead to immune complex formation and tissue damage. Impaired clearance by the mononuclear phagocyte system promotes immune complex deposition. APC, antigen-presenting cell. (**Source:** Modified from Rahman and Isenberg[32])

Genetic Factors, Breed, and Sex Predispositions

Genetic Factors

Susceptibility loci with confirmed linkage to SLE have been identified in humans and mice. These loci contain genes encoding molecules involved in immune response and apoptosis and most are associated with the major histocompatibility complex (MHC). The multitude of distinct genetic associations suggests a complex genetic predisposition for the disease, perhaps explaining the variable courses and organ system involvement.

The influence of genetic background in canine SLE is supported by studies of inbred lines and evidence of familial aggregation.[18,23,27,38] SLE is associated with the dog leukocyte antigen (DLA) system: the MHC class I DLA-A7 allele confers risk for disease expression, whereas dogs possessing the DLA-A1 and DLA-B5 (a MHC class II allele) are at decreased risk.[38] Dogs with a specific complement C4 allotype, a class III gene, may be predisposed to a lupus-like syndrome characterized by nonerosive polyarthritis, pyrexia, and ANAs.[11]

Nova Scotia Duck Tolling Retriever (NSDTR) dogs are at increased risk of autoimmune disorders, particularly for immune-mediated rheumatic disease,[16] which is considered to be an SLE-related disorder. In this breed, immune-mediated rheumatic disease is associated with the *DRB1* locus (DLA class II).[40] In addition to association to certain MHC alleles and haplotypes, a similarity between human and dog SLE at the genetic and functional levels is highlighted through an association with the *BANK1* gene (B-cell scaffold protein with ankyrin repeats 1). [41]

Feline SLE may occur more frequently in purebred cats, particularly those with a Siamese or Persian background. This suggests a genetic influence in this species.

Sex Hormones

Since 90% of human patients with lupus are women, an important role for female hormones seems probable. A protective role for male hormones or an effect of genes of the X chromosome is also possible. Such gender predisposition is not clearly seen in dogs.

Environmental and Exogenous Factors

A higher risk of developing ANA or SLE in pet dogs owned by human patients with SLE has been occasionally reported and supports the idea that a common environmental factor or zoonotic agent may have a role in causing SLE.

Light

Ultraviolet radiation is the most obvious environmental factor linked to SLE. Sunlight is known to be capable of triggering, or aggravating, SLE in humans, dogs, and cats. A photosensitive rash is a criterion of the American College of Rheumatology (ACR) for the diagnosis of the disease.[37]

Infectious Agents

While a microbial etiology for autoimmune diseases remains a popular theory, supportive evidence in SLE is limited. A role for viruses in the induction of canine SLE has been proposed. Antigens that cross-react with C-type retroviral particles have been identified on the cell membrane of blood lymphocytes from affected dogs, and cell-free extracts were able to transfer the serological abnormalities of SLE from affected to normal dogs.[31] However, these findings have not been widely corroborated and no direct virus isolation from canine tissues has been described. In cats, the retroviruses feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV) can induce disease with similarities to SLE (e.g., immune-mediated hemolytic anemia), and ANAs develop in some cats infected with FeLV. However, most cases of feline SLE are seronegative for both viruses.[29]

Geographic Influence

Canine SLE is rarely diagnosed in the United States, Northern Europe, and Australia, but seems more frequently recognized in Mediterranean countries. This may reflect an association with different factors including breed distribution, regional gene pools, sun exposure, and prevalence of infectious diseases (e.g., leishmaniasis).

Drugs and Food

In humans, many drugs cause a variant of SLE called drug-induced lupus. The best known of these drugs are procainamide, hydralazine, and quinidine. Lupus-like syndromes have been induced by hydralazine in the dog,[3] and propylthiouracil in the cat.[2] Methimazole has been associated with the development of ANA in cats, but clinical signs of SLE have not been reported.[30]

Other possible factors implicated in humans include foods (celery, parsley) containing psoralens that could potentially increase photosensitivity, l-canavanine (found in alfalfa seeds, sprouts, and vegetables) that is structurally related to hydralazine, hair dyes, exposure to vinyl chloride, and implantation of silicone polymers.

Canine Experimental Model

An experimental model of canine SLE has been developed.[8] SLE was induced in mongrel and mixed-breed dogs by immunization with heparan sulfate, the major glycosaminoglycan of the glomerular basement membrane. The dogs developed a lupus-like syndrome with proteinuria, skin lesions, and ANAs. Gene therapy has been applied to this model using a nonviral peptide vector encoding canine CTLA-4-Ig gene.[9]

CLINICAL MANIFESTATIONS

SLE is a chronic, multifaceted inflammatory disease that can affect every organ system of the body. The symptoms of lupus can vary widely. They may come and go or stay constant; they can be mild, moderate, or severe; they may start suddenly or develop slowly over time.

Hematological Involvement

A history of multiple cytopenias such as leukopenia, lymphopenia, anemia, or thrombocytopenia may suggest SLE. Hemolytic anemia and thrombocytopenia were the main clinical signs together with nephritis cited in the initial description of canine SLE.[23] In more recent canine and feline studies, hemolytic anemia is a moderately frequent finding as it is in human SLE, whereas thrombocytopenia is rare. The Coombs test is positive in 17% of canine SLE cases, even occasionally in the absence of clinical signs of hemolysis. A mild nonregenerative, Coombs-negative anemia associated with chronic inflammation can also be present.

Leukopenia, due to lymphopenia, is not uncommon in SLE. During active disease, there is marked lymphopenia, presumably secondary to the presence of anti-T cell antibodies. An analysis of canine lymphocyte subsets in the peripheral blood reveals a striking decrease in the CD8+ population, which is directly related to disease severity. By contrast, although the percentage of CD4+ cells increases in SLE, the absolute number decreases. Consequently, the CD4:CD8 ratio may rise as high as 6 compared with a normal value of 2.3 in control dogs. Another important finding is the intense staining of T cells by activation markers.[7]

Abnormalities in coagulation pathways can be identified in some patients, resulting in prolongation of the partial thromboplastin time, which fails to be corrected with the addition of normal plasma.[24,36] These abnormalities are due to the presence of "lupus anticoagulant," an antibody directed against phospholipids. In human patients, it defines the phospholipid antibody syndrome, which is associated with a thrombotic disorder.

Overview of the Disease

Canine SLE

Systemic lupus erythematosus is mainly recognized in middle-aged dogs. The clinical expression of canine SLE encompasses a wide range of clinical features (Table 52.2).[12,14,35] Symptoms come and go unpredictably, and are observed successively rather than simultaneously as the disease evolves, except in advanced cases.

Constitutional manifestations such as loss of body mass, inappetence, fatigue, and fever are frequent when the disease is active. Musculoskeletal symptoms are the most frequent primary signs recognized in the dog, usually manifesting as polyarthritis, whereas polymyositis is occasionally identified. Joints most frequently involved are the distal joints (e.g., carpi, tarsi, elbows, and stifles). The vertebral articular facets could also be affected and, later in the course of the disease, the temporomandibular joints. The arthritis is usually symmetrical, nonerosive, and nondeforming. The arthritis/arthralgia may be evanescent, and recurrent. Synovial fluid analysis shows an increased nucleated cell count

TABLE 52.2 Clinical signs in canine SLE.

Signs	Percent positive
Fever	70–100
Nonerosive polyarthritis	80–90
Renal disorders	55–65
Mucocutaneous lesions	45–60
Lymphadenopathy and/or splenomegaly	40–50
Hemolytic anemia	15
Thrombocytopenia	5–15
Leukopenia	15–20
Myositis	5–10
Pleuropericarditis	<10
Central nervous system disorders	<5
Polyneuritis	<2

Source: Fournel et al.[12]; Grindem and Johnson[14]; Stone[35]

with predominance of neutrophils. Renal involvement is frequent, but can be asymptomatic initially. Proteinuria, or microhematuria, is the earliest manifestation and may be the only presenting renal symptom. Immune complex glomerulonephritis can range in severity from minor mesangial changes to diffuse proliferative lesions. Skin manifestations are highly variable and involve both cutaneous and mucous membranes. The lesions usually are characterized by photosensitization, with selective localization in areas exposed to sunlight (the dorsal region of the anterior limbs, the face with lips, nose pad, external ears in dogs with erect ears), and also in areas of thin skin poorly protected by the hair coat (axilla, inguinal region, ventral abdomen). Oral ulcers are infrequent, but palatal ulcers are highly suggestive of SLE. Subacute cutaneous lupus erythematosus in humans defines a subgroup of SLE patients characterized by photosensitivity, non-scarring dermatitis, mild systemic features, and a low prevalence of nephritis. These patients are frequently ANA-negative, but have antibodies to SS-A/Ro. Studies provide evidence supporting the hypothesis that vesicular cutaneous lupus erythematosus in the Rough Collie and Shetland dogs is a homolog of subacute cutaneous lupus erythematosus.[19] Lymphadenopathy or splenomegaly are common presenting signs (50%). In some cases, there is acute extensive facial or hemifacial edema. Cytological examination of enlarged lymph nodes shows plasma cell hyperplasia with an increase in small nucleolated cells or eosinophilic lymphadenitis. Cardiopulmonary involvement is not commonly documented in canine SLE.[4] Features include serositis (pleuritis, pericarditis), and myocarditis.[25] Although neurological manifestations are rare in canine SLE,[21] subtle behavioral disturbances may go unrecognized in the dog. NSDTR dogs predisposed to a SLE-like disease also show strong predisposition to another related immune-mediated disease, steroid-responsive meningitis-arteritis, with clinically relevant neurological signs.

Feline SLE

Systemic lupus erythematosus is a rare disease in the cat. Feline SLE involves the musculoskeletal, urinary, cutaneous, and hematopoietic systems with approximately equal frequency.[30,36,39] Fever is frequently reported in cats. Although the true prevalence of polyarthritis in feline SLE may be underestimated because it tends to be subclinical, articular symptoms seem to be common. Proteinuria or glomerulonephritis are frequently reported. Cutaneous manifestations include generalized alopecia, erythema, scaling, and crusting involving the face, ears, neck, ventrum, and limbs, and crusting of all digital pads. Neurological and behavioral disturbances may be not uncommon in feline SLE and include racing around the house; twitching of the ears, tail, or hind limbs; repeated licking; hyperesthesia along the dorsum; restless crying; disorientation; ataxia; and generalized seizures.[30,36]

Equine SLE

Equine lupus presents as generalized skin disease (alopecia, dermal ulceration, and crusting), accompanied by an antiglobulin-positive anemia. The disease is remarkable insofar as affected horses may be almost totally hairless. Affected horses may also have glomerulonephritis, synovitis, and lymphadenopathy.[13]

DIAGNOSIS

SLE must be diagnosed based on a proper constellation of clinical features and laboratory evidence.

Laboratory Investigations

Routine Laboratory Evaluation

Laboratory tests to diagnose possible SLE should include: a complete blood count to screen for leukopenia, lymphopenia, anemia, and thrombocytopenia; urinalysis to evaluate for proteinuria or hematuria; kidney and liver serum chemistry tests to monitor kidney disease activity and response to immunosuppressive and anti-inflammatory drugs; and inflammatory markers (e.g., c-reactive protein). The latter may be elevated in any inflammatory condition, including SLE, but level changes could be interesting for monitoring disease activity.[5,15]

Antinuclear Antibodies

ANAs are found in over 95% of dogs with SLE. The highest titers are seen in the most severe clinical cases, and diminish progressively with treatment. The decline consistently follows clinical remission, but with a certain time lag. ANAs can be encountered in other diseases,[33] and even in healthy animals (up to 15% of healthy dogs, and approximately 20% of dogs with various infectious diseases). In the majority of cases, their

titers are low, but titers higher than 1:1000 are occasionally found in healthy dogs, notably in German Shepherd dogs.[28] The most frequently observed staining pattern is homogeneous, followed by speckled. Among NSDTR dogs positive for ANA, 61% showed the speckled pattern, whereas 39% displayed homogeneous phenotype.[41] The significance of these staining patterns is currently unclear in animals. Although the ANA test is very sensitive, it has a low specificity, but is useful as a screening test. In NSDTR dogs with immune-mediated rheumatic disease, 30% displayed a negative ANA test.

In canine patients with high clinical suspicion or high ANA titer, additional testing is indicated for evaluation of ANA subtypes (such as histones, Sm, SS-A/Ro, hnRNP G). A number of methods are used to identify antibodies specific for the nuclear components (immunoprecipitation, ELISA, radioimmunoassay, western blotting). Unfortunately, these assays are rarely available outside the research setting. There are two categories of anti-ENA antibodies that, although their sensitivity is low, seem to be particularly interesting because of their high specificity for canine SLE. These categories are anti-Sm antibodies (found in 16% of patients) and anti-hnRNP G (found in 20% of patients).[28]

Healthy cats may have positive ANA (up to 30%): 10% had weak titers (1:40–1:80), but 20% had strong titers (1:160–1:320).[1] Titers greater than or equal to 1:1280 are occasionally found [personal observation].

Other Immunodiagnostic Tools

Formerly, the lupus erythematosus (LE) cell test was used for diagnosis of SLE. LE cells are neutrophils that have phagocytosed nuclei from dead and dying cells. Their presence may be detected in bone marrow and occasionally in buffy coat preparations. It is usually necessary to produce them in vitro using heparinized or clotted blood from patients. There is a high incidence of both false-positive and false-negative results. Because of this, the LE cell test is mostly of historic significance. LE cells rarely can be seen on smears of fluids (pericardial, pleural, or peritoneal effusions, bronchoalveolar lavage,[4] joint, cerebrospinal, and blister fluids) and, when present, are considered as highly suggestive of SLE.

A wide variety of autoantibodies can be detected in sera or tissue: rheumatoid factors (seen at low titers in 20% of patients);[6] antiphospholipid antibodies;[24,36] Coombs test to demonstrate anti-RBC antibodies; tests for antiplatelet antibodies (see Chapter 143).

A skin biopsy can help to diagnose SLE with histopathologic evidence of an interface dermatitis or vasculitis. The demonstration of immune deposits at the basement membrane zone (the "lupus band test") by immunofluorescence or immunohistochemistry in both affected and unaffected sites that have been exposed to sun, although not specific, is supportive of the diagnosis of SLE. A renal biopsy is useful in identifying the specific type of glomerulonephritis present.

CIC levels, serum complement function, and concentration of specific complement components can also be measured. CIC concentration will decrease and complement levels will rise to normal during clinical remission or after therapy. Reduced levels of complement in canine SLE are less likely to reflect inherited complement deficiency than utilization in immune complex clearance.

Diagnostic Criteria and Differential Diagnosis

Many diagnostic criteria have been proposed for SLE in veterinary medicine, and have been modified from those given for humans. The ACR established eleven criteria in 1982 that were updated in 1997.[17,37] These criteria summarize features necessary to diagnose SLE. However, more than diagnostic criteria, they were established for classification and use in scientific research (i.e., inclusion in randomized controlled trials); therefore, patients may have lupus but not meet all of the criteria.

In dogs and cats, the most frequently accepted and simplest diagnostic criteria include the presence of two separate manifestations of autoimmunity, together with significantly elevated titers of serum ANAs. For a more stringent diagnosis, it would be recommended to use the 1982 revised ACR criteria that have been proposed and adapted for dogs (Table 52.3).[35] A rigorous approach (Figure 52.2) is necessary for recognition of this complex disease and assessment of particular cases of SLE.

Systemic lupus erythematosus is a challenging disease whose wide variety of manifestations makes it part of the differential diagnosis for patients with many different presenting complaints (e.g., fever of unknown origin, polyarthritis, anemia, and nephritis). Infectious and neoplastic diseases must be excluded through imaging and appropriate biological testing, such as serology or polymerase chain reaction for vector-borne diseases (e.g., ehrlichiosis, leishmaniasis).

PROGNOSIS AND TREATMENT

Prognosis

The disease is progressive, so the severity of lesions and the number of organ systems involved increase gradually in untreated cases. Many patients die from renal failure. The earlier a diagnosis is made, the better the chance in-depth treatment will lead to a cure. Secondary infections are an ever-present risk in animals on immunosuppressive therapy.

Prognosis must take both disease activity and severity into account. A dog suffering from mild polyarthritis cannot be compared to one that is in critical condition, with a combination of severe anemia and renal failure. Various parameters have been suggested to evaluate disease severity and to monitor patients throughout therapy and during periods of remission: clinical criteria, as in the indices of activity defined for humans by the ACR; the ANA titer, which is in general proportional to the seriousness of the condition, but goes down slowly by comparison with clinical improvements; the serum levels of complement components, and

TABLE 52.3 Proposed criteria for the diagnosis of canine SLE.

1. **Arthritis:** nonerosive arthritis involving two or more peripheral joints, characterized mainly by pain during movement (progressive forced flexion–extension), where swelling or effusion is often not very marked
2. **Renal disorder**: persistent proteinuria in absence of urinary tract infection; or cellular casts (red blood cell, hemoglobin, granular, tubular, or mixed cellular casts)
3. **Cutaneous lesions**: depigmentation, erythema, erosions, ulcerations, crusts, and keratotic scaling which selectively affect the areas of thin skin, or skin poorly protected by the hair-coat (in particular, the face: nose pad, forehead, lips, and the periocular region)
4. **Oral ulcers**: oral or nasopharyngeal ulceration, usually painless
5. **Hematologic disorder**: hemolytic anemia; or leukopenia, <3.0 × 10^9/L on two or more occasions; or lymphopenia, <1.0 × 10^9/L on two or more occasions; or thrombocytopenia, <100 × 10^9/L in the absence of offending drugs
6. **Serositis:** presence of a nonseptic inflammatory cavity effusion (pleuritis or pericarditis)
7. **Neurologic disorders**: seizures or behavioral disturbances in the absence of offending drugs or known metabolic disorders (e.g., uremia, ketoacidosis, or electrolyte imbalances)
8. **Idiopathic inflammatory disease of the skeletal or cardiac muscles**: polymyositis or myocarditis
9. **Antinuclear antibody**: an abnormal ANA titer by immunofluorescence or an equivalent assay at any time and in the absence of drugs known to be associated with their formation
10. **Immunologic disorder**: antibody to histone; or antibody to the Sm nuclear antigen; or antibody to the hnRNP G nuclear antigen; or decrease in the CD8 population, <0.2 × 10^9/L; or CD4/CD8 ratio higher than 4; or positive finding of anti-phospholipid antibody

Note: ANA, antinuclear antibody; RNP, ribonucleoprotein. Any combination of four or more of 10 criteria, well-documented at any time during a patient's history, makes it likely that the dog has SLE
Source: See Stone.[35]

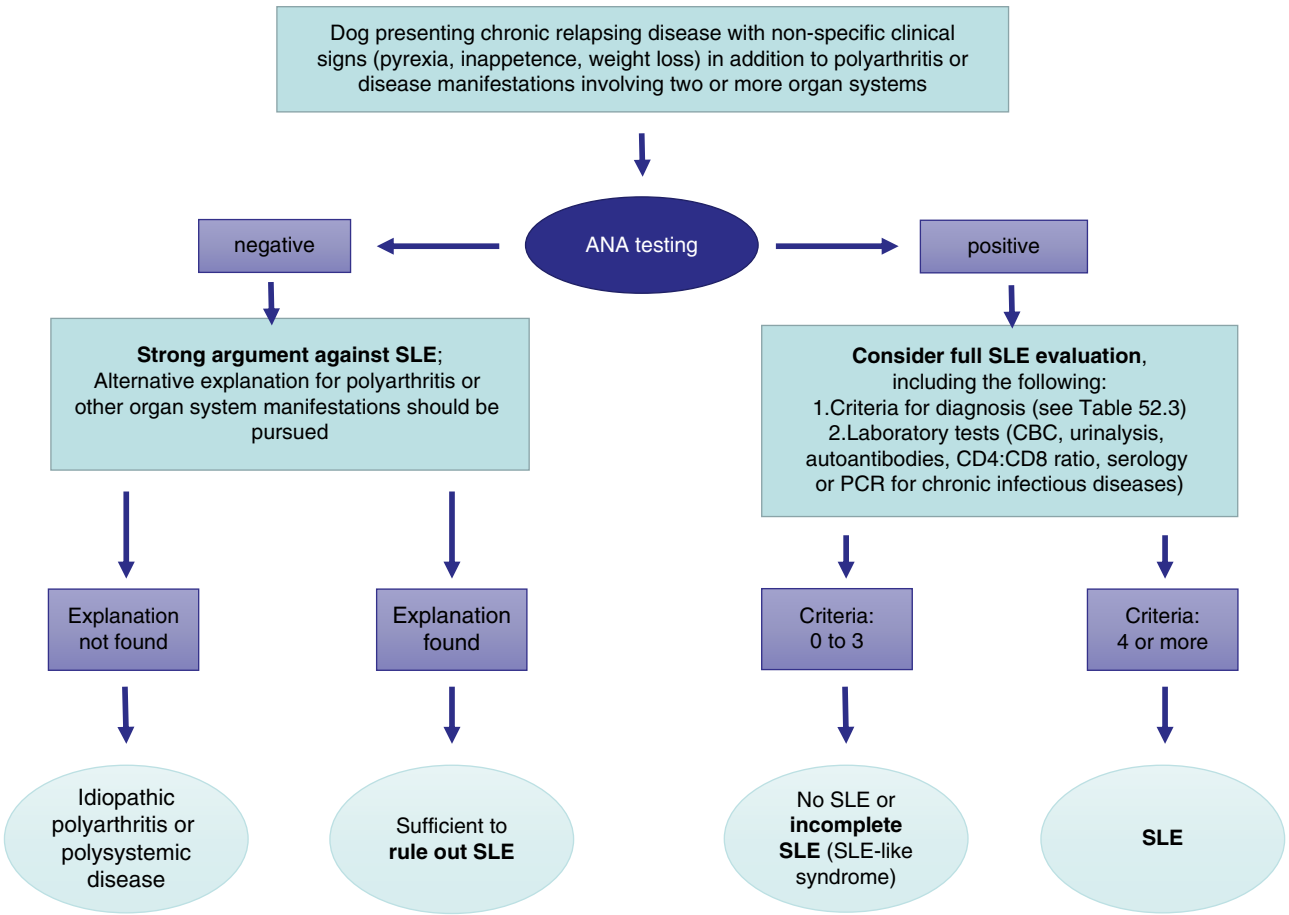


FIGURE 52.2 An algorithm for the diagnosis of systemic lupus erythematosus in the dog. ANA, antinuclear antibody; CBC, complete blood count; PCR, polymerase chain reaction; SLE, systemic lupus erythematosus.

TABLE 52.4 Treatment regimen of canine SLE.

Phase/regimen	With MMF[35]	With Levamisole[7,12]
Induction	Prednisone (1 mg/kg PO q 12–24h) and MMF (10 mg/kg PO q 12h) administered in combination. The dosage of prednisone is tapered every 2 weeks, while MMF is continued at the same dosage	Prednisone (0.5 mg/kg PO q 12–24h) and levamisole (5 mg/kg PO q 48h—a maximum dose of 150 mg/dog is used) administered in combination. The dosage of prednisone is tapered every 2 weeks, while levamisole is continued at the same dosage
Maintenance	MMF alone. If remission is obtained on MMF alone for 2 months, the dosage is halved for an additional 2 months, and then discontinued	Levamisole is given continuously for 4 months and then stopped
Relapse	MMF is reinduced with a short course of prednisone, and MMF (10 mg/kg PO q 12–24h) is then continued for life	Levamisole alone is reinstituted for a further 4-month period.
Side effects	Side effects of MMF are generally mild (diarrhea) and dosage-related; hematologic side effects are uncommon	Side effects of long-term levamisole treatment are rare in dogs (neutropenia, aggressiveness, excited behavior)

evaluation of cellular immunity (e.g., enumeration of the circulating CD8+ lymphocytes, CD4:CD8 ratio, degree of lymphocyte activation).[7]

Therapy

Owing to the wide spectrum of clinical manifestations, treatment of SLE should be tailored to the severity and extent of disease in individual patients. Medications are used to control the disease, prevent recurrence of symptoms, and reduce their severity and duration. Sunlight should be avoided if photosensitization occurs.

Traditional Immunosuppressive Drugs

The standard primary therapeutic approach in veterinary medicine involves the use of tapered high-dose corticosteroid therapy. Prednisone or prednisolone at doses of 2–2.2 mg/kg/day is recommended in most instances. Full doses are administered until remission is attained, and then tapered slowly. Remission is usually brief and permanent corticosteroid therapy at the lowest effective dose is generally required. Dexamethasone has been used in treatment of equine SLE, but treatment of reported equine cases has been unsuccessful so far.

Additional immunosuppressive drugs, such as cyclophosphamide or azathioprine in dogs or cyclophosphamide or chlorambucil in cats, are used in refractory cases or to reduce the corticosteroid dosage because of unacceptable side effects.

Nowadays, standard treatment for active lupus nephritis in humans is mycophenolate mofetil (MMF) or cyclophosphamide combined with high-dose corticosteroids, while calcineurin inhibitors (including cyclosporine) are promising agents for the treatment of refractory cases. Studies in lupus nephritis have established the equivalence of MMF to intravenous cyclophosphamide and its equivalence or superiority to azathioprine in the maintenance phase of treatment.[10] The clinical utility of MMF is increasingly being explored in companion animals,[20] and its use in canine SLE as an adjunctive immunosuppressive agent with corticosteroids can be recommended (Table 52.4).[35] In our experience, we also use cyclosporine (5 mg/kg PO q 12h) for canine SLE in cases refractory to levamisole, with pharmacodynamics testing to adjust the dosage.

Levamisole

Levamisole is an anthelmintic drug, also used as an immunoadjuvant that stimulates T cell responses. It probably enhances suppressor T cell function. Levamisole has been used with success in canine SLE.[7,12] This regimen (Table 52.4) is reported to produce long-term remission in over 50% of dogs with SLE.

Other Alternatives

In human SLE, antimalarial agents (hydroxychloroquine) remain as first-line treatment for patient with mild SLE along with nonsteroidal anti-inflammatory drugs. Hydroxychloroquine is used to control skin and joint symptoms and protect against the development of systemic disease. Its use has not been reported in dogs and cats.

According to the expansion of knowledge and understanding of disease mechanisms over the past two decades, biologic therapies have been proposed for human SLE. These targeted therapies in use or under evaluation (Table 52.5) are designed to block pathways involved in disease pathogenesis and re-establish self-tolerance.[10] The use of CTLA-4-Ig is one of the targeted therapeutic approaches in SLE and has been used in the canine SLE experimental model.[9] CTLA-4 (CD152) fusion protein outcompetes the CD28 receptor for binding to CD80 (B7-1) and CD86 (B7-2) ligands expressed by antigen-presenting myeloid cells, including B cells (Figure 52.3). CD28 is a key costimulatory molecule that delivers the second signal required for antigen-induced T cell activation. The CTLA-4-Ig blocks the

TABLE 52.5 Examples of targeted therapies for SLE.

Target	Agent type	Effect
B cell	Anti-CD20 mAb	B-cell depletion
	Anti-CD19 mAb	B-cell and plasma cell depletion
	Anti-CD22 mAb	Inhibition of B-cell activation and proliferation
Cytokine	Anti-CD257 (BAFF) mAb	Inhibition of B-cell survival and differentiation
	Anti-IFN□	Blocking of inflammation induced by type I IFNγ
	Anti-IL-6R	Blocking of IL-6 binding to the IL-6R to decrease inflammation
	Anti-IL-6	Neutralization of IL-6 to decrease inflammation
Treg	Recombinant IL-2 (low-dose)	Induction and expansion of Tregs
Costimulators	CTLA-4-Fc fusion protein	Blocking of CD28 binding to CD80/86, blocking of T- and B-cell costimulation
	Anti-CD275 mAb	Blocking of T follicular helper cell activation/differentiation
	Anti-CXCR5 mAb	Blocking of CXCR5 binding to CXCL13 and cell migration to lymph nodes and target organs
	Anti-C5 mAb	Blocking of C5, preventing of C5a, membrane attack complex formation (C5b–C9)

Note: BAFF, B-cell-activating factor of the TNF family; CD, cluster of differentiation; CTLA-4, cytotoxic T lymphocyte-associated protein 4; CXCL, chemokine ligand; CXCR, chemokine receptor; IFN, interferon; IL, interleukin; mAb, monoclonal antibody; Tregs, Regulatory T cells.
Source: Adapted from Davis and Reimold[10].

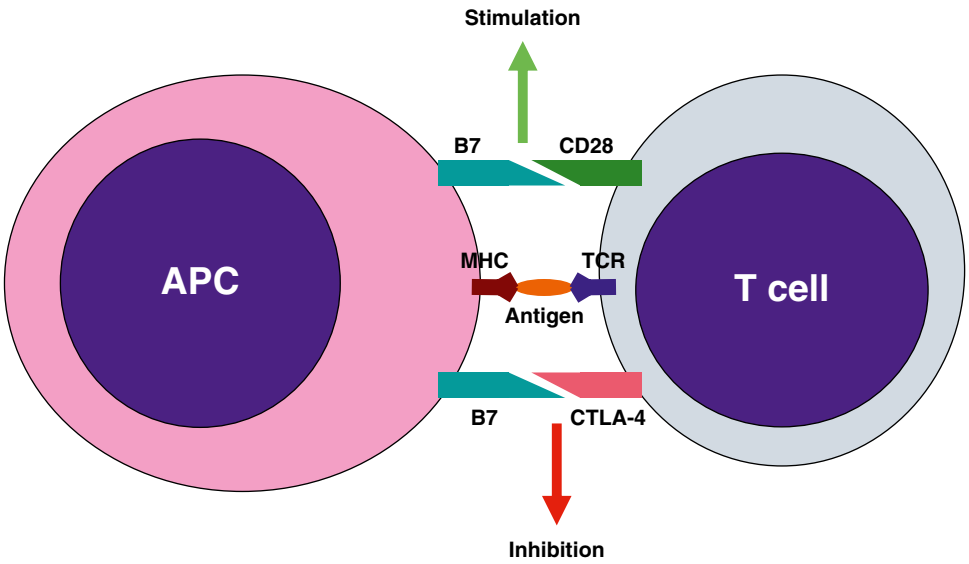


FIGURE 52.3 Interaction between a T cell and an antigen-presenting cell. The antigen-presenting cell (APC) binds antigen in a complex with a molecule from the MHC on its surface. This complex interacts with the T-cell receptor (TCR). The effect on the T cells depends on the interaction between other molecules on the surfaces of the two cells. Two alternative interactions are shown: B7 with CD28, which is stimulatory, and B7 with cytotoxic T lymphocyte-associated protein 4 (CTLA-4), which is inhibitory. If the positive signal caused by the CD28–B7 interaction dominates, the T cell is activated, leading to cytokine release, B cell help, and inflammation. If the negative signal caused by the CTLA-4–B7 interaction dominates, activation is suppressed.

CD28–B7 interaction and thus directly targets the interaction between T cells and antigen-presenting cells by inhibiting costimulation. The CTLA-4-Ig treatment can result in diminished T cell responses and T-dependent B-cell antibody production.

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CHAPTER 53

Feline Immunodeficiency Virus

MARGARET J. HOSIE and HANS LUTZ

Prevalence and Epidemiology
Pathogenesis
Clinical Disease
Hematologic Disorders
Immune Reaction
Diagnosis
Direct Detection of FIV
Virus Isolation
Polymerase Chain Reaction
Detection of Antibodies to FIV
Treatment
Supportive Treatment
Antiretroviral Drugs
Immune Modulators
Prognosis

Acronyms and Abbreviations

3TC, 2′,3′-dideoxy-3′-thiacytidine; AIDS, acquired immunodeficiency syndrome; AZT, azidothymidine; CNS, central nervous system; CRD2, cysteine-rich domain-2; ELISA, enzyme-linked immunosorbent assay; FCV, feline calicivirus; FCoV, feline coronavirus; FHV-1, feline herpesvirus-1; FeLV, feline leukemia virus; FIV, feline immunodeficiency virus; FPV, feline panleukopenia virus; HIV, human immunodeficiency virus; IFN-α, interferon-α; MDA, maternally derived antibodies; PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; PMEA, 9-(2-phosphonomethoxyethyl)adenine; rHuEPO, recombinant human erythropoietin; rHuGM-CSF, recombinant human granulocyte macrophage colony-stimulating factor; TM, transmembrane.

Feline immunodeficiency virus (FIV), discovered in 1987,[36] shares a similar structure, genetic organization, lymphocyte tropism, and pathogenesis to the human immunodeficiency virus (HIV).[26] Species-specific FIV strains have been isolated from many species of non-domestic Felidae, including the puma, lion, leopard, and Pallas cat.[35] FIV is a lentivirus, containing accessory genes in addition to gag, pol, and env. The FIV gag gene encodes, among others, the capsid protein p24; the pol gene encodes the protease, integrase, and reverse transcriptase and additional enzymes important to virulence of FIV. Both gag and pol are relatively conserved between strains. The env gene encodes the viral surface glycoprotein (gp120) and the transmembrane (TM) protein (gp41), with the gp120 carrying the major determinants of viral diversity among isolates. Six genetically distinct subtypes or clades (designated A to F) have been defined, which exhibit considerable sequence diversity within the env genes. The majority of viruses identified so far belong to either subtype A or B. Although multiple subtypes have been documented in cats from the same continent, there tends to be geographic clustering of subtypes.

PREVALENCE AND EPIDEMIOLOGY

FIV is endemic in domestic cats worldwide; the seroprevalence varies between regions, with estimates of 1–14% in cats with no clinical signs and up to 44% in sick cats. Sick adults, males, and free-roaming cats are most likely to be infected. The major route of natural transmission occurs via the inoculation of saliva during fighting.[55] Vertical transmission and transmission between socially well-adapted cats are relatively uncommon. Transmission from mother to kittens can occur; the proportion of kittens infected depends on the viral load of the queen during pregnancy and birth.[9] If the queen

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became infected at the time of mating (being bitten by an FIV-infected tom cat), the viral load may be high and up to 70% of the kittens may become infected at birth or via milk.[11–13] By contrast, if the queen was infected before conception and was clinically healthy during gestation, her viral load would be low and kittens would generally not become infected.

PATHOGENESIS

FIV gp120 binds CD134 expressed on activated CD4+ T lymphocytes resulting in a conformational change occurring in gp120 that leads to binding to the viral co-receptor, CXCR4.[44] These steps mediate fusion of viral and cell membranes (Figure 53.1) and viral entry. The viral reverse transcriptase enzyme synthesizes a DNA strand, complementary to the viral RNA, which is inserted into the host DNA to form the provirus. As reverse transcriptase lacks a proofreading function, its fidelity is low, and, as a consequence, FIV has a tendency to mutate. This, together with the development of the immune reaction, is the basis for genetic diversity, resulting in the generation of variants and immune evasion.

In the first days after infection, FIV replicates in dendritic cells, macrophages, and CD4+ T lymphocytes. Within 1–2 weeks, a low-level viremia develops.[3] The loads of viral RNA in plasma and provirus present in blood mononuclear cells increase with time, with the highest loads found between 8 and 12 weeks post infection.[8] This "acute phase" of infection is overcome by a functioning immune system, giving rise usually to a long asymptomatic phase during which plasma viral loads are stable at low levels (Figure 53.2). As infection progresses, the number of CD4+ T lymphocytes gradually decreases, resulting in a decreased CD4:CD8 T lymphocyte ratio and immune deficiency.[25] Immunodeficiency with high viral loads is accompanied by the clinical signs of acquired immunodeficiency syndrome (AIDS) and death.[28] FIV strains isolated during the early or primary stage of infection display a distinct receptor usage phenotype, requiring the CRD2 domain of the CD134 molecule for infection; the CRD2 domain is not required by strains isolated during the terminal/AIDS phase.[53] The switch in phenotype from CRD2-dependent to CRD2-independent strains coincides with the onset of immunodeficiency[6,7] and can be used to stage the infection (Figure 53.2).

CLINICAL DISEASE

During the acute phase of infection in experimentally infected cats, lymphadenopathy, pyrexia, anorexia, and lethargy can be observed. Lymphadenopathy may persist for many weeks or months and is associated with increased number and size of germinal centers. A decrease in plasma viral load marks the beginning of the "asymptomatic" phase that can last for many years or can be lifelong.[5] Progression to immunosuppression probably depends on the virulence of the infecting strain and cat age at the time of infection.[28,47] In addition, the higher genetic stability of FIV compared to HIV could explain why many cats naturally infected with FIV do not show evidence of disease progression.

Immunosuppression usually manifests as stomatitis/gingivitis, chronic rhinitis, skin disease, lymphadenopathy, or weight loss and infected cats are prone to secondary or opportunistic infections and neoplasia. B cell lymphosarcomas, myeloproliferative disease, and squamous cell carcinomas have been reported, as well as many concurrent viral, bacterial, fungal, and protozoal infections, although

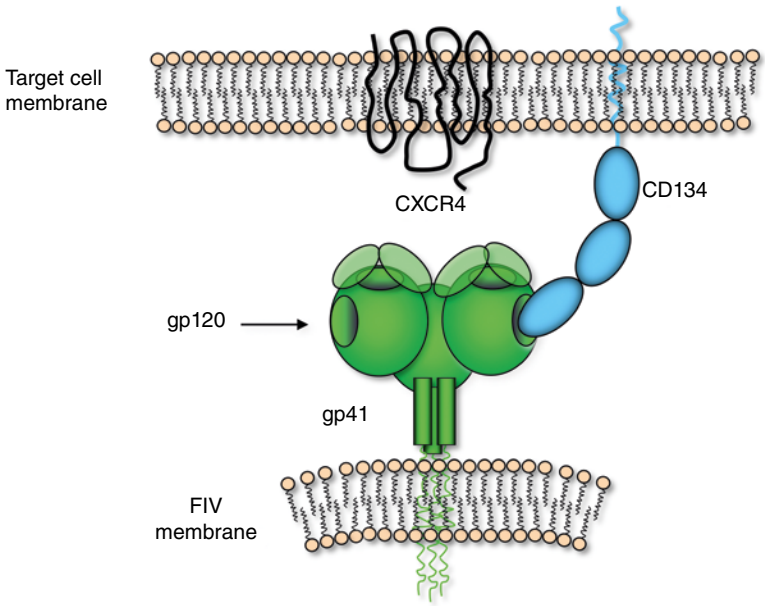


FIGURE 53.1 Schematic representation of the two-receptor mechanism for FIV infection, showing the interaction between FIV gp120 and the primary receptor molecule CD134 and the co-receptor CXCR4. (**Source:** Provided by Prof. B. Willett, MRC-University of Glasgow Centre for Virus Research, Glasgow, UK)

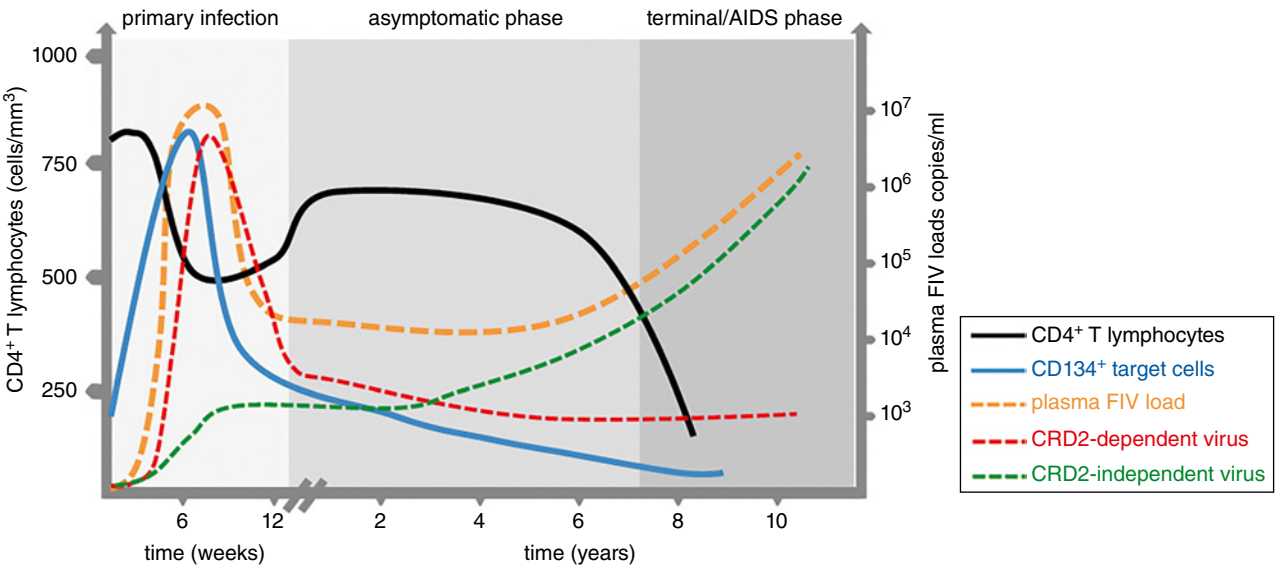


FIGURE 53.2 The course of FIV infection, showing CD4+ T lymphocytes, CD134+ activated T cells, plasma virus load, and levels of CRD2-dependent and CRD2-independent variants of FIV during the primary, asymptomatic, and terminal phases of infection. (**Source:** Provided by Dr. N. Techakriengkrai, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand)

some studies have found no significant association between FIV infection and *Cryptococcus*, *Cryptosporidium*, or *Mycoplasma haemofelis / Mycoplasma haemominutum*.[10,27,45,50]

FIV also infects the central nervous system (CNS),[14] which can lead to unnatural behavior.[38] Disrupted reproduction has been reported in FIV-infected cats when placental and fetal tissues are affected.[51] Also, an association between FIV infection and glomerular and tubulointerstitial lesions combined with proteinuria has been documented.[39] However, many of these reported outcomes were observed for cats that had been infected experimentally with FIV, often using specific strains of the virus. By contrast, clinical signs in cats with naturally acquired FIV infection are often mild or inapparent. Cats housed in single-cat households remained free of clinical signs during a 22-month study period, while cats kept in crowded shelter conditions developed clinical signs, showed more pronounced declines in CD4+ T lymphocyte numbers, and were more likely to die during the follow-up period.[5]

HEMATOLOGIC DISORDERS

During the acute phase of experimental FIV infection, mild lymphopenia and later lymphopenia and severe neutropenia have been described.[22] The most frequent hematologic abnormalities include anemia, neutropenia, lymphopenia, and monocytosis.[28,31,43]

IMMUNE REACTION

After infection, cats readily develop high titers of antibodies recognizing different FIV components. Antibodies can be detected by immunofluorescence assays, ELISAs, or immunochromatography tests.[32,36] Antibody specificity is usually determined by western blots or radioimmunoprecipitation.[16] Anti-FIV antibodies are detectable from 2–4 weeks post infection, or later if cats are exposed to low doses of virus.[24] Antibodies recognizing gp41 appear first, within a few weeks after infection, whereas those recognizing gag and gp120 appear later.[11] Virus-neutralizing antibodies develop a few weeks after infection. However, cats cannot clear FIV infection, even if high titers of virus neutralizing antibodies are present, suggesting that cellular immunity might have a role in suppressing FIV replication in cats displaying low viral loads.[6] FIV-specific CD8+ T cells were detected as early as 2 weeks after infection.[4] A population of CD8+ T cells termed CD8low was observed in early FIV infection, which act as a marker of immune activation by more virulent strains of FIV and might contribute functionally to the noncytolytic CD8+ T cell activity against FIV.[17]

DIAGNOSIS

FIV infection can be diagnosed either directly, by detecting the virus, or indirectly, by demonstrating the presence of antibodies.

Direct Detection of FIV

Virus Isolation

Isolation of FIV in cell culture was the first method used to demonstrate FIV infection in infected cats.[36] Peripheral blood lymphocytes are prepared from fresh samples of heparinized blood and are co-cultivated with primary feline T cells for up to 4 weeks. The presence of virus in culture fluids can be confirmed by measuring the levels

of FIV core proteins in the culture fluids or by quantitation of provirus or FIV RNA using real-time PCR or RT-PCR.[30] Virus isolation in culture is laborious and tends not to be used routinely.

Polymerase Chain Reaction

PCR-based assays that detect viral RNA or proviral DNA[42,49] led to use of real-time PCR to quantify FIV-DNA and RNA.[30] PCR-based FIV detection is sensitive, but false negatives occur. Seropositive results in PCR-negative cats can also occur following vaccination. In addition, false positive results have been described in cats living in close contact with FIV-infected seropositive cats. It is suspected that such cats have become infected at low levels and will seroconvert when viral loads have increased sufficiently to induce antibody production.

Detection of Antibodies to FIV

During FIV infection cats develop antibodies to all components of FIV.[6,11,34] Antibodies to the TM protein gp41 appear first, followed by antibodies to gag and gp120.[11] For routine detection of antibodies to FIV, purified whole FIV, FIV-infected cells, and purified proteins produced by recombinant DNA technology have been described.[41] Serological tests used in diagnostic laboratories are mostly based on a combination of p24 and TM peptides, while immunochromatography tests usually contain synthetically produced peptides from the TM region. Western blots are considered the "gold standard" for FIV serology and are used to confirm inconclusive results.[34] ELISA and immunochromatography tests are widely used under practice conditions, but false positive results can occur. This is especially important in populations with low prevalence, such as in many countries in Central Europe. Therefore, any positive result in a cat without risk factors (e.g., young, healthy, indoor, purebred cats) must be confirmed. A positive result in a cat from a high-risk group (e.g., free roaming, aged, male with scratch wounds) is more likely to be a true positive as the frequency of true positives will exceed the frequency of false positives in this population. Negative results in low-prevalence populations are generally very accurate, but false negative results may be obtained early in infection because cats remain seronegative for weeks to months. False negative results may be also obtained in the terminal stages of disease due to immunosuppression and when high viral titers may lead to sequestration of anti-FIV antibodies in virus–antibody complexes.

Kittens born to FIV-infected queens can test seropositive as a result of passively acquired maternally derived antibodies (MDA) and should be retested after approximately 16 weeks of age, when MDA will have declined to undetectable levels. However, in rare cases, antibodies may persist up to 6 months. If an earlier result is required, virus isolation or PCR could be used; however, the queen should be tested in parallel to ensure that the PCR can detect the strain of virus causing the seropositive reaction.

Vaccination of cats against FIV infection with an inactivated, whole virus vaccine results in rapid and persistent production of antibodies that are indistinguishable from those used for diagnosis of FIV infection. This vaccine is available in Australia, New Zealand, and Japan, but is not licensed in Europe and has been withdrawn from use in the USA. Vaccinated cats may be imported from a country where the vaccine is (or was) used and will test positive for antibodies to FIV. Antibodies can usually be detected within a few weeks after vaccination, and they can persist for more than 7 years. In such cases, a positive antibody test cannot distinguish between infection and vaccination.

TREATMENT

Supportive Treatment

Symptomatic treatment of infected cats should be directed at the clinical signs that arise from secondary infections. During the early phase of FIV infection, cats usually respond, as well as FIV-negative cats to appropriate medications, although a longer or more aggressive course of therapy (e.g., antibiotics) may be required. There are reports of clinical benefits using corticosteroids and other immune suppressive drugs in FIV-infected cats with chronic stomatitis, but their use is controversial.

Antiretroviral Drugs

There is no treatment of FIV infection that leads to complete clearance of the virus. Several drugs have proven antiretroviral activity against FIV, their modes of action and efficacies being similar for FIV and HIV.

Azidothymidine (AZT) or zidovudine, 3′-azido-2′,3′-dideoxythymidine, is a nucleoside analog reverse transcriptase inhibitor that inhibits the replication of both HIV and FIV. Treatment with AZT led to significant clinical improvements in FIV-infected cats.[2,21] Side effects of AZT (nonregenerative anemia) are seen in some cats, especially at higher doses.

Other nucleoside analogs with a similar mode of action to AZT have been used to treat FIV. Stampidine, 2′,3′-didehydro-3′-deoxy-5′-thymidylyl-L-alanine methyl ester, inhibited FIV replication in vitro and was well tolerated by cats.[48] Lamivudine (2′,3′-dideoxy-3′-thiacytidine), 3TC, was evaluated in vitro and in vivo and inhibited FIV replication. The combination of 3TC and AZT was highly effective for prophylaxis; however, the combination was only marginally effective in chronically infected cats.[2] The acyclic purine nucleoside analog 9-(2-phosphonomethoxyethyl)adenine (PMEA) efficiently inhibited FIV replication and improved the clinical signs of FIV infection, but with strong side effects, especially at higher doses, precluding its use in clinical cases. Enfuvirtide or T20 is a fusion inhibitor that binds gp41 and prevents fusion between the virus and the host cell, thereby blocking infection. Similarly, an analog to the FIV TM protein was shown to inhibit

FIV infection in vitro and to lead to a reduced viral load in vivo.[18] Although well tolerated by cats, its use is limited as it must be administered by subcutaneous injection because of its short half-life.

Bicyclams inhibit FIV as well as HIV infection,[15] blocking the primary receptor molecule CXCR4 molecule.[52] Plerixafor (1,1′-(1,4-phenylenebismethylene)-bis(1,4,8,11-tetraazacyclotetradecane)-octachloride dehydrate, AMD3100) is the prototype bicyclam drug and is active against FIV in vitro 90 when administered to cats at 0.5 mg/kg q12h. Magnesium and calcium levels should be monitored during treatment. In a placebo-controlled double-blind clinical trial of naturally FIV-infected cats, treatment with plerixafor resulted in a significant decrease in provirus load, but also a decrease in serum magnesium levels, although the latter was not clinically significant.

Immune Modulators

The use of recombinant human granulocyte macrophage colony-stimulating factor (rHuGM-CSF) in FIV infection is not advised because the peripheral blood mononuclear cells (PBMC)-associated FIV load in some treated cats increased by one to two logs.[1] In trials, significant increases in neutrophils and myeloid:erythroid ratios of the bone marrow cells were observed without increased stimulation of FIV expression, but 14–21 days after initiation of treatment, anti-rHuGM-CSF antibodies were induced in some cats, neutralizing its effect.[37] Recombinant human erythropoietin (rHuEPO) led to a gradual increase in red and white blood cell counts in FIV-infected cats and no increase in virus load was observed.[1] Insulin-like growth factor-1 (IGF-1) is available as recombinant human product (rHuIGF-1). Besides other actions, it has the ability to induce thymic growth and to stimulate T-cell function. In cats experimentally infected with FIV, the use of rHuIGF-1 resulted in increased thymus size and thymic cortical regeneration, leading to an increase in peripheral T cell counts.[54] Therefore, rHuIGF-1 could be considered as a supportive treatment in younger cats with FIV infection.

Interferon-α (IFN-α) is active against FIV in vitro[46] and has been used to treat FIV-infected cats.[12,20] In a placebo-controlled double-blind study, low-dose human IFN-α treatment significantly prolonged the survival of FIV-infected cats and improved their clinical signs compared to cats in a placebo group. Feline interferon-ω (IFN-ω) is licensed for use in veterinary medicine in some European countries and Japan, as it inhibits FIV replication in vitro[46] and does not stimulate the development of antibodies. In a placebo-controlled multicenter trial treating 62 naturally FIV-infected cats (106 IU/kg SC q24h on 5 consecutive days), there was no difference in survival of IFN-ω-treated cats compared to the placebo group, although some clinical improvements were noted.[12] In naturally FIV-infected cats housed in a shelter, clinical improvement was observed, but there was no control group. Oral administration of a lower dose of rfIFN-ω (105 U/cat PO q24h for 90 days) to clinically affected, naturally FIV-infected cats was associated with improvement of clinical scores.[20] The improvement was similar to that observed in a historical control group treated with SC rfIFN-ω, suggesting that PO administration of the lower dose of rfIFN-ω could be used as an alternative to the licensed protocol at reduced cost. In a follow-up study of naturally FIV-infected cats treated either with PO or SC rfIFN-ω, there was no change in viremia and most cytokine concentrations measured,[29] raising questions about the efficacy noted in the previous trials that lacked placebo-treated controls; the clinical improvement observed might have resulted from an effect of rfIFN-ω on secondary bacterial infections rather than on the primary FIV infection. Indeed, 16 naturally FIV- and or FeLV-infected cats that were followed for 65 days during rfIFN-ω treatment had reduced excretion of co-infecting viruses (feline calicivirus (FCV), feline herpesvirus-1 (FHV-1), feline coronavirus (FCoV), feline panleukopenia virus (FPV)).[19] Additional randomized, placebo-controlled, and double-blinded studies are required to fully determine the efficacy of IFN-ω.

PROGNOSIS

Under natural conditions, clinical signs may take many months to years to develop and many FIV-infected cats have an apparently normal life expectancy.[40] Nevertheless, FIV-positive cats are more likely to develop clinical signs, often associated with secondary infections, immune-mediated disease, or neoplasia.[33] Therefore, surrogate markers are required to provide an objective assessment of FIV progression in individual cats. FIV strains that dominate in early infection display a distinct receptor usage phenotype[53] and the emergence of viruses with an altered receptor usage phenotype coincides with the onset of immunodeficiency.[7] Therefore, it might be possible to develop viral phenotyping tests to assist in the clinical staging of individual cats diagnosed with FIV infection.

The clinical course of FIV infection depends on the sex, age, roaming habits, and the immune status of the affected cat.[13] Co-infections, especially with feline leukemia virus, accelerate disease progression.[23] Prospective studies of FIV-infected cats kept in stable, single-cat households showed that disease progression was delayed or even prevented, whereas FIV-infected cats kept in overcrowded conditions were more likely to show signs of disease progression.[5]

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CHAPTER 54

T Cell, Immunoglobulin, and Complement Immunodeficiency Disorders

PETER J. FELSBURG

Immunodeficiency Disease
Diagnosis of an Immunodeficiency
Diseases of the B Cell (Humoral) Immune System
  Agammaglobulinemia
  Selective IgM Deficiency
  Selective IgA Deficiency
  Common Variable Immunodeficiency
  Transient Hypogammaglobulinemia of Infancy
  Failure to Receive Colostrum
Diseases of the T Cell (Cell-Mediated) Immune System
  Hypotrichosis with Thymic Aplasia (Nude Kittens)
  T Cell Deficiency in Growth-Hormone-Deficient Dogs
  Lethal Acrodermatitis in Bull Terriers
  Viral Infections
Diseases of the Complement System
  C3 Deficiency

Acronyms and Abbreviations

C3, the third component of the complement system; CVID, common variable immunodeficiency; sIgA, secretory IgA; IgAD, IgA deficiency.

IMMUNODEFICIENCY DISEASE

Immunodeficiency diseases, characterized by an increased susceptibility to infection, are a diverse group of diseases that result from abnormalities in one or more components of the immune system. Immunodeficiency diseases can be broadly divided into either primary or acquired immunodeficiencies. Primary immunodeficiencies are diseases in which the animal is born with a genetic defect involving the immune system and clinical disease is a direct consequence of the hereditary defect with the majority of affected patients being neonates or young animals. Acquired immunodeficiencies are diseases in which the animal is born with an intact immune system, but as a result of some underlying disease process, their immune system becomes transiently or permanently impaired. Conditions that may indicate immunodeficiency include chronic or recurrent infections, infection with usually nonpathogenic organisms (opportunistic infections), infections nonresponsive to appropriate antibiotic therapy, failure to respond to immunization, adverse reactions to modified live vaccines, and growth retardation.

Defects in the B cell (humoral immune) system usually result in increased susceptibility to bacterial infections. Animals with T cell (cell-mediated immune) deficiencies usually have an increased susceptibility to intracellular microorganisms such as fungal, protozoal, and viral infections. Since a humoral immune response is highly dependent on the T cell system, certain T cell deficiencies may also present as humoral immunodeficiencies. Complement deficiencies are usually associated with recurrent infections with pyogenic bacteria.

DIAGNOSIS OF AN IMMUNODEFICIENCY

Although many tests of immune function are available, the clinical immunologic tests listed in the following text are commonly used to initially determine the competence of the various components of the immune system. Once an immunodeficiency has been established,

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other more sophisticated tests can be performed to localize the defect more precisely.

1. B cell system
   - Quantitation of serum IgA, IgG, and IgM.
   - Phenotypic evaluation of B cells by flow cytometry (see Chapter 142).
2. T cell system
   - T cell proliferation assay.
   - Phenotypic evaluation of T cells by flow cytometry (see Chapter 142).
3. Complement system\
   - Quantitation of serum C3.

The most practical test for evaluating the humoral immune system is to quantitate the serum concentrations of IgG, IgM, and IgA. Since the only published normal values are from normal adult animals, it is important to compare the immunoglobulin concentrations of young animals with age-matched healthy animals. The T cell proliferation assay is the simplest and most widely accepted in vitro test to evaluate the competence of the T cell system. This test evaluates the capacity of T cells to proliferate after stimulation. Animals with T cell deficiencies have an absent or a significantly reduced response to stimulation.

DISEASES OF THE B CELL (HUMORAL) IMMUNE SYSTEM

Agammaglobulinemia

Agammaglobulinemia is a rare immunodeficiency that has been reported in Thoroughbreds, Quarter Horses, and Standardbreds.[4] In a study of 2516 horses, approximately 0.16% were diagnosed with agammaglobulinemia.[23] It has only been reported in males, suggesting an X-linked mode of inheritance with many similarities to X-linked agammaglobulinemia in humans.

Affected horses suffer from recurrent bacterial infections, such as pneumonia, enteritis, laminitis, and arthritis. Clinical signs begin between 2 and 6 months of age with most affected horses dying before 2 years of age from generalized infections. The two major laboratory findings are the absence of peripheral B cells and low or absent serum IgA, IgG, and IgM. Specific antibody responses also are severely depressed T cell function in these horses is normal. Treatment is symptomatic including antibiotic therapy. Although administration of plasma may temporarily control infections, the long-term prognosis is poor.

Selective IgM Deficiency

IgM deficiency is characterized by reduced or absent serum IgM with normal or elevated serum concentrations of other immunoglobulin classes. IgM deficiency has been reported in foals 2–8 months of age and adult horses.[3,24] Although it occurs in different breeds of horses, it is most frequent in Arabians and Quarter Horses. The mode of inheritance and genetic cause is unknown.

Affected foals are often small for their age, and they experience repeated episodes of bacterial infections including pneumonia, arthritis, enteritis, and possibly septicemia. The bacterial infections are frequently caused by *Klebsiella sp*. Most of these foals die before 10 months of age. IgM deficiency in adult horses has been associated with lymphoma/lymphosarcoma.

The only significant laboratory abnormality is an absent or low serum IgM and is the basis for the diagnosis of this disease. T cells in horses with IgM deficiency have normal T cell function. In contrast to equine agammaglobulinemia, the proportion and absolute numbers of both B cells and T cells are normal. Other than supportive care and antibiotic therapy, there is no effective treatment for IgM deficiency.

Selective IgA Deficiency

The function of serum IgA remains unclear, but a specialized form of IgA, secretory IgA (sIgA), that is produced locally in various mucosal organs is found in saliva, tears, colostrum, nasal and bronchial secretions, gastrointestinal fluids, and urine. A deficiency of IgA results in failure to mount a local immune response to bacteria and viruses, predisposing the individual to infections particularly of mucosal organs. IgA-deficient individuals also have an increased absorption of antigens, predisposing them to potential development of allergies and autoimmune diseases. IgA deficiency has only been documented in humans and dogs.

IgA deficiency (IgAD) is the most frequent primary immunodeficiency in humans with a reported prevalence of approximately 1/600 in Western populations based upon screening large populations of healthy adult blood donors.[30,31] IgADs consist of two main types—severe IgAD defined as undetectable IgA, and partial IgAD defined as low IgA. Although individuals with IgAD are frequently asymptomatic, reduced levels of IgA predispose to a variety of diseases, including upper respiratory infections, gastrointestinal infections including giardiasis, skin infections, allergies, and autoimmune diseases. Approximately 50% of symptomatic IgAD patients present with recurrent infections, particularly of the upper respiratory tract. Autoimmune diseases have been reported to occur in 25–30% of symptomatic IgAD patients, and atopic disease has been reported to occur in 10–15% of symptomatic IgAD patients.

The initial descriptions of IgAD in the dog were in the Beagle and Shar Pei.[13,21] The most frequent clinical problems in dogs with IgAD include recurrent upper respiratory infections due to *Bordetella bronchiseptica* and canine parainfluenza virus, otitis, staphylococcal dermatitis, and atopic dermatitis. The infections associated with IgAD in the dog begin in the first few months of life, but are usually not life-threatening. In a prospective

study of Beagle puppies diagnosed with IgAD, the incidence of upper respiratory disease was 2.4 times greater and infection with *Giardia* was three times greater in the IgAD dogs than in age-matched dogs with normal IgA concentrations.[14] A relative IgA deficiency has been reported in German Shepherd dogs, which is associated with gastrointestinal disorders.[5,29]

Screening of 829 clinically normal adult Beagles revealed the presence of IgAD in approximately 9% of the dogs, 1% with severe IgAD and 8% with partial IgAD.[14] A recent study of 1247 dogs, both healthy and diseased, representing 22 different breeds over 1 year of age from the United States and Europe revealed that 15% of the dogs were considered IgA-deficient.[22] This study also showed a strong association of canine IgAD and atopic dermatitis, and breed predilection for IgAD not only in the previously described Shar Pei and German Shepherd, but also Golden Retriever and Labrador Retriever.

The diagnosis of IgAD, both in humans and dogs, is based upon the quantitation of serum IgA. It is important to compare a young, potentially IgAD dog with values for age-matched normal dogs. Treatment of IgAD dogs is limited to symptomatic treatment of the various infections, allergies, and/or autoimmune diseases. No specific immunotherapy exists for the treatment of IgAD. Immune globulin is contraindicated because many IgAD patients may possess or produce anti-IgA antibodies that could result in anaphylactic reactions. In addition, it would not replace sIgA that is the cause of IgAD.

Common Variable Immunodeficiency

Common variable immunodeficiency (CVID) in humans is a late-onset hypogammaglobulinemia with onset of symptoms between 20 and 30 years of age.[10,11] CVID patients suffer from recurrent upper respiratory and gastrointestinal infections and have an increased incidence of autoimmune disorders and malignancies, primarily lymphoma and gastrointestinal adenocarcinomas. The major immunologic finding is low concentrations of two or all three of the immunoglobulin isotypes, reduced or absent specific antibody production, and abnormal T cell function in approximately 40–50% of patients. CVID has been reported in dogs and horses.

CVID has been documented in two breeds of dogs with *Pneumocystis carinii* pneumonia. *Pneumocystis* pneumonia has been reported in a small number of human CVID patients. The first report was in seven Miniature Dachshunds.[19] The immunologic abnormalities in these dogs were low levels of serum IgG, IgM, and IgA, decreased T cell function as measured by the lymphocyte transformation test, and the absence of B cells with the presence of T cells in lymphoid tissues. The second report involved a Pomeranian with *Pneumocystis* pneumonia.[18] In this dog, serum IgG and IgA were low, but serum IgM was normal. Immunohistochemical examination of lymphoid tissue revealed markedly decreased numbers of B cells with abundant T cells. T cell function was not evaluated in this dog.

A late-onset immunodeficiency that has striking similarities to CVID has been described in Shar Pei dogs.[25] The disease in Shar Peis is characterized by intermittent fever and recurrent infection of skin, respiratory system, and gastrointestinal system, including ulcerative colitis. The mean age at clinical onset is 3 years. In several reported cases, death occurred as the result of intestinal adenocarcinoma and malignant lymphoma. The mean age of dogs with malignant tumors was 6 years. Although affected dogs have normal proportions of peripheral B and T cells, they do demonstrate abnormalities in B and T cell function. The major laboratory findings are low serum concentrations of one or more of the serum immunoglobulins. Most affected dogs had low serum concentrations of IgM and IgA, and some dogs also had low levels of serum IgG. Approximately half of affected dogs had depressed T cell proliferative responses following mitogenic stimulation that correlated, like human CVID patients, with decreased serum concentrations of IgG.

CVID has been reported in a total of 14 horses.[15] The average age of the affected horses was 10.7 years (range = 2–19 years) with essentially an equal number of males and females. The most common clinical signs are recurrent fever and respiratory infections, and meningitis in a large number of patients. The immunologic abnormalities included low serum IgG and IgM, low peripheral B cells and normal peripheral T cells, normal proliferative response of peripheral T cells to mitogenic stimulation, poor specific antibody response following immunization, and absence of B cells in lymphoid tissues. Although a few horses have been managed for 1–5 years on continuous or intermittent antibiotic therapy, in the absence of intensive therapy survival past 6 months after diagnosis is rare.

Transient Hypogammaglobulinemia of Infancy

Transient hypogammaglobulinemia of infancy is a self-limited immunoglobulin deficiency in dogs and horses resulting from a delayed onset of IgG and IgA production by the young puppy or foal.[12,20] Affected puppies and foals are clinically normal during the time they possess maternal antibody. However, when the maternal antibody disappears, they have increased susceptibility to infection, primarily chronic or recurrent bacterial infections of the respiratory tract. Spontaneous recovery occurs between 5 and 7 months of age when the animal's own humoral immune system begins to produce sufficient immunoglobulin. Symptomatic treatment may be needed during this period. The only significant laboratory finding is markedly reduced concentrations of serum immunoglobulin after the disappearance of maternal antibody, at around 2–3 months of age that persists until 5–7 months of age. It is essential to monitor the immunoglobulin concentrations of puppies and foals diagnosed as immunoglobulin-deficient to determine if the defect is permanent or transient.

Failure to Receive Colostrum

Although the immune system of the normal newborn is competent at the time of birth, they have never been exposed to antigens in the environment and any immune response they mount following their initial antigenic challenge has to be a primary immune response. This makes them vulnerable to infectious disease agents during the first weeks of life. Antibodies from the mother (maternal or passive immunity) help protect the newborn while they develop their own immune response to the antigens in their environment.

The newborn receives maternal antibody through either the colostrum or placenta that is directly influenced by the type of placenta in a particular species.[8] In humans, placental transfer is the primary source of maternal antibody in newborns. In dogs, only 5–10% of maternal antibody is transferred through the placenta, while most must be obtained through colostrum. Placental transfer of maternal antibody is prevented in most other domestic animals, including the horse, making the newborn entirely dependent upon colostrum for maternal immunity. It is important that newborns receive colostrum within the first 12–24 hours after birth, since the ability of the intestinal epithelium to absorb the maternal antibody ceases around 24 hours after birth. This phenomenon is referred to as "intestinal closure."

Newborns that fail to obtain colostrum have abnormally low levels of IgG for the first 2–3 months of life. They may have problems with bacterial infections during this time until their own humoral immune system has produced sufficient amounts of antibody to protect them. These animals may have to be maintained on supportive antibiotic therapy.

DISEASES OF THE T CELL (CELL-MEDIATED) IMMUNE SYSTEM

Diseases associated with isolated T cell defects are rare in domestic animals. The major T cell deficiencies in animals also involve the B cell system and are referred to as combined immunodeficiencies including X-linked severe combined immunodeficiency in dogs, severe combined immunodeficiency in Jack Russell Terriers, and severe combined immunodeficiency in Arabian foals. These diseases are discussed in Chapter 55.

Hypotrichosis with Thymic Aplasia (Nude Kittens)

An autosomal recessive disease has been described in Birman cats that appears to be the homolog of the T-cell-immunodeficient nude mouse and, more recently, T-cell-immunodeficient human nude patients.[9,16] The disease is characterized by lack of hair growth and thymic development, and severe immunodeficiency resulting in increased susceptibility to infections. Kittens are born with no hair and fail to thrive, which results in early death. Necropsy findings include lack of a thymus and severe depletion of lymphocytes in the paracortical (T-cell-dependent) regions of the lymphoid tissue in the spleen, Peyer's patches, and lymph nodes. The genetic defect in these kittens has recently been shown to be due to mutations in the FOXN1 gene, which is the same gene involved in T-cell-deficient nude mice and human patients.[1,16] FOXN1 encodes a transcription factor important in the development of keratinocytes in the skin and maturation of thymic epithelial cells that are important in the development of T cells.

T Cell Deficiency in Growth-Hormone-Deficient Dogs

A growth hormone deficiency has been reported in an inbred family of Weimaraner dogs that also had an associated T cell deficiency.[27] This is not surprising because there is increasing evidence that the thymus is a target organ for growth hormone.[27,28] Growth hormone is produced in the thymus by both thymic epithelial cells and thymocytes and appears to enhance secretion of cytokines by thymic epithelial cells, and proliferation and differentiation of thymocytes. This is most likely mediated by interleukin-7 secreted by thymic epithelial cells.

Affected puppies appear normal at birth, but at 6–7 weeks of age they develop a wasting syndrome that is characterized by anemia, emaciation, lethargy, growth failure, and persistent infections, resulting in death. The peripheral T cells from affected dogs fail to respond to mitogenic stimulation. Serum immunoglobulin concentrations in affected dogs are within the reference interval, and the dogs are capable of producing specific antibody following immunization. The thymus is small and lacks cellularity. This syndrome is similar to that seen in several strains of immunodeficient mice. Replacement therapy with growth hormone results in marked increase in size and cellularity of the thymus and marked clinical improvement.[26]

Lethal Acrodermatitis in Bull Terriers

An acquired T cell deficiency has been described in Bull Terriers secondary to an abnormality in absorption and metabolism of zinc.[17] The clinical signs include skin lesions (acrodermatitis, chronic pyoderma, and paronychia), hyperkeratosis of foot pads, poor growth, diarrhea, and recurrent respiratory infections. Affected dogs also have a lighter pigmentation at birth that becomes progressively more noticeable with age. Growth retardation is noticeable by 5–6 weeks of age when compared with their unaffected littermates. Affected dogs rarely survive past 1–2 years of age with bronchopneumonia as the major cause of death. Immunological evaluation of affected dogs reveals normal serum IgM and IgG, low normal serum IgA, and severely depressed T cell response after mitogenic stimulation. Pathologic findings, in addition to skin pathology, include a small, frequently unidentifiable thymus, severe depletion of lymphocytes in the T cell areas of the lymph nodes and spleen, and dilatation of the cerebral ventricles. Unlike other zinc deficiencies, affected dogs show little clinical

improvement after zinc supplementation. The mode of inheritance is autosomal recessive. A recent study has reported a mutation in the MKLN1 (muskelin 1) gene in 46 Bull Terriers with the disease that was not present in 191 normal Bull Terriers.[6]

Viral Infections

Many viral infections are capable of producing an acquired immunodeficiency in dogs and cats, especially those that affect the lymphoid tissue. The viruses include canine distemper virus, canine parvovirus, feline leukemia virus, feline immunodeficiency virus, and feline panleukopenia virus. These viral infections cause primarily a T cell deficiency as the result of a direct lytic effect on lymphocytes or disruption in the normal immunoregulatory cytokine network.

DISEASES OF THE COMPLEMENT SYSTEM

C3 Deficiency

C3 is a component of the complement system important in the opsonization of bacteria. A C3 deficiency, with an autosomal recessive mode of inheritance, has been reported in Brittany Spaniels.[7] Dogs that are homozygous for the trait have no detectable C3, whereas dogs that are heterozygous have C3 concentrations that are approximately 50% of normal and are clinically normal. The genetic defect is a deletion of cytosine at position 2136 causing a frameshift and generation of a premature stop codon resulting in a truncated protein <40% of the wild-type protein.[2] Clinical signs are observed only in dogs that are homozygous for the C3 deficiency and are related to an increased susceptibility to bacterial infections, including septicemia, and renal disease. Older C3-deficient dogs may develop type 1 glomerulonephritis and amyloidosis. The major immunologic abnormality is the absence of serum C3.

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CHAPTER 55

Severe Combined Immunodeficiencies

STEVEN E. SUTER

Severe Combined Immunodeficiency in Arabian Foals
- Molecular Basis of Disease
- Clinical Features
- Diagnosis
- Treatment
- Control of SCID in the Arabian Horse Population

Fell Pony Syndrome
- Clinical Presentation
- Immunologic Abnormalities
- Diagnosis
- Treatment

X-Linked Severe Combined Immunodeficiency
- Incidence of Canine XSCID
- Molecular Basis of Canine XSCID
- Clinical Presentation
- Immunologic Abnormalities
- Treatment of XSCID

Severe Combined Immunodeficiency in Jack Russell Terriers
- Molecular Basis of Disease
- Clinical Features
- Diagnosis
- Treatment

Acronyms and abbreviations

ADA, adenosine deaminase; BCR, B cell receptors; DNA-PK$_{CS}$ IL2RG, DNA-dependent protein kinase; FPS, Fell pony syndrome; JAK3, Janus kinas 3; MHC, major histocompatibility; RAG, recombination activating gene; SCID, severe combined immunodeficiency; SLC, sodium/myo-inositol cotransporter; XSCID, X-linked severe combined immunodeficiency.

Severe combined immunodeficiency (SCID) disorders are a rare (approximately 1/100,000 live births) heterogeneous group of at least 11 congenital genetic conditions in humans characterized by profound defects in both humoral and cell-mediated immune responses, most often leading to infantile death.[4,8,16] SCID classification is based on four main disease mechanisms: (1) Premature cell death due to accumulation of purine metabolites, seen in adenosine deaminase (ADA) deficiency. (2) Defective cytokine-dependent survival signaling in T cell precursors, seen in common gamma (γ_c) chain or janus kinas 3 (JAK3) mutations. (3) Defective V(D)J rearrangements of T cell (TCR) and B cell receptors (BCR), seen in recombination activating gene (RAG)1 or RAG2 mutations. (4) Defective pre-TCR and TCR signaling, seen in CD3δ or CD45 mutations.

Regardless of the mechanism, affected individuals are characterized by: (1) severely reduced numbers and functional capacities of T cells and, in some cases, B cells and natural killer (NK) cells; (2) failure to thrive; and (3) presentation with diarrhea and chronic infections after 2 months of age as protective maternal antibody titers decline. Most forms of SCID exhibit autosomal recessive inheritance with the X-linked recessive form of SCID (XSCID) being the most common (approximately 50%). Both SCID and XSCID have been reported in the veterinary literature.

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SEVERE COMBINED IMMUNODEFICIENCY IN ARABIAN FOALS

Severe combined immunodeficiency in Arabian foals, a uniformly fatal disease first reported in 1973,[19] is an autosomal recessive disorder limited to foals in which both dam and sire are descendants of Arabian horses.[22] SCID foals appear deceptively normal at birth, but they cannot generate antigen-specific immune responses,[7] since they lack mature B and T cells. They have profound lymphopenia and hypoplasia of their lymphoid organs.[17,18,33]

Molecular Basis of Disease

SCID in Arabian foals is caused by a mutation in a gene located on chromosome 9 encoding the catalytic subunit of DNA-dependent protein kinase (DNA-PK_{CS}),[1] leading to a complete lack of expression of an active version of this enzyme.[26] DNA-PK_{CS} is an integral enzyme involved in the V(D)J recombination events of B and T lymphocyte antigen receptor rearrangements. A lack of V(D)J recombination results in B cells lacking expression of cell surface IgM and T cells lacking cell surface expression of antigen-specific receptors. These defective progenitors, which are unable to complete the maturation process, are eliminated resulting in profound lymphopenia. Therefore, this disease is similar mechanistically to human RAG1- or RAG2-deficient SCID where both B and T cells are affected with sparing of NK cells.

Sequence analysis revealed the same five base pair deletion in all cases.[26] The deletion causes a frameshift mutation at codon 3155 and formation of a premature stop codon at amino acid position 3160 (of 4127 amino acids). The resulting 967 amino acid loss eliminates the protein kinase active sites, which renders the enzyme functionally inactive.

Clinical Features

Most affected foals remain free of infections and grow well for 3–8 weeks if adequate colostrum is ingested. As maternal antibodies wane, respiratory infections become increasingly evident. Although initially responsive to antimicrobial therapy, the respiratory infections tend to increase in severity and frequency. Affected foals lose body condition and inevitably die between 2 and 5 months of age.

A number of opportunistic infectious agents have been isolated from SCID foals, with equine adenovirus being the most common and important. Infection usually starts in the upper respiratory tract or intestinal epithelial cells with spread to other sites including bronchiolar, renal, and pancreatic duct epithelium. The pancreas from a SCID foal infected with adenovirus exhibits marked reduction in exocrine tissue and an increase in fibrous connective tissue. Notable opportunistic respiratory bacteria cultured from SCID foals include *Pneumocystis carinii* and *Rhodococcus equi*. *Cryptosporidium parvum* infection can cause severe, life-threatening diarrhea. Because SCID foals have normal NK cell numbers and activity, equine herpesviruses rarely cause serious infections.

Diagnosis

Foals with selective IgM deficiency (see Chapter 54) or failure to absorb adequate maternal immunoglobulin from colostrum can exhibit clinical signs similar to SCID foals, although profound lymphopenia (<1000 lymphocytes/μL of blood) and lymphoid tissue hypoplasia are not seen. Therefore, before 1997, a definitive diagnosis of SCID required demonstration of (1) profound lymphopenia,[27] (2) absence of IgM in serum, and (3) hypoplasia of the thymus and spleen. A definitive diagnosis could not be confirmed until postmortem histologic examination of the spleen and lymphoid tissue revealed marked hypoplasia.

The definitive molecular criteria of SCID diagnosis in Arabian foals were published in 1997.[27] DNA isolated from blood or buccal swabs from horses of any age is subjected to PCR using primers that produce a product that spans the area of the five base pair deletion. DNA from a normal horse produces a 130 bp amplicon, while DNA from a SCID horse produces a 125 bp amplicon. Horses heterozygous for the disease will produce amplicons of both sizes (Figure 55.1). Importantly, this test can be used to unequivocally determine the definitive genotype of any horse and rule in or out SCID in foals exhibiting clinical signs such as recurrent infections or failure to thrive. The diagnostic test for SCID is available from VetGen, Inc. (www.vetgen.com). SCID test kits can be ordered from the Arabian Foal Association (www.foal.org/TestKitsandForms.html).

Treatment

Unfortunately, SCID is uniformly fatal, with all foals incapable of generating antigen-specific immune responses to prevent or recover from opportunistic bacterial and or viral infections. Although the clinical course of the disease can be prolonged with plasma transfusions as a source of antibodies in conjunction with antimicrobial therapy, SCID foals do not survive past 5 months of age.

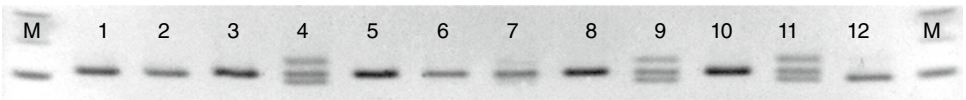


FIGURE 55.1 Genomic DNA PCR analysis for normal and SCID DNA-PK_{cs} sequences. Lanes 1–9 represent clinical blood samples; lane 10, known uninfected foal; lane 11, known heterozygous foal; lane 12, known SCID foal; M is a DNA marker. The foals in lanes 4 and 9 are heterozygous SCID carriers.

Bone marrow transplantation of a 32-day-old SCID foal using histocompatible, mixed-leukocyte-culture-nonreactive, sex-matched, full sibling marrow cells resulted in successful engraftment and reversal of the SCID phenotype.[21] Peripheral blood lymphocyte counts increased within 2 weeks of transplantation and, although a full year was required for normalization of immune functions, the foal developed the ability to respond to vaccine antigens and resist infections, indirectly implying normal B and T cell function. The horse survived for 5 years before succumbing to an unrelated intestinal disorder. Similar to human and canine XSCID, this experiment demonstrated that hematopoietic precursors in the donor marrow with a normal copy of the DNA-PK_{CS} gene enjoy a selective advantage in the SCID recipient with a rapid expansion of functional, mature B and T cells within the bone marrow, thymus, and peripheral lymphoid organs.

Control of SCID in the Arabian Horse Population

SCID in Arabian horses could be completely eliminated through careful genetic screening and selective breeding based on these results. Since only SCID foals can be produced when both the sire and dam are heterozygous for the mutant DNA-PK_{CS} gene, breeding programs can be structured to ensure that heterozygous stallions are never mated to heterozygous mares. Therefore, Arabian mares and stallions should be tested and classified as homozygous, heterozygous, or normal for the DNA-PK_{CS} gene. Theoretically, barring any new spontaneous mutations, Arabian SCID could be completely eliminated from the population within a few generations. Canine XSCID was eliminated from the canine breeding population using this strategy.

Although not recommended, an alternative to breeding only homozygous normal horses would be the continued use of known heterozygous horses under carefully controlled conditions based on genetic screening. For example, a heterozygous SCID stallion could breed with a homozygous normal mare with subsequent testing of all the foals to determine the genotype. The homozygous normal foals could be selected for future breeding programs, while the heterozygous colts could be gelded and all heterozygous offspring be used for nonreproductive activities. Indeed, the finding that the carrier prevalence of SCID in Arabian foals in South Africa was reduced from 6.4% in 2004/2005 to 3.4% in 2009/2010 is suggested to be the result of genetic screening followed by selective breeding.[31]

FELL PONY SYNDROME

Fell pony syndrome (FPS), also called foal immunodeficiency syndrome (FIS), first described in 1998 in an ancient breed of ponies bred mainly in upland areas of the UK,[25] is a uniformly fatal disease seen in Europe[3] and the USA.[11] Similar to other primary immunodeficiencies, affected foals are usually normal at birth, but then fail to thrive as maternal antibodies begin to wane. Unpublished genealogical studies have suggested a possible autosomal recessive mode of inheritance, which has been confirmed with the recent identification of a recessive mutation associated with the disease.

Clinical Presentation

At 4–8 weeks of age, affected foals begin to lose body condition and develop multiple, chronic infections that do not respond appropriately to antimicrobial therapy. Weight loss, diarrhea, bilateral nasal or oculonasal mucopurulent discharge, hypersalivation, coughing, and bronchopneumonia are the most common clinical signs. Other notable abnormalities include pyrexia, frequent chewing movements, halitosis, and a pale, friable, pseudomembranous and hyperkeratotic lingual coating. Severe, normocytic, normochromic anemia and lymphopenia are the most common blood dyscrasias. The bone marrow typically has a reduction in erythrocyte precursors. The foals deteriorate progressively and die or are euthanized, usually between 1 and 3 months of age.

Postmortem examination invariably reveals multisystemic necrotizing and inflammatory lesions associated with opportunistic infections with a wide variety of bacteria.[24] Rhinitis, sinusitis, bronchitis, pneumonia, and glossal hyperkeratosis are the most common respiratory abnormalities. The most common gastrointestinal abnormalities include hepatitis, typhlocolitis, adenovirus-induced pancreatitis, and enteritis. Most ponies also have inconspicuous lymphoid tissue including small thymuses, a lack of lymph node follicles and germinal centers, and a paucity of splenic corpuscular tissue. Peripheral gangliopathy, characterized by neuronal chromatolysis and nuclear pyknosis of dorsal root ganglia, has also been documented.[25]

Immunologic Abnormalities

FPS foals have low IgM and IgA immunoglobulin levels,[25] while IgG levels can be variable, most likely reflecting the presence of maternally derived IgG antibodies from colostrum. Importantly, FPS foals have significantly reduced peripheral B cell numbers (<10%) when compared to age-matched normal Fell ponies. This correlates with immunohistochemical data showing a severe reduction of CD79a+ B cells in the lymph nodes, spleen, and bone marrow from FPS foals.[25] Lymphocyte function tests and the distribution of CD4+ or CD8+ T cells are typically normal, although depressed mitogenic responses have been described.[25] Lymphocyte cell surface expression of major histocompatibility (MHC) class II is variable.

Diagnosis

The genetic mutation associated with FPS in Fell and Dales ponies has been reported.[9] A point mutation in a single exon of the sodium/myo-inositol cotransporter (SLC5A3) gene leads to a Pro446 to Leu446 amino acid

change in the protein. To date, the altered phenotype in affected foals is thought to be the result of either alteration or loss of SLC5A3 activity that is deleterious to B cell and erythroid development. Using a DNA-based carrier test, approximately 18% of Dales ponies and approximately 38% of Fells ponies were carriers of the mutation.[10] No alterations of gene expression relating to B cell development, signaling, and maturation have been identified.[30]

Treatment

As with most primary immunodeficiencies in animals, there is no treatment for FPS. This disease is most likely the result of a severe defect in B cell development at the level of the bone marrow, which contributes to the failure of B cell expansion in the periphery. Similar to SCID in Arabian foals, implementation of appropriate breeding programs to reduce the incidence of FPS can now be considered in light of an available DNA-based carrier test (http://www.fellponysociety.org.uk/health.htm).

X-LINKED SEVERE COMBINED IMMUNODEFICIENCY

XSCID, the most common SCID in humans,[4] has also been reported in dogs.[15,23] Both species share common immunologic abnormalities including: peripheral T cell lymphopenia with variable numbers of phenotypically normal B cells that do not undergo isotype class-switching to IgG in vivo peripheral T cells that do not proliferate in response to mitogens; and reduced production of NK cells. Human and canine XSCID patients exhibit a reduced ability to prevent or combat opportunistic infections resulting in infantile death.

Incidence of Canine XSCID

Canine XSCID was first reported in a litter of Basset Hounds in 1989.[15] Breeding studies confirmed the disease was X-linked. Additional cases of XSCID in the general Basset Hound population have not been reported. Canine XSCID was again recognized in 1997 in closely related Cardigan Welsh Corgi dogs.[23] Pedigree analysis of this family was consistent with X-linked inheritance, and the definitive diagnosis of XSCID was confirmed with DNA analysis. Subsequent pedigree DNA analysis identified the gene mutation in the affected male dog and related obligate carrier females and grandmother of the affected dog. Genetic testing identified carrier females so they could be removed from the breeding population.

It is believed that the genetic mutations responsible for XSCID in Basset Hounds and Cardigan Welsh Corgis were due to random chance. Since genetic testing followed by controlled breeding has eliminated the disease from the Corgi and Basset populations, XSCID is no more likely to occur in these two breeds than in any other breed.

Molecular Basis of Canine XSCID

Similar to XSCID-affected boys, canine XSCID is caused by mutations in the gene encoding the third or gamma chain of the interleukin-2 (IL-2) receptor.[14] The IL-2 receptor gamma chain is also a component of additional cytokine receptors, including the receptors for IL-4, IL-7, IL-9, and IL-15. The normal canine γc protein, which is 373 amino acids in length and 84% and 71% identical to the human and mouse protein sequences, respectively,[14] is composed of several evolutionarily conserved potential functional domains. Unlike human XSCID where hundreds of different γc mutations have been identified, only two mutations have been identified in dogs. Basset hounds have a four base pair deletion in the signal peptide region of the γc gene, which causes a shift in the translation reading frame and premature truncation of the γc protein during its synthesis. The mutant, nonfunctional protein is only 21 amino acids in length.[14] Cardigan Welsh Corgi dogs have a single nucleotide insertion following nucleotide 582 that also causes a shift in the translation reading frame and premature truncation of the γc protein at amino acid 196.[23] This effectively eliminates the transmembrane and the Src homology domains, resulting in a nonfunctional protein.

Clinical Presentation

Similar to XSCID boys, canine XSCID is characterized by a failure to thrive and an increased susceptibility to bacterial and viral infections at a young age.[5] Puppies appear normal at birth and are presumably protected by maternal antibodies for several weeks. As maternal antibodies wane, XSCID puppies fail to thrive and infections begin to develop. These infections include bacterial pyoderma, otitis, and cystitis, as well as diarrhea. Affected puppies lack palpable peripheral lymph nodes and no thymic shadow is visible on thoracic radiographs. Antimicrobial therapy is generally ineffective in combating these recurrent infections and puppies die or are euthanized by 5–6 months of age.

The thymuses of XSCID puppies are often less than 10% of the weight of age-matched normal littermates and are usually obscured by adipose tissue. Histologically, thymuses from XSCID puppies can range from simple dysplasia with no Hassall's corpuscles to relatively normal looking with many Hassall's corpuscles, although total thymocyte numbers are always severely reduced.

Immunologic Abnormalities

The most significant immunologic problem in XSCID is the defect in T cell function and development.[28] XSCID puppies are variably lymphopenic, with normal or increased percentages of B cells, and usually decreased percentages of peripheral T cells. XSCID dogs can develop variable numbers of phenotypically mature peripheral T cells; their ability to proliferate in response to mitogenic stimulation or IL-2 binding is negligible due to a dysfunctional IL-2 receptor.

Treatment of XSCID

In humans, bone marrow transplantation is the only treatment for XSCID, with success ranging from 50% to nearly 100%. Gene therapy using retroviral vectors harboring a normal copy of the γc cDNA has cured a number of affected boys, although two patients later developed leukemia, most likely the result of insertional mutagenesis.[12]

XSCID puppies have received bone marrow transplants using unfractionated whole marrow collected from both normal and carrier littermates.[6] The transplanted dogs, none of which received pretransplant cytoablative therapy, developed completely engrafted donor T cells, partially engrafted donor B cells, and were maintained in a conventional environment. XSCID puppies have also been transplanted with highly purified populations of CD34+ hematopoietic progenitor cells harvested from dog leukocyte antigen (DLA)-matched littermates.[13] All six dogs in this study went on to develop completely engrafted donor T cells from 1 to 3 months after transplantation, variable engrafted donor B cells, and were maintained in a conventional environment for over 6 months.

Retroviral gene therapy has also been used successfully to treat canine XSCID. In an initial study, a murine retroviral expression vector harboring enhanced green fluorescent protein (EGFP) and γc cDNAs was used for the ex vivo transduction of normal DLA-matched CD34+ cells followed by transplantation into XSCID-affected littermates.[29] Three of the five transplanted puppies went on to develop durable immune reconstitution with up to 28% of peripheral blood T cells expressing EGFP. A more recent study used in vivo retroviral gene therapy whereby XSCID puppies were directly injected with a retroviral vector encoding a normal copy of the γc cDNA.[32] Three of the four injected dogs attained normal numbers of gene-corrected peripheral T cells by 8 weeks following treatment.

SEVERE COMBINED IMMUNODEFICIENCY IN JACK RUSSELL TERRIERS

SCID has also been reported in a single family of Jack Russell Terriers. Twelve of 32 siblings from this single pair breeding succumbed to opportunistic infections within 8–14 weeks of age.[3] Additional breedings allowed the elucidation of the underlying genetic defect responsible for the immunodeficiency.

Molecular Basis of Disease

Jack Russell Terriers SCID, which is inherited as an autosomal recessive trait, is a non-B/non-T SCID most likely caused by a genetic DNA-PK_{CS} deficiency, similar to Arabian foal SCID.[20] A single nucleotide polymorphic transition (A/G) at position 95 of intron 5 results in a stop codon at nucleotide 10,828 and a premature termination at amino acid 517. This functionally null allele is responsible for lack of DNA-PK_{CS} activity and subsequent defective V(D)J recombination. The carrier frequency of the Jack Russell Terriers SCID allele is less than 1.1%.

Clinical Features

The clinical features of SCID in Jack Russell Terriers mirror those seen in most other SCID-affected animals.[2] Puppies typically appear normal until maternal antibodies begin to wane, with most succumbing to a wide variety of opportunistic infections between 8 and 14 weeks of age. Extreme lymphopenia and agammaglobulinemia are seen, while thymic dysplasia and peripheral lymphoid aplasia are the most common findings at necropsy.

Diagnosis

A DNA-based carrier test is available to screen Jack Russell Terriers whose matings produce offspring with an unusually high puppy mortality rate (http://www.animalabs.com/shop/dogs/severe-combined-immunodeficiency-terrier-type/).

Treatment

There are no published reports of Jack Russell Terriers SCID treatment, although bone marrow transplantation using DLA-matched siblings is, at least theoretically, a possibility.

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CHAPTER 56

Lymphadenopathy Not Caused by Lymphoma

HAROLD TVEDTEN

Lymph Node Anatomy
Lymphadenopathy
Cytological Evaluation
  Lymph Node Histopathology
Selected Disorders Associated with Nonmalignant Lymphadenopathy in Dogs and Cats
  Bacterial Lymphadenitis
  Idiopathic Lymphadenopathies
  Mineral-Associated Lymphadenopathy

Acronyms and Abbreviations

FNA, fine needle aspiration biopsy.

LYMPH NODE ANATOMY

The focus of this chapter is on lymphadenopathy not caused by lymphoma and other hematopoietic neoplasia, which is described in Section V and elsewhere. The functional anatomy and histology of the lymph node are well described in Chapter 9.

LYMPHADENOPATHY

The term lymphadenopathy means an abnormal process (disease) has occurred in one or more lymph nodes. Lymphadenopathy is usually interpreted to mean enlargement of lymph nodes. A distinction, not in common use, is to restrict the term lymphadenopathy for changes without an infectious cause. This relates to the difference in the two suffixes: *opathy* and *itis*. Lymphadenitis denotes infiltration of inflammatory cells into the node rather than identifying an infectious agent. It is frequently not possible with cytologic or histologic examination to identify an infectious agent. Solitary lymphadenopathy refers to enlargement of a single lymph node. Regional lymphadenopathy describes the enlargement of lymph nodes draining a specific anatomic region. Generalized lymphadenopathy refers to enlargement of lymph nodes in more than one anatomic area. The distribution of lymphadenopathy aides in diagnosis. Solitary or regional lymphadenopathy suggests a lesion in the area drained by the lymph node. By contrast, generalized lymphadenopathy suggests systemic infections or hematopoietic neoplasia.

The type of animal affected, anamneses, and frequency of different diseases in a geographical area affect the diagnosis. For descriptions of changes seen with the many diseases of lymph nodes and other lymphoid organs, use references such as *Veterinary Comparative Hematopathology*.[9] Bacterial and viral infections may initially cause edema and swelling of lymph nodes early, and later cause variable patterns of necrosis, atrophy, hemorrhage, or infarction with or without lymphoid hyperplasia. Chronic stress may cause atrophy of lymph nodes.

CYTOLOGICAL EVALUATION

Abnormal lymph nodes are usually initially evaluated by cytologic evaluation of fine needle aspiration (FNA) biopsies as a minimally invasive test with rapidly available interpretations.[1] Impression smears may also be made from the cut surface of excised lymph nodes. FNA should indicate the relative numbers of different cell

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types found in the node if adequate numbers of cells with good morphology are obtained. Lymphoid cells are fragile and are easily and often damaged beyond recognition. Take great care to be gentle with collection of a FNA and with spreading of the sample into a thin monolayer.

The cells found normally are mainly small to medium lymphocytes (75–95%) with few plasma cells (0–3%), and a small number of large lymphoid cells and blast cells (3–15%). Occasional other cells are present and include macrophages, neutrophils, eosinophils, and mast cells (Figure 56.1a). The percentages of different cell types are given to help describe expected populations, but should not be used as absolutes when making cytological diagnoses. The author usually combines the larger lymphoid cells (with a more open chromatin pattern) and lymphoblasts (with a nucleolus) together as "immature cells" in a differential cell count when both are few in number, but will add different categories to a differential cell count, such as intermediate cells, hand-mirror cells, plasmacytoid cells, etc., when they represent a dominant cell type.

Younger, more actively dividing cells have more euchromatin. Euchromatin is not stained. The increased area of unstained euchromatin gives the nucleus a more "open" appearance. Heterochromatin is the resting DNA that appears as stained granules of different sizes. The expected cell populations will vary among different lymph nodes. For example, "normal" intestinal, submandibular, and popliteal lymph nodes often look more reactive with a higher percentage of plasma cells or more immature-appearing lymphoid cells.

If the FNA indicates a similar population of cells to those normally seen in a lymph node and the lymph node is enlarged, a diagnosis of hyperplasia is appropriate. A prominent increase in plasma cells indicates a B cell response, while mainly small lymphocytes may suggest a T cell response. The numbers of lymphoblasts and larger lymphoid cells are often increased in hyperplastic lymph nodes and may be so greatly increased that differentiation of hyperplasia from lymphoma cannot be made with certainty. A cutoff of more than 50% immature lymphoid cells/lymphoblasts is usually used to indicate lymphoma. Similarly, a prominent increase in plasma cells in some infections may be difficult to distinguish from plasma cell neoplasia. Fortunately, the most common type of canine lymphoma has clearly over 50% large blast cells with large nucleoli, many mitotic figures, and tingible body macrophages to allow a definitive lymphoma diagnosis. However, nuclei in many types of lymphoma lack nucleoli and may be small to medium in size, which makes cytological diagnosis more difficult and uncertain. When lymphoid cells are very similar in appearance (monomorphic), lymphoid neoplasia is more likely. With hyperplasia, the appearance of lymphoid cells is typically more diverse.

An increase in nonlymphoid leukocytes indicates inflammation (lymphadenitis). The predominant cell type is used to classify the inflammation. A prominent increase in neutrophils (>15–25%) indicates neutrophilic inflammation (purulent or suppurative lymphadenitis). A predominance of neutrophils indicates severe purulent inflammation or even abscess formation. The amount of blood contamination should be considered because of the contribution of neutrophils and other leukocytes found in blood to the FNA sample especially in patients with leukocytosis. A small number of neutrophils (3–8%) may be normal or only indicate blood contamination especially in samples with a small number of nucleated cells.

This author uses the "reactive lymph node" classification as a more general diagnosis to include changes too mild to consider as lymphadenitis. This includes mild increases in neutrophils (10–15%), eosinophils (2–7%), and macrophages (3–8%). These increases are inflammatory changes, but mild. Hyperplasia of normal-appearing lymphoid cells is a reactive change, but suggests no increase in other types of leukocytes.

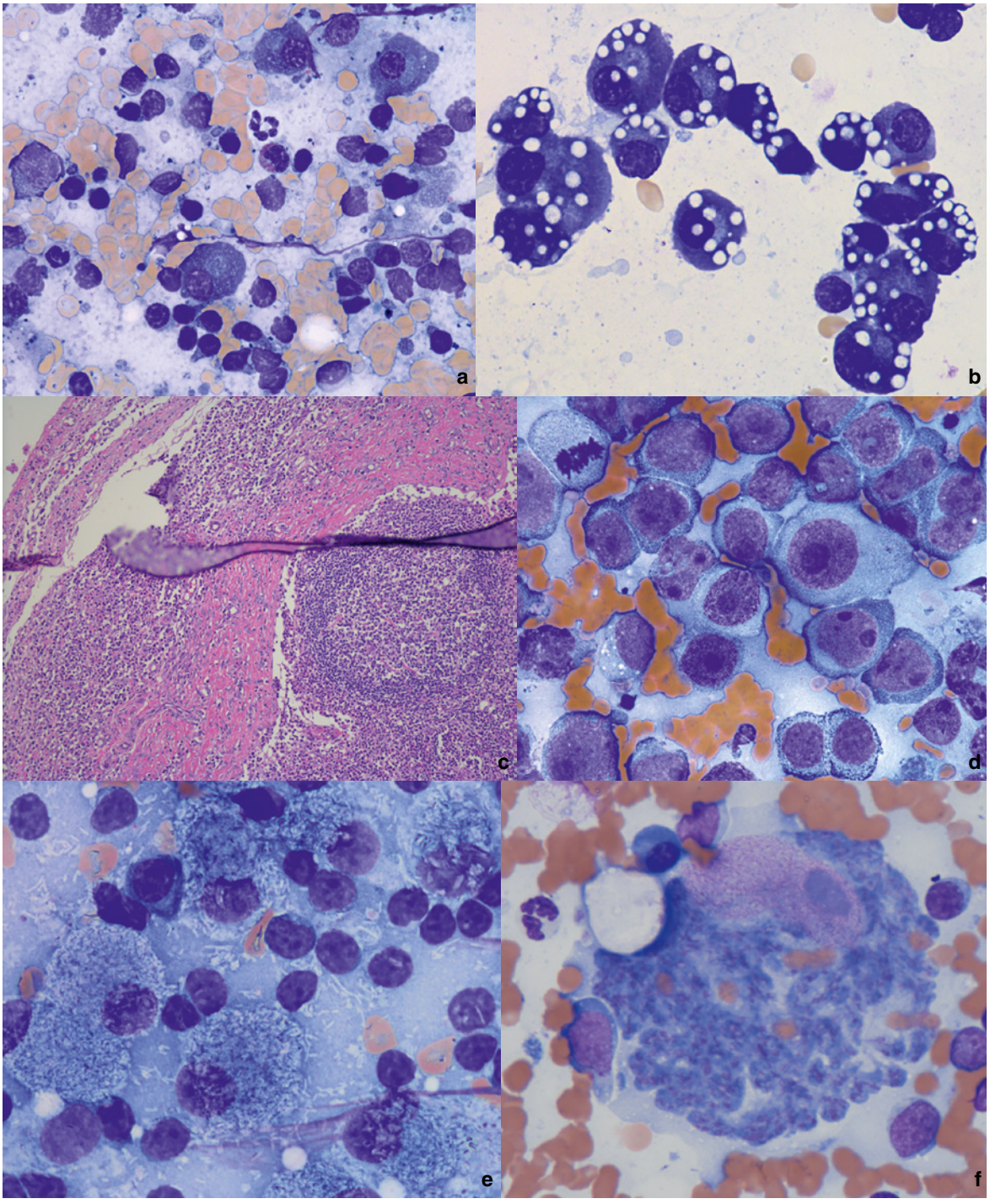
Macrophages occur normally in medullary sinuses and other areas of lymph nodes. A mild increase (3–8%) in macrophages or finding focal accumulations of macrophages may indicate a hyperplastic change due to some stimulus such with sinus histiocytosis. Moderate to large numbers (10–80%) of macrophages should indicate macrophage (granulomatous) inflammation (Figure 56.1e). Macrophages should be evaluated for phagocytosis and the phagocytic material may indicate the cause. Erythrophagocytosis and hemosiderin indicate pathological hemorrhage in the drainage field. Melanin may be seen in macrophages in lymph nodes draining an area with dermatitis.

If both neutrophils and macrophages are increased in number, the term pyogranulomatous lymphadenitis is used. Neutrophils are less fixed in the tissue, which allows relatively more neutrophils to be taken up in a FNA so the percentage of neutrophils usually exceeds that of macrophages. Larger numbers of macrophages may be seen in histological sections.

Organisms, when found, clearly indicate the diagnosis (Figure 56.1e,f). Organisms include various bacteria (e.g., *Mycobacterium*), fungi (including the classical dimorphic *Histoplasma*, *Blastomyces*, *Cryptococcus*, and *Coccidioides*), protozoa (e.g., *Leishmania*, *Cytauxzoon*), and even parasites (*Demodex*).[4]

Eosinophilic inflammation is fairly common.[2] The percentage of eosinophils required to classify the change as eosinophilic inflammation is less (>10–20 %) than that needed for neutrophils. Eosinophils may accompany neoplasia including mast cell tumors and lymphosarcoma. Basophilic inflammation is rare. The cytological pattern seen in lesions in the draining field may aid in diagnosis (e.g., allergic dermatitis).

Metastatic neoplasia in a lymph node sample is a common consideration/question. Metastasis can be early with only occasional neoplastic cells in a FNA or the entire lymph node can be replaced by neoplastic cells. Diagnosis is easier if the abnormal cells in a FNA are different from those normally seen in lymph nodes (Figure 56.1d). Occasional mast cells may be seen normally. Alternatively, large numbers of mast cells in a FNA indicate mast cell neoplasia. Mast cell hyperplasia is usually mild with few mast cells, but even a few mast


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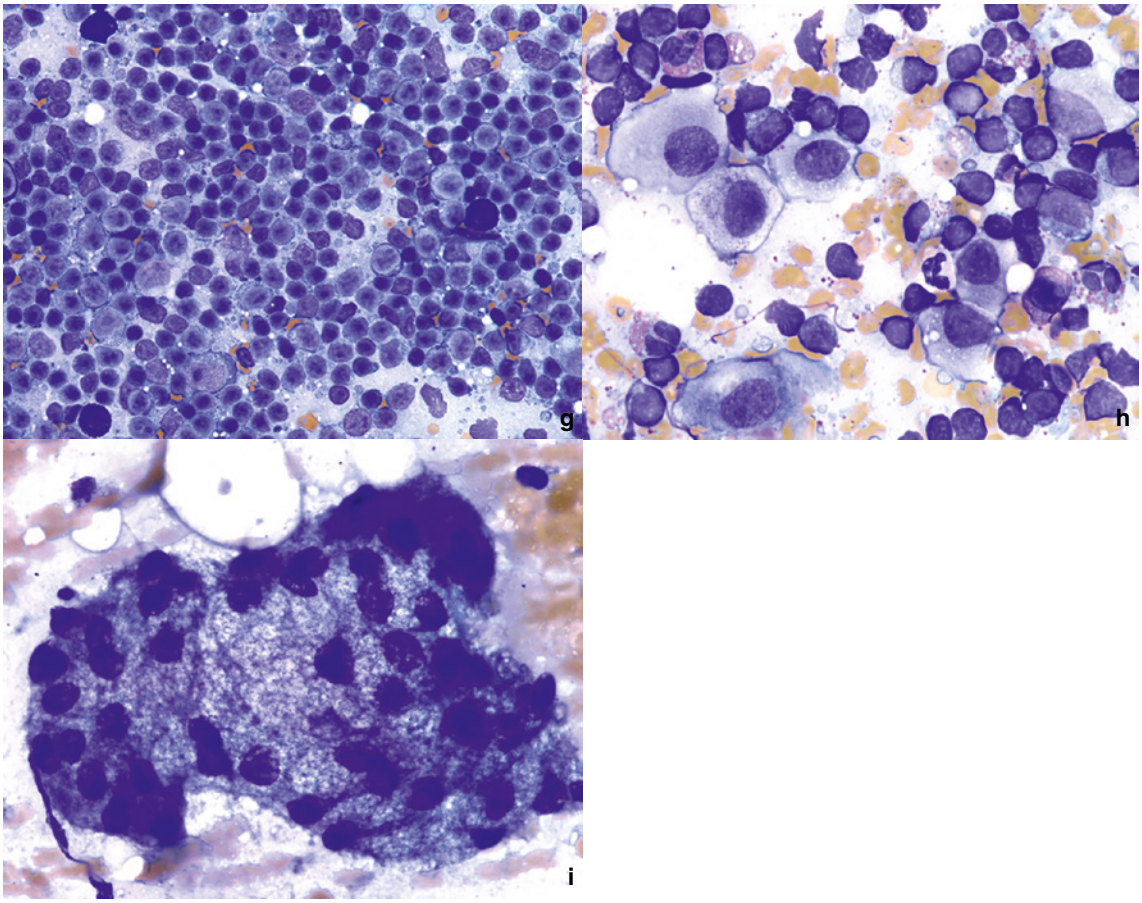


FIGURE 56.1 Lymph node aspirates and histological sections. (a) Reactive lymph node illustrating the types of cells seen in a normal lymph node aspirate. There are three plasma cells with abundant dark blue cytoplasm and a perinuclear halo. Three plasma cells on one field could suggest plasma cell hyperplasia. Most of the cells are small lymphocytes. One lymphoblast on the left side has a nucleus that is about 2.5 times the diameter of an RBC and has an open chromatin pattern with several nucleoli (light blue color). Lymphoblasts have larger nuclei, more euchromatin, and usually have one or more nucleoli. There is also a neutrophil and an eosinophil (Giemsa stain, original magnification 600×); (b) A lymph node aspirate with 15 plasma cells containing Russell bodies. Russell bodies are clear vacuoles in the cytoplasm representing excessive antibody in the endoplasmic reticulum. It is unusual to see this many plasma cells containing Russell bodies and a monomorphic population can suggest a lymphoproliferative disorder (Giemsa stain; original magnification 600×); (c) A histological section of an intestinal lymph node from a cat with gastrointestinal eosinophilic sclerosing fibroplasia. The purpose of the photo is to illustrate the architectural information available from histology that is not appreciated with cytology. A round germinal center is present on the center right. A bit of the capsule is at the upper left. Between these is of fibroplasia and an infiltrate of eosinophils and other leukocytes. (H&E stain; original magnification 100×); (d) An abdominal lymph node aspirate illustrates that not all large round, blast cells in a lymph node indicate lymphoma. This dog had metastatic testicular seminoma. The cells are often round with large round nuclei and very large nucleoli. One normal-appearing mitotic figure is present in the upper left (Giemsa stain; original magnification 1000×); (e) A lymph node aspirate from a Miniature Schnauzer with a *Mycobacterium* infection. There are six to seven large macrophages filled with negatively staining, white rods, which are also seen in the background. This indicates a granulomatous lymphadenitis with *Mycobacterium*-type bacteria (Giemsa stain; original magnification 600×); (f) Lymph node aspirate from a cat with *Cytauxzoon* infection. There is a huge macrophage (schizont) filled with many developing merozoites. (Giemsa stain; original magnification 600×); (g) A lymph node aspirate from a cat illustrate three well-granulated mast cells seen as individual cells widely separated from each other. Low numbers of mast cells are present within normal and inflamed lymph node aspirates and do not necessarily indicate the presence of a mast cell tumor. (Giemsa stain; original magnification 100×); (h) Mandibular lymph node aspirate from a dog illustrating metastasis of a mast cell tumor. Most cells are lymphocytes. There are six or more large mononuclear cells which are malignant mast cells. One of these cells to the upper left has a small number of granules that indicate it is of mast cell origin. The presence of increased numbers of eosinophils supports a conclusion that these undifferentiated cells are mast cells (Giemsa stain; original magnification 600×); (i) A salivary gland aspirate. Cells consist of an acinus of mature salivary gland cells with abundant foamy cytoplasm. The salivary gland is fairly often sampled with FNA when it is mistaken for an enlarge lymph node (Giemsa stain; original magnification 600×).

cells can cause difficulty in ruling out metastasis of mast cell tumors to a lymph node. The pattern seen with mast cell hyperplasia in a FNA smear or fragments of lymph node tissue in a FNA smear is the presence of individual mast cells separated from each other (Figure 56.1g). Aggregates of mast cells are more consistent with mast cell neoplasia. The presence of poorly differentiated mast cells is a stronger indication of mast cell tumor metastasis (Figure 56.1h). Cytologic diagnosis of histiocytic neoplasia provides similar challenges. Macrophage inflammation and hyperplasia are frequently associated with large and immature macrophages which may resemble histiocytic sarcoma.

Increased size of a lymph node can be due to noncellular elements such as edema or other causes of increased lymph. There is normally fat around a lymph node which can be a prominent part of the FNA. FNA of nonneoplastic, noninflamed submandibular salivary glands are relatively commonly seen in cytological practice (Figure 56.1i). Tattoo ink may be found in lymph nodes. Calcium oxalate crystals may be seen rarely in lymph nodes with pyogranulomatous inflammation.

Lymph Node Histopathology

Histopathology reveals abundant information not available from cytology.[8] Architectural patterns are important in differential diagnosis of hyperplasia from neoplasia (Figure 56.1c). There are often more special stains available to histopathologists than to cytologists to more precisely identify various cell types.

SELECTED DISORDERS ASSOCIATED WITH NONMALIGNANT LYMPHADENOPATHY IN DOGS AND CATS

Bacterial Lymphadenitis

Two syndromes presumptively associated with pyogenic bacterial infections and lymphadenitis, contagious streptococcal lymphadenitis of cats and puppy strangles, have been described in small animals.[3] Also, localized streptococcal and staphylococcal infections may result in solitary or regional lymphadenopathy in these species.

Contagious streptococcal lymphadenitis was described in several kittens and one adult cat in a cat colony.[3] The kittens developed diarrhea and fever at 4 weeks of age and cervical lymphadenopathy 2 weeks later. The lymph nodes in these cats were significantly enlarged and developed draining purulent lesions. All the cats tested negative for circulating feline leukemia virus (FeLV) p27 antigen. Lancefield group G beta-hemolytic streptococci were isolated from abscessed lymph nodes and from heart blood in one kitten that died of septicemia. Most cats responded to treatment with penicillin.

Puppy strangles (i.e., juvenile cellulitis) is a disorder associated with cutaneous cellulitis of the head and neck, and mandibular and cervical lymphadenopathy in 4–12-week-old puppies. Pups usually present with fever and facial skin lesions including edema, pustules, and crusts on the chin or muzzle, or in the ears, along with lymphadenopathy. Sometimes the lymphadenopathy is not associated with skin lesions. More than one pup in the litter is frequently affected. Fine needle aspiration of affected lymph nodes reveals purulent lymphadenitis, and staphylococcal or streptococcal organisms may be cultured. However, due to lack of response to antibiotic therapy in a high proportion of cases, the absence of bacteria in most cultures obtained from affected areas, and the subsequent response to immunosuppressive doses of corticosteroids, a hypersensitivity reaction to staphylococcal antigens is suspected.

Idiopathic Lymphadenopathies

A syndrome, called idiopathic lymphadenopathy, has been described in the cat.[6,7] Some cats are clinically normal on initial physical examination, except for the presence of lymphadenopathy; clinical signs in the other cats included fever, lethargy, anorexia, pallor, hematuria, eczema, vomiting, and mastitis. In one study, six of nine cats evaluated for FeLV antigens in peripheral blood were positive, while in another study they were negative.[7]

In one study, histologic changes in affected lymph nodes included distortion of the architecture; proliferation of histiocytes, lymphocytes, plasma cells, and lymphoblasts in paracortical regions; and numerous prominent postcapillary venules.[7] Histological features in the FeLV-negative cats were suggestive of lymphoma and included loss of normal lymph node architecture, presence of a uniform population of lymphoid cells in the paracortical areas, capsular and perinodal infiltration, and presence of large follicular structures without germinal centers.[6] However, other features were not compatible with lymphoma. Resolution of the lymphadenopathy occurred in the FeLV-negative cats within 5–120 days and all cats were alive 12–84 months after initial diagnosis.

The clinical and pathologic features in cats that have idiopathic reactive lymphadenopathy are similar to those observed in cats naturally or experimentally infected with feline immunodeficiency virus (FIV). It is improbable that all cats that have idiopathic reactive lymphadenopathy are infected with FIV; more likely, FIV represents one of the agents that can induce these changes in lymph nodes of domestic cats.

A clinical syndrome characterized by solitary cervical or inguinal lymphadenopathy in cats ranging in ages from 3 to 14 years, and referred to as plexiform vascularization of lymph nodes has been described.[5] The lesions were unilateral in seven of nine cats studied. Most cats were asymptomatic and the FeLV status was

not reported. Surgical removal of the affected nodes resulted in uneventful recovery in most cats. Histologically, the nodes showed replacement of the interfollicular pulp by a plexiform proliferation of small capillary-sized vascular channels and lymphoid atrophy.

Mineral-Associated Lymphadenopathy

A primary lymph node disease termed mineral-associated lymphadenopathy was described in the 1980s and has been reported since exclusively in dogs in the United Kingdom.[1] Affected dogs have nonspecific clinical signs (e.g., malaise, pyrexia, inappetence) in combination with generalized lymphadenopathy. Histologic examination of lymph nodes reveals a granulomatous lymphadenitis with accumulation of large quantities of crystalline-appearing, mineral material in the node. Electron microprobe analysis of this mineral reveals it consists of a complex mixture of elements including aluminum, silicon, titanium, and nickel, suggesting an environmental origin. In affected lymph nodes, inflammatory cytokines (IL-1, TNF-α) are produced causing the clinical symptoms. In several cases, concurrent diseases, like lymphosarcoma and immune-mediated diseases, are present.

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SECTION V
Hematologic Neoplasia

CHAPTER 57

Cell-Cycle Control in Hematopoietic Cells

JAIME F. MODIANO and CATHERINE A. ST. HILL

The Cell-Cycle Engine
- Cyclins
- Cyclin-Dependent Kinases
- Cyclin-Dependent Kinase Inhibitors
- Regulation of Cyclin-Dependent Kinase Activity

Maintenance of G0
- Negative Regulation of Cell-Cycle Entry

The G0 to G1 Transition and G1-Phase Progression
- The G1 Restriction Point
- Regulation of the G1 to S Transition by Rb

S-Phase Progression
- Checkpoint Transit
- Regulation of the Origin or Replication

G2-Phase Progression
- DNA Damage Checkpoint
- Preparation for Mitosis

M-Phase Progression
- Prophase
- Metaphase
- Anaphase

The Cell Cycle and Cancer

Acronyms and Abbreviations

APC, anaphase-promoting complex; CAK, CDK-activating kinase; CDK, cyclin-dependent kinase; CDKI, CDK inhibitor; DNA, deoxyribonucleic acid; G(0), gap (0) phase; HDAC, histone deacetylase; IL, interleukin; MHC, major histocompatibility complex; MPF, maturation-promoting factor; ORC, origin of replication complex; TGF-β, transforming growth factor-β.

In the late 1980s, there was a confluence of several fields of research. Scientists studying cell division in budding yeast (*Saccharomyces cerevisiae*), in fission yeast (*Schizosaccharomyces pombe*), and in frog eggs (*Xenopus laevis*) discovered a group of proteins that were responsible for cell-cycle oscillation.[4,8,16] Because the proteins were expressed cyclically, they were named cyclins. In short order, a "cell-cycle engine" that functions in all eukaryotic organisms was defined. This mechanism consists of cyclin-regulated cell-cycle kinases that provide the crucial checkpoints and feedback controls that regulate cell division.

THE CELL-CYCLE ENGINE

To simplify its study, the cell cycle has been subdivided into various steps (Figure 57.1). These steps include gap (G0), or the phase at which cells are in a resting or nonproliferative phase; G1, which defines entry into the cell cycle and is characterized by increased cellular metabolism and growth; synthesis (S), in which the whole genetic complement of the cell is replicated; G2, where the cell finishes the growth processes necessary for division; and finally mitosis (M), where division of one cell into two daughter cells finally takes place.[9] In normal cells, transitions among these states are defined functionally and are exquisitely fine-tuned. The balanced action of proliferative and antiproliferative signals ultimately determines whether cells enter the cell cycle, exit the cell cycle, or undergo programed cell death (i.e., apoptosis).[7]

The cell-cycle engine defines a regulatory network whose key components include proteins from two unrelated gene families, the cyclins and the cyclin-dependent kinases (CDKs). These proteins control phase transition and are regulated transcriptionally and post-transcriptionally. In this chapter, we will use T cells to

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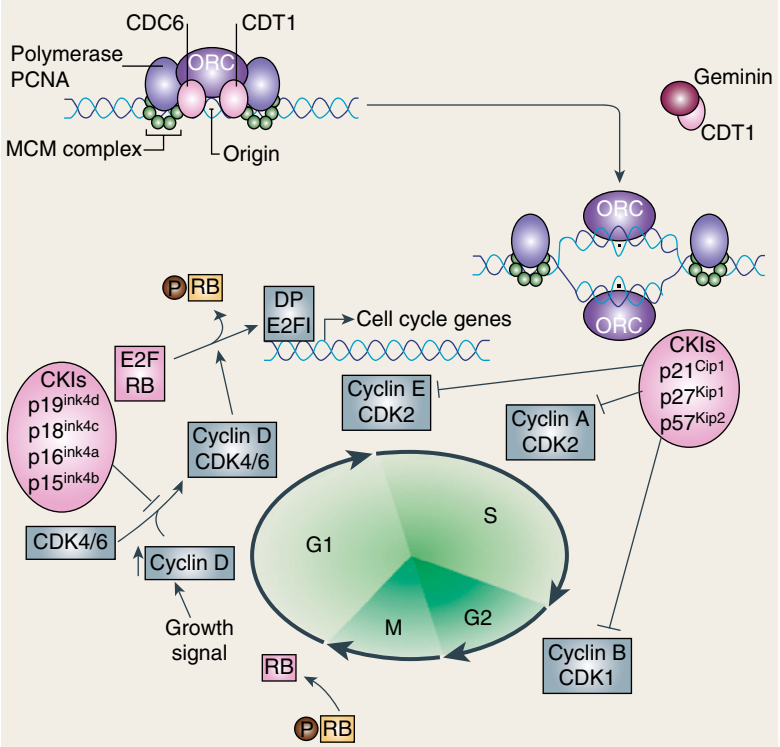


FIGURE 57.1 A schematic representation of the cell cycle. Cells enter the cell cycle in the G1 phase. G0 is a specialized form of G1 (highly differentiated cells, which are unlikely to divide unless provoked, remain in G0). During late G1, the cell commits to divide, typically marked by phosphorylation of Rb, and activation of E2F, which in association with DP-1 binds to the promoters of various cell-cycle genes. The process of replication of the entire genome is completed during the S phase, resulting in doubling of DNA content of the cell. The mechanical components that will organize chromosomes and physically divide the cell are assembled during the G2 phase, and a complex cytological event that accurately divides the duplicated sets of chromosomes and coordinates the events of fission to produce two cells from one is executed during the M phase. Many events take place during M phase, including dephosphorylation of RB. Upon completion of M phase, both daughter cells re-enter G1 and a new cell cycle is set to begin. Proteins that drive or inhibit the cell cycle are shown in gray or pink boxes, respectively. Cyclins are activator proteins that are upregulated or downregulated depending on the phase of the cell cycle. CDKs are serine/threonine kinases that require the binding of a cyclin (or related protein) for full activity. Their range of substrates is not fully defined, but interfering with their activity arrests or slows the cycle. CDKIs are small peptides that block cyclin/CDK activity either by forming an inactive complex or by acting as a competitive CDK ligand. DNA polymerases, proliferating cell nuclear antigen (PCNA), and minichromosome maintenance (MCM) proteins initiate replication only once per cycle. The checkpoint proteins are members of a network of proteins that monitor DNA integrity and arrest the cell cycle until DNA damage can be repaired. (**Source:** Reproduced from Herrup and Yang[9] with permission. ©Macmillan)

illustrate the transition steps that regulate cell-cycle entry, progression, and exit.

Cyclins

Cyclins are highly conserved in all eukaryotic species studied. More than 12 mammalian cyclin homolog genes have been characterized, but here we will focus only on those genes that control cell-cycle transitions. Cyclins function as regulatory subunits for the CDKs because monomeric CDKs have essentially no catalytic function.[19] However, the association of a CDK and a cyclin into a complex is only one regulatory step in the activation of these protein kinases. The cyclins are divided into groups according to the phase of the cell cycle in which they are expressed. Examples include the mitotic cyclins (B-type cyclins), S-phase cyclins (cyclins A and E), and G1 cyclins (cyclins D1–D3, cyclin E). Cyclin H is part of an enzyme complex called CDK-activating kinase (CAK). The nucleotide and amino acid sequence homology among cyclin proteins is limited (<30% at the amino acid level) and is restricted to a highly conserved region called the cyclin box. This cyclin box is required for association with a CDK, along with an independent, protein-specific sequence that mediates binding to small molecules called cyclin-dependent kinase inhibitors (CDKIs) that facilitate assembly of the cyclin–CDK complex.[11]

Cyclin-Dependent Kinases

In higher eukaryotes, the CDKs comprise a family of more than 12 proline-directed, serine and threonine protein kinases that share a PSTAIRE motif. Those members of the CDK family that control cell-cycle transitions are subdivided into proteins closely related to CDK1 (the mammalian Cdc2 homolog) that include CDK2, CDK3, CDK 4, CDK5, and CDK6.

Cyclin-Dependent Kinase Inhibitors

The CDKIs are products from various distinct gene families, the prototypes of which are p16 (CDKN2A), and the closely related p21 (CDKN1A) and p27 (CDKN1B).[17,20] Proteins in the p16 family (p15, p16, p18, p19) are specific inhibitors of CDK4 and CDK6, and their expression and function are dependent on a functional *RB*1 gene product (Rb). Indeed, the absence of Rb appears to make these CDKIs, as well as CDK4 and CDK6, inconsequential in their cell-cycle regulatory function. In normal cells, the upregulation of p16-related proteins is inducible by transforming growth factor-β (TGF-β). The expression of p21 is closely regulated by another unrelated tumor suppressor gene called p53. The p21 gene may be inducible as an effector of growth arrest before S-phase entry if the cell must undergo DNA repair. The function of members of the p27 family (p27, p57) appears to be regulated by various signaling pathways that affect cell-cycle progression, including those that are mediated by TGF-β or by cyclic AMP.

Regulation of Cyclin-Dependent Kinase Activity

Each CDK can associate with multiple cyclins: among the G1 CDKs (CDK2, CDK4, CDK6), CDK2 associates with A, E, and D-type cyclins, whereas CDK4 and CDK6 form complexes only with D-type cyclins. The cyclin partner is required for activation of the kinase, it confers substrate specificity, and it regulates the interactions of the complex with other proteins.

Inactivation of the kinases occurs in part due to the proteolytic degradation of the cyclin partner. CDK activity also is controlled by other kinases and phosphatases. Phosphorylation and dephosphorylation reactions occur at defined residues that are highly conserved throughout the CDK family; e.g., phosphorylation of a distal threonine residue (T160/T172 in CDK2 and CDK4, respectively) by CAK is an important activating event for all CDKs. Another critical event is dephosphorylation of N-terminal threonine (T14) and tyrosine (Y15/17) residues near the ATP-binding site by a bifunctional protein phosphatase of the Cdc25 family. Conversely, phosphorylation of T14 and Y15/Y17 by the Wee1 bifunctional protein kinase and dephosphorylation of T161/T172 by a protein like protein phosphatase 1 are important to inactivate the CDKs. The kinases and phosphatases that carry out this phase of CDK regulation provide additional links between environmental signals and activation of the cell-cycle machinery.

MAINTENANCE OF G0

Cell growth and proliferation are integral parts of the immune response. Mature peripheral T cells generally maintain homeostasis in a true resting (G0) state where the cell-cycle kinases (CDK1, CDK2, CDK4, or CDK6) and their cyclin partners (cyclin D2, cyclin D3, cyclin E, cyclin A, or cyclin B) are not expressed.[15]

Negative Regulation of Cell-Cycle Entry

The process of thymic education dictates that naïve T cells must interact with self-peptides presented by major histocompatibility complex (MHC) (i.e., potential autoantigens) to survive. Naïve T cells express high levels of T cell receptors, so the avidity for self-MHC might trigger their activation and result in autoimmunity. To prevent such activation, several interrelated negative regulatory pathways in lymphocytes enforce quiescence. Regulatory molecules in these pathways include those that dampen signaling such as IAN5 and negative transcriptional regulators such as the transducer of ErbB2-1 (Tob-1), nuclear factor of activated T cells-c2 (NFATc2), and Smad3, which is primarily responsible for integrating antiproliferative and prosurvival signals delivered through the TGF-β receptor.[12,23] Mutations in these genes result in T cells that survive poorly (IAN5 knockout), fulminant autoimmunity (TGF-β receptor dominant-negative), or cells that show cell-cycle dysregulation with eventual tumor formation (Tob-1 and NFATc2 knockouts). Intriguingly, the tumor phenotypes for Tob-1 and NFATc2 deficiency are B-cell lymphomas, and a recent study suggests that these proteins contribute to this subtype of lymphoma in dogs.[6]

THE G0 TO G1 TRANSITION AND G1-PHASE PROGRESSION

Antigen binding to the T-cell receptor complex triggers a series of events that give rise to several interactive, nonlinear biochemical cascades that disengage negative regulation and activate the cell-cycle machinery. Events during the G1 phase are essential to integrate environmental signals with cellular responses. In some instances, cell proliferation may be separated from other biological responses. For example, stimuli that incite production of interleukins can promote lymphocyte maturation and differentiation, immunoglobulin production, or enhanced cytotoxic or antigen-presenting functions, and may not lead to cell division. Conversely, exogenously provided cytokines may promote proliferation of receptive lymphocytes in a paracrine or endocrine fashion.

Unlike embryonic cells where the length of the cell cycle is temporally fixed, transit time through the G1 phase is variable in adult somatic cells. Potentially transforming events can significantly shorten the G1 phase. In most cases, this prompts activation of checkpoints that prevent transition to the S phase or leads to apoptosis. Cancer cells must, therefore, acquire mutations that allow them to overcome these safeguards.[20]

The G1 Restriction Point

Cell-cycle entry, defined as the transition from G0 to G1, is reversible. This transition point can be defined by partial activation of the CDK4/cyclin D2 holoenzyme complex and phosphorylation of the retinoblastoma susceptibility protein Rb. Transition into G1 also requires activation of CDK6 and CDK2. The CDKI p27 protein

may be especially important in control of G1 progression. At least part of the observed increase in CDK2 activity in activated T cells may be due to IL2-induced dissociation of p27 from CDK2 kinase complexes, and T cells from p27-knockout mice have exaggerated T-cell responses.[5] Cells that reach this stage of activation acquire the capability to respond to proliferative signals, but are not committed to continue transit into the S phase until they cross the restriction point, R.[15] This occurs at least in part by activation of CDK4/cyclin D3 and CDK2/cyclin E complexes.

Regulation of the G1 to S Transition by Rb

The proteins of the Rb family promote (or suppress) the expression of genes that control the G1 to S transition, including members of the E2F family of transcription factors and histone deacetylases (HDACs). In the hypophosphorylated state, modulation of these proteins by Rb acts as a cell-cycle brake. Phosphorylation of Rb by the CDKs leads to functional collapse of the pocket, releasing latent transcription factors and allowing their activation. It appears that phosphorylation by CDK4 releases HDACs, relieving transcriptional repression for some genes such as cyclin E, and opens the conformation of Rb, making it permissive for phosphorylation by CDK2. Subsequent Rb phosphorylation by CDK2/cyclin E releases E2F transcription factors. The activation of E2F factors requires their association with DP-1 or DP-2 to form heterodimers that orchestrate transcription of genes necessary for DNA synthesis (Figure 57.2). In lymphocytes, activation of Aurora B in conjunction with Survivin and mTOR is an essential step to complete the transition from G1 to the S phase.

S-PHASE PROGRESSION

In mammalian cells, an important control element in the process of DNA replication is assembly and activation of pre-replication complexes. Initiation sites (the replication origin) interact with a group of proteins called the origin of replication complex (ORC).[23] This allows helicases and other enzymes to unwind the double strand and recruit polymerases. Replication forks then move away from the origin in either direction (Figure 57.1).

As is true with abnormal G1 transit, partial re-replication triggers checkpoints that arrest cell-cycle progression or promote apoptosis. Re-replication is in some ways unavoidable and may be a driver of evolution. Indeed, it is now clear that subtle differences in DNA copy number exist among individuals within a species, and in dogs these might be conserved within members of the same breed, similarly to microsatellites and single-nucleotide polymorphisms.[3,13]

Checkpoint Transit

The S-phase checkpoint relies on detection of stalled replication forks (usually due to insufficient trinucleotide building blocks) or DNA damage. Members of the ataxia-telangiectasia-mutated and Rad3-related (ATM/ATR) kinase families act as sensor kinases, which in turn activate the effector kinase Chk2. Chk2 activation delays or arrests S-phase progression. Persistent stalled forks or DNA damage that cannot be repaired triggers apoptosis of normal cells.

Regulation of the Origin or Replication

Cells must avoid extensive re-replication during DNA synthesis. The ORC contains six stable subunits that bind to DNA in the transition from mitosis to G1 (or G0), followed by additional proteins, such as minichromosome maintenance (MCM) helicases, that are synthesized or stabilized at or near the G1 to S transition to form the pre-replicative complexes. CDK2/cyclin E (and other S-phase kinases) primes these pre-replicative complexes to form preinitiation complexes that are necessary for activation of DNA polymerases. Progression through S phase requires transition from CDK2/cyclin E to CDK2/cyclin A complexes, which are followed by the appearance of CDK1 complexed to cyclin A and cyclin B. Substrate specificity is probably important to prevent re-replication and to maintain the fidelity of DNA polymerases.

G2-PHASE PROGRESSION

Transition from S to the G2 phase occurs upon completion of DNA replication. At this point, somatic cells are tetraploid (i.e., have four copies of DNA) and are destined to divide.

DNA Damage Checkpoint

The S-phase checkpoint is fallible, so a partially redundant checkpoint exists during the G2 phase. ATM/ATR kinases also act as G2-DNA damage sensors. Lymphocytes that undergo physiologic DNA double strand breaks during antigen receptor gene rearrangement are particularly susceptible to chromosomal instability, which is why patients with ataxia telangiectasia have hypofunctional adaptive immune responses and a significantly higher risk of developing cancer.[2] The primary G2-DNA damage effector kinase is Chk1, which controls G2-phase progression and M-phase entry, primarily by phosphorylating the CDK-regulatory Cdc25 phosphatases.

Preparation for Mitosis

Proper chromatid alignment and association are necessary for normal separation during mitosis. Spindle assembly and the spindle checkpoint are regulated by CDK1 in conjunction with the Aurora A and Aurora B kinases, respectively. Aurora C kinase controls chromosomal segregation in mitosis.

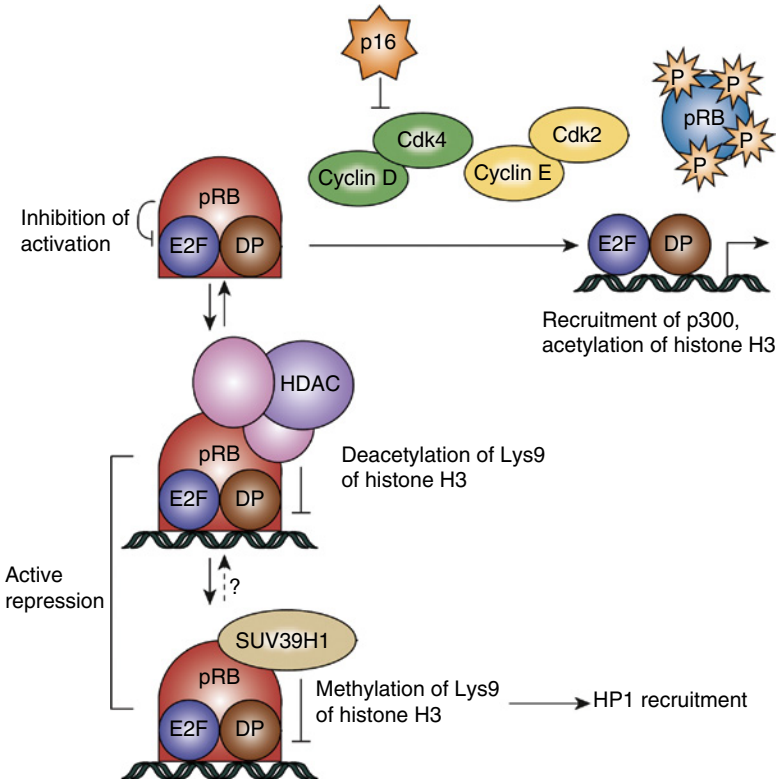


FIGURE 57.2 General mechanisms that control the repressive activity of E2F. The retinoblastoma protein binds to an E2F–DP complex in G0/G1 cells and this leads to repression of E2F-responsive genes through two possible mechanisms. First, phosphorylated RB (pRB) inhibits E2F from activating transcription by binding to its transactivation domain and preventing its interaction with the core transcriptional machinery. Second, the resulting complex binds to the promoters of E2F-responsive genes and enforces their "active repression" through recruitment of either histone deacetylases (HDACs), which remove the acetyl group from lysine 9 (Lys9) of the histone H3 tail and thereby facilitate nucleosome packing, or SUV39H1, which methylates the same lysine residue to create binding sites for HP1, leading to transcriptional silencing. Cell-cycle entry is dependent on the sequential activation of the cell-cycle-dependent kinases, CDK4/CDK6–cyclin D and CDK2–cyclin E, which phosphorylate RB and cause it to release E2F. The resultant activation of E2F-responsive genes seems to be at least partially dependent on the ability of E2F to recruit p300, leading to the acetylation of Lys9 of histone H3. Several key components of the pRB regulatory pathway are known to be tumor suppressors (pRB, p16) or oncogenes (cyclin D, CDK4). DP, differentiation-regulated transcription factor (DRTF) protein; pRB, retinoblastoma protein. (**Source:** Reproduced from Trimarchi and Lees[21] with permission. ©Macmillan)

M-PHASE PROGRESSION

Chromatin condensation occurs throughout the cell cycle until the onset of mitosis, when the chromatin is packaged into species-specific units called chromosomes. M-phase progression begins with the dissolution of the nuclear membrane, continues with the assembly of the mitotic spindle, and culminates with division into two daughter cells, each with a diploid complement of DNA.

Prophase

Breakdown of the nuclear envelope proceeds at the end stage of chromosome condensation by phosphorylation of lamins. This precedes spindle formation, which is mediated by complex interactions of microtubules and kinetochores. Centromeres allow chromosomes to attach to the spindle, a process that also may be controlled through Aurora B kinase and Survivin.[1]

Metaphase

As cells approach metaphase, chromosomes align along the spindle at equal distance from each pole to form the traditional metaphase plate in a process called *congression*.

Anaphase

Cyclin B is rapidly degraded by the anaphase-promoting complex (APC), which functions as an E3 ubiquitin ligase; this is followed by separation of sister chromatids. Anaphase-promoting complex activity is restrained by the spindle checkpoint until kinetochore alignment is complete. Sister chromatid separation is itself a complex process and is controlled at least in part by DNA topoisomerase II. The importance of this enzyme in mitosis is underscored by the exacting sensitivity of cancer cells to camptothecin and other topoisomerase II inhibitors.

After the separation of sister chromatids, there is little resistance for chromosome segregation toward the

poles. Vesicles on the chromosomes promote reformation of the nuclear envelope and cytokinesis ensues as CDK1/cyclin B activity subsides. Each daughter cell can then resume its normal function or re-enter the cell cycle.

THE CELL CYCLE AND CANCER

Cancer is a condition where cells divide uncontrollably, making it easy to see how it can be linked to abnormal cell-cycle function. Common abnormalities include inactivation of CDKIs through mutation or deletion, inappropriate expression of D-type cyclins, inactivation of Rb, and malfunction of DNA damage checkpoints, among others.[10] Not surprisingly, these gross abnormalities are prognostic, but even subtle quantitative differences in cyclin expression, Rb phosphorylation, etc., can influence tumor progression and serve as useful indicators of prognosis for hematopoietic tumors.[14] Indeed, studies from our laboratory suggest that gene expression signatures that are indicative of aberrant cell-cycle regulation are hallmarks of canine high-grade B-cell malignancies and canine osteosarcomas that respond poorly to conventional therapy.[6,18]

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CHAPTER 58

Epidemiology of Hematopoietic Neoplasia

MICHELLE G. RITT

Hematopoietic Neoplasia Epidemiology Based on a Reference Population
- Reference Population Information for Dogs
- Reference Population Information for Cats
- Reference Population Information for Horses
- Reference Population Information for Cattle

Hematopoietic Neoplasia Risk Assessment in Dogs
- Lymphoma
  - Environmental Risk Factors
  - Genetic/Heritable Risks
  - Geographic Risk Factors
- Leukemia
- Histiocytic Sarcomas
- Plasma Cell Tumors
- Mast Cell Tumors
- Hemangiosarcomas

Hematopoietic Neoplasia Risk Assessment in Cats
- Lymphoma/Leukemia (Lymphoid Neoplasms)
  - Environmental
  - Infectious Diseases
  - Genetic/Heritable
- Mast Cell Tumors
- Hemangiosarcoma

Hematopoietic Neoplasia Risk Assessment in Horses

Hematopoietic Neoplasia Risk Assessment in Cattle
- Lymphoma

Conclusions

Acronyms and Abbreviations

2,4-D, 2,4-dichlorophenoxyacetic acid; BLV, bovine leukemia virus; FeLV, feline leukemia virus; FIV, feline immunodeficiency virus; VDL, Veterinary Diagnostic Laboratory.

The simplest definition of epidemiology is the study of factors which define a population's risk of susceptibility to a given disease. Both genetic and environmental influences are usually important, particularly when identifying risk factors for neoplasia. Epidemiology data have inherent uncertainty, as they seek to identify the probability that disease will develop within a given population or the risk of one population to develop a particular disease as compared to another population without that risk. The predominant statistical terms used in cancer epidemiology are relative risk and absolute risk. Relative risk is the comparison between two groups: one group has a given risk factor and the other group does not. Absolute risk is the probability that cancer will develop within a defined period of time. If the period of time is from birth to death, it is referred to as lifetime risk (Figure 58.1). In order to establish risk factors, data from a test population are compared to a reference population. Replication of the data is required for validation. The ultimate goal is to have the most representative group to which other populations can be objectively compared. There are vast amounts of information of this type available for neoplasia in human beings; however, it is significantly lacking for other species.

Epidemiology of hematopoietic neoplasia in domestic animals is an understudied area. By far, the most information available is for dogs. Yet, even in dogs, the most current database regarding a reference population is 40 years old. The reference population itself has changed considerably and needs to be updated to reflect: (1) breed popularity changes; (2) changes in breeding practices to produce different desirable traits; (3) changes in production management and husbandry practices; (4) popular sire effects; (5) development of new breeds, and breeds from narrow gene pools; (6)

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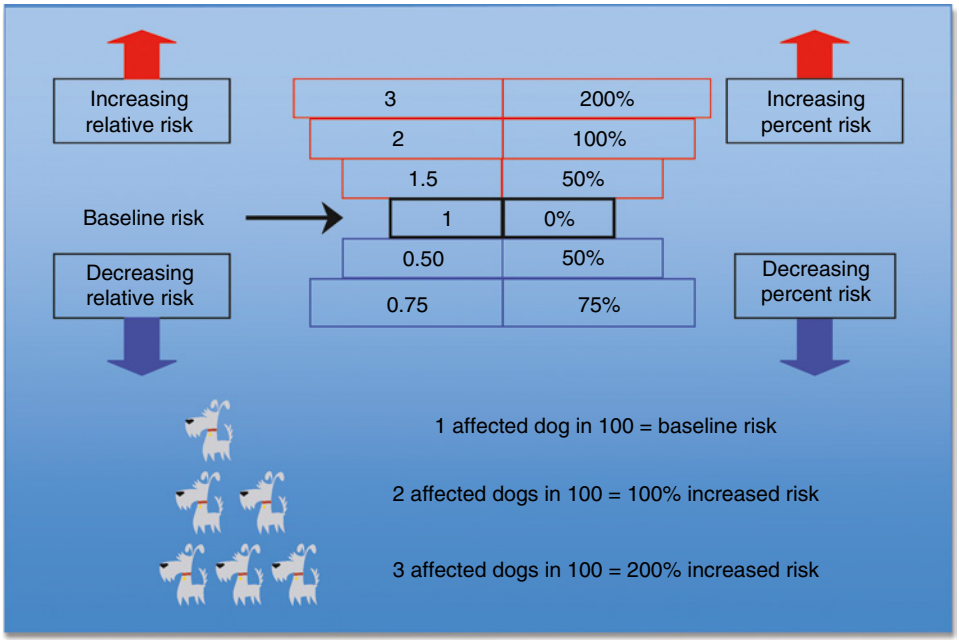


FIGURE 58.1 Two different ways of looking at cancer risk. In the upper portion of the figure, baseline relative risk is equivalent to 1% or 0% risk in the population. When risk increases to 50%, relative risk increases by 1.5× the baseline. When the overall risk decreases by 50%, relative risk decreases to 0.5× the baseline. In the bottom portion of the figure, this is represented by the example of a baseline risk of one affected dog in a given population of 100 dogs: when two dogs are affected, the risk increases by 100%; when three dogs are affected, the risk increases by 200%.

geographical changes; and (7) changing infectious disease influences. More recent studies generally have involved significantly smaller numbers of animals and have not been based on reference populations. This means many specific risk factors will be difficult to identify.

HEMATOPOIETIC NEOPLASIA EPIDEMIOLOGY BASED ON A REFERENCE POPULATION

A pioneering study on cancer epidemiology in dogs and cats was the first of its kind to investigate the implications of sex and age in a defined population in 1968.[4] Although the data encompassed a limited geographical region, this paper was among the first to describe a higher incidence of cancer in purebred dogs compared to mixed breeds. Above all, this paper underscored the need for a more geographically comprehensive reference regarding the susceptibilities and risks of domestic animals for developing neoplasia. A much broader study and the only animal cancer reference population still available today comes from a compilation of data from 15 veterinary schools in the USA and Canada through the years 1964–1977.[22] The geographical bias is somewhat minimized, however, by comparing animals from schools from a diverse group of geographic locations.

Reference Population Information for Dogs

In dogs with hematopoietic neoplasms, lymphoma represented the most cases (1324), followed distantly by hemangiosarcoma of the spleen (referred to as angiosarcoma) with 165 cases, and leukemia (subclassifications not defined) at 151 cases. Breeds that represented the highest risk for lymphoma, leukemia, and hemangiosarcoma (spleen) included Boxers, Basset Hounds, and St. Bernards, which were three to four times as likely as all breeds combined (reference population) to develop these cancers. Breeds twice as likely to develop lymphoma, hemangiosarcoma, and leukemia included Scottish Terriers, Bulldogs, Airedales, Weimaraners, Golden Retrievers, Doberman Pinschers, Labrador Retrievers, English Setters, and Great Danes. Dogs considered less likely to develop these tumors (relative risk <1) were mixed breeds, Miniature and Toy Poodles, Pomeranians, Chihuahuas, Boston Terriers, Cocker Spaniels, and Dachshunds.

When hemangiosarcoma was evaluated separately from leukemia and lymphoma, breeds with the highest risk (two to three times greater) included: Boxers, Great Danes, German Shepherds, English Setters, Golden Retrievers, Pointers, and German Shorthaired Pointers. Breeds with decreased risk included Miniature Schnauzers, Brittany Spaniels, Cocker Spaniels, Beagles, Boston Terriers, Miniature and Toy Poodles, Dachshunds, and Pekingese, with Chihuahuas showing the lowest risk.

There were 202 cases of mast cell tumors in the skin and 162 cases of cutaneous hemangiosarcoma in the database. The relative risk was similar to hematopoietic and lymphatic neoplasms; however, spayed females were 1.3 times more likely to develop these tumors. Also, it should be noted that the incidence of mast cell tumors was only reported in dogs.

Hematopoietic tumors not considered in this study included plasma cell tumors and histiocytic sarcomas (with the exception of histiocytomas which were considered along with benign neoplasia). Histiocytomas were by far more prevalent in dogs than cats.

An independent study that reviewed the database of the Veterinary Medical Data Program from 1964 to 1989 to determine the incidence of neoplasia in dogs of 6 months of age or younger showed that cancers of the hematopoietic system were most frequent in this population.[13]

Reference Population Information for Cats

Lymphoma was reported in 657 cats. Manx cats were 4.6 times more likely than non-Manx cats, and Burmese were 3.1 times more likely than non-Burmese to develop lymphoma. Females, neutered and intact, had slightly lower relative risk than males. Four-hundred-and-seventeen cases of leukemia were identified. Mixed breed cats were 1.3 times as likely to develop leukemia as purebred cats, and Persians were 1.2 times as likely as non-Persians to develop leukemia. Siamese cats had a lower relative risk than the average for the reference population. Feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV) status was not evaluated.

Reference Population Information for Horses

Forty-eight cases of equine lymphoma were recorded, as were five cases of leukemia. Appaloosas, mixed breeds, and Standardbreds were 1.7, 1.5, and 1.2 times as likely to develop lymphoma, respectively. Mares were 1.6 times as likely as geldings and stallions to develop lymphoma.

Reference Population Information for Cattle

There were 332 cases of bovine lymphoma and 56 cases of bovine leukemia recorded in the database. Gender was found to be the most significant risk factor. Steers were 3.8 times as likely as bulls, and cows were twice as likely as bulls to develop lymphoma. Bovine leukemia virus (BLV) status was not evaluated. Breeds at highest risk for lymphoma included Angus and Ayrshire (1.6×), Jerseys (1.5×), mixed-breed dairy and shorthorns (1.3×), mixed-breed beef and Holsteins (1.2×), while Guernseys, Herefords, and Charolais had lower relative risk.

HEMATOPOIETIC NEOPLASIA RISK ASSESSMENT IN DOGS

Lymphoma

Environmental Risk Factors

Because it is one of the most common malignant neoplasms affecting domestic dogs, lymphoma has been very extensively studied in dogs (see Chapters 62, 67, and 70). Many attempts have been made to associate lymphoma with environmental risk factors; however, none has been conclusively verified. One environmental risk factor evaluated was exposure to the lawn herbicide 2,4-dichlorophenoxyacetic acid (2,4-D). Although one study found a relationship, a subsequent re-evaluation was unable to confirm the association.[10,12] An association between exposure to electromagnetic fields and the subsequent development of lymphoma in dogs was published, but follow-up studies have not been performed to validate that connection.[24]

A study retrospectively performed in the Tuscany region of Italy evaluated dogs with residential exposure to environmental pollutants from 1996 to 1998.[6] The results of this study suggested that there might be an increased risk of lymphoma in dogs living in or near industrial areas and dogs that may have had environmental exposure to household paints and solvents. However, the study's findings were subject to bias recall on the part of the dogs' owners (information obtained by questionnaires and telephone interviews), and specific information regarding types of chemical exposure and amount of exposure was not investigated. The authors of this study affirm that additional investigation is warranted to validate these potential associations.

Genetic/Heritable Risks

It has been postulated that perhaps no other species shows more variability and flexibility of its genome than the domestic dog.[20] *Canis familiaris* has, through selective breeding practices by humans, had its gene pool manipulated to produce more than 400 distinct breeds ranging in size from 2 kg to over 100 kg. Life expectancy, hair coat, skull and skeletal conformation, and behaviors all have been diversified in just 2800 years.[20] There is abundant evidence that humans also have altered dogs' susceptibility to cancer. Epidemiologic evidence for genetic risk factors for lymphoma in domestic dogs is currently limited to breed associations. The occurrence of tumors is sporadic; however, their development is affected to an extent by heritability. The prevalence of lymphoid neoplasms in dogs differs among breeds as does the frequency of B-cell and T-cell neoplasms; however, heritable risk factors have not been identified.

More recent information regarding hematopoietic cancers in dogs can be obtained from health surveys privately commissioned by specific American Kennel Club National Breed Clubs. This information is collected via voluntary recruitment of members of the national breed clubs who complete a written survey. The data are then compiled by statisticians and are presented as a health report for the specific breed club. These surveys are not normalized to a reference population and may contain considerable bias. Diagnoses are unable to be directly confirmed by the investigators and sometimes by a veterinarian, so there is considerable variation in the numbers of responses obtained. Nevertheless, taken within the context of their limitations, breed health surveys can provide further insight into the epidemiology of hematopoietic neoplasms in dogs.

Based on analysis of data for Golden Retrievers, Irish Setters, Rottweilers, Portuguese Water Dogs, English

Cocker Spaniels, and Gordon Setters, the risks for hematopoietic neoplasia were combined to include lymphoma, mast cell tumors, and hemangiosarcoma.[7,8,27–30] A meta-analysis of the available data, using the chi-squared test, indicates Golden Retrievers have the highest risk of the three hematopoietic tumors combined, followed distantly by Gordon Setters, which, in turn, were at higher risk than Portuguese Water Dogs, Rottweilers, and Irish Setters.[27–30] The last three breeds were roughly equivalent in prevalence for these hematopoietic tumors. When the prevalence of lymphoma was considered separately from mast cell tumors and hemangiosarcoma, the rank order of apparent breed risk did not change. When a nonhematopoietic neoplasia such as osteosarcoma was evaluated using the same breed survey information, the rank order of breed risk changed considerably. Irish Setters had the highest absolute number of affected dogs followed by Golden Retrievers, Rottweilers, Gordon Setters, and, more distantly, by Portuguese Water Dogs. English Cocker Spaniels had an insignificant risk for osteosarcoma.

Geographic Risk Factors

There may be geographic risk factors; however, data are regionalized and sporadic. For example, Jagielski et al. found in a study of dogs admitted to the Warsaw Veterinary College between 1998 and 2000 that the odds ratio for Rottweilers to be diagnosed with multicentric lymphoma was sixfold greater than dogs from other breeds.[11] However, the study populations may not accurately reflect the general dog population in Poland or elsewhere. This information certainly is a valuable starting point for more thorough and controlled epidemiological studies; however, one must guard against overinterpretation of this type of data.

Similarly, a study comparing the lifetime occurrence of neoplasia in German Shepherd dogs and Belgian Malinois military working dogs (United States Department of Defense) during the year 1992 reported that more Belgian Malinois were diagnosed with lymphoma than were German Shepherd dogs and that more German Shepherd dogs were diagnosed with hemangiosarcoma.[21] The authors remind the readers that the study's limitations include that these dogs spend a proportion of their lives outside of the USA in various regions around the world, and that these dogs have a standard high-nutritional plane and level of fitness as well as handlers that are extremely vigilant over their welfare. Thus, these groups are being evaluated, as they relate to each other and the study's findings should not necessarily be generalized to the Belgian Malinois and German Shepherd dog breeds as a whole.

Investigators in Austria[9] classified subtypes of canine lymphoma and then compared their results with findings in other countries in Western Europe and found no difference in morphology and immunophenotype of lymphomas. They concluded from this information that Austrian dogs are unlikely to have significantly different risk factors than other canine populations in Western Europe.

Leukemia

Leukemia appears to be less common than lymphoma in the canine population and, although leukemia may be underdiagnosed, there is no new information on the specific epidemiology of this neoplasm since the study by Priester and McKay (see Chapters 63 and 65).[22]

Histiocytic Sarcomas

Cutaneous histiocytomas are associated with young dogs (<6 years of age) and are typically benign in nature. Breeds historically associated with a higher incidence of cutaneous histiocytoma include Boxer dogs and Bulldogs. Scottish Terriers, Doberman Pinschers, and Cocker Spaniels also have been suggested as breeds with higher risk to develop this neoplasm.[5]

The malignant counterparts of cutaneous histiocytic sarcoma are collectively known as the histiocytic sarcoma complex, which comprises a spectrum of malignancy rather than a static neoplastic process (see Chapter 71). The histiocytic sarcoma complex includes both localized and disseminated forms. Breeds at highest risk are reported to be Bernese Mountain Dogs, Golden and Flat-coated Retrievers, and Rottweilers.[5] It should be noted that histiocytic sarcomas are not related to malignant fibrous histiocytomas, which are classified with the tumor group of soft-tissue sarcoma.

Plasma Cell Tumors

An increased frequency of lingual plasma cell tumors was found in Cocker Spaniels.[3] This study evaluated 1196 cases of lingual lesions that were submitted to the Colorado State University Veterinary Diagnostic Laboratory (VDL) during the years 1995–2004. Previously, Cocker Spaniels were suspected of an increased risk to develop mucocutaneous plasmacytomas in a study of 75 cases published in 1989.[23]

Mast Cell Tumors

Mast cell tumors can occur in any breed of dog and mixed-breed dogs; however, there have been associations made with specific dog breeds that seem to have increased susceptibility (see Chapter 66). A study from Sydney, Australia considering data from 1989 to 2001 at the University Veterinary Center found that Boxers, Australian Cattle Dogs, and Staffordshire Bull Terriers were at a significantly higher risk for mast cell tumors.[1] Pugs, Boston Terriers, and other brachycephalic breeds also have been present in statistically significant numbers in other studies.

Hemangiosarcomas

Hemangiosarcoma has recently been suggested to originate from progenitor cells in the bone marrow rather than from endothelial lining cells of the organs in which the solid tumors have originated (see Chapter 72).[15] Recent evidence suggests that these tumors may merit

reclassification from the soft-tissue sarcoma group into the hematopoietic neoplasms. Interestingly, Priester and McKay also categorized lymphoma, leukemia, and hemangiosarcoma together in their publication in the late 1970s.[22]

Hemangiosarcoma is more common in dogs than any other species. Data from six consecutive years were used to evaluate the prevalence of canine hemangiosarcoma. Two-hundred-and-twenty-two canine samples of nonvisceral hemangiosarcoma (mostly cutaneous) received by the VDL at Colorado State University in 1996, 1997, and 2002 revealed equal numbers of male and female dogs affected. The most common breeds, when corrected for popularity bias, included Pit Bull Terriers (most common), and sighthounds (Whippets, Greyhounds, and Italian Greyhounds, specifically). The largest number of submissions came from Golden Retrievers and German Shepherd dogs. Lingual hemangiosarcoma risk was shown to be increased in Border Collies, while Beagles were shown to have an increased risk for concurrent lingual and cutaneous hemangiosarcoma.[3] Three-hundred-and-eight tissue samples of canine visceral hemangiosarcoma evaluated exclusively from year 6 of the VDL study showed an approximately equal sex distribution. However, the most common breeds affected were Golden Retriever (56 cases), Labrador Retrievers (45 cases), and then German Shepherd dogs (30 cases).[25]

Srebernik and Appleby[31] reported a retrospective study of 4535 cases of canine vascular tumors evaluated from 1968 to 1989 at the Royal Veterinary College (UK). This study showed a significantly higher risk among German Shepherd dogs for hemangiomas and hemangiosarcomas with roughly 66% of those cases being hemangiosarcomas. They also found that the spleen was the most frequent site in German Shepherd dogs with males being overrepresented. German Shepherd dogs had a lower frequency of cutaneous and subcutaneous hemangioma and hemangiosarcoma.

HEMATOPOIETIC NEOPLASIA RISK ASSESSMENT IN CATS

Lymphoma/Leukemia (Lymphoid Neoplasms)

Environmental

A recent questionnaire-based study suggested that cats with exposure of 5 years or longer to secondhand household tobacco smoke were 3.2 times more likely to develop lymphoma than cats from nonsmoking households.[2] Although some recall bias is inevitable in this type of study, it is more limited here, as the study focused on only one parameter of which the owners would have a firsthand knowledge. Nevertheless, this study needs to be further validated.

Infectious Diseases

Feline retroviruses as risk factors have had a significant impact on the development of lymphoid neoplasms in the cat. The so-called "feline leukemia virus infection era" comprises the late 1970s through 1983.[17] This period of time showed a high incidence of lymphoma, in particular, associated with FeLV infection. A retrospective study from 1968–1988 of 53 cats from the Pacific Northwest region with matched controls showed that cats infected with the FeLV virus had a 62.1 times greater risk of developing leukemia or lymphoma than cats that were uninfected.[26] Cats infected with both FeLV and FIV had an increased risk of 77.3 times greater than the control population for these neoplasms, and cats infected with FIV alone had a 5.6 times greater risk than uninfected cats.[26] After 1983, the impact of vaccination against FeLV and culling of infected animals determined by FeLV testing resulted in a significant reduction in FeLV infection. Cases of non-FeLV-associated lymphoma (i.e., thymic and alimentary lymphomas) began to rise in the feline population.[26]

Genetic/Heritable

Siamese cats and other related Oriental breeds have demonstrated an increased risk for development of mediastinal lymphoma at younger than 4 years of age in multiple studies.[17,26] Some cats were infected with FeLV.[26] Nevertheless, this information indicates a potential shift in breed risk in feline lymphoma compared to the reference population evaluated 40 years ago.[22] In that population, Siamese cats had a lower-than-average risk of developing lymphoma.

Mast Cell Tumors

Siamese cats are predisposed to mast cell tumors compared with other breeds.[19] In an assessment of 41 cats with mast cell tumors published in 2006, young Siamese cats were shown to be more likely to present with a military histiocytic form of cutaneous mast cell tumors.[16] It appears that visceral mast cell tumors (majority in the spleen) are more common in cats. Currently, there is no known association of infectious disease with mast cell tumor development.

Hemangiosarcoma

Hemangiosarcoma continues to be a rare tumor in cats and no specific risk factors have yet been identified in this species. Evaluation of 72 histopathological samples of hemangiosarcomas in cats submitted to the VDL at Colorado State University over a 6-year period showed that the majority of these tumors were cutaneous (59 out of 72), there was no noted sex predilection, and the average cat age was 10 years.[25]

HEMATOPOIETIC NEOPLASIA RISK ASSESSMENT IN HORSES

The only current information on epidemiology of lymphoma suggests that T-cell multicentric lymphomas are

more common than B-cell multicentric lymphomas in horses.[18] Hemangiosarcoma in horses is rare and no specific risk factors have yet been identified. A total of 11 samples of equine hemangiosarcoma were identified in the previously mentioned study by Schultheiss through the VDL at Colorado State University in a 6-year period.[25]

HEMATOPOIETIC NEOPLASIA RISK ASSESSMENT IN CATTLE

Lymphoma

The only hematopoietic neoplasia of clinical importance in cows, based on the literature to date, is lymphoma. One risk factor identified in cows for lymphoma is infection with BLV. It is estimated that 2–5% of BLV cows develop lymphoma, representing about 5200 cows/year in the state of California alone.[14]

CONCLUSIONS

The paucity of specific data based on an updated reference population regarding hematopoietic neoplasia in domestic animals underscores the need to define specific risk factors for these diseases. By linking large databases with updated communication technology, and by addressing factors affecting populations of domestic animals that have relevance to development of hematopoietic cancer, more accurate perceptions of risk can be ascertained. Changes in breeding practices and husbandry based on better-defined risk factors can then be implemented to help reduce the incidence of hematopoietic neoplasia for those animals that are significantly threatened. In the big picture, by deepening our understanding of susceptibility to cancer, we also come to understand its nature more profoundly, which will lead us to earlier diagnosis and, ultimately, more effective therapy.

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CHAPTER 59

Genetics of Hematopoietic Neoplasia

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Genes and Cancer Risk
  Heritable Cancer Syndromes
  Genetic Influence in Sporadic Cancers
The Hallmarks of Cancer
  Sustaining Proliferative Signaling
  Evading Growth Suppressors
  Resisting Cell Death
  Enabling Reproductive Immortality
  Inducing Angiogenesis
  Invasion and Metastasis
Molecular Events in Carcinogenesis
  Initiation, Promotion, Progression
  Genetic Instability
  Epigenetic Events
Cancer Stem Cells
Adaptive Evolution in the Tumor Microenvironment

Acronyms and Abbreviations

DNA, deoxyribonucleic acid; HIF, hypoxia-inducible factor; IL, interleukin; LOH, loss of heterozygosity; LTR, long terminal repeat; mRNA, messenger RNA; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; RCND, renal carcinoma and nodular dermatofibrosis; VEGF, vascular endothelial growth factor-A; VEGFR, vascular endothelial growth factor receptor; VHL, von Hippel–Lindau (tumor suppressor gene).

It is now clear that cancer is a disease driven by the accumulation of genetic abnormalities.[41] Hundreds of genetic abnormalities that are peculiarly associated with specific cancers have been identified. In fact, technological advances, such as high-throughput methods, have allowed full sequencing of tumor genomes.[28] During the past decade, a vast amount of sequencing data has been generated. Consequently, the process of statistically integrating diverse sources of information arising from the genome, such as genetic, epigenetic, and transcriptomic data, is becoming increasingly prevalent. The information from these experiments has reinforced current concepts and provided insight into new areas of research. This chapter will focus on how interactions between genes and environment impact the origin, progression, and response to therapy of hematopoietic tumors.

GENES AND CANCER RISK

To understand cancer, one must first realize this is neither a single nor a simple disease. Rather, the term cancer describes a large number of diseases whose only common feature is uncontrolled cell growth and proliferation. An important and universally accepted concept is that cancer is a genetic disease, although it is not always heritable. Tumors arise from the accumulation of mutations that eliminate normal constraints of proliferation and genetic integrity in a somatic cell. Among other causes, mutations can arise due to the inherent error rate of enzymes that control DNA replication, which introduces from one in ten million to one in a million mutations for each base that is replicated during each round of cell division. Most mammalian genomes comprise two to three billion base pairs, so every time a cell divides, each daughter cell is likely to carry at least a few hundred mutations in its DNA. Most of these mutations are silent; i.e., they do not hinder the cell's ability to function. However, others can disable tumor suppressor genes or activate protooncogenes that respectively inhibit or promote cell division and survival.

Heritable Cancer Syndromes

The existence of genetic predisposition to cancer is illustrated by well-defined heritable cancer syndromes.[11] Even though they account for fewer than 5% of all

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human cancers, studies of families with these syndromes provided many of the initial clues to understand the genetic basis of sporadic (nonheritable) cancers. Although inheritance is recessive, these familial cancer syndromes show dominant patterns of inheritance and have high penetrance. All but two of the known familial cancer syndromes are due to mutations that inactivate tumor suppressor genes. As originally proposed by Knudson in his "two-hit" hypothesis from studies in children with retinoblastoma,[24] individuals at risk are obligate heterozygotes (they inherit a mutant allele and a wild-type allele). As it happens, homozygous mutations in critical growth-regulatory genes usually cause embryonic lethality; but in the case where a single allele is affected, the mutation is present in every cell in the body. Given the rate of spontaneous mutation described in the earlier text, the probability that the second, wild-type allele will be inactivated in at least one cell is extremely high, therefore facilitating tumorigenesis. This process is called loss of heterozygosity (LOH).

A mutation in a single gene can predispose individuals to distinct cancer syndromes, while independent, single mutations of different genes can result in virtually the same disease.[11] This is not surprising when we consider that commonly affected genes are multifunctional and are parts of complex interactive networks or circuits, so a mutation may only alter gene function along one biochemical pathway, leaving its interactions with other pathways intact. Moreover, mutations that contribute to most sporadic cancers are restricted to a small subset of genes, many of which also are associated with heritable cancer syndromes.

At least one heritable cancer syndrome (renal carcinoma and nodular dermatofibrosis [RCND]) has been described in German Shepherd dogs.[29] The heritable factor (or *RCND* gene) for this syndrome maps to dog chromosome 5 (CFA 5), and specifically to the folliculin gene, which was recently described as corresponding to the human disease (Birt–Hogg–Dubé syndrome).[37]

Genetic Influence in Sporadic Cancers

Both the environment and the individual's peculiar genetic background influence cancer risk and the natural history of tumors. This is especially clear in mice, where the relative rate of spontaneous cancers and the susceptibility to chemically induced cancers differ according to the genetic background of various inbred strains. Similar evidence exists for humans; for example, the risk of habitual smokers to develop lung cancer is tightly linked to a unique allele encoding the alpha-3 subunit of the high-affinity nicotinic receptor.[1] There also is evidence to suggest that in some cases mutations are "directed" due to the presence of a "mutator phenotype," where the factors that control DNA replication and repair are prone to more errors than would be expected by simple random events. This leads to different rates of cancer predisposition, which would be higher than the mean in individuals bearing this mutator phenotype, and might explain why not all people or animals exposed to similar environmental carcinogens develop the same forms of cancer at the same rate.[30]

In dogs and other domestic animals, the coexistence of genetic isolates in closed populations we call breeds, along with animals of mixed breeding, lends itself to study how a relatively homogeneous background influences cancer in outbred populations. Published data from whole genome association studies suggest there are distinct heritable traits that segregate with common cancer phenotypes in dogs.[22,40,53] Some of these traits are shared between closely related breeds, whereas others seem to contribute to risk independently in different breeds. Perhaps more importantly, dogs are the first species where genetic background has been shown to mold tumor genomes and tumor gene expression profiles.[33,50,52] Together with the demonstration that causal, pathognomonic genetic abnormalities are conserved in homologous human and canine cancers, this opens up a new area to identify the precise contribution of heritable traits to sporadic cancers using comparative systems approaches.

THE HALLMARKS OF CANCER

Six essential, acquired characteristics are necessary for cellular transformation (Figure 59.1).[17] These characteristics are: (1) the ability to sustain proliferative signaling; (2) the evasion of growth suppression; (3) the ability to resist cell death; (4) the capacity to enable reproductive immortality; (5) the ability to induce angiogenesis; and (6) the capacity to invade tissues and metastasize. Some of the important concepts that are clarified by this list include: no single gene is universally responsible for transformation; five or six mutations are the minimum probable number required to endow the cancer phenotype; each step is regulated by multiple interactive biochemical pathways; thus, mutations of different genes along a pathway can result in equivalent phenotypes, and, conversely, mutations of the same gene can result in different cancers with distinct biology; tumors behave as tissues, and the interactions between the tumor and its microenvironment are major drivers of cancer behavior.

Sustaining Proliferative Signaling

The production and release of growth-promoting signals that lead to entrance into and progression through the cell-growth-and-division cycle are accurately controlled by normal tissues, thereby ensuring a homeostasis of number and thus maintenance of normal tissue architecture and function. Cells communicate with each other and integrate environmental signals by sensing cues and gradients. For example, migration, metabolism, and proliferation of mature hematopoietic cells are regulated in autocrine and paracrine fashions by locally secreted cytokines. The same cytokines may travel systemically and act in an endocrine fashion. Generally,

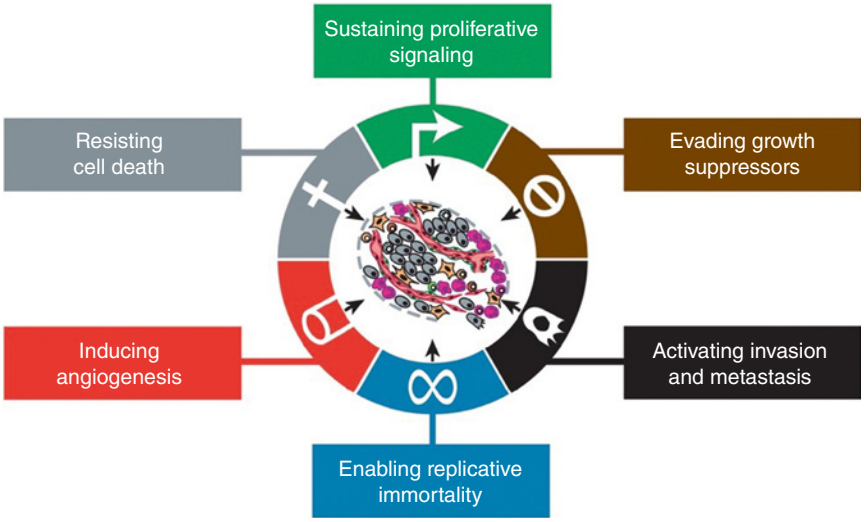


FIGURE 59.1 Acquired capabilities of cancer. Most, if not all, cancers acquire the same set of functional capabilities during their development, albeit through multiple mechanistic strategies. (**Source:** Reproduced from Hanahan and Weinberg[17] with permission. ©Elsevier.)

the cytokines work by binding transmembrane receptors, which in turn initiate signaling cascades that culminate in transcriptional changes. The activities of these cytokines, their receptors, and the corresponding signaling molecules are finely tuned. The system can be shut down when the concentration of the cytokine falls below a threshold that can stably bind the receptor, when the receptor ceases to be expressed, or when signaling molecules are downregulated or otherwise inactivated. High-throughput DNA sequencing analyses of cancer cell genomes have revealed somatic mutations in human and companion animal tumors that predict constitutive activation of signaling circuits, usually triggered by activated growth factor receptors. This capability to sustain chronic proliferation is the most fundamental trait of cancer cells. Among many examples, there is a translocation between chromosome 2 and chromosome 5 (t(2;5)) that is present in almost half of human anaplastic lymphomas. The translocation creates a fusion protein between the nucleophosmin gene (*NPM1*) and the anaplastic lymphoma kinase gene (*ALK*), which aberrantly activates the JAK2/STAT5 signaling pathway[47] that is normally responsive to various interleukins (IL) including IL-2, IL-3, and IL-6. The genes that encode the normal growth-promoting proteins (such as *ALK*, JAK2, and STAT5) are called *protooncogenes*; the mutated versions that allow cells to gain self-sufficiency from the environmental signals are called oncogenes. It is important to note that not all growth-promoting genes have the capacity to become oncogenes and that the outcome of oncogenic activation is most commonly apoptosis, unless there are additional events that promote stable transformation and survival.

Evading Growth Suppressors

To maintain homeostasis, cells also must integrate antigrowth signals from the environment. As noted in the previous section, quiescence in nonhematopoietic cells is enforced by signals delivered by contact inhibition. Hematopoietic cells, on the other hand, utilize cell–cell contacts to maintain interactions within the niche and to regulate the timing and intensity of hematopoiesis, inflammation, and immunity.[35] Stop signals are usually delivered and integrated by the products of tumor suppressor genes, which derive their name largely from the observation that their inactivation facilitates tumor formation. Tumor suppressor genes balance the activity of growth-promoting protooncogenes and tend to act in tandem with these in most biochemical pathways. Loss of function of one or more tumor suppressor genes occurs in virtually every cancer, with inactivation of *p53*, *RB-1*, *PTEN*, or *CDKN2A* each seen in >50% of all tumors. Each of these pathways may contribute to the pathogenesis of bone-marrow-derived tumors in companion animals, and their dysfunction also may be predictive for outcomes.[9,26,27,34]

Resisting Cell Death

Apoptosis, or programed cell death, is the imprinted outcome for every cell in multicellular organisms. Survival requires support from extrinsic (environmental) factors, as well as precise balance of cellular energetics and metabolism. Bone-marrow-derived cells normally undergo apoptosis when concentrations of survival factors (stem cell factor, IL-3, IL-7, etc.) or nutrients are limiting, or when there are severe disruptions to cellular bioenergetics. Evasion of apoptosis is an essential acquired feature of all cancers, and it can result from loss of pro-apoptotic tumor suppressor genes such as *p53* or *PTEN*, or by gain of function of anti-apoptotic genes such as *BCL2*.

Enabling Reproductive Immortality

Immortalization is another essential feature of cancer. The genetic program limits the number of times a cell is

able to replicate, the so-called Hayflick limit, that when reached induces replicative senescence. Induction of replicative senescence does not induce death; cells maintain energetic homeostasis and remain functional, but they undergo significant genetic changes characterized by telomere erosion. Cells that are able to replicate must maintain the integrity of telomeres, "caps" made of repetitive DNA sequence that protect chromosomes from destruction. Solid tumors acquire immortalization predominantly by activation of the telomerase enzyme system and the consequent maintenance of telomere integrity. In hematopoietic cells, telomerase activity seems to be retained longer than in other somatic cells, probably facilitating immortalization in lymphoma and leukemia.[39] Indeed, critically shortened telomeres, telomerase enzyme activation, and altered expression of telomere-associated proteins are common features in hematopoietic malignancies and are related with progression or chemotherapy drug resistance in these diseases.[55]

Inducing Angiogenesis

The process of angiogenesis requires the coordinated action of a variety of growth factors and cell-adhesion molecules in endothelial and stromal cells. So far, vascular endothelial growth factor-A (VEGF) and its receptors comprise the best-characterized signaling pathway in tumor angiogenesis.[12] VEGF binds two receptor tyrosine kinases, VEGFR-1 (Flt-1) and VEGFR-2 (KDR, Flk-1). VEGFR-2 is the major mediator of the angiogenic effects of VEGF. However, VEGFR-1 is expressed by some tumor cells and may mediate chemotactic signals, thus potentially having a role in cancer growth. The expression of VEGF is upregulated by hypoxia and inflammation. The transcription factor hypoxia-inducible factor-1 (HIF-1), which is part of a pathway that also includes regulation by the von Hippel–Lindau (VHL) tumor suppressor gene, is a major regulator of VEGF expression. Under conditions or normal oxygen tension, VHL protein targets HIF for degradation; under low oxygen conditions, HIF increases and VHL-mediated degradation is reduced, permitting upregulation of VEGF. Other signaling molecules that contribute to angiogenesis include platelet-derived growth factor-β (PDGF-β) and its receptor (PDGFR), and the angiopoietins. PDGF-β is required for recruitment of pericytes and maturation of new capillaries. Recent studies also document the importance of tumor-derived PDGF in recruitment of stroma that produces VEGF and other angiogenic factors (Figure 59.2).

Angiogenesis appears to be important in hematopoietic tumors. Patients with chronic lymphocytic leukemia responded to treatment with anti-angiogenic compounds,[36] followed by similar success for some patients with multiple myeloma.[11] Furthermore, different histologic types of human non-Hodgkin lymphoma show different patterns of angiogenesis, and these can predict outcomes.[46] Plasma VEGF levels have proved to be significantly higher in B- and T-cell lymphoma compared with healthy controls.[2] Notably, high-grade T-cell lymphomas, that usually harbor a poor prognosis, and high-stages B-cell lymphomas revealed higher VEGF mRNA and protein expression levels compared to low-grade T-cell lymphomas and low-stages B-cell lymphomas, respectively.[4] One study has shown that microvessel density is similarly correlated with aggressive behavior of canine lymphoma,[44] and similar findings have been reported for other blood-derived and solid tumors of dogs such as mast cell cancer and mammary cancer.[43,45]

Invasion and Metastasis

The role of genetic events in invasion and metastasis is still incompletely understood. The classical model of metastasis proposed by Fidler[14] suggests a stepwise acquisition of assets that enables cells to leave the primary tumor site, travel through blood or lymph, invade stroma in favorable locations, and thus become re-established at distant sites. More recent work suggests that most tumors possess the ability to dislodge cells that travel to distant sites, and the ability of such cells to survive in capillary beds may be the most important step in the metastatic process.[23]

Bone-marrow-derived cells have intrinsic properties that allow them to travel throughout the body, traffic through all major organs, and home to areas of inflammation. Thus, bone-marrow-derived tumors are inherently metastatic. Nevertheless, hematopoietic tumors that are cytologically indistinguishable can have distinct and preferential tissue distribution. We do not fully understand what events make leukemic cells stay in the circulation, while cells from corresponding lymphomas (or myeloid sarcomas) with virtually identical molecular signatures stay confined to lymphoid or visceral organs.

MOLECULAR EVENTS IN CARCINOGENESIS

Various models have been proposed to explain how cells acquire the features that lead to neoplastic transformation and eventually to clinical cancer. One model describes the process as stepwise accumulation of mutations that reduce constraints on growth and eventually promote transformation. Although this model is overly simplistic and technically flawed, it is nevertheless useful to convey the events that lead to carcinogenesis.

Initiation, Promotion, Progression

Unlike diseases due to single-gene defects, cancer is a complex, multigenic disease. The initiation, promotion, and progression model was among the first to propose a sequential progression of mutations that could account for cancer.[54] In this model, a genetic event would endow a somatic cell with limitless replicative potential or another growth or survival advantage from other cells in its environment (initiation). Alone, this would not be sufficient to give rise to a tumor, as the cell would remain

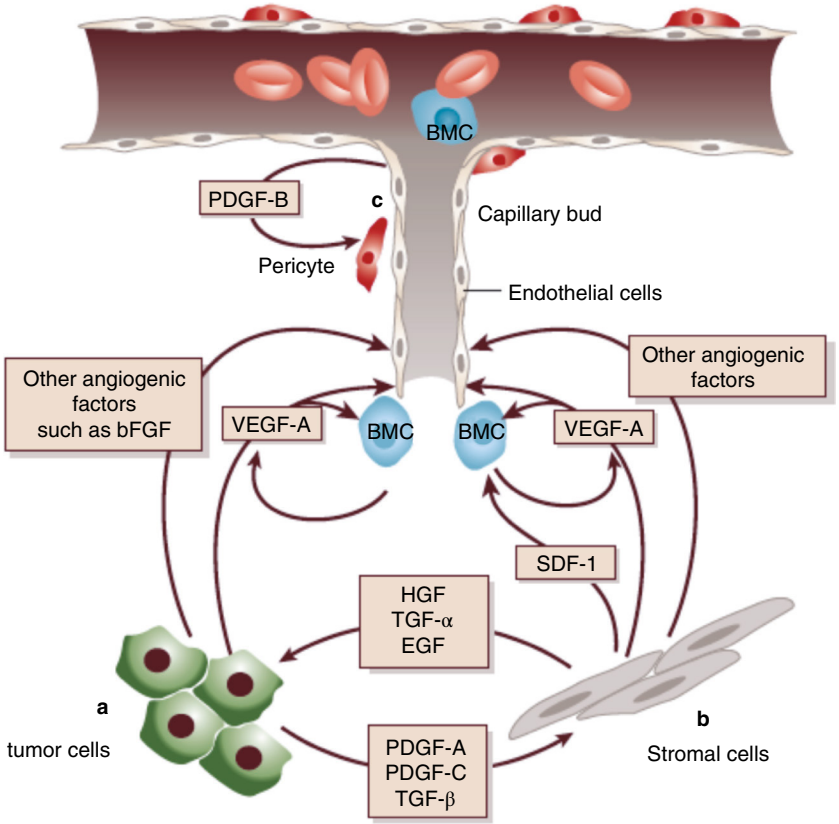


FIGURE 59.2 Molecular and cellular components of the tumor microvascular environment. (a) Tumor cells produce VEGF and other angiogenic factors such as basic fibroblast growth factor (bFGF), angiopoietins, and interleukin-8. These stimulate resident endothelial cells to proliferate and migrate; (b) An additional source of angiogenic factors is the stroma. This is a heterogeneous compartment, comprising fibroblastic, inflammatory, and immune cells. Recent studies indicate that VEGF, as well as chemokines produced by tumor-associated fibroblasts, recruit bone-marrow-derived angiogenic cells (BMC). A well-established function of tumor-associated fibroblasts is the production of growth/survival factor for tumor cells such as epidermal growth factor (EGF), transforming growth factor-α (TGF-α), hepatocyte growth factor (HGF), and heregulin; (c) Endothelial cells produce PDGF-β, which promotes recruitment of pericytes in the microvasculature after activation of PDGFR. Tumor cell death will lead to dissolution of blood vessels and release of endothelial cells into the circulation. (**Source:** Reproduced from Ferrara and Kerbel[12] with permission. ©Macmillan.)

constrained by environmental factors. A second event would further add to the cell's ability to outcompete its neighbors in this environment, leading to its potential expansion into a recognizable tumor mass (promotion). Finally, a third event would reinforce the cell's malignant potential (invasion, tissue destruction, and metastasis), leading to clinical disease (progression). It is important to note that an "event" is not equivalent to a single mutation, but rather is more likely to represent a series of mutations that act in concert to alter the cell's functional and morphological phenotype.

In recent years, mimicking human studies, significant efforts have been directed at identifying key mutations in canine osteosarcoma,[15] melanoma,[19] ameloblastoma,[41] hemangiosarcoma,[32] and lymphoma,[7,10] also assessing the evolution of the mutational pattern from initiation to progression and metastasis.[15,16,57] Two studies investigated somatic mutations by exome sequencing in canine lymphoma. The first study[7] identified TRAF3 gene as being commonly mutated in both dog and human B-cell lymphomas. The second study[10] detected the presence of recurrent mutations on TRAF3–MAP3K14, FBXW7, and POT1 gene loci in Cocker Spaniels and Golden Retrievers, highlighting the crucial role of breed predisposition and genetic risk factors.

Genetic Instability

The stepwise model of clonal evolution is satisfying because it can be correlated with discrete pathological changes in tumor progression, especially for epithelial tumors where such progression can be seen in lesions that go through stages of hyperplasia, atypical hyperplasia (dysplasia), adenoma, carcinoma in situ, invasive carcinoma, and metastatic carcinoma. However, analysis of tumor genomes even in early stages usually shows aneuploidy (abnormal DNA copy number) as well as chaotic changes indicative of multiple numerical and structural DNA abnormalities. Similar abnormalities first noticed by Boveri more than 100 years ago in studies of sea urchin cells, which are described in a recently reprinted, annotated version of the original 1914 article translated into English,[6] led him to formulate the aneuploidy theory of cancer. We know now that

aneuploidy is especially evident in solid tumors; based on this, Loeb proposed the existence of a "mutator phenotype" where cells are predisposed to undergo multiple mutations, some of which inevitably lead to cancer.[30] Some tenets of his hypothesis appear to be correct; however, direct measurements of mutation rates of sporadic tumors are much lower than those predicted if a "mutator phenotype" was operative in these tumors.

Still, genetic instability is a hallmark of most tumors, and while it can be partly explained by increased errors in DNA replication and chromosomal segregation in cells that are rapidly dividing, other mechanisms are clearly operative, involving telomeres and telomerase, as well as mechanisms of DNA replication and repair and chromosomal segregation. Although many of these changes are not recurrent and appear to be random products of instability, some may in fact contribute to proliferative crisis.[31] It is possible that the initiation events for many tumors occur early in life during highly proliferative stages of tissue growth and remodeling, but they become evident late in life when a last series of mutations allows the transformed cell to reach this crisis stage. As we inferred in the earlier text, human hematopoietic tumors seem to avoid the chaotic chromosomal instability associated with solid tumors: the same trend has also been observed in canine non-Hodgkin lymphomas.[3,51] We do not fully understand the reasons for this, although it may be partly due to intrinsic protective mechanisms associated with the proliferative rate of bone marrow precursor cells.

Epigenetic Events

Another observation is that events leading to cancer need not necessarily be caused by mutational events, and advances in epigenetic analysis of tumor genomes using high-throughput DNA sequencing have provided the compelling demonstration that cancer cells harbor global epigenetic abnormalities, in addition to numerous genetic alterations. Epigenetic events are those that can alter phenotype without changing the genotype. Two well-characterized epigenetic mechanisms regulate gene expression. Gene silencing can occur by methylation of CpG residues in promoter regions, as well as by histone deacetylation. Both of these events interfere with the transcriptional machinery and repress gene expression. Silencing specific genes by methylation is implicated in numerous cancers of humans and animals.[42] One important observation is that most (or all) genes that are subject to silencing by methylation in specific cancers (e.g., *CDKN2A* in T-cell leukemia) are commonly inactivated by mutation or deletion in other cancers (e.g., *CDKN2A* in melanoma). DNA methylation profiling studies in canine diffuse large B-cell lymphomas have revealed tumorigenic and stem-cell-like signatures, highlighting the presence of putative subgroups with prognostic relevance.[3,13]

Genomic imprinting presents a unique example where heritable epigenetic changes influence cancer predisposition. Genomic imprinting refers to a pattern of gene expression that is determined by the parental origin of the gene; in other words, unlike most genes where both parental alleles are expressed, only one allele of an imprinted gene is expressed and the other one is permanently repressed. Epigenetic changes in Wilms' tumor and in heritable colon cancer (among others) alter the expression of the imprinted allele, leading to loss of imprinting that causes overexpression of the insulin growth factor-2 (*IGF2*) gene.[42]

CANCER STEM CELLS

Unlike diseases that arise from single-gene defects, cancer is a complex, multigenic disease. In fact, a sequential progression of mutations is required to produce a tumor cell. We can think of many genes contributing to, rather than causing, the origin and progression of cancer. This model is consistent with many aspects of the natural behavior of tumors, and, to some extent, it can predict risk and outcome.

A competing theory now exists whose main tenet is that self-renewal is limited to a small population of cancer stem cells.[5,8,20] The existence of cancer stem cells is documented; they are characterized both by peculiar phenotypes and by defined sets of mutations of a small number of genes.[20] Other mutations then endow their progeny with limited or extensive capacity to undergo programed differentiation, hence resulting in distinct clinical phenotypes such as acute and chronic leukemias or high-grade and low-grade solid tumors. The strongest evidence for cancer stem cells comes from hematopoietic tumors, but cancer stem cells also have been identified in various solid tumors.[48] The origin of cancer stem cells is among the most important contemporary topics of cancer investigation. Cancer stem cells may arise from mutations that occur in bona fide stem cells, they may arise by de-differentiation of somatic cells that acquire mutations endowing them with stem-cell-like properties, or they may develop by fusion of a transformed cell and a bone-marrow-derived stem cell. In companion animals, putative cancer stem cells have been identified in hemangiosarcoma,[25] osteosarcoma,[56] brain tumors,[49] and lymphoma.[18]

ADAPTIVE EVOLUTION IN THE TUMOR MICROENVIRONMENT

The clonal evolution theory[38] addresses the significance of sequential genetic changes providing growth and survival advantages, but to this we must add the fact that, in addition to these self-sufficient events that influence growth and survival, tumor cells must also evade predators, such as inflammation and the immune system. Tumors have the capacity to modulate stromal cells to support their own growth by providing a suitable matrix and an abundance of nutrients, while maintaining antitumor responses at bay. In addition, the immune inflammatory cells can also be recruited by the tumor and act as tumor-promoting agents. Tumors that

outgrow the capability of their immediate surroundings to support their growth will seek to become established in other favorable locations. Thus, adaptive evolution likely contributes to the anatomic distribution of hematopoietic tumors much as it does to the metastatic spread of solid tumors.

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CHAPTER 60

Transforming Retroviruses

MARY JO BURKHARD

Retroviridae
  General Retroviral Structure
Retroviral Life Cycle
Classification of the Family Retroviridae
  Alpharetroviruses
  Betaretroviruses
  Gammaretroviruses
  Deltaretroviruses
  Epsilonretroviruses
  Lentiviruses
  Spumaviruses (Foamy Viruses or Syncytial Viruses)
Overview of Retrovirus-Induced Disease
  Immunodeficiency
  Leukemogenesis
  Direct Cytopathicity
  Host Determinants of Pathogenicity
  Stimulation of Host Cell Proliferation
  Oncogenic Transformation
Retrovirus-Induced Oncogenes
  Role in Cancer
    Nonacute
    Acute
  Mechanisms of Action
    Transduction
    Cis-Activation
    Trans-Activation
Prototypical and Well-Characterized Oncoretroviruses
  Avian Leukosis and Sarcoma Viruses
  Bovine Leukemia Virus
  Feline Leukemia Virus
  Gibbon Ape Leukemia Virus
  Human T-Cell Lymphotropic Viruses
  Jaagsiekte Sheep Retrovirus and Enzootic Nasal Tumor Virus
  Mouse Mammary Tumor Virus
Murine Leukemia Viruses
  Reticuloendotheliosis Virus
  Simian T-Cell Lymphotropic Virus
  Walleye Dermal Sarcoma Virus

Acronyms and Abbreviations

ALSV, avian leukosis and sarcoma viruses; ALV, avian leukosis virus; ASV, avian sarcoma virus; ATL, adult T-cell leukemia; BLV, bovine leukemia virus; CAEV, caprine arthritis encephalitis virus; CIS, common insertion or integration site; DNA, deoxyribonucleic acid; EIAV, equine infectious anemia virus; ENTV, enzootic nasal tumor virus; FeLV, feline leukemia virus; FIV, feline immunodeficiency virus; GALV, gibbon ape leukemia virus; HAM/TSP, HTLV-associated myelopathy/tropical spastic paraparesis; HIV, human immunodeficiency virus; HTLV, human T-cell lymphotropic virus; IL, interleukin; JSRV, jaagsiekte sheep retrovirus; LRT, long terminal repeats; MHC, major histocompatibility complex; MMTV, mouse mammary tumor virus; MPMV, Mason-Pfizer monkey virus; MuLV, murine leukemia virus; RSV, Rous sarcoma virus; Sag, superantigen; SIV, simian immunodeficiency virus; STLV, simian T-cell lymphotropic virus; WDSV, Walleye dermal sarcoma virus.

Oncogenic retroviruses were first identified near the beginning of the 20th century when Rous and colleagues discovered that certain cancers could be transmitted between animals by the inoculation of cell-free extracts. Studies of these agents have resulted in many critical breakthroughs in the field of oncogenesis.

RETROVIRIDAE

Retroviruses are enveloped RNA viruses that replicate via a DNA intermediate that becomes integrated into the host genome. Seven genera of retroviruses are currently recognized.[5] Retroviruses exist both as exogenous

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(nonintegrated) and endogenous forms (integrated provirus). Endogenous retroviruses integrated into the host DNA comprise 5–8% of the human genome, and perhaps more than 10% of the genome in other mammals.

General Retroviral Structure

Retroviruses are single-stranded, linear, positive-sense RNA viruses with 7–13 kb genomes.[2,5] All retroviruses contain *gag*, *env*, *pol*, and *pro* genes, while complex retroviruses also encode a variety of regulatory proteins. Viral RNA is characterized by the presence of R (repeated) and U (unique) regions at both ends of the genome that, once transcribed, result in identical copies being placed at each end of the newly produced DNA in a U3–R–U5 sequence to create long terminal repeats (LTRs). Long terminal repeats are used for integration into the host genome and serve as regulatory sequences for virus production.

Virions are approximately 100 nm in diameter. At the center is a highly condensed form of the genomic RNA in association with the nucleocapsid protein. The virion core also contains viral enzymes such as reverse transcriptase, integrase, and protease. The core is contained within a structural icosahedral capsid and then a matrix shell, both encoded by gag. The virion is enveloped by a lipid bilayer, encoded by env, which also produces distinctive glycoprotein cell-surface spikes that are critical for virus binding and entry.

RETROVIRAL LIFE CYCLE

The retroviral life cycle is complex and includes variations between different retroviruses; however, the basic template is similar.[2] Virus binds via a specific cell-surface receptor. Retroviral receptors are diverse and include ion transporters, cell differentiation markers, chemokine receptors, the transferrin receptor, and Fas-like receptors. Internalization and uncoating occur through fusion of the viral and plasma membrane or via endocytosis.

Reverse transcription of the RNA genome into double-stranded DNA uniquely defines the retroviridae. Reverse transcription is a complex, multistep process initiated by the creation of a negative-sense DNA strand. Following degradation of the viral RNA by RNase H, the negative strand serves as a template to create a double-stranded linear DNA. The double-stranded linear DNA is then transported to the cell nucleus and integrated into the host DNA as a provirus.

Viral RNA and protein are expressed from the integrated provirus using the normal cellular processes of transcription and translation. Transcription by the RNA polymerase II system results in a complete unspliced viral genomic RNA that can serve as the genetic material to be packaged into progeny virions or as the template for production of viral proteins. The latter may require RNA splicing and processing of precursor proteins.

RNA packaging into developing virions is driven through Gag interactions. Gag also controls the assembly of the viral protein products whether it occurs at the plasma membrane or in the cytoplasm. Virions assembled in the cytoplasm are then transported to the membrane for envelopment and release. During the process of release, host membrane proteins are frequently incorporated into the virion envelope and several have been shown to be functionally significant.

CLASSIFICATION OF THE FAMILY RETROVIRIDAE

The current classification by the International Committee on Taxonomy of Viruses uses physical and morphologic properties of the virion, organization and replication of the genome, and biologic properties such as host range to classify viruses.[5] The family Retroviridae contains two subfamilies. The Orthoretroviruses contain six of the seven genera, while Spumaretroviruses comprise only the spumaviruses.

Alpharetroviruses

Alpharetroviruses are simple retroviruses that encode only *gag*, *pro*, *pol*, and *env*. The *pro* and *gag* genes utilize the same reading frame. These C-type virions are assembled at the plasma membrane and do not appear to produce intermediate-stage cytoplasmic particles. The virion has a centrally located, condensed, spherical core and surface projections of intermediate prominence. Key examples of this genera include avian leukosis virus (ALV), Rous sarcoma virus (RSV), and avian sarcoma virus (ASV).

Betaretroviruses

Betaretroviruses are also simple retroviruses but have a B- or D-type virus morphology; they are assembled within the cytoplasm, transported to the plasma membrane, and become enveloped during budding. B-type virions are characterized by a condensed, eccentric core and have prominent surface envelope projections. The *gag*, *pro*, *pol*, and *env* genes are found in different reading frames. Viruses of significance include mouse mammary tumor virus (MMTV), Mason-Pfizer monkey virus (MPMV), jaagsiekte sheep retrovirus (JSRV), and enzootic nasal tumor virus (ENTV) of sheep.

Gammaretroviruses

Gammaretroviruses are also simple C-type retroviruses; however, the *pol*, *gag*, and *pro* sequences share a reading frame. Gammaretroviruses comprise the largest number of species and infect mammalian, avian, and reptilian hosts. This genus also contains a number of replication-defective endogenous viruses. Key gammaretroviruses include feline leukemia virus (FeLV), the murine leukemia viruses (MuLV), and the avian reticuloendotheliosis virus (REV) group.

Deltaretroviruses

Deltaretroviruses are complex C-type retroviruses that contain *tax* and *rex* regulatory genes in addition to *gag*, *pro*, *pol*, and *env*. Protein expression requires frame shifts over several different reading frames. Bovine leukemia virus (BLV) and human T-cell lymphotropic virus (HTLV) are the best-known examples.

Epsilonretroviruses

Epsilonretroviruses are complex C-type retroviruses that contain one or more additional open reading frames. The prototype virus is walleye dermal sarcoma virus (WDSV). Two other walleye viruses are included in the genus and two additional fish viruses are proposed for inclusion.

Lentiviruses

Lentiviruses are complex retroviruses that are further categorized into five groups based on the host species they infect: primates, cats (domestic and nondomestic), horses, cattle, and sheep/goats. Virions have a distinctive conical morphology. Genomes contain a variable number of accessory genes that regulate viral replication. This genus includes several well-studied viruses including human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), equine infectious anemia virus (EIAV), caprine arthritis encephalitis virus (CAEV), and Maedi-visna virus. Lentiviruses are characterized by the relatively long period between infection and disease development.

Spumaviruses (Foamy Viruses or Syncytial Viruses)

Spumaviruses are complex retroviruses with immature C-like virions that have a less-condensed core and more prominent surface spikes. Virions are assembled in the cytoplasm and then bud through the endoplasmic reticulum as well as the plasma membrane. Newly formed virions uniquely contain large amounts of reverse transcribed DNA. While spumaviruses establish persistent infection in a number of species including humans, nonhuman primates, cattle, horses, and cats, they are not typically associated with clinical disease.

OVERVIEW OF RETROVIRUS-INDUCED DISEASE

Immunodeficiency

Modification of the immune system by viral proteins can enhance carcinogenesis through several mechanisms including disruption of tumor surveillance, atypical stimulation of the immune system, and alteration of the microenvironment (see Chapter 55). The association of lentivirus-induced immunodeficiency and cancer has been well established in several systems such as HIV infection and the development of Kaposi sarcomas and B-cell lymphomas.

Leukemogenesis

One of the most common mechanisms of retroviral-induced leukemogenesis is through insertional activation, which is the activation of proto-oncogenes following proviral integration.

Direct Cytopathicity

Several viruses produce gene products that are directly cytopathic to the host. Examples include the neurologic damage induced by the Env SU subunit of some MuLVs, wasting syndrome induced by many of the avian viruses, and induction of cytopathicity by FeLVs with mutation in the Env SU subunit.

Host Determinants of Pathogenicity

Alterations in host genes can influence the progression of retroviral infection, particularly those that control the target cell availability through cell cycling, tumor suppression genes, and growth factors. Similarly, mutations within the genes responsible for the immune response can alter pathogenicity.

Stimulation of Host Cell Proliferation

While the primary means of inducing host cell proliferation is through insertional activation, some oncogenic retroviruses use other mechanisms. One of the best understood is the superantigen (Sag) protein of MMTV that binds and activates T cells through common (nonantigen-specific) areas on major histocompatibility complex (MHCII) molecules. Alternatively, the spleen focus-forming viruses and the Env helper protein of MuLV activate erythroid proliferations through binding of the erythropoietin receptor.

Oncogenic Transformation

Retroviruses can induce oncogenic transformation through a number of mechanisms covered in detail in the next section.

RETROVIRUS-INDUCED ONCOGENES

Oncogenes are genes whose products contribute to the formation of cancer. Oncogenes arise as mutated forms of normal cellular genes (i.e., proto-oncogenes) coding for key proteins involved in growth control, development, and differentiation.

Role in Cancer

Carcinogenesis is a multistage process. Oncogenes may have direct or indirect roles at several stages in this

process. Direct transformation occurs when a viral gene product interacts with a cellular gene or gene product resulting in loss of internal regulation of normal proliferation, cell cycle, or cell death and neoplastic transformation in that cell. Indirect transformation occurs when the virus alters cells other than the tumor cell resulting in loss of external regulation. The oncogene may transform only a specific cell type or it may have more global effects.

Nonacute

Nonacute transforming retroviruses are replication-competent viruses that induce neoplasia after a latent period. These viruses do not typically induce morphologic transformation in culture. Oncogenesis is often initiated, as proto-oncogenes are activated after viral integration. There are two general mechanisms by which this occurs. In promoter insertion, viral transcription "reads through" to the proto-oncogene each time the virus is replicated resulting in overexpression of the host gene. The second mechanism is enhancer activation in which the proto-oncogene promoter is activated by a viral enhancer.

Acute

Acute transforming retroviruses derive from nonacute retroviruses but have acquired an altered host proto-oncogene. Because they carry oncogenes, these viruses induce rapid transformation and tumor growth. Most acute transforming retroviruses are replication-defective and require replication-competent viruses for propagation. These host-derived oncogenes include growth factors, tyrosine and serine/threonine kinases, transcription proteins, and G-protein signaling proteins.

Mechanisms of Action

The primary mechanisms of oncogenic transformation are through transduction, cis-activation, and trans-activation. Less commonly, viral proteins such as Env may directly stimulate cell proliferation, interfere with cell cycle checkpoints, or inhibit surveillance for tumor cells.

Transduction

During replication, retroviruses can acquire host sequences through recombination errors. These viruses are usually defective and require helper viruses for replication. The transforming power of recombinant viruses depends on the cellular gene they have acquired, but many viruses with rapid transformative ability utilize this mechanism.[11] In general, transduction is uncommon in natural infections, but is a useful research tool for studying oncogenesis in the laboratory.

Cis-Activation

Cis-activation occurs when a provirus inserts near a proto-oncogene. Activation of the proto-oncogene occurs by promoters or enhancer elements in the viral LTR. Cis-activation is commonly used by simple retroviruses.

Trans-Activation

Trans-activation occurs as a virally encoded protein such as Tax interacts with host transcriptional factors, thereby altering expression of normal cellular genes.

PROTOTYPICAL AND WELL-CHARACTERIZED ONCORETROVIRUSES

Avian Leukosis and Sarcoma Viruses

The avian leukosis and sarcoma viruses (ALSVs) cause tumors in a variety of avian species. ALVs are simple retroviruses that are transmitted both horizontally and vertically. However, while a large percentage of the flock may be infected, neoplasia develops in only a small percentage. Vaccination strategies have not been successful, making eradication of ALV from the breeding stock a critical control step. In the early 1980s, eradication of the five primary envelope subgroups had been reasonably successful. However, in the mid-1980s, a highly pathogenic myeloid tumor-inducing subgroup J virus emerged to become one of the major infectious diseases facing the poultry industry.

Exogenous ALVs are transmitted as infectious virions, while endogenous ALVs are incorporated into the host genome and transmitted genetically. The number of endogenous viruses varies among avian species and the biological significance of endogenous viruses is not entirely clear. Some forms appear to protect the host from infection by newly acquired ALV infection through receptor interference. However, endogenous viruses may also have a negative effect on the host.

ALVs are well-described oncogenic viruses. Both nonacute and acute transforming forms are recognized. Nonacute transforming viruses induce tumors that appear late in the period of infection. Typically, oncogenesis is induced through the insertion of the ALV genome near a proto-oncogene resulting in cis-activation. Lymphoid leukosis is one of the best-described examples. In lymphoid leukosis, the B-cell c-*myc* gene is activated resulting in a multistage lymphomagenesis that produces neoplastic lymphoblasts resistant to apoptosis. Because multiple follicles can be affected nearly simultaneously, the disease can appear to be polyclonal.

By contrast, acute transforming ALVs contain one or more viral oncogenes. These ALVs tend to be replication-incompetent due to mutations (usually deletions) within the structural genes and require coinfection with a nondefective helper virus. Several ALV strains carry the *myc* oncogene that tends to induce myelomonocytic neoplasia. Avian sarcoma viruses also tend to be acutely transforming. The best characterized is Rous sarcoma virus which encodes a protein kinase through *src* viral oncogene.

In addition to their known neoplastic induction, certain ALVs serve as a model of viral-associated obesity. For example, the Rous-associated virus type 7 ALV induces a syndrome of hypertriglyceridemia and hypercholesterolemia.

Bovine Leukemia Virus

BLV is a type-C deltaretrovirus that infects cattle and sheep. Bovine leukemia virus replication is primarily dependent on cell proliferation and mitotic division. Virus infection stimulates a polyclonal expansion of B cells, called persistent lymphocytosis, in approximately one-third of infected animals. In cattle, only a small percentage of infected animals develop bovine enzootic leukosis, a polyclonal, but neoplastic, B-cell expansion. A higher rate of neoplasia is seen in experimentally infected sheep. Transmission is primarily horizontal through the transfer of infected cells by direct contact and possibly through blood-sucking insects. Vertical transmission is less common, but may occur in utero, at parturition, or through milk ingestion.

Bovine leukemia virus is related to the primate lymphotropic viruses, but has over 40% phylogenetic divergence. While the primate viruses infect T cells, BLV primarily infects B cells, particularly those expressing surface IgM, but also can infect monocytes and macrophages. Bovine leukemia virus encodes both Rex and Tax proteins, Tax being the primary oncogenic protein. Transmission by cell-free virus is inefficient, and cell-to-cell transmission by cell fusion predominates. After infection of a new cell, BLV integrates randomly into the host genome. Virus replication is primarily through the production of daughter cells via mitotic division.

Feline Leukemia Virus

FeLV is a simple retrovirus that infects domestic cats as well as nondomestic felids. FeLV has high sequence homology with MuLV, supporting the hypothesis that infection of cats resulted from MuLV cross-species transmission. Three genotypes of FeLV have been described: types A, B, and C. Type A FeLV is the dominant virus in nature. This exogenously transmitted retrovirus is ubiquitous, readily transmitted, and highly conserved. Types B and C result from mutation and recombination of type A FeLV with endogenous retroviral sequences. The variability in viral recombination corresponds with variability in the pathogenesis and disease outcomes including aplastic anemia, T-cell lymphoma/leukemia, myeloid leukemia, and immunosuppression.

Feline leukemia virus is transmitted horizontally, primarily through saliva, biting, and other close contact. Vertical transmission occurs both in utero and by ingestion of infected milk. Susceptibility is inversely correlated with age; thus, the highest potential for transmission and infection occurs during the first 4–6 months of age. Approximately 30% of infected cats develop persistent viremia and secondary diseases including immunodeficiency, hematopoietic and other neoplasia, and opportunistic infections. The majority (60–65%) of all infected cats rapidly or eventually clear the virus after infection due to a robust immune response, minimal virus exposure, or a combination of the two, and remain antigen-negative by ELISA. A small percentage of cats develop a transient antigen-positive viremia, but then become persistently aviremic and antigen-negative. Finally, 5–10% of all infected cats may develop an "atypical" infection due to localized or sequestered infection and have mixed or inconsistent test results. These cats may maintain a lifelong latent infection, but may progress to outright viremia or viral clearance. Latent FeLV may be reactivated through secondary diseases or treatments that result in impairment of the immune system.

Gibbon Ape Leukemia Virus

Gibbon ape leukemia virus (GALV) is genetically related to simian-sarcoma-associated virus, murine leukemia virus, and feline leukemia virus. Gibbon ape leukemia virus is a highly oncogenic C-type retrovirus that induces myeloproliferative disorders, particularly in juvenile animals. To date, four strains of GALV have been identified from captive gibbons. Because the virus has not been identified in wild animals, it is uncertain if GALV is of simian origin or represents an endogenous retrovirus transferred between species. Because GALV infects a wide variety of cell types from different species, several GALV-based retroviral vectors have been developed for gene transfer.

Human T-Cell Lymphotropic Viruses

Type 1 and type 2 HTLVs are closely related viruses that share significant nucleotide similarly, but also have important differences. Recently, additional members of the HTLV family, HTLV-3 and HTLV-4, have been identified.[6] The HTLVs are complex retroviruses with several regulatory and accessory genes. The HTLVs are genetically stable viruses whose replication is limited by the immune response. HTLV replication primarily occurs through clonal replication of infected cells. HTLV-1 is endemic in Southeast Asia including Japan, the Caribbean, Melanesia, as well as parts of South America, Central Africa, and the Middle East. HTLV-1 infection can result in adult T-cell leukemia (ATL) and HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP); however, only a small percentage of infected individuals develop clinical disease.

Jaagsiekte Sheep Retrovirus and Enzootic Nasal Tumor Virus

JSRV is the causative agent of pulmonary adenomatosis in sheep. Pulmonary adenomatosis serves as a transmissible model of human bronchioloalveolar carcinoma. It is a simple retrovirus, but includes an additional gene, *orf-x*. The mechanism of JSRV-induced oncogenesis is not well defined, but both *orf-x* and *env* have been proposed to play a role. The virus spreads

horizontally through infection of type II pneumocytes and Clara cells in the lower respiratory tract. The time to tumorigenesis ranges from months to years, but disease progresses rapidly once clinical signs develop.

ENTV has >95% genetic similarity with JSRV. While JSRV induces tumors in the lower respiratory tract, ENTV causes tumors in the nasal cavity.

Mouse Mammary Tumor Virus

MMTV induces mammary adenocarcinomas and, less commonly, T-cell lymphoma. MMTV is transmitted genetically through the germ line and via infected breast milk. MMTV has been classified as a simple betaretrovirus, but encodes Rem, a self-regulatory mRNA export protein related to the Rev protein of HIV.[3,7] MMTV also encodes *sag*, a superantigen that uniquely stimulates viral replication. B cells in the gut are the initial target for viral infection. B-cell expression of the superantigen activates bystander CD4+ T cells that, in turn, induce clonal expansion of Sag expressing B cells responsible for trafficking to the mammary gland and infecting mammary epithelial cells.

Mouse mammary tumor virus tumorigenesis occurs primarily through viral insertion near a proto-oncogene and subsequent activation. Endogenous viruses are frequently truncated or defective; however, those mice with a functional *sag* gene have some degree of protection against infection with exogenous MMTV because the T cells that would normally react against the superantigen have been deleted during thymic maturation.

Recently, several sequences have been detected within the human genome and human cancers that have very high sequence homology with MMTV. Although somewhat controversial, this has led to speculation that these elements may play a role in human breast cancer and lymphoma as well as other diseases such as primary biliary cirrhosis.[1,4,8,9,10,12] While additional work is necessary to establish a causative link between MMTV-like sequences and these diseases, current studies are shifting our understanding of the role of endogenous retroviruses and retroelements.

MURINE LEUKEMIA VIRUSES

There are several strains of well-characterized MuLVs including Friend, Moloney, and Rauscher viruses. MuLVs induce a wide spectrum of malignancies including T-cell lymphoma/leukemia, myeloid leukemia, and erythroleukemia. These viruses can also affect the nervous system. The tumorigenic effects are driven by the LTR U3 region, while neuropathicity is mediated by the Env SU. These simple retroviruses are further classified by host range and replication capacity. Ecotropic viruses replicate only in their natural host. Amphotropic or polytropic viruses replicate in both rodent and nonrodent species. Xenotropic viruses infect only nonmurine cells. Wild rodents frequently harbor non-ecotropic viruses. Because these non-ecotropic viruses efficiently infect human cells, they make efficient gene delivery vectors.

Reticuloendotheliosis Virus

REV is a simple gammaretrovirus that infects a number of avian species. Serologic studies suggest that the virus is prevalent in both chickens and turkeys, and appears to be transmitted vertically and horizontally. The name reticuloendotheliosis virus arises from the acute reticulum cell neoplasia seen in chickens experimentally inoculated with a unique replication-defective laboratory strain containing the *rel* oncogene. However, most clinical isolates lack oncogenes and are replication-competent. Reticuloendotheliosis virus induces three distinct clinical syndromes including a non-neoplastic runting syndrome associated with bursal and thymic atrophy, a variety of acute neoplastic diseases involving multiple organs, and chronic B- and T-cell lymphomas.

Simian T-Cell Lymphotropic Virus

The simian T-cell lymphotropic viruses (STLVs) are complex deltaretroviruses closely related to HTLV. The genetic relatedness between STLV-1 and HTLV-1 supports the hypothesis that HTLV-1 originated from cross-species STLV transmission. STLV-1 infects over 20 species of Old World primate species, while STLV-2 has a much more limited species range. Clinical disease is uncommon in STLV-infected primates and, when seen, tends to occur following chronic infection.

Walleye Dermal Sarcoma Virus

WDSV is the best-characterized piscine retrovirus and is endemic in walleye throughout North America. It is a complex retrovirus with at least three accessory genes. Walleye dermal sarcoma virus replication and the induced dermal sarcomas have a unique cyclical pattern. Walleye-dermal-sarcoma-virus-induced dermal sarcomas are benign tumors that develop in the fall when virus production is low. In the spring, virus replication increases and these tumors concurrently regress. These findings suggest that virus replication is dependent on host endocrine or immunologic changes associated with spawning and/or changes in water temperature.

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CHAPTER 61

Cytochemical Staining and Immunocytochemistry

ROSE E. RASKIN, KELLY SANTANGELO, and KLAUDIA POLAK

General Principles of Cytochemistry
General Considerations for Staining Neoplastic Hematopoietic Cells
Reactions of Normal Hematopoietic Cells
  Neutrophils/Heterophils
  Eosinophils
  Basophils
  Monocytes/Azurophils
  Lymphocytes
  Platelets, Thrombocytes, and Megakaryocytes
  Erythroid Cells
  Mast Cells
Cytochemical Staining Principles and Diagnostic Application
  Myeloperoxidase
  Sudan Black B
  Chloroacetate Esterase
  Leukocyte Alkaline Phosphatase
  Periodic Acid–Schiff
  Nonspecific Esterases
  Acid Phosphatase
  Beta-Glucuronidase
  Miscellaneous Stains
    Toluidine Blue
    Omega-Exonuclease
    Synacril Black AN
    Acetylcholinesterase
    Luna
General Principles of Immunocytochemistry
Reactions of Normal Hematopoietic Cells
General Considerations for Diagnosing Neoplastic Hematopoietic Cells

Acronyms and Abbreviations

ACH, acetylcholinesterase; ACP, acid phosphatase; ALL, acute lymphoblastic leukemia; ANAE, alpha-naphthyl acetate esterase; ANBE, alpha-naphthyl butyrate esterase; βG, β-glucuronidase; CAE, chloroacetate esterase; IHC, immunohistochemistry; LAP, leukocyte alkaline phosphatase; NK, natural killer; NSE, nonspecific esterase; OEN, omega-exonuclease; PAS, periodic acid–Schiff; PER, peroxidase; SBB, Sudan Black B; TB, toluidine blue.

GENERAL PRINCIPLES OF CYTOCHEMISTRY

Cytochemistry is the use of special stains for the microscopic detection of cellular constituents such as lipids, carbohydrates, and enzymes.[97] These stains may detect differences between normal cell types and identify the lineage of poorly differentiated blast cells. Cytochemistry is valuable because it involves minimal cost and is readily performed at diagnostic referral centers. This technique may be used with hemolymphatic tissues (blood, bone marrow, lymph nodes, spleen, and thymus) or with liver.[82] These specimens may include fresh imprints or aspirates, frozen tissues, or plastic-embedded tissues.[15,136] Notably, cytochemistry can also detect substrates that may be lost during the preparation of paraffin-embedded tissues (e.g., nonspecific esterase (NSE) activity) because the enzyme is unstable during tissue processing. In addition to light microscopic preparations, cytochemical staining also has been applied to electron microscopic specimens.[9,85]

To ensure proper sample handling, submitters should contact the laboratory prior to sample collection.

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Multiple unfixed slides usually are preferred, so a panel of stains can be performed. During cytochemical staining, appropriate control slides are used as positive and negative controls to assure quality control for the procedure(s). Although cytochemistry is applicable to the cells of various species, it is noteworthy that incubation times, staining patterns, and the presence of substrates may differ, even among the various avian, reptile, and fish species.

GENERAL CONSIDERATIONS FOR STAINING NEOPLASTIC HEMATOPOIETIC CELLS

The first step in the identification and classification of hematopoietic neoplasia is the morphologic characterization of the blast cell population. This may be followed by special tests to better define the cell of origin (e.g., cytochemistry, immunocytochemistry (ICC), and flow cytometry). Poorly differentiated cells are indistinguishable by morphology alone, but may be better identified by their cytochemical characteristics. This is critical for acute leukemias and especially useful in domestic animals like the dog, cat, and horse. The use of cytochemical staining can improve the diagnosis and sometimes change the initial morphologic interpretation.[53,67]

The general premise of cytochemistry is that the neoplastic cells contain the same enzymes or cellular products as cells from healthy individuals. However, occasional irregularities may occur related to the altered development of neoplastic or dysplastic cells. Cytochemical staining is best interpreted when a panel rather than one or two stains is performed, since discrepancies may occur with neoplastic cells.

In general, lymphoid cells do not stain with many cytochemical stains; however, negative staining does not automatically indicate a lymphoid neoplasm. Stain intensity is often very weak or absent in the most undifferentiated cells because they contain few of the necessary enzymes or cellular constituents. By contrast, some enzymes (e.g., myeloperoxidase (MPO)) are highly expressed in the immature cells. In cytochemistry, it is best to interpret the results of positive staining rather than the lack of staining.

In addition to stain intensity, the pattern of staining may be helpful. For example, T-lymphocytes containing focal lysosomal granules often produce a focal localization of NSE enzyme activity compared with the diffuse or granular nature found in some nonlymphoid cells. Lymphoid cells are best classified using immunologic markers, but lineage- and species-specific monoclonal antibodies may be limited in availability or by cost. Therefore, particularly for unique species, cytochemical staining can be very helpful for T-cell identification.[9]

In the interpretation of cytochemical staining data, it is important to note that reactions seen within one species cannot be extended to other species. For example, feline eosinophils do not stain with either peroxidase (PER) or Sudan Black B (SBB), but such staining can be seen in other species. It is also important to note the extent of staining within the sample. For example, the percentage of PER- or SBB-positive blast cells in the bone marrow should be greater than 3% to identify human myeloblastic leukemia (without maturation).[16] It is best to count a minimum of 200 bone marrow cells when calculating this figure to avoid an overestimation or underestimation.[79] A simplified classification of acute leukemias using cytochemical staining is shown in Figure 61.1.

REACTIONS OF NORMAL HEMATOPOIETIC CELLS

A summary of cytochemical staining profiles for selected stains and leukocytes in several domestic and exotic animal species is provided in Tables 61.1 and 61.2.

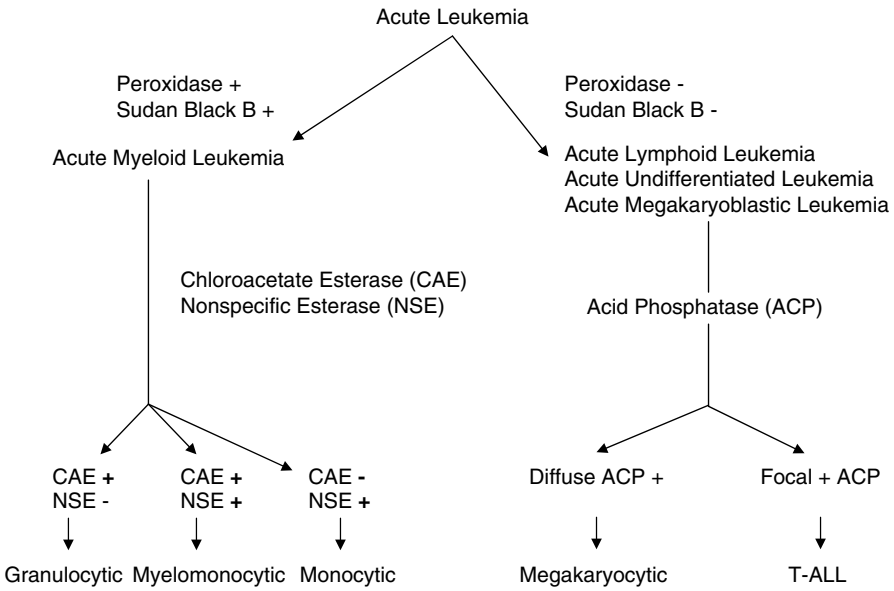


FIGURE 61.1 Simplified algorithm for the classification of acute leukemia from blast cells in blood or bone marrow using cytochemical stains.

TABLE 61.1 Summary of selected cytochemical staining reactions for normal blood cells in various domestic animal species.[a]

Species/cell type	PER	SBB	CAE	LAP	PAS	NSE	NSE/FL	ACP	ACP/T	BG
Bovine										
Neutrophil	pos	pos	pos	pos	pos	neg	–	pos/neg	–	–
Eosinophil	pos	pos	neg	pos	pos/neg	pos/neg	–	pos/neg	–	–
Basophil	neg	pos/neg	pos/neg	pos/neg	pos/neg	neg	neg	neg	neg	–
Monocyte	pos/neg	pos/neg	pos/neg	neg	pos/neg	pos/neg	pos/neg	pos/neg	–	–
Lymphocyte	neg	neg	neg	neg	pos/neg	pos/neg	pos	pos/neg	neg	–
Cat										
Neutrophil	pos	pos	pos	neg	pos	neg	neg	pos/neg	neg	neg
Eosinophil	neg	neg	neg	pos/neg	pos/neg	neg	neg	pos	pos	neg
Basophil	neg	neg	pos	pos/neg	pos/neg	neg	neg	pos	neg	–
Monocyte	pos/neg	pos/neg	neg	neg	pos/neg	pos/neg	neg	pos/neg	neg	pos
Lymphocyte	neg	neg	neg	neg	neg	pos/neg	pos/neg	pos/neg	neg	pos/neg
Platelet/ megakaryocyte	neg	neg	pos/neg	neg	pos/neg	pos/neg	neg	pos/neg	neg	pos/neg
Dog										
Neutrophil	pos	pos	pos	neg	pos	neg	neg	pos/neg	neg	–
Eosinophil	pos	pos	neg	pos/neg	pos/neg	neg	neg	pos	pos	–
Basophil	neg	neg	pos	pos/neg	pos/neg	neg	neg	pos/neg	pos	–
Monocyte	pos/neg	pos/neg	neg	neg	pos/neg	pos/neg	neg	pos	neg	–
Lymphocyte	neg	neg	pos/neg	pos/neg	pos/neg	pos/neg	pos	pos/neg	neg	–
Platelet/ megakaryocyte	neg	neg	pos/neg	neg	pos	pos	pos/neg	pos	neg	–
Horse										
Neutrophil	pos	pos	pos	pos	pos	pos/neg	–	pos/neg	–	–
Eosinophil	pos	pos	neg	pos/neg	pos/neg	pos/neg	–	pos/neg	–	–
Basophil	neg	neg	pos/neg	neg	pos	neg	–	neg	–	–
Monocyte	pos/neg	pos/neg	pos/neg	neg	pos/neg	pos/neg	–	pos/neg	–	–
Lymphocyte	neg	neg	neg	neg	pos/neg	pos/neg	–	pos/neg	–	–
Platelet/ megakaryocyte	neg	neg	–	neg	–	–	–	pos	–	–
Chicken										
Heterophil	neg	neg	neg	neg	pos/neg	neg	–	neg	–	–
Eosinophil	pos	pos	neg	neg	neg	neg	–	pos	–	–
Monocyte	pos/neg	pos/neg	–	neg	pos/neg	pos	–	pos	–	–
Lymphocyte	neg	neg	–	neg	neg	pos/neg	–	pos/neg	–	–
Thrombocyte	–	–	–	–	pos	–	–	–	–	–

[a]PER, peroxidase; SBB, Sudan Black B; CAE, chloroacetate esterase; LAP, leukocyte alkaline phosphatase; PAS, periodic acid–Schiff; NSE, nonspecific esterase; NSE/FL, nonspecific esterase with fluoride; ACP, acid phosphatase, ACP/T, acid phosphatase with tartrate; BG, beta-glucuronidase; neg, no reaction; pos, easily detectable; pos/neg, weak or focal or occasional reactivity; –, undetermined.

Neutrophils/Heterophils

Primary or azurophilic lysosomal granules contain myeloperoxidase. PER activity is readily demonstrated from the promyelocyte stage through mature segmented forms (Figure 61.2a,b). In electron microscopic preparations, PER activity also may be found in the nuclear membrane, rough endoplasmic reticulum, and Golgi apparatus of myeloblasts. PER reactivity generally is absent in heterophils of rabbits, certain birds, and reptiles.[88,3,178] Iguana heterophils, in contrast to many reptiles, have a significant positive PER reactivity as do two species of frog.[27,28] Neutrophils with eosinophilic prominence, such as those of the manatee, demonstrate intense PER staining.[64]

SBB intensely stains the lipids of secondary or specific granules (Figure 61.3a), but can also be found to a lesser extent within primary granules (Figure 61.3b). As such, all stages of neutrophil maturation are detected. Primary and secondary neutrophilic granules also contain chloroacetate esterase (CAE) activity (Figure 61.4a–c), which results in moderate to strong reactions in all neutrophil maturation stages (Figure 61.5a–c). Notably, in frogs and cod, CAE is found in monocytes.[27,148]

Leukocyte alkaline phosphatase (LAP) is found predominantly in the secondary granules of most species including humans, horses, rabbits, and ruminants (Figures 61.5 and 61.6). However, LAP staining is absent in the mature neutrophils of dogs and cats, is variable in the neutrophils of horses and goats, and is absent from the neutrophils from most other species.[169] Acid phosphatase (ACP) activity of modest staining intensity is present in the primary granules of neutrophils. The highest ACP activity is detected in immature neutrophils as opposed to bands and segmented forms.

TABLE 61.2 Summary of selected cytochemical staining reactions for normal blood cells in various exotic animal species.[a]

Species/cell type	PER	SBB	CAE	LAP	PAS	NSE	ACP	BG	TB
Elephant									
Heterophil	pos	pos/neg	pos	pos	pos/neg	pos/neg	–	pos/neg	–
Eosinophil	pos	neg	neg	neg	neg	pos	–	pos/neg	–
Basophil	neg	neg	pos/neg	–	neg	pos	–	pos	–
Monocyte	pos	pos/neg	pos/neg	neg	–	pos/neg	–	pos	–
Lymphocyte	neg	neg	pos/neg	neg	neg	pos/neg	–	pos	–
Platelet	neg	neg	neg	–	neg	pos/neg	–	neg	–
Channel catfish									
Neutrophil	pos	pos	neg	neg	pos	neg	pos	pos/neg	–
Basophil	neg	neg	neg	neg	pos	neg	neg	neg	–
Monocyte	neg	neg	neg	neg	neg	pos/neg	pos	neg	–
Lymphocyte	neg	pos/neg	neg	neg	neg	neg	pos/neg	neg	–
Thrombocyte	neg	neg	neg	neg	pos/neg	neg	neg	neg	–
Murray cod									
Heterophil	pos	pos	pos	pos	pos	pos/neg	pos	pos/neg	–
Basophil	neg	neg	pos	neg	pos	pos/neg	pos	pos	–
Monocyte	neg	neg	neg	neg	neg	pos/neg	pos	pos	–
Lymphocyte	neg	pos	pos	pos/neg	pos/neg	pos/neg	pos	pos	–
Thrombocyte	neg	neg	neg	neg	pos	neg	neg	neg	–
Bullfrog									
Neutrophil	pos	pos	pos	neg	–	pos	–	–	–
Eosinophil	pos	pos	neg	neg	–	neg	–	–	–
Basophil	neg	neg	neg	neg	–	neg	–	–	–
Monocyte	neg	neg	pos	neg	–	pos	–	–	–
Lymphocyte	neg	neg	pos/neg	neg	–	neg	–	–	–
Thrombocyte	neg	neg	neg	pos/neg	–	neg	–	–	–
Alligator									
Heterophil	neg	–	neg	pos	pos	pos	pos	–	neg
Eosinophil	pos	–	neg	pos	pos	neg	neg	–	neg
Basophil	neg	–	neg	neg	pos	neg	pos	–	pos
Monocyte	neg	–	pos/neg	neg	pos	neg	neg	–	neg
Lymphocyte	neg	–	pos	pos	pos	pos	pos	–	neg
Thrombocyte	neg	–	neg	neg	pos	neg	neg	–	neg
Iguana									
Heterophil	pos	pos	neg	neg	pos	pos	pos	–	neg
Eosinophil	neg	neg	neg	neg	neg	neg	neg	–	neg
Basophil	neg	neg	neg	neg	pos/neg	neg	neg	–	neg
Monocyte/ azurophil	neg	neg	neg	neg	pos/neg	pos	pos	–	neg
Lymphocyte	neg	neg	neg	neg	neg	neg	neg	–	neg
Thrombocyte	neg	neg	neg	neg	pos	pos	neg	–	neg
Green sea turtle									
Heterophil	neg	neg	neg	–	pos	pos	neg	–	neg
Eosinophil	neg	neg	pos	–	pos/neg	pos/neg	neg	–	neg
Basophil	neg	neg	neg	–	neg	neg	neg	–	pos
Monocyte	neg	neg	pos/neg	–	pos	pos/neg	pos	–	neg
Lymphocyte	neg	neg	neg	–	neg	neg	neg	–	neg
Thrombocyte	neg	neg	neg	–	pos	pos	neg	–	neg

[a]PER, peroxidase; SBB, Sudan Black B; CAE, chloroacetate esterase; LAP, leukocyte alkaline phosphatase; PAS, periodic acid–Schiff; NSE, nonspecific esterase; ACP, acid phosphatase; BG, beta-glucuronidase;TB, toluidine blue; neg, no reaction; pos, easily detectable; pos/neg, weak or focal or occasional reactivity; –, undetermined.

Eosinophils

The PER in eosinophils differs both structurally and biochemically from that found in neutrophils and megakaryocytes. In eosinophils, PER is present in the matrix of large eosinophilic granules, but not within the crystalloid core (Figure 61.2a). Notably, and unlike most species, feline eosinophils do not stain with either PER or SBB techniques. However, PER and SBB reactivity may be observed in avian and reptilian eosinophils.[3,6,104] In general, eosinophils do not stain with

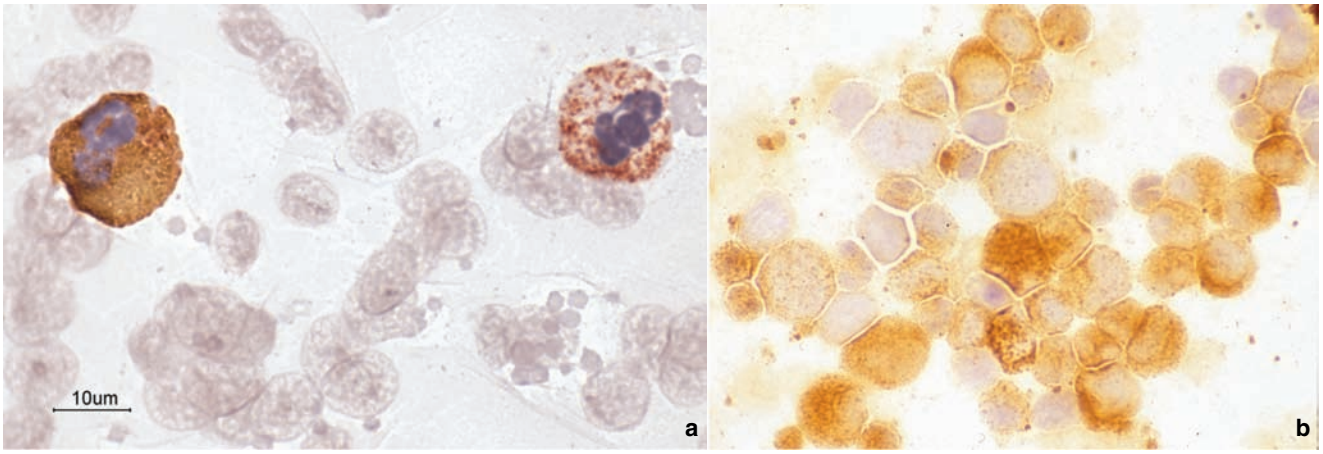


FIGURE 61.2 (a) Peroxidase (aminoethylcarbazole) reaction in elephant blood showing strong reaction in an eosinophil (left) and moderate reaction in a segmented heterophil (right). 100× objective; (b) Diffuse PER (benzidine) staining of immature cells in canine acute myelogenous leukemia. Bone marrow cytology. 100× objective.

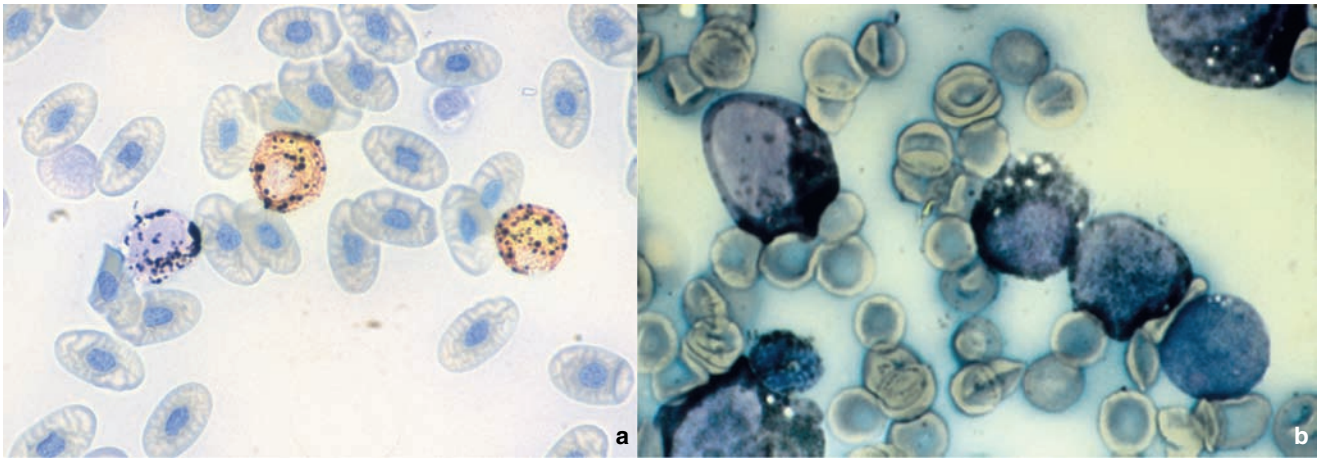


FIGURE 61.3 (a) SBB reaction in two heterophils (right) and one azurophil (left) from boa snake blood. 100× objective; (b) Diffuse SBB staining of blast cells in canine acute myelomonocytic leukemia. Bone marrow cytology. 100× objective.

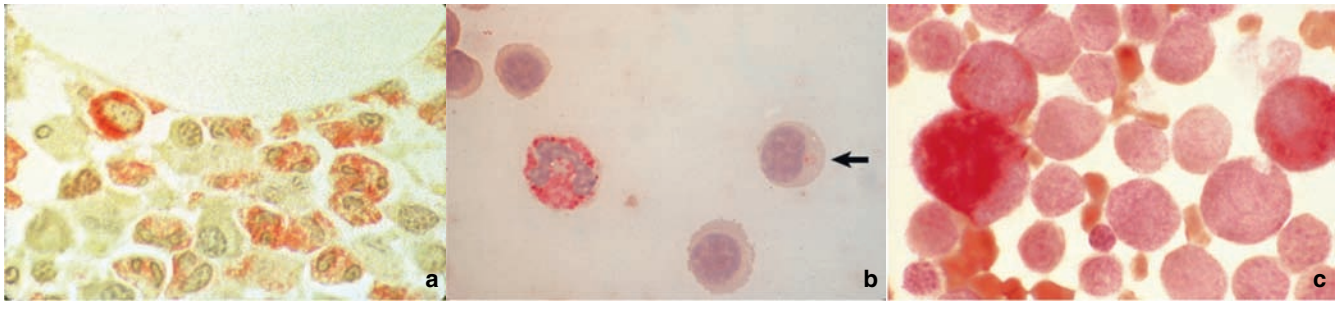


FIGURE 61.4 (a) Canine bone marrow with diffuse positive CAE reaction in granulocytic precursors. Plastic-embedded section. 100× objective; (b) Canine blood stained with CAE showing strong positive reaction in a neutrophil and focal reaction (arrow) in a lymphocyte. 100× objective; (c) Granulocytic blast cells stain CAE positive in feline acute myelomonocytic leukemia. Bone marrow cytology. 100× objective.

CAE technique; however, reactivity has been observed in these cells from the green turtle, yellow-bellied slider, and koi.[69,168,178] NSE activity is absent in canine and feline eosinophils, but may be observed in equine and ruminant eosinophils. Both alpha-naphthyl butyrate esterase (ANBE) and alpha-naphthyl acetate esterase (ANAE) activities are noted to be strong in elephant eosinophils. Eosinophils, especially those of the cat, stain strongly for ACP activity within the intergranular areas and is tartrate-resistant.[53,54] LAP activity

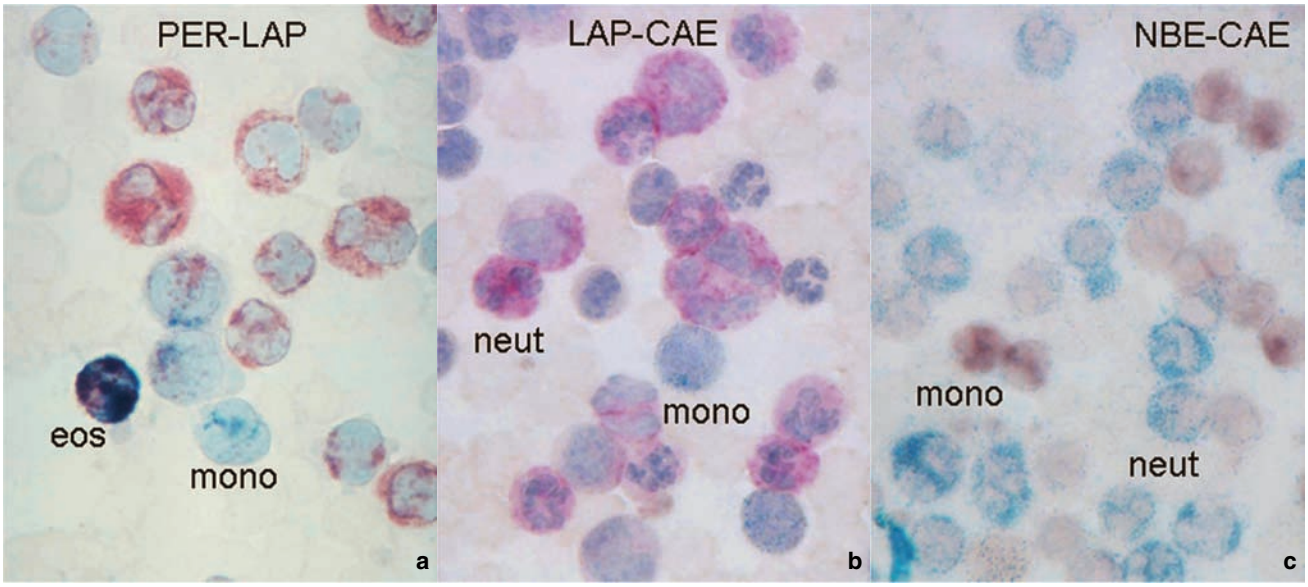


FIGURE 61.5 Double staining technique for canine bone marrow with chronic myelomonocytic leukemia. (a) Peroxidase activity (red) in neutrophils is coupled with leukocyte alkaline phosphatase (blue) that produces a strong reaction in between the eosinophil granules along with a mild reaction in monocytes; (b) Mild leukocyte alkaline phosphatase activity (blue) in monocytes along with chloroacetate esterase activity (red) that reacts strongly with variable stages of neutrophils; (c) Chloroacetate esterase activity (blue) in neutrophils is performed along with alpha-naphthyl butyrate esterase (red-brown) indicating activity in monocytes. (**Source:** Modified from cytochemical images provided by Dr. Julia Blue.)

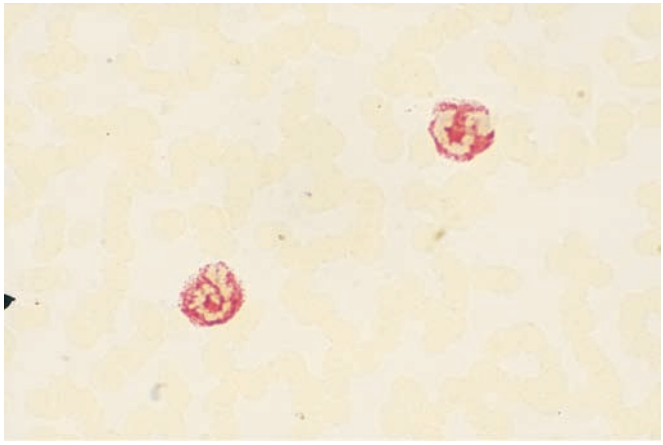


FIGURE 61.6 Equine blood with positive LAP in two neutrophils. 100× objective.

is found intracellularly between the specific granules of eosinophils (Figures 61.5 and 61.6) with the strongest reactivity seen in the horse, whereas feline eosinophils may lack or have weak reactivity.[82] Specific eosinophil stains, such as luna and luxol fast blue, may also be helpful in eosinophil identification.[183]

Basophils

Basophils are negative for SBB and PER reactivity. Feline and opossum basophils may demonstrate occasional positive LAP staining.[39] Canine and feline basophils demonstrate moderate CAE staining, whereas bovine and equine basophils are weakly positive. Basophils of the dog, cat, horse, and cow are negative for NSE activity. Tartrate-resistant ACP activity may be observed in canine and feline basophils.[53,54] Alcian blue stains the sulfated mucosubstances in the basophil granules of many species.[135] Omega-exonuclease (OEN) activity is present in basophils and mast cells of dogs and cats (Figure 61.7a,b), but it is absent in both equine and bovine basophils.[81,135] Toluidine blue (TB) staining may be metachromatic (purple) for basophil granules of domestic animals under acidic conditions, but this characteristic is not observed at neutral pH. TB staining also has been demonstrated in basophils from several reptiles.[3,69,104] Basophils from Asian elephants are strongly positive to β-glucuronidase (βG).

Monocytes/Azurophils

Monocytes and azurophils of some species show diffuse, weakly positive reactivity for SBB and PER (Figure 61.5).[2,4,24] PER activity may be more evident when copper nitrate is used to intensify the enzymatic reaction within the lysosomes. Generally, monocytes do not contain CAE activity; however, trace positive reactivity may be observed in bovine, equine, ovine, caprine monocytes, as well as the bilobed monocyte of elephants. NSE is present in cells of monocytic origin, resulting in diffuse, finely granular reactivity (Figure 61.5) that is most intense in macrophages.[76,180] NSE reactivity (Figure 61.8) is inhibited with sodium fluoride due to the presence of the enzymes on the plasma membrane.[26,47] ACP reactivity is present in a diffuse manner, similar to that observed for NSE. ACP reactivity is of moderate intensity in monocytes and increases with

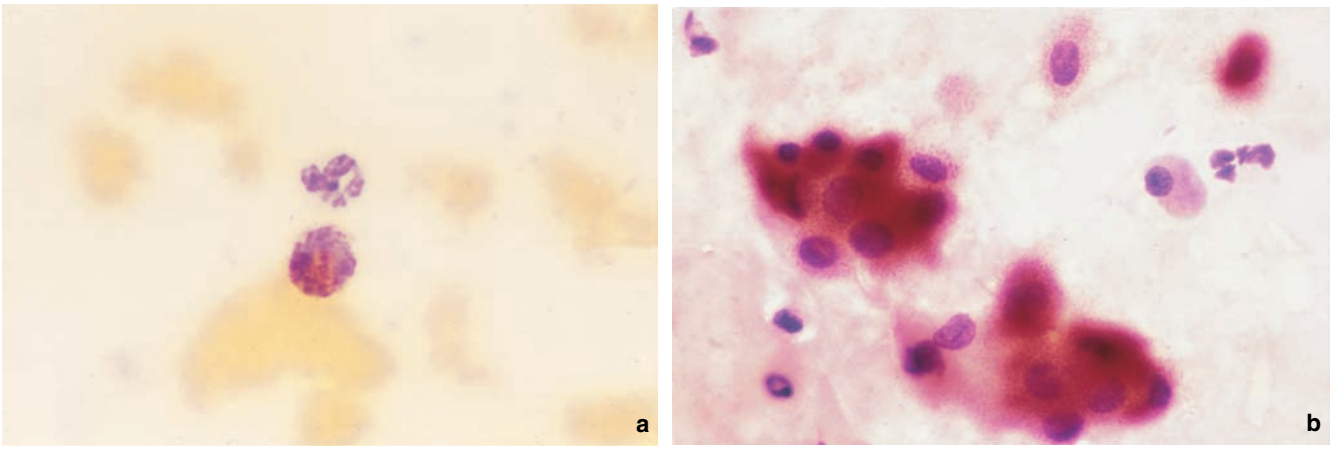


FIGURE 61.7 (a) Positive stain reaction with OEN in a feline basophil. Blood. 100× objective; (b) Strong cytoplasmic staining of cells from a canine mast cell tumor with OEN. Skin mass cytology. 100× objective.

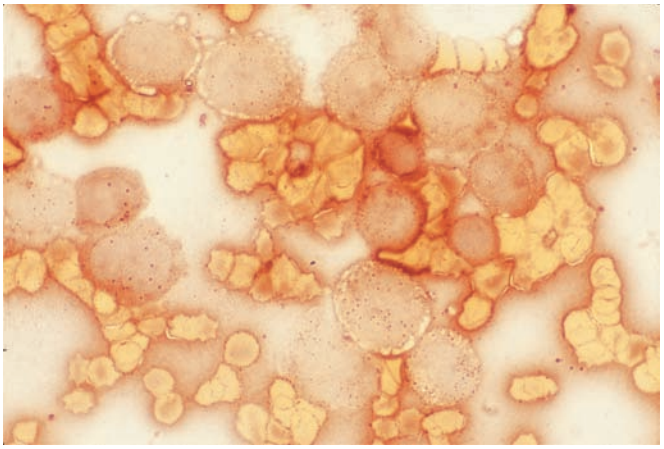


FIGURE 61.8 Diffuse ANAE staining of monocytoid blast cells that were fluoride-sensitive in equine acute monoblastic leukemia. Bone marrow cytology. 100× objective.

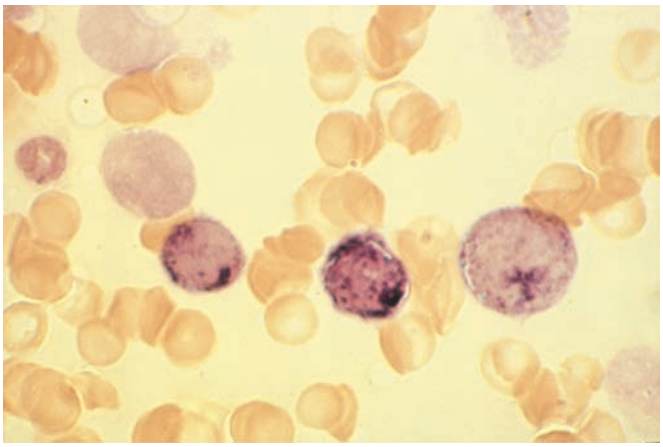


FIGURE 61.9 Diffuse LAP staining of blast cells in canine acute myelomonocytic leukemia. Bone marrow cytology. 100× objective.

maturity into macrophages. LAP may be found in canine monocytes and monoblasts (Figures 61.5 and 61.9). Koi monocytes are noted to have naphthyl butyrate esterase (NBE), as well as beta-glucuronidase activities.[168]

Lymphocytes

Traditionally, lymphocytes lack reactivity to many of the cytochemical stains used to identify granulocytes and monocytes. PER activity is absent, although rare PER expression detected by molecular studies has been documented in humans.[74] LAP activity, present as a fine and diffusely granular pattern involving the plasma membrane, may be observed in a B-cell subtype associated with the mantle zone of lymph nodes in dogs.[136] CAE reactivity is rare in lymphocytes (Figure 61.4b), but weak staining has been reported in bovine and equine lymphocytes, as well as elephants. Occasional punctate staining has been associated with non-T-cell large granular lymphocytes in people.[71] NSE activity can be seen as a focal, discrete staining reaction within the intracellular organelles of normal and neoplastic T-lymphocytes from humans, dogs, cats, cattle, horses, sheep, elephants, and chickens.[9,47,53,54,71,130,136,175,179,181] Weak periodic acid–Schiff (PAS) staining has been demonstrated in lymphocytes from cattle, dogs, horses, and sheep. Positive reactivity for ACP has been observed in canine natural killer (NK) cells with and without tartrate incubation (Figure 61.10).[90]

Platelets, Thrombocytes, and Megakaryocytes

Platelets and their precursors contain, in the endoplasmic reticulum and nuclear membrane of megakaryocytes and in the tubular system of the platelets, a specific PER which is only apparent in ultrastructural preparations. Slight CAE reactivity in canine and feline megakaryocytes has been reported, similar to that observed in human cells.[53,54] PAS staining is often apparent within platelets and megakaryocytes of many animal species, including thrombocytes of birds and reptiles (Figure 61.11), but not in some fish.[69,104,148,159] Although megakaryocytes and platelets may exhibit reactivity for

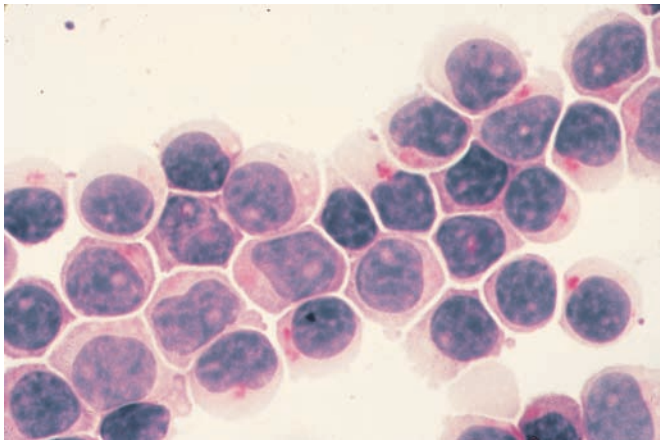


FIGURE 61.10 Focal stain reaction with ACP in canine lymphocytes having NK-cell activity. 100× objective.

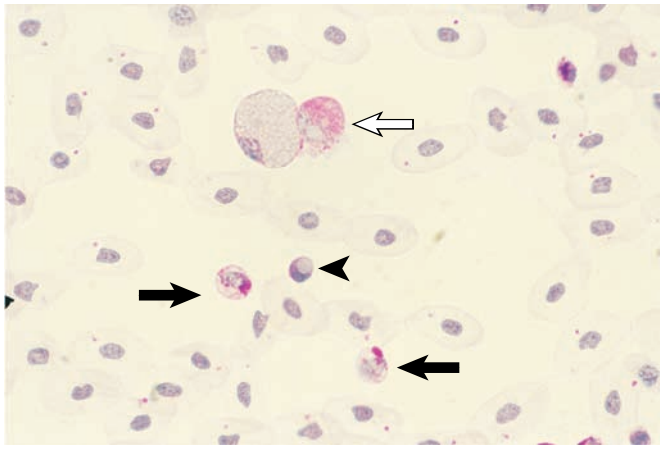


FIGURE 61.11 PAS reaction in boa snake blood cells showing strong reaction in thrombocytes (black arrows), moderate reaction in an azurophil (white arrow), trace reaction in a heterophil, and punctate reaction in erythrocytes. Note lymphocyte with inclusion body (arrowhead). 100× objective.

NSE, these reactions are mildly to totally inhibited with sodium fluoride.[67] Both megakaryocytes and platelets are strongly reactive for ACP activity. Synacril black AN, a textile dye, yields selectively metachromatic staining of the acid mucopolysaccharides in megakaryocyte cytoplasm (Figure 61.12a,b).[87] Acetylcholinesterase (ACH) is another marker that primarily identifies megakaryocytes and platelets in dogs, cats, and rats.[84] Platelets are negative for LAP and SBB.

Erythroid Cells

Reactivity of erythroid cells to routine cytochemical stains is observed infrequently. Examples include a diffuse blush of positive reactivity to PER and SBB techniques. ACP, beta-glucuronidase, PAS (Figure 61.11), and alpha-naphthyl butyrate esterase techniques may result in diffuse granular staining of various stages of erythroid precursors.[53,54,170]

Mast Cells

Canine and feline mast cells exhibit variable reactivity with CAE, PAS, Alcian blue, TB, and OEN (Figure 61.7b).[81] Mast cells, like basophils, are negative for PER and SBB reactivity. LAP activity is not present in canine and feline mast cells, whereas ACP reactivity results only in weak staining. NSE reactivity may have variable intensity in mast cells from some species like the pig, dog, and cat.

CYTOCHEMICAL STAINING PRINCIPLES AND DIAGNOSTIC APPLICATION

A listing of selected cytochemical stains and their methodologies can be found in the 5th edition (2000). Notably, other methods and kits may be used to obtain similar results, including double staining techniques (Figure 61.5). The cytochemical principle and diagnostic

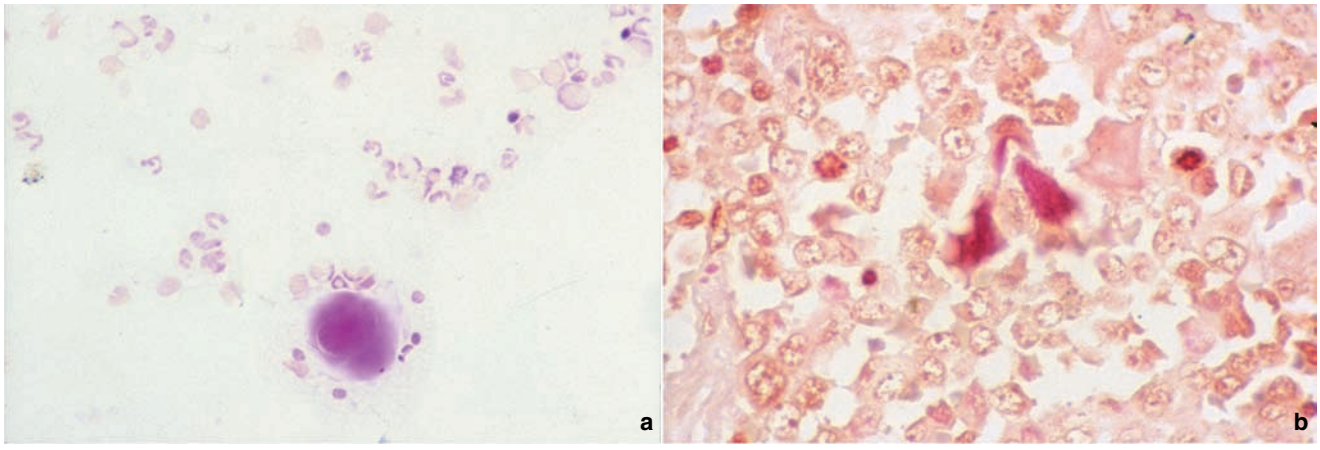


FIGURE 61.12 (a) Feline megakaryocyte with magenta reaction stained with Synacril black AN (Megacolor). Bone marrow. 40× objective; (b) Immature and mature megakaryocytic precursor cells stain positive with Synacril black AN (Megacolor) in canine acute megakaryoblastic leukemia. Bone marrow histopathology. 100× objective.

application for each staining target are described in the following text and summarized in Table 61.3.

Myeloperoxidase

Peroxidase staining is used to identify myeloid cells, particularly neutrophils, eosinophils, and monocytes in most mammals (Figure 61.2a). The PER enzymes are sensitive to tissue processing; therefore, enzymatic reactivity is demonstrable only in fresh tissues and not in paraffin-embedded tissues. PER reactivity diminishes over time, especially in samples older than 2 weeks of age. PER reactivity may be less intense if smears are over 3 days old.[66] The most sensitive method to detect PER activity involves benzidine compounds that are potentially carcinogenic.(39) Copper salts enhance the benzidine reaction producing a gray-black product rather than an orange-brown result. An acceptable alternative substrate to diaminobenzidine tetrahydrochloride is 3-amino-9-ethylcarbazole.[80]

TABLE 61.3 Summary of cytochemical staining in 171 selected cases of nonlymphoid malignancies.[a]

Disease/subtype/ species	# cases*	PER	SBB	CAE	LAP	NAE	NBE	Fluoride	ACP	ACP/T	BG	PAS
Acute undifferentiated leukemia												
Cat	1	neg	neg	ND	ND	ND	pos/neg	ND	pos/neg	ND	pos	ND
Myeloblastic												
Dog	5	pos	pos/neg	pos	pos	neg	neg	ND	pos	neg	ND	neg
Cat	7	pos/neg	pos	pos	pos/neg	neg	neg	neg	pos	pos	ND	pos
Horse	1	pos	pos	pos	neg	ND	neg	ND	pos	ND	ND	pos
Swine	6	pos	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Eosinophilic												
Cat	2	neg	neg	pos/neg	pos	ND	pos/neg	ND	pos	pos	ND	pos
Horse	1	ND	pos	ND	ND	ND	ND	ND	ND	ND	ND	ND
Basophilic												
Dog	2	neg	neg	pos/neg	neg	pos	neg	ND	pos	ND	ND	pos/neg
Cat	1	neg	neg	pos	pos/neg	pos	ND	ND	pos	ND	ND	ND
Myelomonocytic												
Dog	20	pos/neg	pos	pos	pos	pos	pos	neg	pos	neg	ND	pos/neg
Cat	2	pos	pos	pos	pos/neg	pos	pos	neg	neg	ND	ND	pos/neg
Horse	4	pos	pos	pos	neg	pos	pos	neg	ND	ND	ND	ND
Bovine	1	neg	pos	pos	ND	pos	ND	ND	ND	ND	ND	ND
Monoblastic												
Dog	10	pos/neg	pos/neg	neg	pos/neg	pos	ND	neg	ND	ND	ND	pos/neg
Cat	3	neg	neg	neg	neg	pos	pos	neg	neg	neg	ND	pos
Horse	1	ND	ND	pos/neg	pos/neg	pos	ND	ND	ND	ND	ND	ND
Erythroleukemia												
Cat	4	neg	pos	pos	pos	neg	pos/neg	neg	pos	pos	ND	pos/neg
Cat (erythroid predominance)	4	neg	neg	pos/neg	pos/neg	ND	neg	ND	pos/neg	pos/neg	ND	pos/neg
Dog (erythroid predominance)	1	neg	pos/neg	neg	pos/neg	ND	ND	ND	pos	pos	ND	neg
Megakaryoblastic or ET												
Dog	10	neg	pos/neg	pos/neg	neg	pos	pos/neg	pos/neg	pos/neg	neg	ND	pos
Cat	2	neg	ND	neg	neg	neg	neg	ND	neg	ND	ND	pos
Histiocytic sarcoma												
Dog	3	neg	pos/neg	pos	neg	pos	neg	ND	pos	pos/neg	ND	pos
Horse	1	ND	ND	ND	ND	ND	pos	ND	ND	ND	ND	ND
Cat	2	ND	ND	ND	ND	ND	pos	neg	pos	ND	ND	ND
Mast cell neoplasia												
Dog	39	neg	neg	pos	neg	ND	pos/neg	ND	pos/neg	ND	ND	pos/neg
Cat mast cell leukemia	2	neg	neg	pos	neg	ND	pos/neg	ND	pos/neg	pos/neg	ND	pos/neg
Goat	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	pos
Cow	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Pig	4	ND	ND	pos	ND	ND	pos	ND	pos	ND	ND	pos/neg
Horse	30	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a]PER = peroxidase; SBB = Sudan Black B; CAE = chloroacetate esterase; LAP = leukocyte alkaline phosphatase; NAE = naphthyl acetate esterase; NBE = naphthyl butyrate esterase; ACP = acid phosphatase; ACP/T = acid phosphatase with tartrate; BG = beta-glucuronidase; PAS = periodic acid–Schiff; ET = essential thrombocythemia; neg = no reaction; pos = easily detectable; pos/neg = weak or occasional reactivity.

The blast cells within the bone marrow may stain positive with PER for myeloblastic leukemia (with or without maturation), myelomonocytic leukemia, and erythroleukemia. Rare positive staining in monoblastic leukemia (without maturation) is possible. In humans, PER activity may be used as a monitoring tool during the treatment for myeloid leukemia. In a subset of human patients, neutrophil MPO activity can be used as a surrogate for MPO activity in neoplastic myeloblasts, and, in these patients, an increase in neutrophil MPO activity may presage emergence from remission.

Lymphoid malignancies traditionally lack PER reactivity; however, occasional reports in human medicine have identified molecular expression of the enzyme.[74] There are limited reports of PER activity in veterinary hematopoietic neoplasia. One case detected myeloperoxidase positivity in a dog with acute megakaryoblastic leukemia.[55] PER staining was helpful in the diagnosis of several canine, feline, equine, and porcine cases of myeloblastic leukemia (Figure 61.2a) and myelomonocytic leukemia (Figure 61.5).[28,37,65-67,86,116,138]

Sudan Black B

Lipids (including phospholipids, neutral fats, and sterols) within neutrophils/heterophils, eosinophils, and occasional monocytes/azurophils are identified by a gray-black reaction using SBB dye (Figure 61.3a). The mechanism of cellular staining may involve an enzymatic reaction because eosinophils from human and feline patients that have eosinophil PER deficiency do not stain after SBB application. The sudanophilic material within cells is stable with heat or storage. SBB activity has been demonstrated in cases of canine, feline, equine, and bovine leukemia, including eosinophilic leukemia, monocytic leukemia, myelomonocytic leukemia, and erythroleukemia.[28,53,54,64,100,117,134,138,177] It is also expected that the blast cells in myeloblastic leukemia (with or without maturation) should stain positively with SBB (Figure 61.3b). Lymphoid malignancies are traditionally negative for SBB staining; however, one case of canine acute lymphoblastic leukemia displayed slight sudanophilia.[54] One case of canine malignant histiocytosis (now termed disseminated histiocytic sarcoma) had positive SBB reactivity.[54]

Chloroacetate Esterase

Referred to as specific esterase, the CAE reaction is likely produced by a group of enzymes. This reaction is considered more specific for neutrophils than PER or SBB, although the CAE technique is less sensitive (Figure 61.4b). CAE reactivity is strongest from the promyelocyte to the segmented stage with weaker reactivity observed in myeloblasts (Figure 61.4a). This enzyme is found within the primary and secondary granules of neutrophils and within the specific granules of basophils. Although CAE is generally specific for neutrophils, other cell types have weak reactions. For example, CAE activity is found in mast cells and basophils and mild positivity may be seen within the monocytes of horses and ruminants. CAE positivity is described in the monocytes, heterophils, eosinophils, and basophils of the yellow-bellied slider, and mild reactions are described in normal canine and feline megakaryocytes.[53,54]

In disease, CAE activity can be detected in myeloblastic leukemia (with or without maturation), myelomonocytic leukemia (Figures 61.4a and 61.5), erythroleukemia, basophilic leukemia, and mast cell tumors. One case of feline eosinophilic leukemia was positive for CAE, and occasional weak to moderate staining was noted in three cases of canine megakaryoblastic leukemia.[40,53,109] One case of weak CAE staining occurred in a horse with monocytic leukemia.[31] Moreover, CAE activity was helpful in the diagnosis of several canine, feline, bovine, equine, porcine, camelid, and saurian cases of myeloblastic leukemia, basophilic leukemia, myelogenous leukemia, myelomonocytic leukemia, malignant histiocytosis, mast cell tumor, and erythroleukemia.[12,14,19,25,28,53,54,64,106,116,134,135,138,150,154,161,165,176] Although lymphoid malignancies generally lack positive reactions with CAE, one case each of canine and feline acute lymphoblastic leukemia (ALL), similar to human ALL, displayed a slight reaction.[53] In addition, CAE staining was noted in several feline lymphoma cases, many of which involved large granular lymphocytes.[141,175]

The enzymatic reactivity detected during the CAE staining results from proteases such as chymotrypsin and elastase. During staining, these enzymes hydrolyze esters linked to the substrate (naphthol AS-D chloroacetate) which then complex with a diazonium salt to produce an insoluble, colored product.[113] The CAE esterase group can be distinguished from NSE using gel electrophoresis and is considered specific due to its resistance to sodium fluoride inhibition. CAE may be performed together with a nonspecific esterase to simultaneously detect neutrophils (CAE-positive) and monocytes (NSE-positive) (Figure 61.5).[180] The CAE cytochemical reaction is very stable and may be used in paraffin-embedded tissues.

Leukocyte Alkaline Phosphatase

Enzymes with LAP activity hydrolyze naphthol AS-BI phosphate at an alkaline pH, releasing naphthol to complex with a diazonium salt, forming an insoluble, colored product. This group of enzymes is present in granulocytes (Figure 61.5), heterophils, osteoblasts, histiocytes, vascular endothelial cells, and a subset of lymphocytes.[11,51,120,148] The extent and distribution of LAP reactivity in neutrophils vary with the species. In human neutrophils, LAP activity is present in a subset of plasma-membrane-associated granules and is detectable only in mature cells. In humans, LAP reactivity can be increased in leukocytosis or leukemoid reaction, but decreased in neoplastic neutrophils. Similar LAP reactivity is found in rabbits, rats, guinea pigs, horses, cattle, sheep, goats, and monkeys. In dogs and cats, mature neutrophils are devoid of LAP activity, whereas enzymatic activity is present in myeloblast and promyelocyte stages. LAP activity is absent in human eosinophils,

but is strong in equine eosinophils, moderate in bovine eosinophils, and negative to moderate in canine and feline eosinophils (Figure 61.5). In each instance, enzymatic activity is usually observed in the cytoplasm between the specific granules. A subset of B-lymphocytes, present in the mantle zone of lymph nodes and spleen, exhibits cellular membrane staining with LAP technique in people, dogs, rats, and mice.[120,136,156]

Although LAP may be found in neutrophil precursors, it is not considered lineage-specific in the characterization of canine and feline hematopoietic neoplasia. LAP activity has been described in myeloblastic leukemia (with or without maturation), myelomonocytic leukemia, erythroleukemia, eosinophilic leukemia, and basophilic leukemia (Figure 61.9).[25,53,54,64–66,101,156,160] Additionally, LAP staining has been described in CD34+ canine acute lymphoblastic leukemia, (presumed monoblastic) myeloid leukemia, canine monocytic leukemia, and equine monocytic leukemia.[31,79,156(47)] Lymphoid malignancies generally lack LAP activity; however, rare positive reactions have been described in both B-cell and T-cell lymphoid malignancies from dogs and pigs.[54,136,156,161,174] In summary, LAP activity is not lineage-specific and other diagnostic methods, including morphology, cytochemistry, and flow cytometry, are necessary for lineage assignment.

Periodic Acid–Schiff

In the PAS technique, periodic acid oxidizes carbohydrates to form aldehydes that react with Schiff's reagent to produce a dye product. The bright red or magenta reaction product is focal, diffuse, or granular. Within cells, stainable substrates include glycoproteins, mucoproteins, glycolipids, and high-molecular-weight carbohydrates. By including a diastase digestion step, glycogen staining can be eliminated, which indicates that a positive reaction reflects the presence of mucoproteins or other materials. Depending on the species, PAS may be found within neutrophils, heterophils, eosinophils, basophils, monocytes, azurophils, lymphocytes, platelets, thrombocytes, and erythrocytes (Figure 61.11). In circulating cells, glycogen is the most stained substance. Mature forms of neutrophils, platelets, and megakaryocytes stain the most intense, whereas monocytes, eosinophils, and basophils produce a weak positive reaction. Myeloblasts and promyelocytes are PAS-negative and PAS-weakly positive, respectively.[53,54] Lymphocytes usually do not stain positive.

Diagnostically, PAS staining is used to differentiate granulocytic or megakaryocytic precursors from lymphoid precursors. Specific subtypes of PAS-positive myeloid leukemia include myeloblastic leukemia, myelomonocytic leukemia, monocytic leukemia, eosinophilic leukemia, basophilic leukemia, erythroleukemia or erythroleukemia with erythroid predominance, and megakaryoblastic leukemia.[40,53,54,67,106,134,138,143] Mast cell neoplasia in the dog, cat, goat, pig, and gecko are PAS-positive.[14,135,150] Infrequent or weak PAS staining has occurred with some feline and equine lymphoid malignancies.[53,175] Positive staining has been documented in canine histiocytic sarcoma.[54]

Nonspecific Esterases

NSE comprises a group of enzymes capable of hydrolyzing esters to liberate naphthol products that bind with a coupler dye. These esterases demonstrate a wide range of substrate specificity and stain pH environment for various blood cells. Therefore, they are termed nonspecific. However, many of the esterases, as determined by polyacrylamide gel electrophoresis, are cell-specific. Monocytes, granulocytes, and a subset of lymphocytes exhibit unique patterns of reactivity. A coupler-dependent, red to pink-brown diffusely granular pattern is reported in monocytes (Figure 61.5) and macrophages, whereas focal to punctate staining is seen in T-lymphocytes.[9,179,181] The NSE activity in monocytes, histiocytes, macrophages, megakaryocytes, platelets, and plasma cells is inhibited by the addition of sodium fluoride, whereas T-lymphocytes remain stained.[47,67] The difference in inhibition relates to the location of the enzyme in these cells.[26,46,71,174] The activity of these enzymes is unstable and sensitive to heat, fixatives, and storage. Accordingly, fresh blood smears or tissue imprints are preferred to paraffin-embedded tissues.

Cell specificity for naphthyl acetate esterase (NAE) activity may be demonstrated by altering the pH of the medium, the staining temperature, or the length of incubation.[26,97] For example, monocytes are best stained in an acidic medium, although both granulocytes and monocytes may stain in a neutral medium. Lymphocytes stain best in acid conditions with prolonged incubation. Compared with NBE, NAE is more sensitive and less specific in identifying mammalian monocytes, macrophages, and T-lymphocytes. For example, in Murray cod, NAE was positive in heterophils, basophils, lymphocytes, and monocytes, while NBE was negative for all blood cells.[148] Sodium fluoride inhibition is necessary to differentiate the cell types when using NAE in mammals; however, it is rarely used with NBE owing to the latter's greater specificity for monocytes. Notably, in the case of heterophils, basophils, and thrombocytes from yellow-bellied sliders, these cells remained positive following fluoride inhibition with NBE.[69] Caution should be used with interpretation of nonmammalian blood cells, as the presence of enzymes does not necessarily relate to cell function or identification.

Specific subtypes of myeloid leukemia that demonstrate NSE activity include myelomonocytic leukemia, monoblastic leukemia, megakaryoblastic leukemia, basophilic leukemia (using ANAE), eosinophilic leukemia (using NBE), undifferentiated leukemia, and erythroleukemia (using NBE).[25,53,61,106] In addition, occasional positive NBE reactions have been documented in canine, feline, and porcine mast cell tumors.[14,53,135] Positive staining has been documented in cases of canine, feline, and equine histiocytic neoplasia.[42,79,96,176] Positive staining is occasionally reported in lymphoid neoplasms from dogs, cats, cattle, and sheep.[30,53,54,62,66,130,136,141,161,174,175] In summary, NAE has been helpful in diagnosing mammalian myelomonocytic leukemia, monocytic leukemia (Figure 61.8), megakaryoblastic leukemia, blast cells of essential

thrombocythemia, and histiocytic neoplasia, whereas NBE has been helpful in diagnosing myelomonocytic leukemia, monocytic leukemia, and histiocytic neoplasia.[19,28,31,33,37,42,53,54,64,66,67,72,96,112,116,127,134,136,171,176,177]

Acid Phosphatase

ACP comprises a group of enzymes that hydrolyze phosphate esters in an acidic environment. Many cell types stain in the ACP reaction, including lymphocytes, monocytes, histiocytes, granulocytes (particularly eosinophils), platelets, megakaryocytes, plasma cells, and erythroid precursors.[15,54] The reaction product is diffuse in monocytes, whereas histiocytes stain more intensely. A single focal or punctate reaction product in the Golgi area occurs in canine T-lymphocytes and lymphocytes with NK-cell activity (Figure 61.10).[181] Tartrate-resistant acid phosphatase was found in eosinophils and basophils from normal dogs and cats.[53,54] As all ACP isoenzymes are sensitive to tissue processing and heat, the ACP technique is recommended only for fresh cytologic or plastic-embedded materials (Figure 61.13) and not for paraffin-embedded tissues.

Specific subtypes of myeloid leukemia that display ACP positivity include myeloblastic leukemia, myelomonocytic leukemia, eosinophilic leukemia, basophilic leukemia, erythroleukemia, megakaryoblastic leukemia, and undifferentiated leukemia(37).[53,54,109,138] Additionally, occasional positive ACP reactions have been documented in canine, feline, and porcine mast cell tumors and histiocytic neoplasia.[14,42,53,54,135,175] Positive ACP staining may also be found in lymphoid malignancies from dogs, cats, cattle, sheep, and pigs.[30,53,54,130,136,141,151,161,175,182]

Beta-Glucuronidase

βG is a hydrolytic enzyme with a mechanism and staining pattern similar to the esterases. βG hydrolyzes the staining substrate and liberates naphthol compounds which subsequently bind to a colored dye. Enzymatic activity is found in blood lymphocytes, monocytes, and granulocytes (Figure 61.14). Both monocytes and neutrophils display weak, diffuse, cytoplasmic reactivity, whereas T-lymphocytes produce a focal or distinct granular reaction.[99] In cats, βG activity appears in monocytes (which demonstrate the strongest staining), granulocytes, lymphocytes, and platelets.[170] Weak positive reactivity has been observed in neutrophils/heterophils of channel catfish and Murray cod.[148,185] Murray cod fish demonstrate βG reactivity in lymphocytes, monocytes, and basophils.[148] In disease, βG staining has been documented in a case of feline acute undifferentiated leukemia and in cats with large granular lymphocyte lymphoma.[30,61,175]

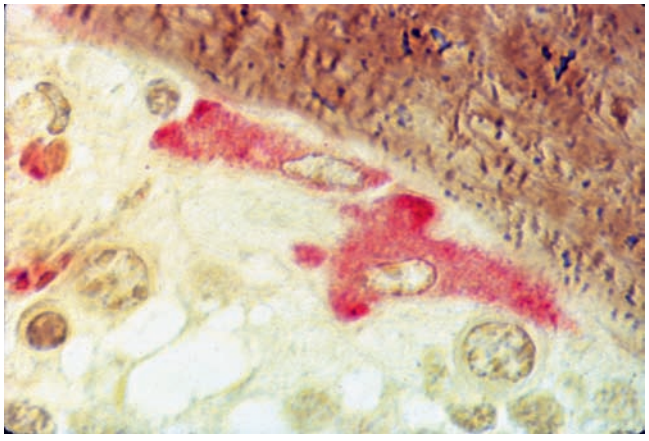


FIGURE 61.13 Bovine bone marrow with diffuse positive ACP reaction in fibrohistiocytic cells. Plastic-embedded section. 100× objective.

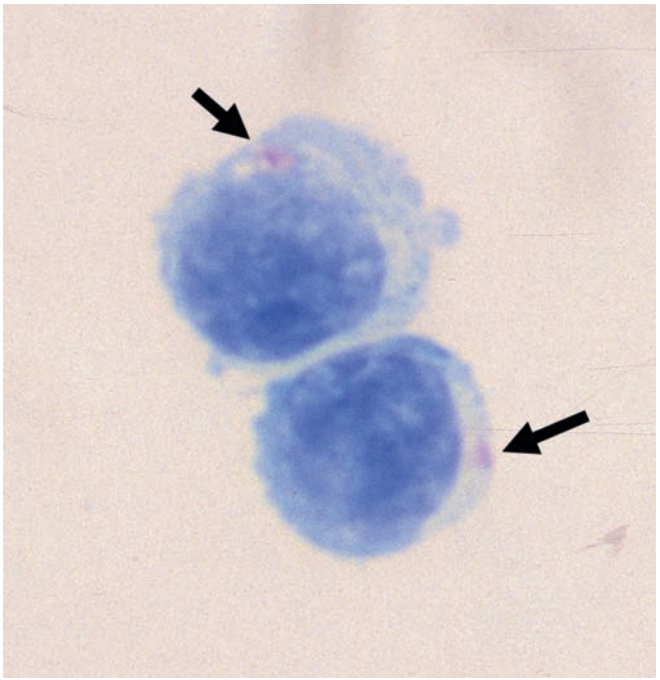


FIGURE 61.14 Focal stain reaction (arrows) with beta-glucuronidase in two canine lymphocytes. 100× objective.

Miscellaneous Stains

Toluidine Blue

This common histologic stain is helpful for the evaluation of basophil and mast cells from many species.[69,81] TB is a basic dye that reacts with the acid mucopolysaccharides to form metachromatic complexes that appear red-purple. Basophils stain readily in an acidic TB, but not in a neutral TB.

In disease, TB staining aided in the diagnosis of two cases of canine basophilic leukemia, a case of basophilic leukemia in a snake, and for the diagnosis of mast cell tumors from the dog, cat, goat, cow, pig, and horse.[14,29,102,105,106,135,143,147,150] Several cases of large granular lymphoma and leukemia, including in the dog, cat, horse,

and ferret, were evaluated with TB, but only feline case demonstrated a positive reaction.[23,30,174,175]

Omega-Exonuclease

OEN is an alkaline phosphodiesterase found within the cytoplasm of basophils and mast cells and their precursors in several species, particularly humans, dogs, cats (Figure 61.7a), and primates. Positive OEN reactivity produces diffuse, red-brown, granular cytoplasmic deposits, but does not stain the specific granules; therefore, it is said that OEN activity is more helpful than TB in identifying poorly differentiated or degranulated mast cells. OEN staining has been used to determine the extent of involvement in one case of basophilia leukemia in the dog.[106] Also, OEN was used to identify mast cells in tumors (Figure 61.7a) from a dog and cat.[135] OEN staining is not found in normal basophils of the horse, cow, goat, pig, or alpaca.[81](17)

Synacril Black AN

Synacril black AN, which was originally a commercial textile dye (Megacolor from Cytocolor, Inc.), stains the acid mucopolysaccharides found in megakaryocytic cytoplasm.[87](35) After fixation with formaldehyde acetic acid ethanol, specimens are pretreated with ribonuclease A (bovine pancreas type III-A) to remove ribonucleic acid. The dye stains the cytoplasm of mature and immature megakaryocytes with a red to violet, metachromatic reaction product (Figure 61.12a). All other cells are pale yellow to brown except eosinophils whose granules appear pink. In one report, normal equine megakaryocytes along with normal and neoplastic canine megakaryocytes (Figure 61.12b) stained magenta.[96]

Immunohistochemical staining using antibodies against human von Willebrand factor (Factor VIII-related antigen) and human platelet glycoprotein (GP IIIa) helped support the characterization of the neoplastic cells in several cases of canine and feline megakaryoblastic leukemia.[32,40,109,128]

Acetylcholinesterase

The enzymatic detection of ACH can be used to identify mature megakaryocytes and platelets from dogs, cats, and rats.[78,84] In addition, ACH is found in human and murine erythroid precursors.[91] ACH staining is accomplished using acetylthiocholine iodide as a substrate and the subsequent reaction product is brown. ACH staining was helpful in the identification of megakaryocytic precursors in canine megakaryoblastic leukemia and essential thrombocythemia.[33,72]

Luna

Luna is a specific stain for eosinophil granules used to identify atypical granulocytes or blast cells with eosinophilic differentiation. It was helpful in confirming the presence of eosinophilic precursors in a case of equine eosinophilic leukemia.[117]

GENERAL PRINCIPLES OF IMMUNOCYTOCHEMISTRY

As with cytochemistry, ICC is a useful ancillary tool to assist with: (1) identifying cell(s) of origin in ambiguous cases of suspected neoplasia; (2) assessing prognostic features of tumor populations; and (3) defining cellular characteristics that may aid in treatment selection. Like other antibody-based immunostaining techniques, ICC is rooted in the detection of specific antigens of interest using natural or synthetic antibodies via chromogenic or fluorescent markers. Detection of these epitopes can occur individually (single-plexed ICC) or in combination (multiplexed ICC) as part of a panel of antibodies.

compared to immunohistochemistry (IHC), advantages of ICC include: (1) collection of samples via minimally invasive fine-needle aspiration (FNA); (2) resampling is easier than biopsy in the case of nondiagnostic results; (3) shorter turnaround time offers the opportunity for near-bedside results; and (4) recognition that certain antibodies, such as some T-lymphocyte and histiocytic markers, are only effective in cytologic preparations.[8,34,57,146,172] As compared to flow cytometry, ICC allows for the simultaneous visualization of cell morphology and pattern of immunostaining (which can be helpful if the cells of interest are scant) and does not require viable cells (allowing slides for ICC to be stored at room temperature or frozen).[73,152]

In clinical scenarios, ICC is often performed on samples collected by FNA (e.g., direct smears, cytospins, and cell blocks) or biopsy impression smears.[49,58,63,89,140,152,163,173,184] Chromogenic ICC can be performed multiple times on decolorized smears, as well as samples previously visualized with Romanowsky-type stains or Papanicolaou.[1,111,119,149,157] However, previously stained slides are subject to autofluorescence, making them unsuitable for fluorescent ICC. In cases with limited slides, single samples can be divided into multiple zones or the smear can be divided using tissue-transfer techniques.[157]

While the technical details and troubleshooting steps of ICC are beyond the scope of this chapter, it is important to note that specific ICC techniques are dictated by: (1) the subcellular location of the epitope of interest; (2) whether antigen retrieval is necessary; (3) whether direct or indirect methods are employed; and (4) whether chromogenic versus fluorescent detection is utilized. Regardless of staining protocol, positive and negative controls must be included to allow for proper interpretation. Negative controls comprise cells that are not expected to show immunoreactivity when incubated with the specific antibody. If staining is seen on the negative control, it is interpreted as nonspecific antibody cross-reactivity and must be considered in the interpretation of any staining on the patient sample. Positive controls are tissues that are expected to show positive immunoreactivity to the antibody of interest. Positive controls affirm that the assay was performed correctly and allow for the comparison of staining intensity

between the positive control and test sample. Laboratories often maintain a bank of positive and negative controls to facilitate their usage. Formalin-fixed paraffin-embedded sections are considered inappropriate ICC control specimens.[58]

The most common disadvantages of ICC include preanalytical variations in sample quality and inadequate slides to complete a full ICC panel.[58,125,131] While the former is challenging to resolve once the sample has arrived at the laboratory, cytospin and cell block techniques offer a valuable solution to the latter scenario.[58,172] For these techniques, cells are collected into transport media and shipped for slide or block preparation (fluid samples may not require transport media). Liquid transport media also allows for the concentration of low cellular samples and enables the removal of problematic, background-causing substances (e.g., protein, carbohydrate, and lipid).

It is strongly recommended that ICC be utilized only as an adjunct to corresponding cytologic and clinicopathologic data.[125,131] As such, one representative Romanowsky-stained slide should be reserved for comparison with ICC slides. If this is not possible, representative images (photomicrographs or whole slide scanning) should be retained. The interpretation of ICC also requires a strict definition of positive or negative immunostaining, which is reliant on the use of appropriate controls. When reporting ICC findings, the interpreting pathologist should define and record the staining intensity and percentage of positive cells.[131] Finally, it is recommended that a standardized panel of antibodies be used to avoid confirmation bias toward a preliminary diagnosis.[58,125]

REACTIONS OF NORMAL HEMATOPOIETIC CELLS

As described in the cytochemistry section, it is important to be aware of the pattern of marker expression in non-neoplastic hematopoietic cells when interpreting their potentially neoplastic counterparts. This information is provided for five common domestic species seen frequently in clinics or utilized for research purposes: canine (Table 61.4), feline (Table 61.5), equine (Table 61.6), bovine (Table 61.7), and porcine (Table 61.8). As can be inferred from these tables, protocols and antibodies for ICC are most commonly validated in dogs and cats. Further studies are needed to expand ICC options into other veterinary species.

GENERAL CONSIDERATIONS FOR DIAGNOSING NEOPLASTIC HEMATOPOIETIC CELLS

Morphologic assessment, via cytology or histology, is key in the complete characterization of neoplastic hematopoietic cells. This morphologic assessment is complemented by tumor cell immunophenotyping, which can supply important diagnostic and/or prognostic information. Antibody-based assays for the immunophenotyping of hematopoietic neoplasms include IHC, ICC, and flow cytometry. Flow cytometry is generally considered the preferred method to diagnose and immunophenotype hematopoietic neoplasms. The non-antibody-based PCR for antigen receptor rearrangements (PARR) assay distinguishes a monoclonal expansion of lymphocytes from a polyclonal population while also providing a T- or B-cell phenotype. Currently, there are no known antibodies available against erythroid progenitor cells in veterinary medicine.

When only cytology slides are available, ICC can be used to immunophenotype neoplastic hematopoietic cells. Like flow cytometry, ICC utilizes antibody panels, including those directed at surface and intracellular lymphoid and myeloid markers (Tables 61.4–61.8) to assign lineage. According to the American Society for Veterinary Clinical Pathology (ASVCP) Quality Assurance Guidelines, institutions offering ICC are expected to validate their staining protocols for each intended species. Such validation, and the adoption of panel-based staining approach (instead of using a single marker), is particularly important when interpreting results for neoplastic cases. One antigen is rarely sufficient to accurately define cell lineage, and aberrant epitope expression can occur with malignant transformation.[44,156] Comprehensive lineage classification in some cases of hematopoietic neoplasia (such as myeloid versus lymphoid) often requires a combination of morphological, flow cytometric, and cytochemical interpretations.

Beyond determining cell of origin, ICC can be used to provide novel biologic and prognostic information for certain types of canine neoplasia. In dogs with lymphoma, there was a small, but statistically significant relationship between tobacco smoke exposure and ICC-detectable Ki-67.[122] Additionally, ICC has shown that paranuclear CD117 (c-kit) staining pattern was significantly increased in high-grade canine mast cell tumors.[145] Several articles have used a variety of molecular techniques to look at markers of proliferation, multidrug resistance, and apoptotic pathways in dogs with lymphoma to aid with prognostic information.[17,114,158] Although many prognostic indicators have been established for ICC use in human samples, the clinical significance and utility of these antigens with ICC are still in their infancy for our veterinary patients.

Summary

Cytochemical staining can be an inexpensive and convenient tool to identify the chemical constituents of blood cells and characterize poorly differentiated forms of myeloid neoplasia. Given the sparse number of cell-specific antibodies, cytochemistry is particularly useful in nondomestic animal species including birds, reptiles, amphibians, fish, and invertebrates. ICC is also a complimentary diagnostic tool that assists with cell identification, prognostication, and selection of treatment options. In the future, cytological diagnosis of hematopoietic

TABLE 61.4 Canine markers of leukocytes/hematopoietic cells detected using ICC and/or flow cytometry.

Antigen	Cell Population	Technique	Comments	References
CD3	T-cell lymphocytes	FCA, ICC	Membrane/cytoplasm staining pattern	5,20,92,43,57,137
CD4	T-cell lymphocytes, monocytes, DCs, neutrophils*	FCA, ICC	T helper subsets,* neutrophils in dogs	34,43,57
CD5	T-cell lymphocytes	FCA, ICC		57
CD8	T-cell lymphocytes	FCA, ICC	Cytotoxic subset, NK cells	34,57
TCRαβ/γδ	T-cell lymphocytes	ICC	Cytotoxic subsets	125
CD1a	T-cell and B-cell lymphocyte subsets, monocytes, DCs	FCA, ICC		7,10,142
CD34	HSCs	ICC		43,92
CD18	All leukocytes	FCA, ICC	Greater intensity on granulocytes and monocytes	7,34
CD45	All leukocytes	FCA, ICC		10,34,57
CD90 (Thy-1)	Lymphocytes, monocytes, HSCs, eosinophils			155
CD20	B-cell lymphocytes	ICC	Membrane/cytoplasm staining pattern	5,137
CD21	B-cell lymphocytes	FCA, ICC	Absent on plasma cells	34,57,92
CD79a and CD79b	B-cell lymphocytes	ICC	Intracellular antigen	5,7,34,43,57,92
BLA36	B-cell lymphocytes, DCs	ICC		125
IgM, IgA, IgG	B-cell lymphocytes	ICC		92
Pax5	B-cell lymphocytes	ICC	Nuclear staining	137
MUM1/IRF-4	Plasma cells, B-cell lymphocytes	ICC	Nuclear ± cytoplasmic staining	132,137
MHCII	Monocytes, DCs, T- and B-cell lymphocytes	FCA, ICC	Membrane/cytoplasm staining pattern	34,92,137
CD1c	Monocytes, DCs	ICC		43,142
CD11b	Granulocytes, monocytes, macrophages	ICC		43,56
CD11c	Granulocytes, monocytes, DCs	ICC		10,43,57,142
CD11d	T-cell lymphocyte subset, monocytes, and histiocytes	FCA, ICC	T cells in splenic red pulp and large granular lymphocytes	10,43,142
CD14	Monocytes, macrophages	FCA, ICC		7,143,155
CD41	Platelets, megakaryocytes	ICC		125
CD42b	Megakaryocytes	ICC		56
CD61	Platelets, megakaryocytes	FCA, ICC		56,155
Lysozyme	Granulocytes, monocytes, macrophages	ICC	Cytoplasm (granular) staining	73,137
MPO	Granulocytes, monocytes	ICC		43,56
CD117 (c-kit)	Mast cells	ICC		145

*FCA, flow cytometric analysis; HSCs, hematopoietic stem cells; DCs, dendritic cells; MPO, myeloperoxidase.

TABLE 61.5 Feline markers of leukocytes/hematopoietic cells detected using ICC and/or flow cytometry.

Antigen	Cell population	Technique	Comments	References
CD3	T-cell lymphocytes	ICC	Membrane/cytoplasm staining pattern	60,133,137
CD4	T-cell lymphocytes, activated monocytes, macrophages, DCs, neutrophils	FCA, ICC		59,60,133
CD5	T-cell lymphocytes	FCA, ICC		21
CD8	T-cell lymphocytes	FCA, ICC	Cytotoxic subset, NK cells	489
CD103	Intra-epithelial lymphocytes (IELs)	FCA		139
CD1a	T-cell and B-cell lymphocyte subsets, monocytes, DCs	ICC		133
CD1c	Monocytes, DCs	ICC		60,133
CD14	Monocytes, macrophages	FCA, ICC		59,133

TABLE 61.5 (Continued)

Antigen	Cell population	Technique	Comments	References
CD18	All leukocytes	FCA, ICC	Greater intensity on granulocytes and monocytes	59,60,133
CD45	All leukocytes	FCA		21
CD20	B-cell lymphocytes	ICC	Membrane/cytoplasm staining pattern	133,137
CD21	B-cell lymphocytes	FCA, ICC	Absent on plasma cells	60,133
CD22	B-cell lymphocytes	FCA	Absent on plasma cells	21
CD79a and CD79b	B-cell lymphocytes	ICC	Intracellular antigen	60,133
BLA36	B-cell lymphocytes, dendritic cells	ICC		60,133
Pax5	B-cell lymphocytes	ICC	Nuclear staining	133,137
MUM1	Plasma cells, B-cell lymphocytes	ICC	Nuclear ± cytoplasmic staining	137
MHCII	Monocytes, DCs, T- and B-cell lymphocytes	FCA, ICC	Membrane/cytoplasm staining pattern	59,60,133
CD11b	Granulocytes, monocytes, macrophages	FCA, ICC		21
CD11d	T-cell lymphocyte subset, monocytes, and histiocytes	ICC		133
Lysozyme	Granulocytes, monocytes, macrophages	ICC	Cytoplasmic (granular) staining	137
MPO	Granulocytes, monocytes	ICC		22
CD117/c-kit	Mast cells	ICC		22
Tryptase	Mast cells	ICC		22

FCA, flow cytometric analysis; DCs, dendritic cells; MPO, myeloperoxidase.

TABLE 61.6 Equine markers of leukocytes/hematopoietic cells detected using ICC and/or flow cytometry.

Antigen	Cell population	Technique	Comments	References
CD2	T-cell lymphocytes	FCA		12,126
CD3	T-cell lymphocytes	FCA, ICC		12,38,50,167
CD4	T-cell lymphocytes, DCs	FCA		12,38,167
CD5	T-cell lymphocytes, B-cell lymphocytes*	FCA	*Considered pan-T-cell marker, but shown to be variably expressed in B cells in certain species	12,21,38,126,167
CD8	T-cell lymphocytes	FCA	Cytotoxic subset	12,38,167
FoxP3	T regulatory cells	FCA		22,126,167
CD21	B-cell lymphocytes	FCA	Membranous antigen	12,107,110,126
CD79a and CD79b	B-cell lymphocytes	FCA, ICC	Intracellular antigen	38,50,107
BLA36	B-cell lymphocytes, macrophages, DCs	ICC		94
IgM, IgG	B-cell lymphocyte subset(s)	FCA		12,126
MHCI	All leukocytes	FCA		12
MHCII	Lymphocytes, monocytes, DCs	FCA		12,110,126
CD11a	All leukocytes, DCs	FCA		12
CD14	Monocytes	FCA		12,110,153
CD44	Monocytes, DCs, lymphocytes	FCA, ICC		98,103
CD68	Macrophages	ICC		18
CD163	Monocytes	FCA, ICC	Variable expression	94,108,153
CD172a	Monocytes, granulocytes	FCA		12
CD204	Monocytes	FCA, ICC		94,153
CD41/61	Platelets	FCA		77,157
CD13	Granulocytes, monocytes	FCA		48,129
CD90 (Thy-1)	Granulocytes	FCA		129
MPO	Neutrophils	FCA	Intracellular expression	70

*FCA, flow cytometric analysis; DCs, dendritic cells; MPO, myeloperoxidase.

TABLE 61.7 Bovine markers of leukocytes/hematopoietic cells detected using flow cytometry that may be applicable to ICC.

Antigen	Cell population	Technique	Comments	References
CD3	T-cell lymphocytes	FCA		20
CD4	T-cell lymphocytes	FCA		13,20,75
CD5	T-cell lymphocytes, B-cell lymphocytes*	FCA	* Variably expressed in B-cell lymphocytes	20,118
CD8	T-cell lymphocytes	FCA		13,20,118
CD335	NK cells, some γδT cells	FCA		13,75,83
WC1	γδT-lymphocytes	FCA		13,75,164
CD21	B-cell lymphocytes	FCA		13,20,75
CD40	APCs	FCA		41
CD80	APCs	FCA		41
MHCII	APCs	FCA		41
CD11b	Monocytes, neutrophils	FCA		20
CD11c	Monocytes	FCA		52
CD14	Monocytes	FCA		20,52,121
CD16	Monocytes	FCA	Variable expression	52
CD163	Monocytes	FCA		52
CD172a	Monocytes, granulocytes	FCA	Considered to be superior monocytic marker over CD14	45,52,75,124
CD209	Monocytes	FCA		52
CD44	Neutrophils, monocytes	FCA		20
CH138A	Granulocytes	FCA		20,45,121
MPO	Monocytes, lymphocytes*	FCA	* Source suggests distinguishing monocytes that have a higher MPO-fluorescence from lymphocytes with a lower MPO signal	45

*FCA, flow cytometric analysis; NK, natural killer; APCs, antigen-presenting cells; MPO, myeloperoxidase.

TABLE 61.8 Porcine markers of leukocytes/hematopoietic cells detected using flow cytometry that may be applicable to ICC.

Antigen	Cell population	Technique	Comments	References
CD2	T-cell lymphocytes,* NK cells, some B-cell lymphocytes	FCA	*TCR-αβT cells	123
CD3	T-cell lymphocytes	FCA		36,115,165
CD4	T-cell lymphocytes	FCA		36,165
CD5	T-cell lymphocytes, B-cell lymphocytes*	FCA	* Variably expressed in B-cell lymphocytes	165
CD8α	T-cell lymphocytes, NK cells	FCA		36,115,165
CD18	All leukocytes	FCA		36
CD45	All leukocytes	FCA		36,165
CD45RA and CD45RC	T-cell lymphocytes, B-cell lymphocytes	FCA		115,165
WC1	γδT lymphocytes	FCA		35
CD21	B-cell lymphocytes	FCA		165
CD163	Monocytes	FCA	Variable expression	115
CD14	Monocytes	FCA		36
CD16	Monocytes	FCA		36
SLA-II	Monocytes	FCA	Variable expression	36,115
Tük4	Monocytes	FCA	Variable expression	115
SWC1 (CD52)	Monocytes,* granulocytes, some T-lymphocytes	FCA	*Expressed at higher levels on monocytes than mature macrophages, used as marker of differentiation	36,95,144
SWC3 (CD172a)	Monocytes, granulocytes	FCA	Identified as pan monocyte marker in swine	36,75,165
CD11a	Leukocytes	FCA		36
CD11b	Monocytes, macrophages, granulocytes, NK cells	FCA		165
CD62	Platelets			93

*FCA, flow cytometric analysis; SWC, swine workshop cluster; SLA, swine leukocyte antigen (major histocompatibility complex); NK, natural killer.

neoplasms in animals may benefit from the development and validation of novel ICC assays (when flow cytometry is not a possibility) for subtyping and characterizing the biologic behavior of neoplastic cells.

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CHAPTER 62

Determination of Clonality

YUKO GOTO-KOSHINO and HAJIME TSUJIMOTO

Introduction
Polymerase Chain Reaction for Antigen Receptor Rearrangement (PARR)
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  Procedures for PARR
  Samples for PARR
  Interpretation of the Results of PARR
  Sensitivity and Specificity of PARR
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X-Chromosome Inactivation Pattern (XCIP) Analysis
  Principles of XCIP Analysis
  Procedures for XCIP Analysis
  Application of XCIP Analysis for Diagnosis

Acronyms and Abbreviations

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; AR, androgen receptor; CDR3, complementarity-determining region 3; CIAR, corrected inactive allele ratio; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; DLBCL, diffuse large B-cell lymphoma; EATL, enteropathy-associated T-cell lymphoma; ET, essential thrombocythemia; FCM, flow cytometry; FeLV, feline leukemia virus; FFPE, formalin-fixed paraffin-embedded; FNA, fine-needle aspiration; HUMARA, human androgen receptor gene assay; IGH, immunoglobulin heavy chain; IGK, immunoglobulin kappa light chain; IGL, immunoglobulin lambda light chain; IHC, immunohistochemistry; Kde, kappa-deleting element; MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasm; NK, natural killer; PARR, polymerase chain reaction for antigen receptor rearrangement; PCR, polymerase chain reaction; PV, polycythemia vera; TCRG, T-cell receptor gamma chain; WHO, World Health Organization; XCIP, X-chromosome inactivation pattern.

INTRODUCTION

Clonal cell proliferation is a fundamental characteristic of neoplasms. Neoplastic cells are essentially derived from a single transformed cell, whereas normal or reactive tissues are generally composed of polyclonal cell populations. Assessment of clonality can provide information that may be useful for diagnosis and management of proliferative disorders.

Several types of molecular analytical systems have been developed for assessment of cell clonality in human neoplastic diseases. When a specific karyotype aberration (resulting in fusion gene synthesis, etc.) or gene mutation fundamentally associated with the tumorigenesis is identified in a neoplasm, detection of such an aberration provides a precise tool with which its clonal origin may be demonstrated. The Philadelphia chromosome carries the *BCR–ABL1* fusion gene, a representative genetic aberration typical of chronic myeloid leukemia (CML) and several other hematologic malignancies in humans, and is utilized for the diagnoses as well as the marker of progression/regression.[33] Genetic aberrations in each subtype of lymphoid and hematopoietic neoplasms in humans have been extensively explored and integrated into the World Health Organization (WHO) classification system.[74]

Similar genetic mutations have been identified in veterinary hematopoietic neoplasms. *TP53*, which is the most commonly mutated gene in human malignancies, has been described as a target of mutations in canine lymphoma.[41] Deletion or mutation of *p16* has also been reported in a large proportion of canine lymphomas.[18] Moreover, recent studies using RNA-seq and exome sequencing have revealed mutations in *TRAF3*, *FBXW7*,

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POT1, and *PTEN* in dogs affected with lymphoma.[6,15] Meanwhile, several other studies have reported the presence of karyotype aberrations[3,4,12] and gene mutations[83] in a variable proportion of dogs with hematopoietic tumors. However, specific genetic aberrations that may be used as diagnostic tools have not yet been identified in lymphoid and hematopoietic tumors in dogs. Studies related to genetic changes in these tumors in cats are scant.

Based on the abovementioned research background, molecular clonality assessments in dogs and cats have been conducted using PCR for antigen receptor rearrangement (PARR) and X-chromosome inactivation pattern (XCIP) analyses, because knowledge of specific genetic aberrations is not required to perform these tests. In this chapter, clinical utility of PARR and XCIP analyses in the determination of cell clonality in lymphoid and hematopoietic neoplasms of dogs and cats is described.

POLYMERASE CHAIN REACTION FOR ANTIGEN RECEPTOR REARRANGEMENT (PARR)

Principles of PARR

The PARR assay determines the clonal or heterogeneous (polyclonal) nature of lymphoid cell populations. It is based on the detection of V–D–J rearrangement of antigen receptor genes such as T-cell receptor gamma chain (*TCRG*) and immunoglobulin heavy chain (*IGH*), which results in the generation of a unique sequence with the ability to bind numerous antigens (Figure 62.1).[35]

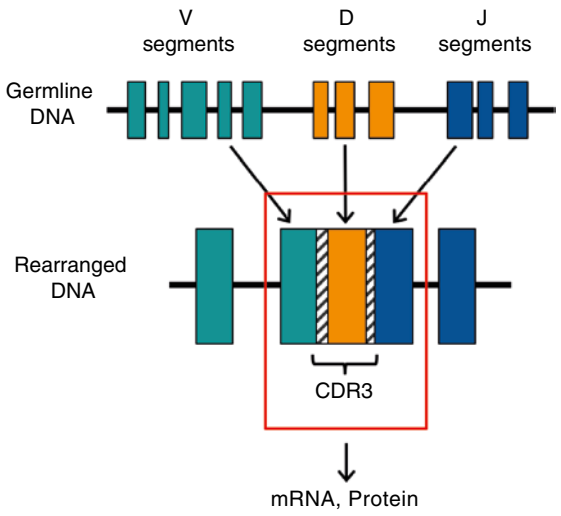


FIGURE 62.1 Schema of antigen receptor (*IGH*) gene rearrangement. *IGH* locus on germline DNA contains multiple different variable (V), diversity (D), and joining (J) segments. The sequences of randomly selected V, D, and J segments are rearranged. At the junction site of each segment, deletion and random insertion of nucleotide(s) occurs during rearrangement, leading to the development of the complementarity-determining region 3 (CDR3). The rearranged antigen receptor gene (e.g., *IGH*) is transcribed and translated to the protein as an antigen receptor.

Diagnoses of lymphoproliferative diseases are traditionally based on histopathology supported by immunohistochemistry (IHC). Cytology, preferentially used in a clinical environment, is useful for diagnosing typical subtypes of lymphoma. However, in a certain number of cases, resection biopsy of the lymph node or other lesions to obtain the histopathological finding is needed for a definitive diagnosis and subtype classification. Flow cytometry (FCM) is increasingly demonstrating utility for both the diagnosis and immunophenotyping of certain lymphoid neoplasms.

PARR combined with conventional examination tools, such as cytology or histopathology, provides information regarding cell morphology, tumor architecture, cell clonality, and lymphocyte lineage (e.g., T or B). PARR has been used as a supporting analytical tool, in the diagnosis of lymphoid malignancies in humans, when the abovementioned examination results are ambiguous. PARR is especially useful for demonstrating the clonal nature of small lymphoid cells in indolent lymphomas of the lymph nodes, spleen, and intestine, as well as chronic lymphocytic leukemia (CLL).

PARR can provide information regarding lymphoid cell lineage (e.g., T, B) from a rearranged gene, such as *IGH* or *TCRG*. However, it should be noted that discrepancies between protein expression evaluated by IHC or FCM and gene rearrangement evaluated by PARR have been reported often.[79,87,88] Furthermore, cross-lineage rearrangement suggested from the rearrangements of both *IGH* and *TCRG* has been observed on occasion in lymphoid malignancies.[39,87,88]

Procedures for PARR

Evaluation of the rearrangement of *TCRG* and *IGH* in canine and feline lymphoid tumors by Southern blot hybridization has been reported previously.[16,59] Southern blot is an authentic method which is useful for investigating gene rearrangement. However, a negative aspect of this method is that it requires a relatively large amount of sample DNA. Following the introduction of PCR techniques for the assessment of antigen receptor gene rearrangement in the veterinary field,[5,14,85] PARR is commonly used to support the diagnosis of lymphoid tumors in dogs and cats.

Primers are designed at variable (V) and joining (J) segments for fragment amplification including complementarity-determining region 3 (CDR3) within antigen receptor genes. Differing lengths of V, diversity (D), and J segments, as well as random insertions and deletions of a few nucleotides occurring in the CDR3 region, lead to the generation of numerous amplicons of different sizes in polyclonal samples (Figure 62.2). On the contrary, since lymphoid neoplasms are fundamentally composed of clonally expanded cells, amplicons of a uniform size are generated by monoclonal samples in PARR (Figure 62.2).

IGH and *TCRG* are the most commonly used targets of PARR analysis. During the differentiation of B cells, rearrangement of *IGH* is followed by the rearrangement of immunoglobulin light chains (kappa and lambda

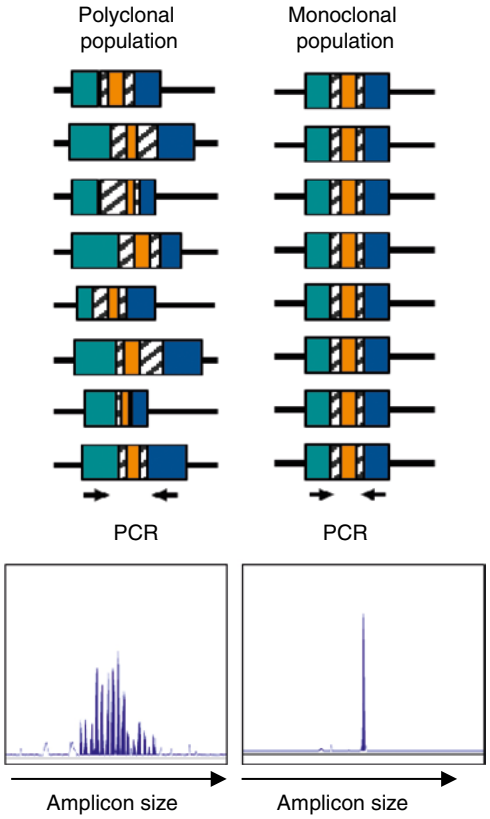


FIGURE 62.2 Schema of PARR analysis results. Forward and reverse primers for PCR are designed within the V and J regions, respectively. (Left) Many differently sized PCR products are amplified from a polyclonal sample resulting in Gaussian distributed multiple peaks. (Right) On the contrary, a single PCR product is amplified from a monoclonal sample resulting in a single peak. Representative results of GeneScan analysis are shown.

chains).[35] During T-cell differentiation, the *TCRG* locus is rearranged early in the differentiation stage and retained throughout the life span of both αβ and γδ T cells.[80] In order to amplify the CDR3 region, forward primers are designed within the V region, while reverse primers are set within the J region, in both *IGH* and *TCRG* loci. In order to improve the sensitivity and specificity of PARR analytical systems, many types of primer sets have been designed in various laboratories for dogs[5,9,22,23,37,38,78,84,85,91] and cats.[28,57,58,62,88,90] In addition to PARR evaluating complete *IGH–VDJ* rearrangement, use of PARR for incomplete *IGH–DJ*, kappa-deleting element *(Kde)*, and immunoglobulin lambda light chain *(IGL)* gene rearrangement was recently reported for detecting clonality in feline B-cell neoplasms.[70]

Three types of analytical procedures have been developed for PARR. These are gel electrophoresis, melting curve analysis, and GeneScan. Gel electrophoresis is a standard procedure which is commonly used for the analysis of PCR products. Polyacrylamide gel electrophoresis is usually performed following heteroduplex procedures.[77]

Melting curve analysis is another method used to analyze PARR products in dogs and cats. Real-time PCR systems enable the separation of contiguous PCR products via melting temperatures. Since this method does not utilize electrophoresis, rapid analysis with a reduced risk of cross-contamination is made possible.[29,44]

GeneScan was introduced for PARR tests in dogs and cats.[22,23,57,58] DNA fragments amplified using fluorochrome-labeled primers are analyzed in denatured condition using a capillary-sequencing polymer. Since the GeneScan method possesses high-resolution capabilities, it is suited for the detection of clonal peak(s) in a polyclonal background. Furthermore, the exact sizes of PCR products can be determined using this method. GeneScan analysis followed by DNA sequencing was used to demonstrate the identity of lymphocyte clone during the progression from nodal marginal zone lymphoma to diffuse large B-cell lymphoma in a dog.[71]

Samples for PARR

Any genomic DNA sample extracted from cells/tissues may be used for PARR analysis. Samples that are commonly used for PARR include fine-needle aspiration (FNA) samples of lymph node/mass/organ, endoscopic biopsied tissues, peripheral blood, bone marrow, biopsied samples of skin/tissue/organ, and abdominal/thoracic/cerebrospinal fluid. Freshly isolated or rapidly frozen tissues/cells can be a source of ideal samples. Meanwhile, cells smeared on glass slides (stained or not) or formalin-fixed paraffin-embedded (FFPE) tissues can also be used for PARR. However, since DNA fragmentation may occur due to storage on glass slides at room temperature for a long time, or due to formalin fixation,[25,45] amplification of control gene(s) should be concurrently performed to ensure the integrity of DNA extracted from these samples.

Interpretation of the Results of PARR

Since one somatic cell contains a pair of chromosomes, amplification of one or two distinct band(s)/peak(s) is usually interpreted as clonal.[22,57,84,90] Although normal mature lymphocytes possess only one rearranged *IGH* or *TCRG* allele following differentiation, as described in the allelic exclusion theory,[35] two distinct bands (or peaks) are often observed in lymphoid malignancies. Furthermore, the presence of multiple rearrangements in the *TCRG* locus (4.6 rearrangements per lymphoma in average) has been detected in canine T-cell malignancies.[37] Recent reports have revealed that a canine *TCRG* locus includes seven complete V–J–C cassettes,[37,48] enabling multiple rearrangements in each allele, which is topologically different from that of human genomes. The topology of the feline *TCRG* locus is not known. However, three to five distinct bands (or peaks) are frequently amplified in feline T-cell lymphomas.[62]

As for the rearrangement of *IGH*, amplification of one or two distinct bands (or peaks) is usually interpreted as an indication of clonal rearrangement. On the other hand, the criteria for clonal rearrangement of *TCRG*

differ between studies, wherein some studies define one or two distinct bands (or peaks) as clonal,[20,32,63,84,91] whereas others may consider samples with three or more bands (or peaks) as clonal.[9,22,38,61]

Clonal results indicate the proliferation of a particular lymphocyte clone. Clonally expanded lymphocytes would be most likely indicative of a neoplastic disease, such as lymphoma, lymphoid leukemia, or plasma cell tumors. However, clonal results may also be a result of reactive proliferation against a specific antigen.[5,52] Lymphocyte populations that infiltrated regressing histiocytomas were also shown to be clonal by PARR.[39]

When a clonal pattern is not detected via PARR analysis, the lymphoid cell population in the sample is generally assumed to be normal, or one generated by reactive proliferation. However, irrespective of the actual presence of a neoplastic cell population, if the rate of tumor cells in the sample is below the detection limit of PARR (approximately 10% in lymphoid tissues),[5] a clonal result cannot be confirmed. Moreover, because the rearrangement process of antigen receptor genes is quite complex and variable, any of the primers designed in many laboratories do not have a 100% ability to amplify a rearranged antigen receptor gene, such as *IGH* or *TCRG*. Therefore, even when a clonal pattern is not indicated by PARR analysis, a diagnosis of lymphoma, or other form of lymphoid malignancy, cannot be excluded.

Sensitivity and Specificity of PARR

The overall sensitivity of PARR analysis for detection of lymphoid tumors is reported to be in the range of 60–100% for dogs[5,22,23,38,43,44,79,87] and 30–90% for cats.[26,28,29,57,58,61,62,88,90] Specificity is reported to exceed 90% in both dogs and cats. Sensitivity and specificity of PARR vary with the type of malignancy.

Of these PARR analyses in dogs and cats, sensitivity for detecting *IGH* rearrangement among feline B-cell neoplasms has been low, with sensitivity values ranging from 30% to 50% in several studies.[26,29,61] In addition to the PARR evaluating complete *IGH–VDJ* rearrangement, use of PARR for incomplete *IGH–DJ*, *Kde*, and *IGL* rearrangement was shown to increase the sensitivity (up to 87%) for detecting clonality in feline B-cell neoplasms.[70]

Application of PARR for Diagnosis

A large proportion of dogs with multicentric lymphoma are afflicted with high-grade B-cell lymphoma, histologically classified as DLBCL. FNA of enlarged lymph nodes of a dog with DLBCL usually shows a uniform population of large lymphoid cells (centroblasts, immunoblasts, etc.). In such cases, diagnosis of lymphoma may be made via cytology. However, when cytological examinations show the lymphoid cells to be small, or not typical of centroblasts, resection biopsy followed by histological examination may be indicated. Although PARR does not provide the scope of information provided by histopathology, PARR analysis of FNA samples may detect clonality and define T- or B-cell lineage. Demonstration of cell clonality via PARR has been helpful for the diagnosis of several subtypes of lymphoma with a relatively indolent biologic behavior, such as nodal and lingual T-zone lymphomas (Figure 62.3a),[27,54,84] splenic marginal zone lymphoma,[64,73,84] and colorectal follicular lymphoma.[68]

Various types of lymphomas are observed in cats, including alimentary, nasal, mediastinal, peripheral nodal, laryngeal/tracheal, renal, central nervous system, cutaneous, and hepatic forms. Histopathology is a standard procedure used for diagnosis. However, sufficient tissue samples cannot be obtained in some cats suspected of having lymphoma. In such cases, findings of PARR combined with those of cytological examinations of FNA specimens or body cavity fluids may be utilized to support the diagnosis.

PARR analysis using endoscopically biopsied specimens has been utilized for the assessment of gastrointestinal diseases in cats. In feline gastrointestinal lymphoma, its sensitivity was shown to be 80–90% for T-cell lymphoma,[24,61,62] and 50% for B-cell lymphoma.[61] A stepwise diagnosis algorithm, integrating the results of immunohistochemistry and PARR with those of histopathological findings, was proposed to differentiate between intestinal lymphoma (especially small cell lymphoma) and inflammatory bowel disease in cats.[40]

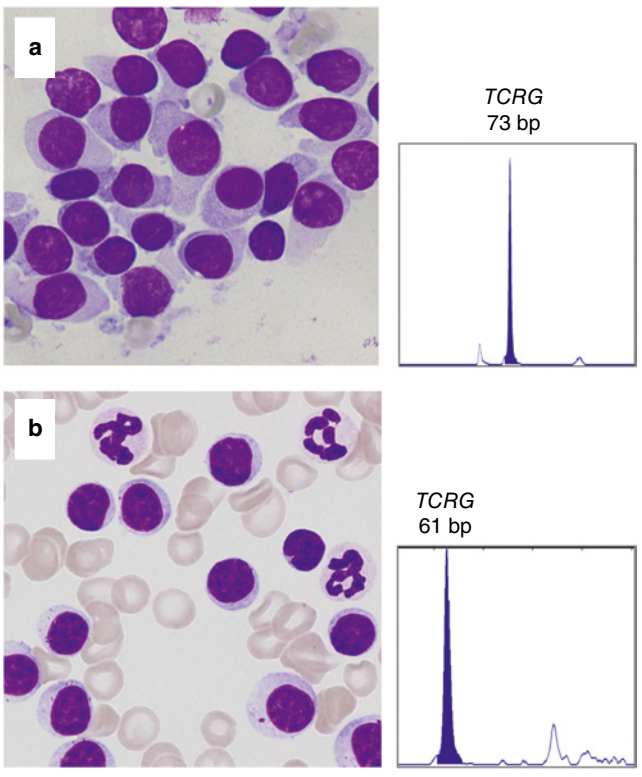


FIGURE 62.3 Examples of the results of PARR analysis for (a) a lymph node tissue sample from a dog with T-zone lymphoma and (b) a peripheral blood sample from a dog with T-cell chronic lymphocytic leukemia (T-CLL). Sizes of the monoclonal peaks are indicated above the charts.

In canine gastrointestinal lymphoma, detection sensitivity of PARR is reported to be 65–85%.[20,23,32,66,75] Canine gastrointestinal T-cell lymphoma is divided into two major subtypes: large cell lymphoma resembling human enteropathy-associated T-cell lymphoma (EATL) type I; and small cell lymphoma resembling human EATL type II.[7,11,49,50,51] Distinguishing between small cell lymphoma and chronic enteritis via histopathology alone is problematic. Moreover, 20–50% of tissue samples diagnosed as chronic enteritis via histologic findings were found to show clonal patterns by PARR analysis,[32,50,63,67] making differential diagnosis of small cell lymphoma and chronic enteritis even more difficult. Caution is required when interpreting PARR results of biopsied gastrointestinal tissue samples. Some studies have indicated an association between clonality and prognosis for canine small cell lymphoma and chronic enteritis.[63,65,67] Further studies may be needed to disclose the relationship between chronic enteritis and small cell lymphoma.

PARR of canine epitheliotropic cutaneous T-cell lymphoma using skin biopsy samples resulted in a sensitivity as high as 95%.[9] On the other hand, PARR of feline cutaneous lymphoma at vaccine injection sites including T, B, and natural killer (NK) types resulted in 60% sensitivity.[69] PARR was also used to indicate clonality in acute lymphoblastic leukemia (ALL) and CLL in dogs, resulting in a sensitivity higher than 80%.[5,91] When an increase in the number of small lymphocytes occurs in peripheral blood in the absence of a possible cause of lymphocytosis, a differential diagnosis of CLL is considered. In such cases, PARR using a sample of peripheral blood or bone marrow may be helpful for identification of clonality and cell lineage (Figure 62.3b). The sensitivity of PARR seems to be lower for leukemias in cats, although the reason for this is not well understood due to the limited case numbers.[57,82] Clonal rearrangement of *IGH* was shown in a large proportion of canine and feline cases with plasmacytoma and myeloma;[5,57,87] however, a low sensitivity (41%) for canine cutaneous plasmacytoma was reported.[76] PARR may also be helpful in the diagnosis of nonlymphoid neoplastic diseases such as thymoma, where polyclonal results were detected in 92% of canine thymoma tissues.[86]

X-CHROMOSOME INACTIVATION PATTERN (XCIP) ANALYSIS

Principles of XCIP Analysis

In mammalian females, either the paternal or maternal X-chromosome generally undergoes random inactivation in each cell by way of epigenetic changes during embryogenesis. This is stably inherited by the daughter progeny of each cell, resulting in a theoretical expectation of an approximately 1:1 inactivation ratio between paternally and maternally derived X-chromosomes in normal or reactive tissues. By contrast, most neoplastic tissues are composed of a cell population clonally derived from a single cell, generally exhibiting a uniform XCIP and a significant deviation from the 1:1 inactivation ratio (Figure 62.4). Therefore, demonstration of a nonrandom XCIP (skewed XCIP) is indicative of a clonal origin of the cells concerned. A widely used method for determining clonality in humans via the X-chromosome inactivation principle is known as the human androgen receptor (AR) gene assay (HUMARA).[10,17]

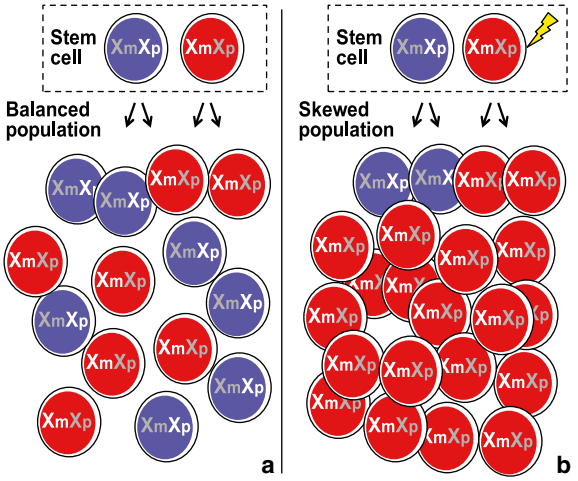


FIGURE 62.4 Principles of XCIP analysis for the clonality assessment. Xm or Xp (characters indicated in the nucleus) indicates maternal or paternal X-chromosome, respectively. White-colored or grey-colored character indicates active or inactive X-chromosome, respectively. (a) In non-neoplastic condition, cells in some organ (e.g., bone marrow) are composed of a balanced population of blue cells (harboring inactive maternal and active paternal X-chromosomes) and red cells (harboring active maternal and inactive paternal X-chromosomes); (b) By contrast, in neoplastic condition, cells in some organ are composed of a skewed population with respect to XCIP because of the clonal expansion originated from a single transformed cell (e.g., red cells harboring active maternal and inactive paternal X-chromosomes).

Procedures for XCIP Analysis

Transcription of human *AR* gene on the inactive X-chromosome is strictly inhibited by methylation of the CpG island in the promoter.[34] Methylation status is revealed whether the restriction site is cleaved by some methylation-sensitive restriction endonuclease. Because of the difference in methylation status between inactive (methylated) and active (nonmethylated) X-chromosomes, DNA from only inactive X-chromosomes can be amplified after digestion with a methylation-sensitive endonuclease (e.g., HpaII). A difference in the number of polymorphic repetitive sequence (e.g., CAG repeat) between two X-chromosomes enables distinguishing one from the other.[1,55,56]

The degree of skewness (imbalanced inactivation) is determined by comparing the amplified DNA amount for each allele of digested and undigested samples, using results of the GeneScan analysis. The corrected inactive allele ratio (CIAR) is defined as the allele ratio of the digested sample divided by the allele ratio of the undigested sample.[55,56] The sample population is judged

as clonal when CIAR exceeds approximately 3 in several reports.[46,53]

To apply the XCIP analysis using *AR* gene for the assessment of clonal origin of cells, at least one of the two CAG tandem repeats should be heterozygous for the repeat number. The frequency of heterozygosity of CAG repeats in feline *AR* gene was shown to be 68%,[55] indicating its availability in more than two thirds of female cat individuals. On the other hand, the frequency of heterozygosity of CAG repeats in canine *AR* gene was found to be lower, 49%.[56] In a recent study, utility of XCIP analysis was improved up to 83% of female individuals by incorporating the examination of AC repeats in *SLIT* and *NTRK-like family member 4* (*SLITRK4*) gene into the previously reported CAG repeats examination of *AR* gene in dogs.[81]

A low tumor-cell purity in the sample (theoretically less than 50%) may lead to false negative results. Therefore, for XCIP analysis to be effective, more than 50% of the cell population should be the cells of interest (i.e., suspected tumor cells).

Application of XCIP Analysis for Diagnosis

XCIP analysis has been used for assessing cell clonality in various neoplastic and preneoplastic conditions of hematopoietic cells in humans such as CML,[53] acute myeloid leukemia (AML),[21] and myelodysplastic syndrome (MDS).[8] Although XCIP analysis is applicable only for females, a major advantage of this analysis is its possible application to any type of neoplastic diseases in which a tumor-specific genetic or phenotypic marker has not been identified.

In myeloproliferative neoplasms (MPNs) (formerly termed chronic myeloproliferative disorders), the morphology of proliferating cells differs little from that of normal blood cells, which makes the differentiation between neoplastic and non-neoplastic cells difficult. This class of diseases includes CML, polycythemia vera (PV), and essential thrombocythemia (ET).[36] Diagnosis of MPNs can be made primarily by excluding other causes via thorough clinical examinations. However, it is sometimes difficult to reach a definitive diagnosis. Although reciprocal chromosomal translocations have been identified in a proportion of canine neoplasms,[3,4,12] their frequency in specific disease is not known. Skewness in XCIP analysis has been shown to be useful for the diagnosis of CML in dogs.[56]

MDSs are a group of clonal hematopoietic stem cell diseases characterized by ineffective hematopoiesis resulting in cytopenia(s) and dysplasia in one or more blood cell lineages. Feline leukemia virus (FeLV) infection has been shown to be associated with the development of MDS in cats;[31] however, MDS is also found in FeLV antigen-negative cats.[36] In humans, MDS is considered to be caused by clonal expansion of bone marrow cells from aberrant pluripotent hematopoietic stem cells with a variety of somatic mutations.[42] Progression to acute myeloid leukemia from MDS has been reported in humans as well as in dogs[2,19,36,89] and cats.[30,31] Distinctly skewed XCIPs were found in bone marrow samples of cats hematologically diagnosed with MDS.[55] When findings based on peripheral blood and bone marrow examinations suggest a diagnosis of MDS, and other disorders are excluded, CIARs obtained via XCIP analysis may substantiate the clonality of cells, leading to a more reliable diagnosis of MDS.

Apart from the lymphoid and hematopoietic diseases, canine cutaneous histiocytoma was shown to be a disease of clonal origin by XCIP analysis.[13] There are several other disorders, such as splenic fibrohistiocytic nodules,[72] various histiocytic proliferative diseases,[13,60] and hypereosinophilic syndromes,[47] in which the neoplastic nature (clonal origin) of the diseases has been argued. XCIP analysis will provide objective data to demonstrate the clonality of the cells of interest, leading to more accurate diagnoses.

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CHAPTER 63

Immunophenotyping

AUSTIN K. VIALL

Immunophenotyping
Indications
Sample Collection and Preparation
Blood and Bone Marrow
Aspirate Material and Cavity Fluids
Flow Cytometry Procedure
Antibody Panels
Reporting of Immunophenotyping Results
Interpretation of Immunophenotyping Results
Differentiating Reactive and Neoplastic Lymphocytes
Leukemia Lineage Determination
Differentiating Leukemia and Lymphoma

Acronyms and Abbreviations

ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; CLL, chronic lymphocytic leukemia; ICC, immunocytochemistry; IHC, immunohistochemistry; MHC, major histocompatibility complex; TCR, T-cell receptor.

IMMUNOPHENOTYPING

In people, well over 80 distinct lymphomas and leukemias are recognized.[46] Historically, specific hematologic malignancies were defined by unique histological, cytological, and clinical features. The advent of diagnostic antibodies for detection of expressed surface and intracellular markers dramatically refined classification of human hematologic malignancies. Through this process of immunophenotyping, the vast majority of human lymphomas and leukemias may be identified by marker expression with concurrent cytological assessment.[4] For animals, there has been a similar expansion in the number of diagnostic antibodies available for tumor immunophenotyping, which has aided the process of diagnosing lymphoma and leukemia along with facilitating identification of novel types and subtypes of hematologic malignancies.

Immunophenotyping methodologies use the same fundamental approach. An antibody targeting a specific membrane or cytosolic protein marker is applied to the tumor cell population, with antibody binding detected either (1) directly through a conjugated fluorochrome or enzyme that facilitates a colorimetric reaction or (2) using a secondary reporting antibody targeting the bound primary antibody. Monoclonal antibodies are preferred over polyclonal antibodies due to variations in the target-binding motifs with polyclonal antibodies; polyclonal antibodies also have a higher tendency for nonspecific binding. Immunophenotyping may be performed on paraffin-embedded tissue specimens through immunohistochemistry (IHC) or on glass slide cytology samples with immunocytochemistry (ICC). IHC on lymph nodes and other tissues histologically suspected to contain lymphoid malignancies is an essential aspect of fully assessing such samples. However, formalin fixation may dramatically alter the physiochemical nature of antibody targets—specifically proteins—and subsequently reduce or nullify the binding capability of the antibody to the protein.[41] Consequently, the number of diagnostic antibodies available for performing IHC for animal lymphomas/leukemias is smaller than those available for immunophenotyping with flow cytometry. While most antibodies available for flow cytometric immunophenotyping also work for ICC, flow cytometry is considered a more flexible and technically forgiving analytic modality and provides a plethora of additional data concerning the target cell population (forward

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scatter/side scatter for cell population differentiation, etc.) As such, immunophenotyping by flow cytometry will be the focus of this chapter.

Indications

For hematologic malignancies, flow cytometric immunophenotyping can be used to accomplish two general diagnostic objectives: (1) determine if a patient has lymphoma or leukemia and (2) determine the specific lymphoma or leukemia present. Furthermore, the presence or absence of certain cellular markers on a tumor population may be predictive of patient survival duration. While hematologic malignancies can manifest as a wide spectrum of clinical presentations, flow cytometric evaluation is most typically indicated when: (1) more cells of a specific type are present than normally expected (e.g., peripheral blood or bone marrow lymphocytosis) or (2) a round cell population has a cytologically malignant appearance. Table 63.1 provides more specific guidelines on scenarios when flow cytometry may be diagnostically beneficial or when the technique may provide no additionally useful information. Please note these are general guidelines and that flow cytometric immunophenotyping could be used diagnostically for a number of clinical scenarios as long as the diagnostician considers the findings in context of the limitations of this technique. Additionally, it is worthwhile to contact the laboratory performing the flow cytometry, as some groups may have additional or novel diagnostic capabilities that are not yet reported in scientific literature.

Sample Collection and Preparation

For flow cytometric immunophenotyping, it is critical that cells be viable during the analysis of their cell surface markers. Nonviable cells may have altered physical properties and decrease/loss of surface markers, which could potentially lead to incorrect or confounding flow cytometric results. Retaining cell viability is paramount and when cells are prepared for analysis measures should be taken to ensure and prolong viability. With refrigeration, peripheral blood and bone marrow samples may retain decent cellular viability for several days. Conversely, fluid preparations made from organs (e.g., lymph node or splenic aspirate material) and low-protein effusions have relatively poor cellular viability without addition of cellular-supportive reagents (e.g., serum or albumin). If not already performed, a concurrent CBC and blood smear review for peripheral blood or cytological/histological evaluation of lymph nodes or other organs is strongly recommended so as to provide complementary diagnostic information to the flow cytometry results. If samples are being submitted to another laboratory for flow cytometry, results for these diagnostic tests should be concurrently submitted with the flow cytometry samples.

The following are generally recommended guidelines for collecting and processing samples for flow cytometric immunophenotyping.

Blood and Bone Marrow

Peripheral blood and bone marrow aspirate samples should be immediately collected into an EDTA vacutainer to the appropriate volume for anticoagulation. 1 mL of whole blood or bone marrow is the minimum volume for analysis for most laboratories. Samples should be kept and/or shipped at 4 °C with cold packs; insulating the sample is recommended so as to prevent freezing. Samples being analyzed by another laboratory should be shipped overnight for analysis. Bone marrow samples usually retain adequate cell viability for 3 days postcollection, while peripheral blood samples may maintain adequate viability for 5 days postcollection. Utilization of commercially available vacutainers with stabilizing reagents, such as Cyto-Chex BCT vacutainers, may extend sample analysis viability out to 7 days postcollection, especially for certain markers (CD4, CD8, and CD21).[7,52]

Aspirate Material and Cavity Fluids

Tissue aspirate material and cavity fluid with a low-protein concentration (<5 mg/dL) should be placed in an aqueous medium with an added protein source (e.g., serum) to preserve cellular viability.[1,54] Tissue aspirate material could be expelled into 1–2 mL Roswell Park Memorial Institute (RPMI) medium, Dulbecco's modified Eagle's medium (DMEM), or other standard cell culture media with addition of fetal bovine serum (FBS) to 10% by volume. In a hospital setting, aspirate material could be placed into 1 mL of an isotonic IV-administered fluid (e.g., lactated Ringer's solution, Normosol-R, 0.9% NaCl) with addition of serum from the patient (or animal from the same species) to 10% by volume into a red-top vacutainer.[28] Cavity fluid should be placed into an EDTA vacutainer and serum similarly added to 10% by volume. The preparation should be immediately kept at 4 °C and if analyzed by another laboratory shipped overnight for evaluation. This stabilization procedure typically preserves cell viability for 2–3 days postcollection.

Flow Cytometry Procedure

Significant variation exists between laboratories for the protocols for preparing and analyzing samples for flow cytometry. That said, most protocols follow a similar procedure: (1) isolate leukocytes, (2) incubate with antibodies and a viability dye, (3) analyze samples with flow cytometer, and (4) evaluate flow cytometer data with flow cytometry standard (FCS) compatible software. Typically, leukocyte isolation, marker staining, and sample analysis by the cytometer are performed in immediate succession; this is the most common approach described by laboratories which report flow cytometric immunophenotyping.[3,19,23,28,47] The simplest method for isolating leukocytes is to treat samples with a hypotonic lysing solution to rupture erythrocytes, followed by centrifugation to generate a leukocyte pellet, and then leukocyte washing with an isotonic solution.[17]

TABLE 63.1 Indications and contraindications for flow cytometric immunophenotyping.

	Blood	Masses/lymph nodes	Bone marrow	Cavity fluids
Indicated	Lymphocytosis Cytologically abnormal cells Monocytosis	Lymphadenopathy Mediastinal masses Splenomegaly with cytologic suspicion of lymphoma	Cytologically abnormal cells Increased lymphocyte percentage	Increased lymphocytes Cells with aberrant morphology suggesting lymphoid or myeloid origin
Not indicated	Neutrophilia[a] Eosinophilia Thrombocytosis Polycythemia vera or cytopenias	Metastatic disease from nonlymphoid tumors[b] Primary tumors of nonhematologic origin Marked lymphoid reactivity	Increased plasma cells or monoclonal gammopathy[c] Loss of a specific lineage or myelofibrosis	Transudates with few cells Suspected septic process Suspect carcinoma/mesothelioma vs reactive mesothelial cells[b]

[a] Neutrophils from patients with chronic myeloid leukemia (CML) and non-neoplastic neutrophilia have identical immunophenotypes.
[b] The use of flow cytometry to detect nonlymphoid tumors in lymph nodes and cavity fluids (e.g., metastatic mast cell tumor or carcinoma) has been reported for certain tumor types. However, such flow cytometric assays are not widely commercially available. Diagnosticians are advised to consult with laboratory performing flow cytometric immunophenotyping to determine if available by the laboratory.
[c] A differentiating immunophenotype between malignant and reactive canine plasma cells has not yet been described. Flow cytometry will not be able to distinguish reactive from neoplastic plasma cells.

As an alternative, density gradient centrifugation to remove erythrocytes, platelets, and dead cells can be performed. However, this approach is more labor intensive, time consuming, and may cause selective loss of certain leukocyte populations. Isolated cells are then incubated with a panel of antibodies, often requiring multiple separate incubations with different antibody combinations designed to identify specific cell types while avoiding fluorochrome crossover. If the sample cannot be analyzed immediately after staining, then stained cells may be placed in a fixative buffer and stored at 4 °C; this can preserve the sample for up to three additional days for cytometer analysis.[22] However, the fixative buffer can potentially alter cell physical properties (e.g., forward scatter and side scatter) and antibody fluorochrome intensity.[17,22] As such, protocols utilizing fixative buffer should thoroughly vet the changes induced by the buffer in context of interpreting flow cytometric results. Specific details on flow cytometry procedural steps are beyond the scope of this chapter, but a number of protocols are reported.[9,11,27,28,37,39,47]

Antibody Panels

As laboratories which perform immunophenotyping use unique antibody combinations tailored to their respective protocols, the reported panels of diagnostic antibodies used for immunophenotyping are highly variable. The central goals of designing an antibody panel are to detect cells with an abnormal immunophenotype, which provides evidence for neoplasia when cytological or histological findings are unclear, and to determine the lineage origin of the malignant population. Currently, there are no standardized antibody panels for immunophenotyping in animals, so following consensus recommendations for immunophenotyping human hematologic malignancies is recommended.[12,43] While no consensus has been published for antibodies for veterinary immunophenotyping, the panel of antibodies for markers of lymphoid malignancies used in a multi-institutional proficiency study of canine immunophenotyping should be considered.[28] Each laboratory should also develop its antibody panel based upon the expertise of the laboratory group, available cytometer, recent literature on analytic antibodies, and commercial availability of antibodies. Furthermore, antibody panels will likely be different for the target species. Many more vetted antibodies are commercially available for dogs relative to cats, horses, ruminants, and other species.

A minimum panel for canine hematologic malignancies should include antibodies targeting surface markers for T lymphocytes (CD3, CD4, CD5, and CD8), B lymphocytes (CD21 or CD22), monocytes (CD14), stem cell antigen for acute leukemia (CD34), pan-leukocyte antigen (CD45), and major histocompatibility complex (MHC) II.[1,9,28] This panel identifies the most common lymphoproliferative disorders in the dog.[28] Addition of antibodies targeting T-cell receptor (TCR)αβ, TCRγδ, IgM, CD25, and CD90 may allow further subcharacterization of lymphoid malignancies along with prognostic information for certain lymphomas and leukemias.[16,23,36,54] Identification of myelomonocytic malignancies can be accomplished with antibodies targeting CD11b (neutrophil and monocyte marker), CD11c, CD11d, and CD1a/b/c (dendritic cell and macrophage markers), and CD18 (β integrin).[28,31,44] Detection of cytoplasmic markers CD3ε and CD79α requires a permeabilization step for staining, but these are essential for detecting the immunophenotype of acute leukemias, as such immature malignancies may not express expected surface antigens for their lineage.[49] The selection of antibodies available for immunophenotyping cats and other mammals is unfortunately limited. Monoclonal antibodies targeting and/or reacting to feline CD4, CD5, CD8, CD18, and CD21 are commercially available and reportedly used diagnostically.[39] For other species, antibodies used to detect CD3ε and CD79α along with the B lymphocyte nuclear transcription factor PAX5 react almost entirely across mammals and could be used to at least establish T or B lymphocyte origins.[24,29,30]

Concerning the source of analytic antibodies, commercially available fluorochrome-conjugated antibodies are generally preferred. A relatively small number of commercial sources provide analytic antibodies for veterinary diagnostics, and many vend the same antibody clones. As such, the production quality control of these antibodies, reagent preparation protocols, and antibody behavior become less variable between laboratories. Additionally, fluorochrome-conjugated primary antibodies permit multiparameter staining of cells, which is essential for identifying cells with aberrant immunophenotype. Concurrent incubation of the cells with a viability identification dye—such as propidium iodide, 7-amino-actinomycin D, or proprietary fixable dyes—is used to exclude dead cells from analysis.[28] Exclusion of nonviable cells is essential, as dead cells can stain falsely positive for markers due to either nonspecifically bind antibodies or being permeable enough for intracellular staining where cytosolic antigens may be present even in the absence of surface expression.[32]

Reporting of Immunophenotyping Results

A useful immunophenotyping report should contain both objective flow cytometric data along with a subjective interpretation of the results.[8,13] Recent consensus recommendations on reporting veterinary flow cytometric results advise the following information be provided: (1) laboratory identification, (2) patient identification, (3) sample type and quality (i.e., percentage of viable cells, (4) cell preparation and staining (i.e., antibodies used and reference intervals for expected positivities for sample type), (5) percentage of positive cells for each antibody (i.e., percentage of positive cells in gated population and/or whole population, absolute count of positive cells for peripheral blood samples), (6) descriptive report, (7) diagnosis and interpretation, (8) comments and references, and (9) pathologist's signature.[8] The consensus statement provides additional details on the specific information to be presented within each section of the report. Laboratories are advised to establish their own positivity reference intervals, either

percentage of lymphocyte subtypes for lymph node/bone marrow or absolute lymphocyte subtype counts for peripheral blood. If laboratory-specific reference intervals cannot be established, then reporting of published values for healthy animals for lymph node, bone marrow, or blood could be reported.[14,15,20,34,39,40,48]

Interpretation of Immunophenotyping Results

In most circumstance, flow cytometric results should be used to answer two key questions: (1) is a hematologic malignancy present? and (2) what is the lineage origin of the neoplastic population and/or the specific lymphoma/leukemia present? Two criteria classically used to determine if cellular population is malignant are (1) the presence of an abnormal phenotype or (2) an expanded population with a homogenous phenotype, especially for lymphocytes.[28] Lineage differentiation can subsequently be determined either by the expressed pattern of markers on the neoplastic population or, for lymphoid malignancies, lymphocyte clonality testing.[47] Furthermore, certain lymphomas and leukemias have phenotypic "calling cards" which permit subclassification using flow cytometric results alone (e.g., loss of CD45 expression in T-zone lymphoma or expression of CD34 for acute leukemias).

Differentiating Reactive and Neoplastic Lymphocytes

Flow cytometry is commonly performed in cases of peripheral blood lymphocytosis or lymphadenopathy when cytologic assessment cannot differentiate neoplastic or reactive processes. Unlike in human medicine, standardized criteria for differentiating lymphoid neoplasia from lymphoid reactivity with flow cytometry have not been reported.[46] That said, the following guidelines have been advocated for differentiating neoplastic lymphocytosis from non-neoplastic lymphocytosis in blood:[2,3]

Lymphocyte count is above the reference interval, and one of the following:

A. 80% of lymphocytes are a single phenotype, or
B. 60% of lymphocytes are a single phenotype with a monoclonal clonality assay result, or
C. presence of lymphocytes with an aberrant phenotype.

Similarly, consensus metrics for diagnosing lymphoma flow cytometrically has not been established, but the following criteria has been used:[9,47,54]

A. Expansion of specific lymphocyte subtype beyond >60% of all cells.
B. More than 1.5 times expansion of a specific lymphocyte subtype above the reference interval for the population.
C. Atypical dual staining phenotype (e.g., CD4+CD8+ or CD3+/CD5+ and CD21+/CD79a+).
D. Atypical null staining phenotype (e.g., CD3+ CD4− CD8−).

A well-documented exception to the idea that a homogenous expansion of lymphocytes is diagnostic for a lymphoid malignancy is that chronic *Ehrlichia canis* infections can induce a CD8+ T-cell lymphocytosis in small portion of infections.[21,35] Consequently, testing for *E. canis* infection should be considered for CD8+ lymphocytosis <30,000 cells/μL.

Cells may also be considered neoplastic if they have an aberrant phenotype, particularly if the antigens expressed are not typically found in normal or reactive lymphoid processes. Atypical phenotypes reported in lymphoid malignancies in the dog include: T-cell lymphomas and leukemias that have lost CD45 (e.g., T-zone lymphoma), CD3+ or CD5+ populations lacking CD4 and CD8, CD79α B-cell tumors with absent CD21 expression, and dual CD4 CD8 expression indicative of lymphoma in a lymph node, thymoma from an intrathoracic mass, or leukemia, or from peripheral blood.[6,25,42,55] The presence of such cells with aberrant immunophenotypes should be considered strong evidence for a lymphoid malignancy.

Less-reported information is present concerning the immunophenotypes of feline lymphomas and leukemias. A relatively homogenous lymphocytosis comprised of CD4+ lymphocytes, CD4− CD8− lymphocytes, or T cells with low CD5 expression is typically neoplastic.[39] Conversely, a peripheral lymphocytosis with B-lymphocyte expansion or heterogeneous population is more likely the result of reactive processes.[39] Cats with large granular lymphocyte alimentary lymphoma may be identified by CD3+ CD8+ population from the intestinal lesion or a peripheral lymphocytosis.[38] Conversely, a population of CD21 cells accounting >90% of lymphocytes in a feline intestinal lesion is strongly supportive of B-cell alimentary lymphoma.[18] The majority of feline nasal lymphomas are also B-cell immunophenotype.[26]

Leukemia Lineage Determination

While the cellular lineage of mature leukemias is relatively easy to identify due to cytomorphological features, leukemias of immature populations are notoriously challenging, if not impossible, to definitively differentiate cytologically.[50] Using flow cytometry, acute lymphocytic leukemia (ALL) may be identified by expression of extracellular/intracellular lymphoid markers along with co-expression of CD34. Historically, acute myeloid leukemia (AML) was diagnosed when a circulating CD34+ neoplastic population failed to express lymphoid markers along with cytomorphological features suggesting myelomonocytic origin.[50] The advent of antibodies targeting myelomonocytic cells (e.g., CD14, CD11b/c/d, CD18, neutrophil surface antibody, myeloperoxidase, and MAC387) has provided more definitive flow cytometric detection of AML.[28,51]

Select cytochemical stains may provide further support for myeloid origin acute leukemias.[44] Acute megakaryoblastic leukemias may express CD41, CD61, and von Willebrand factor in addition to CD34.[10,45]

The expression of CD34 is classically considered a diagnostic hallmark of acute leukemias. However, rare acute myeloid leukemias which meet other criteria for acute leukemia (e.g., >20% blastic cells in blood or bone marrow) have been reported to lack CD34 expression.[51] Furthermore, rarely B-cell lymphomas may express CD34 and could mimic ALL if presenting in advanced leukemic phase.[33] As such, the absence or expression of CD34 may be misleading in terms of establishing a diagnosis of acute leukemia in a minority of cases. MHC II expression may be situational helpful in differentiating such cases, as ALLs are typically MHC-II-negative while CD34+ B-cell lymphoma would likely be MHC-II-positive. Chronic myeloid leukemia (CML) and chronic lymphocytic leukemia (CLL) may be differentiated from their acute variants by the absence of CD34 expression along with cytomorphological features indicating a mature phenotype.

Differentiating Leukemia and Lymphoma

For patients presenting with a neoplastic lymphocytosis, a common diagnostic conundrum is distinguishing a true leukemia from a lymphoma in leukemic phase (e.g., canine stage V lymphoma). Presently, there are no consensus criteria for differentiating lymphoma and leukemia with immunophenotyping alone. As such, differentiating leukemia and lymphoma should be based upon a combination of immunophenotypic, cytologic, clinicopathologic, and clinical data from patients. Patterns suggesting a diagnosis of primary lymphocytic leukemia over leukemic-phase lymphoma in dogs include: CD34+ circulating population for ALL, circulating small cell population expressing CD21 for B-cell CLL, and circulating small cell population of CD8+ T lymphocytes with <30,000 lymphocytes/μL for T-cell CLL.[55] For canine B-cell CLL, alternate criteria includes: >5000 lymphocytes/μL with small cell phenotypes and >60% lymphocytes being CD21+.[5] Patients presenting with marked peripheral lymphadenopathy and lymphocytosis comprised of T or B cells with large lymphocytes suggest leukemic phase large cell lymphoma, although dogs with ALL may also have this clinical presentation. Indeed, the presence of lymphadenopathy—especially for dogs with small cell lymphocytosis—does not reliably differentiate lymphoma from a primary leukemia.[16,19,42] As for cats, criteria for CLL has been reported as >9000 lymphocytes/μL with a small mature phenotype, homogeneous immunophenotype, and concurrent cytopenia.[39] As the vast majority of feline CLLs are CD4+, the presence of such an immunophenotype on the neoplastic cells further supports this diagnosis.[39] Lymphocytosis accompanying lymphoma is relatively uncommon in cats, although low frequency of circulating neoplastic lymphocytes in cats with large granular lymphocyte alimentary lymphoma has been repeatedly reported.[38,53] For some patients presenting with advanced stages of either malignancy, it may be clinically impossible to truly differentiate a primary leukemia from leukemic phase lymphoma.

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CHAPTER 64

Flow Cytometry in Hematologic Neoplasia

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Clinical Flow Cytometry
Indications
Sample Preparation
  Blood and Bone Marrow
  Aspirates and Body Cavity Fluid
Antibody Panels
Technical Considerations
Reporting Flow Cytometry Results
Diagnosing Hematologic Neoplasia by Flow Cytometry
Lymphoproliferative Disease
  Canine B-Cell Lymphoma
    Canine T-Cell Lymphoma
      Multicentric T-Cell Lymphoma
      T-Zone Lymphoma/Leukemia
      Thymoma and Mediastinal Lymphoma
  Feline Lymphoma
Leukemia
  Chronic Leukemia
    Canine B-Cell CLL
    Canine T-Cell CLL
    Feline CLL
  Acute Leukemia
    Histiocytic Neoplasia

Acronyms and Abbreviations

ACK, ammonium-chloride-potassium; ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; AUL, acute undifferentiated leukemia; CBC, complete blood count; CD, cluster of differentiation; CLL, chronic lymphocytic leukemia; CSF, cerebrospinal fluid; DLBCL, diffuse large B-cell lymphoma; FNA, fine needle aspirate; FSC, forward scatter; IHC, immunohistochemistry; LGAL, low-grade alimentary lymphoma; LGL, large granular lymphocyte; LRS, lactated Ringer's solution; MCL, mantle cell lymphoma; MST, median survival time; MHCII, major histocompatibility complex II; MRD, minimal residual disease; NMZL, nodal marginal zone lymphoma; PARR, PCR for antigen receptor rearrangement; PTCL-NOS, peripheral T-cell lymphoma, not otherwise specified; T-LBL, T-cell lymphoblastic lymphoma; TZL, T-zone lymphoma/leukemia.

CLINICAL FLOW CYTOMETRY

The role of flow cytometry as an adjunctive tool for diagnosis and prognosis of hematologic neoplasia in veterinary medicine is rapidly expanding. Flow cytometry allows for the evaluation of thousands of cells in suspension based on light scatter properties and surface or internal protein expression to determine cell size, complexity, and immunophenotype.

There are over 20 recognized subtypes of lymphoma and leukemia in dogs and fewer in cats. Although these different subtypes were originally defined by histologic and/or cytologic appearance and clinical behavior, it is now possible to define some of the most common forms based on the array of proteins expressed on the cell surface using immunophenotyping. Immunophenotyping of hematopoietic cells by flow cytometry utilizes a panel of fluorescently labeled antibodies that bind to cell surface proteins, called cluster of differentiation (CD) markers, to identify cell lineage. Many monoclonal antibodies are now available for dogs, and a more limited number are available for cats.

INDICATIONS

In hematologic neoplasia, flow cytometry can be used to determine if a patient has lymphoma or leukemia, to

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identify the type of lymphoma or leukemia, and to provide prognostic information. Flow cytometry is not a stand-alone diagnostic test. It is most often used in combination with other diagnostic modalities including cytology, CBC, and/or peripheral blood smear exam and less commonly following inconclusive histopathology results.

Hematologic neoplasia can present with a very wide range of clinical symptoms and flow cytometry is generally indicated when: (1) there are more leukocytes present than are expected, (2) there are cells with a cytologically atypical appearance, or (3) there is a cell type inappropriate for the given location. Flow cytometry is performed on peripheral blood, aspirates of lymph nodes, other organs or masses, body cavity fluids, and bone marrow. Flow cytometry may also be useful for evaluating minimal residual disease (MRD) in patients that have undergone chemotherapy.[4,16]

Flow cytometry of peripheral blood is indicated when there is a lymphocytosis, or the presence of cells concerning for neoplasia on blood smear examination. Flow cytometry is not indicated for neutrophilia, monocytosis, or eosinophilia. While persistent elevations in these cell lines may infrequently represent chronic leukemia, these conditions cannot be diagnosed by flow cytometry with currently available antibodies because the non-neoplastic cellular counterparts have the same immunophenotype as the leukemic cells.

Flow cytometry of bone marrow is indicated when increased numbers of blasts/immature cells or small lymphocytes are present, but not for lineage loss or myelofibrosis. Currently, no antibodies that definitively identify plasma cells in either dogs or cats are readily available for flow cytometry. If an expanded plasma cell population is present, and the clinician is trying to determine if it is neoplastic, the PCR for antigen receptor rearrangement (PARR) assay and serum protein electrophoresis/immunofixation would be the most useful tests to answer this question. If a population of clearly neoplastic cells is suspected to be plasma cells but the lineage is not clear, immunocytochemistry/histochemistry using antibodies to lineage-specific antigens (e.g., Mum1) would be appropriate.

Flow cytometry for patients with lymphadenopathy, organomegaly, masses and/or body cavity effusion is indicated if there is a histologic or cytologic interpretation of: (1) lymphoma or probable lymphoma, (2) uniform expansion of small lymphocytes, or (3) immature round cells suspicious for lymphoid or myeloid origin. Flow cytometry is also helpful to distinguish between lymphoma and thymoma for mediastinal masses. The use of flow cytometry to detect nonhematologic tumors in lymph nodes and cavity fluids (such as melanoma in a lymph node or carcinoma in a pleural effusion) is not utilized in veterinary medicine. As antibodies that detect these tumors become available and tested, however, flow cytometry may become an important application for this purpose.

It is also important to contact the laboratory to which you are submitting flow cytometry samples because some laboratories may have additional capabilities that have not yet been reported in the literature.

SAMPLE PREPARATION

Detection of cell surface antigens by flow cytometry requires viable cells. Therefore, when cells are prepared, steps must be taken to ensure they remain viable until they are processed. Peripheral blood and bone marrow samples can retain reasonable viability for several days when kept refrigerated, but samples from effusions, or aspirates of lymph nodes or masses, are less resilient. The following procedures are recommended when collecting samples for flow cytometry.

Blood and Bone Marrow

Collect samples in EDTA (a minimum of 0.5 mL for peripheral blood), store refrigerated, and ship overnight with a cold pack. Testing within 2–3 days of sample collection is preferred, but in some cases, blood samples can remain sufficiently viable for 5 days. A CBC performed within 2–3 days of sample acquisition for flow cytometry is required for interpretation of blood and bone marrow samples.

Aspirates and Body Cavity Fluid

It is essential that tissue aspirates be placed in medium with serum in order to preserve viability. Roswell Park Memorial Institute (RPMI) medium, Dulbecco's modified Eagle's medium (DMEM), and similar cell culture media with 10% fetal bovine serum are ideal. These reagents can usually be found in a university setting. In private practices where this is not readily available, 0.9% saline with 10% conspecific serum can be used. Typically, 1 mL of 0.9% saline lactated Ringer's solution (LRS, Norm R or Norm M may also be used) is added into an additive-free test tube or red-top tube (no serum separator) without anticoagulant, and serum from an animal of the same species (this can be from the patient, but it does not have to be) is added to constitute approximately 10% of the total fluid volume. Solid tissues should be collected using aspiration and gently flushed into the solution until it is cloudy, which requires aspirating about two to three times. For body cavity fluids, provide a minimum of 0.5 mL in an EDTA tube and if available an additional 0.5 mL in a red-top tube (no additive, no serum separator). If the total protein of body cavity fluid is <5 mg/dL or unknown, add two drops of serum (approximately 0.1 mL) from the same patient or another patient of the same species. Aspirates and body cavity fluids should be refrigerated and shipped with a cold pack overnight and tested as soon as possible. This method maintains viability for 2–3 days. Treatment with corticosteroids and/or other chemotherapy at the time of sample collection may alter cell viability or numbers.

The preparation of cells for flow cytometric analysis differs between laboratories. Generally, red blood cells are lysed with a hypotonic solution such as ammonium chloride or water and then stained immediately with fluorescently labeled antibodies.

ANTIBODY PANELS

Veterinary clinical flow cytometry utilizes monoclonal antibodies specific for cell surface (or less commonly cytosolic or nuclear) proteins. The antibodies are either directly conjugated to a fluorescent protein or detected using fluorescently labeled secondary antibodies specific for the monoclonal antibodies (Table 64.1). Each laboratory performing flow cytometry uses different combinations of antibodies and a different approach to immunophenotyping. The goal of designing an antibody panel is to be able to determine the lineage of neoplastic cells and to detect cells with an abnormal immunophenotype, suggesting neoplasia in cases where the diagnosis is not clear. Each laboratory should develop its own panels based on the experience of that laboratory, the most current literature, the technology available to that laboratory (e.g., the capability of the flow cytometer), and the array of antibodies available.

TABLE 64.1 Cell surface markers for immunophenotyping of hematologic neoplasia by flow cytometry.*

CD molecule	Primary cell type
Lymphocytes	
CD3	Pan-T cells
CD5	Pan-T cells
CD4	T helper cells
CD8	T cytotoxic cells
TCRαβ**	T cells
CD21, CD22	Mature B cells
IgM	B cells
Light-chain	Antibody-producing B cells, plasma cells (λ-light-chain expression is most common in cats and dogs)
Myeloid	
CD14	Mature monocytes
CD4	Canine neutrophils
CD1c	Monocytes, dendritic cells
CD11b	Neutrophils, monocytes, some macrophages
CD11c	Neutrophils, monocytes, dendritic cells
CD11d	Monocytes, macrophages, splenic T cells
CD90	Monocytes, dendritic cells, T cells, stem cells
Other	
CD45	All hematopoietic cells (except erythroid)
CD18	Neutrophils^{high}, monocytes^{intermediate}, lymphocytes^{dim}
MHCII	T cells, B cells, monocytes
CD34	Stem cells
CD25	Activated T cells, B cells
CD41, CD61	Platelets, megakaryocytes

* Most common target(s) in the absence of aberrant expression.
** Not commercially available.

It is the authors' opinion that commercially available, directly conjugated antibodies are preferable for several reasons. First, when a small number of commercial sources are used, quality control and reagent preparation (including conjugation) become less of a variable between laboratories. Second, the use of directly conjugated antibodies facilitates multiparameter analysis, which is essential for identifying cells with an abnormal immunophenotype (particularly when such cells are low in numbers). Last, use of directly conjugated antibodies reduces incubation time and nonspecific staining.

A minimum panel for canine hematologic neoplasia should include antibodies to cell surface proteins found on T cells (CD3, CD5, CD4, and CD8), B cells (CD21 or CD22), monocytes (CD14), precursor cells (CD34), and all mature leukocytes (CD18, CD45). An antibody to major histocompatibility complex II (MHCII) should also be included in a canine panel because decreased expression has diagnostic and prognostic utility for subtypes of canine B- and T-cell lymphoma.[6,58] Antibodies to cytoplasmic CD20, CD3e, and CD79a, which require cell permeabilization for staining,[79,86] may be needed to determine the immunophenotype of acute leukemias. Notably, caution is recommended when interpreting intracytoplasmic CD79a (clone HM57) staining for two reasons: (1) nonspecific nuclear staining has been reported that cannot be distinguished from cytoplasmic staining,[60] and (2) CD79a expression has been reported in non-B-cell neoplasms, including T-cell acute lymphoblastic leukemia (LBL) and acute myelogenous leukemia (AML) in people.[38,40] B-cell lineage-specific antigens such as CD20 or Pax5 are preferable to CD79a. The repertoire of monoclonal antibodies available for cats is limited. Directly conjugated antibodies to feline surface CD4, CD5, and CD8 and cytoplasmic CD3 are commercially available, and several additional antibodies react with feline cells, including anti-canine CD21 and CD18, and, possibly, antihuman CD14. The CD18 antibody is helpful to identify CD18 bright neutrophils and to exclude feline platelets, which can be quite large and do not express this antigen.

All flow cytometric panels should contain a cell viability stain (e.g., propidium iodide [PI] or 7-aminoactinomycin D [7-AAD]) to exclude dead cells. The omission of dead cells from analysis is vital, as they can bind antibodies nonspecifically or can inappropriately bind intracellular antigens in cells that lack surface expression of these antigens.[72] In addition, unstained cells and isotype antibodies matching the class and type of the primary antibodies should be included as controls. Fluorescence minus one (FMO) controls are used to identify fluorochrome spread and define gating strategies in a multicolor panel; however, the number of additional tubes required for these controls may be cost-prohibitive for a clinical assay.

TECHNICAL CONSIDERATIONS

As informed by the authors' experiences, there are a number of important, and as yet unpublished, technical considerations in the performance of canine and feline flow cytometry. First, the lysis of feline RBCs using standard ammonium chloride solutions (e.g., ammonium-chloride-potassium, ACK) often results in loss of neutrophils. Additionally, subsets of feline lymphocytes may be lost with water-based RBC lysis approaches. Commercial RBC lysis reagents, which have been reported to have variable success, are available and it is recommended that each laboratory optimize their lysis protocol to minimize nucleated cell loss. Second, anti-CD45 antibody-induced cell death has been observed in canine samples including potentially significant neutrophil loss and minor lymphocyte loss. The cause of the phenomenon has not been defined in dogs, but in mice; cross-linking of CD45 with monoclonal antibodies is reported to induce T-cell death.[28] To minimize this impact, the authors' recommend using the lowest possible concentration of anti-CD45 antibody. In light of this phenomenon, the authors recommend against incorporating this antibody in every tube, which is standard practice in human medicine. Third, autofluorescence from the 633 laser, detected in the 650–670 nm range (allophycocyanin, Alexa Fluor 647, Cy5 fluorochromes), has rarely, but repeatedly, been observed in aspirates of canine tissue or canine blood. Therefore, unstained controls should always be evaluated. Last, artifactual tumor cell binding of a commonly used anti-CD3 antibody (clone CA17.2A12 ST) has been observed in cases of canine B-cell lymphoma/leukemia.

REPORTING FLOW CYTOMETRY RESULTS

A 2017 publication from the European Canine Lymphoma Network provided consensus recommendations for the reporting of flow cytometric findings in canine hematopoietic neoplasms. According to the network, the following details should be reported: tissue and sample type, estimate of sample quality (cell preservation, proportion of viable cells) and method of assessment, proportion or numbers of positive cells, antibodies used, aberrant patterns, qualitative description of immunophenotype of cells of interest, commentary on quantitative expression of relevant markers, diagnosis of immunophenotype, lymphoma subtype (tentative), and party responsible for the service.[18] Any reported patient data should be accompanied by species-specific reference intervals (or ranges). Ideally, each laboratory will establish its own reference data, but flow cytometric reference ranges for hematopoietic cells in canine blood, bone marrow, cerebrospinal fluid (CSF), and lymph nodes have been published.[14,20,23,24,32,67,77,87,89] When size/forward scatter (FSC) of cells in a particular neoplastic population is reported in the literature, it is useful to provide the FSC of a benchmark non-neoplastic population, so that other laboratories can compare their data. To allow for comparisons across individual laboratories and instruments, multiple publications have advocated for using a patient's normal lymphocyte as an internal barometer of cell size. In this work, B cells classified as "large" had a median FSC that was >1.6 times the median FSC of the same patient's $CD5^+$ T cells.[27,58] In addition, the average FSC of neutrophils can be used for comparing the size of neoplastic cell populations between instruments. In one report of canine B-cell chronic lymphocytic leukemia (CLL), cells were categorized as small by flow cytometry if the ratio of the geometric mean of FSC of B cells to neutrophils was <0.55.[11]

Values are reported as percentages of different lymphocyte subpopulations for samples of bone marrow, organ or mass aspirates, and body cavity fluids. For peripheral blood samples it is strongly recommended to report absolute numbers (calculated using a current CBC). Although a similar consensus document has not been published, the chapter authors suggest a similar analytic and reporting approach be adopted for the reporting of feline flow cytometric findings.

DIAGNOSING HEMATOLOGIC NEOPLASIA BY FLOW CYTOMETRY

Objective criteria for defining the presence of a neoplastic cell population by flow cytometry have not been systematically defined within the veterinary community. However, general criteria include: (1) the identification of cells with an aberrant immunophenotype or (2) the identification of a marked expansion of single, immunophenotypically identical, lymphocyte population. The identification of a phenotypically normal lymphocyte population that demonstrates abnormal light scatter properties (e.g., increased FSC or side scatter) should increase suspicion for a neoplastic process. In canine peripheral blood samples, the authors suggest the following diagnostic criteria; namely, an elevated absolute lymphocyte concentration and at least one of the following: (1) >80% of the lymphocytes that share a single immunophenotype, or (2) a immunophenotypically homogeneous lymphocyte expansion of any magnitude and a positive clonality assay (PARR), or (3) the identification of an immunophenotypically aberrant lymphocyte population.[90]

There are important exceptions to these diagnostic criteria. One such is found with *Ehrlichia canis*, in which infected dogs can develop a disproportionate expansion of peripheral blood $CD8^+$ T cells with large granular lymphocyte (LGL) morphology. Young English Bulldogs are another exception to these guidelines with a small B-cell lymphocytosis. This condition was originally described as a unique presentation of B-cell CLL, in which affected dogs were younger and had different antigen expression when compared to the majority of cases.[11] However, further work by one of the authors has demonstrated that these dogs most likely have a non-neoplastic, nonclonal B-cell lymphocytosis (Rout et al., in review). Similar diagnostic criteria are recommended

for flow cytometric evaluation of solid tissues (lymph nodes, tissues, or masses).

Cells can be considered to have an aberrant immunophenotype if the detected phenotype is different from the phenotype expressed by the same cells in health or non-neoplastic disease. This is typified by the inappropriate gain or loss of characteristic antigens. In the dog, such aberrant phenotypes include: T-cell lymphomas/leukemias that lack expression of the pan-leukocyte marker CD45 (i.e., T-zone lymphoma/leukemia),[46,70] $CD3^+$ or $CD5^+$ T cells that lack expression of CD4 or CD8,[19,22,84,90] the coexpression of CD5 and CD21 on neoplastic B-cells,[58] the expression of CD34 on neoplastic B-cells,[89,91] the loss of MHCII expression on both neoplastic B and T cells,[6,22,58] or the loss of CD5 expression on neoplastic T cells.[6] The coexpression of CD4 and CD8 on a T cell can be considered phenotypically aberrant when such cells are found in large proportions in peripheral lymph node aspirates or peripheral blood[19,22,84,89], although a small number of these cells may be consistent with a reactive process.[57,63] $CD4^+/CD8^+$ T cells can be diagnostic for a thymoma when found in a mediastinal mass aspirate.[41] There is significantly less information available for feline leukemia/lymphoma assessed by flow cytometry. The finding of a large proportion of cells in peripheral blood or tissue aspirates that express CD18 but no other lineage antigens is highly suspicious for hematopoietic neoplasia, rather than a reactive process. If intracellular staining indicates that these cells express B- or T-cell antigens (CD3, CD20, Pax5), this could be considered diagnostic for neoplasia.

LYMPHOPROLIFERATIVE DISEASE

While "lymphoma" and "leukemia" are often referred to as separate diseases, the World Health Organization classification system for human lymphoproliferative disorders acknowledges that distinct tumor subtypes may reflect proliferation of an identical tumor cell population and differ only in tissue distribution.[75] For example, B-cell CLL and B-cell small lymphocytic lymphoma (SLL) are considered one disease with variable patterns of tissue distribution (i.e., blood and bone marrow in CLL and tissue in SLL). Neoplasms of precursor lymphocytes can present with either a dominant lymph node or blood/bone marrow disease burden and are referred to as either lymphoblastic lymphoma or leukemia, accordingly. The most defined example in veterinary medicine is canine T-zone lymphoma. In this condition, patients often present with simultaneous peripheral lymph node and peripheral blood involvement, but there is no clinical difference based on tissue of involvement.[25,46,70] Therefore, it is often more clinically useful to define a lymphoproliferative disease based on its phenotype and morphology rather than by the primary site of involvement.

The most common forms of lymphoproliferative disease in veterinary patients are multicentric, peripheral node lymphoma in dogs and intestinal lymphoma in cats.[43,52,56,80,85] Reported prevalence of canine lymphoma subtypes includes 52% diffuse large B-cell lymphoma (DLBCL), 15% peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS), 8% nodal marginal zone lymphoma (NMZL), 3–13% T-zone lymphoma (TZL), 7% other T-cell lymphoma, 6% other B-cell lymphoma, 3% T-cell lymphoblastic lymphoma (T-LBL), 2% mantle cell lymphoma (MCL), and 3% other lymphoma.[7,56,70,81,85] Of these, flow cytometry is most useful in the diagnosis of DLBCL, PTCL-NOS or T-LBL, and TZL (particularly when combined with fine needle aspirate (FNA) cytology). While the literature regarding the flow cytometric profiling of canine lymphoproliferative disease is expanding, limited information is published for cats (Table 64.2).

TABLE 64.2 Immunophenotype of hematologic neoplasia most commonly identified by flow cytometry in cats and dogs.

Neoplasm	Common immunophenotype	Additional considerations
Canine multicentric T-cell lymphoma (PTCL-NOS, T-LBL)	$CD3^+CD4^+CD45^+MHCII^-$	Often exhibit loss of CD5 expression[6]
Canine T-zone lymphoma/leukemia	$CD5^+CD45^-MHCII^{high}$, often $CD3^{dim}$ and $CD25^+$, variably $CD4/CD8^+$ or $^-$, variably $CD21^+$	Loss of CD45 expression and high MHCII expression are consistent features.[46,70] Cells are small to often intermediate (not large)
Canine multicentric B-cell lymphoma	$CD21^+CD45^+MHCII^+$	Low expression of MHCII and large cell size may be negative prognostic indicators[58]
Thymoma	$CD3^-CD5^+CD4^+CD8^+$	>10% of $CD4^+CD8^+$ lymphocytes[41]
Canine B-cell CLL	$CD21^+MHCII^+CD45^+$ often $CD25^+$	Solid tissue involvement is not uncommon[11]
Canine T-cell CLL	$CD3^+CD5^+CD8^+CD45^+$	
Feline T-cell CLL	$CD3^+CD5^+CD4^+$	
ALL	$CD34^+MHCII^-$	Up to 25% of canine ALL may be $CD34^{-}$[1,84,86] Stage V lymphoma may rarely be $CD34^{+}$[31,89]

Canine B-Cell Lymphoma

In dogs with multicentric lymphoma, a B-cell phenotype is more common than a T-cell phenotype, although there are breed-related exceptions.[44,45,50] Of the subtypes described in the earlier text, DLBCL is the most common form of canine multicentric BCL, with less common variants including NMZL, small B-cell lymphoma, Burkitt-like lymphoma, lymphoblastic lymphoma, and follicular lymphoma. Cytologically, canine DLBCL is reflected by a majority (>50%) of large lymphocytes (i.e., larger than a neutrophil). However, there is insufficient published information to broadly characterize the cytology of other subtypes of canine nodal lymphoma. Flow cytometrically, the neoplastic cells in canine BCL are CD21$^+$, CD45$^+$, and MHCII$^+$ (Figure 64.1 and Table 64.2). The size of the neoplastic cells as characterized by FSC is highly variably in canine BCL, including small and large cell variants.[45] Notably, in the authors' experience, cell size assessment by microscopy (particularly FNA cytology) tends to assign larger sizes than cell size assessment (via FSC evaluation) by flow cytometry. As such, although most cases of canine B-cell lymphoma are described as large cells by cytology, they are described as intermediate-sized cells by flow cytometry.[45,58] In light of the overlap in phenotype (and perhaps due to the

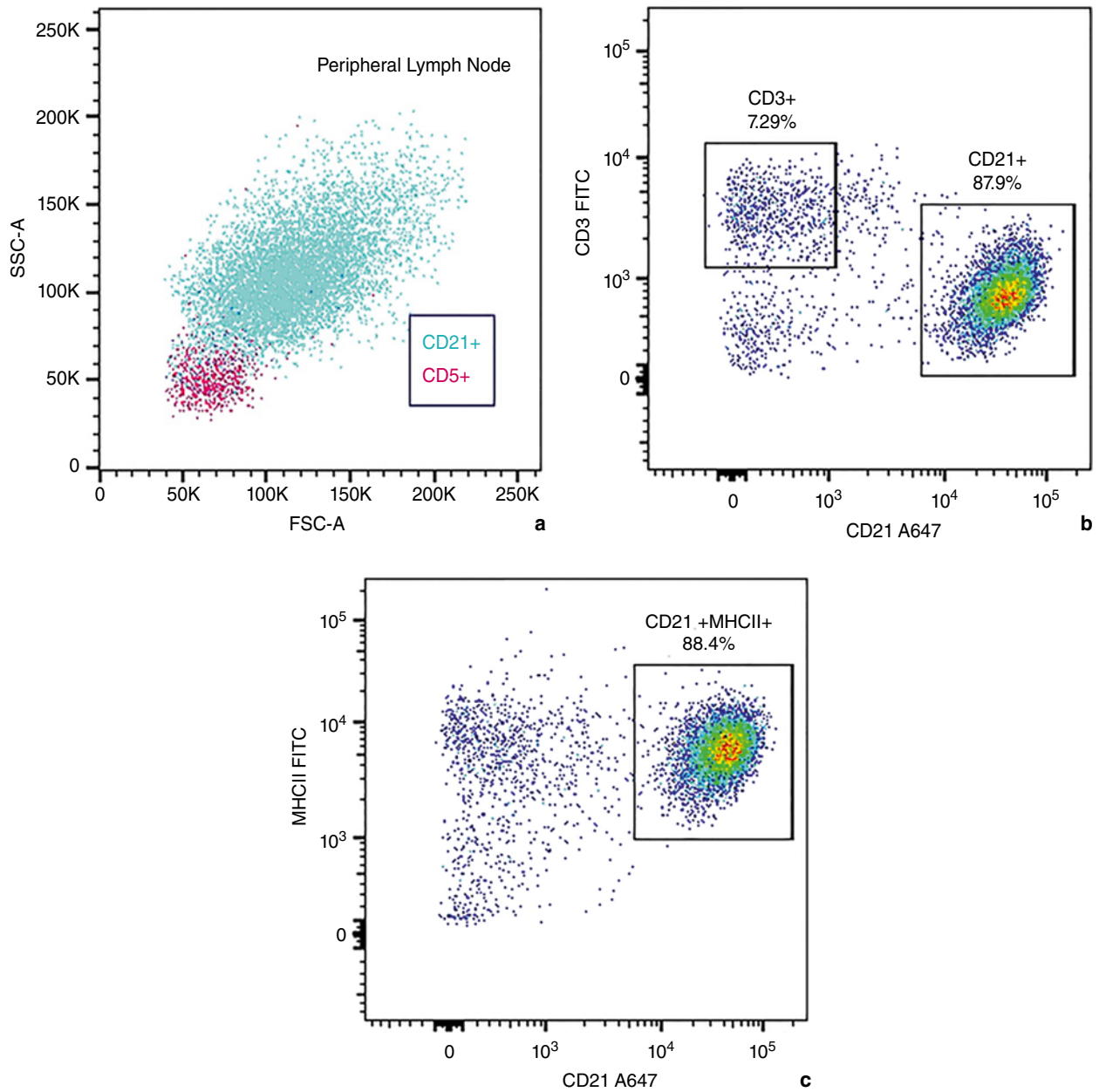


FIGURE 64.1 Flow cytometric analysis of a canine peripheral lymph node with multicentric B-cell lymphoma. (a) Increased light scatter shows an expansion of intermediate to large neoplastic B cells (aqua) with lower numbers of small non-neoplastic T cells (pink); (b) The neoplastic CD21$^+$ B cells comprise approximately 88% of the lymphocyte population and (c) express major histocompatibility complex II.

limited reagents), flow cytometry alone cannot be used to differentiate among the subtypes of BCL.[45]

In designing a flow cytometry panel, the authors recommend including a tube containing antibodies directed against CD21 and MHCII for potential prognostic purposes. In a study of 160 dogs with BCL, tumor cell immunophenotype and forward scatter were reported as independent predictors of outcome.[58] In this series, the majority of cases (93.1%) were composed of intermediate-sized $CD21^+$ B cells with high MHCII expression and a median survival time (MST) of 330 days. By contrast, BCL patients in which the neoplastic cells were defined as large cells or had low MHCII expression had MSTs of 155 days and 120 days, respectively.[58] Notably, expression of CD34 or CD5 in B-cell lymphomas was not correlated with outcome. Recent work focusing on DLBCL only does not demonstrate the prognostic significance of either MHCII expression or tumor cell size.[91] Flow cytometry has also been used to assess MRD in lymph node aspirates from dogs in complete remission following chemotherapy for DLBCL and predicted a shorter time to relapse when >0.5% large B cells were identified.[16]

Canine T-Cell Lymphoma

In the dog, although T-cell lymphoma occurs less frequently, immunophenotyping by flow cytometry is important to identify the two most common biologically distinct subtypes; namely, aggressive multicentric T-cell lymphoma and indolent TZL.

Multicentric T-Cell Lymphoma

Two of the most common forms of canine multicentric T-cell lymphoma (i.e., PTCL-NOS and T-LBL) are composed of $CD3^+$, $CD4^+$, $CD45^+$ lymphocytes that demonstrate variable expression of CD5 and frequent loss of MHCII (Figure 64.2).[6] To facilitate their detection by flow cytometry, the authors recommend combining CD3, CD4, CD8, and CD5 in one tube and CD4 and MHCII in a separate tube. The neoplastic cells are often intermediate to large by cytology, but variable in size by flow cytometry. In one study evaluating the clinical outcome of 61 dogs with multicentric $CD4^+/CD45^+/MHCII^-$ T-cell lymphoma, MST was 159 days.[6] Hypercalcemia and/or mediastinal masses are common findings in dogs with $CD4^+/CD45^+$ T-cell lymphoma; however, several studies have demonstrated that their presence is not a negative prognostic indicator.[6,10,58] An increased incidence of multicentric $CD4^+/CD45^+$ T-cell lymphoma has been reported in Golden Retriever and Boxer dogs as well as other pure breed dogs.[44,45,50]

T-Zone Lymphoma/Leukemia

Canine TZL has an indolent course in dogs and a unique flow cytometric immunophenotype. The neoplastic cells are small to intermediate in size, $CD5^+$ T cells that lack expression of the pan-leukocyte antigen CD45. In addition, the cells are $MHCII^{high}$, variably $CD4^+$ and/or $CD8^+$ ($CD4^+/CD8^-$, $CD4^-/CD8^+$, $CD4^+/CD8^+$, $CD4^-/CD8^-$), $CD3^{dim}$, and variably $CD25^+$ and $CD21^+$ (Figure 64.3).[46,70] To facilitate their identification, the authors recommend including a tube in a diagnostic canine panel containing CD5, CD21, and CD45. Identification of TZL by flow cytometry has resulted in increased recognition such that it represents 3–13% of all canine lymphomas and up to 60% of indolent canine lymphomas.[25,56,82] On cytology, cells are small with expanded cytoplasm that may have an extension creating a "mirror-handle" appearance.[5664,93] TZL is characterized by one or more enlarged lymph nodes and/or lymphocytosis that are often identified in asymptomatic older dogs.[49,70,82,83] Reported MSTs range from 637–1099 days.[25,46,70,83] Lymphocytosis is present in over half of cases and is not associated with a worse prognosis.[25,46,70] A higher incidence of TZL has been observed in Golden Retriever dogs and may be due to an increased frequency of TZ cells in this breed.[36,70] A unique lingual variant of TZL has been reported in 12 dogs with tongue masses, concurrent lymphocytosis and/or lymph node enlargement, and an indolent clinical course.[34] Notably, the lack of CD45 expression on T cells cannot alone be used as an absolute indicator of TZL. One case of suspected canine TZL, based on the flow cytometric identification of a $CD45^-$ T-cell population, was later classified as high-grade canine peripheral T-cell lymphoma by histopathology. Importantly, the neoplastic cells were, according to FSC, larger than those identified in typical TZL.[54] As such, although canine TZL has a unique immunophenotypic signature, if atypical features are identified, including increased cell size or variability in MHCII or CD5 expression, additional assessment using histopathology with immunohistochemistry (IHC) should be performed.

Thymoma and Mediastinal Lymphoma

Flow cytometry can help distinguish between two, cytologically similar mediastinal neoplasms, namely thymoma and mediastinal lymphoma. Thymomas contain thymocytes that express CD4, CD8, and CD5. In one study, the identification of >10% $CD4^+CD8^+$ cells from canine mediastinal mass samples correlated with histologic diagnosis of thymoma.[41] Similarly, a diagnosis of metastatic thymoma was supported by the identification of small $CD4^+CD8^+$ T cells in the liver of a dog.[88] By contrast, canine mediastinal T-cell lymphoma is reported as predominantly a $CD4^+/CD8^-/MHCII^-$, or $CD4^-/CD8^+/MHCII^-$ disease. In one report, a single case of $CD4^+/CD8^+/MHCII^-$ disease was identified, but, importantly, the neoplastic lymphocytes were significantly larger than the $CD4^+/CD8^+$ thymocytes in canine thymoma.[22,41] Paraneoplastic, polyclonal $CD4^-/CD8^-$ lymphocytosis has been reported in association with canine thymoma.[8,13]

Less-common subtypes of canine T-cell lymphoma include hepatosplenic and hepatocytotropic lymphoma. These are distinct and aggressive subtypes that require surgical histopathology for diagnosis. Reported IHC findings include $CD4^-/CD8^+$ or $CD4^-/CD8^-$, and either $CD3^+/TCR\alpha\beta^-/TCR\gamma\delta^+$ or $CD3^-/TCR\alpha\beta^-/TCR\gamma\delta^-$ with

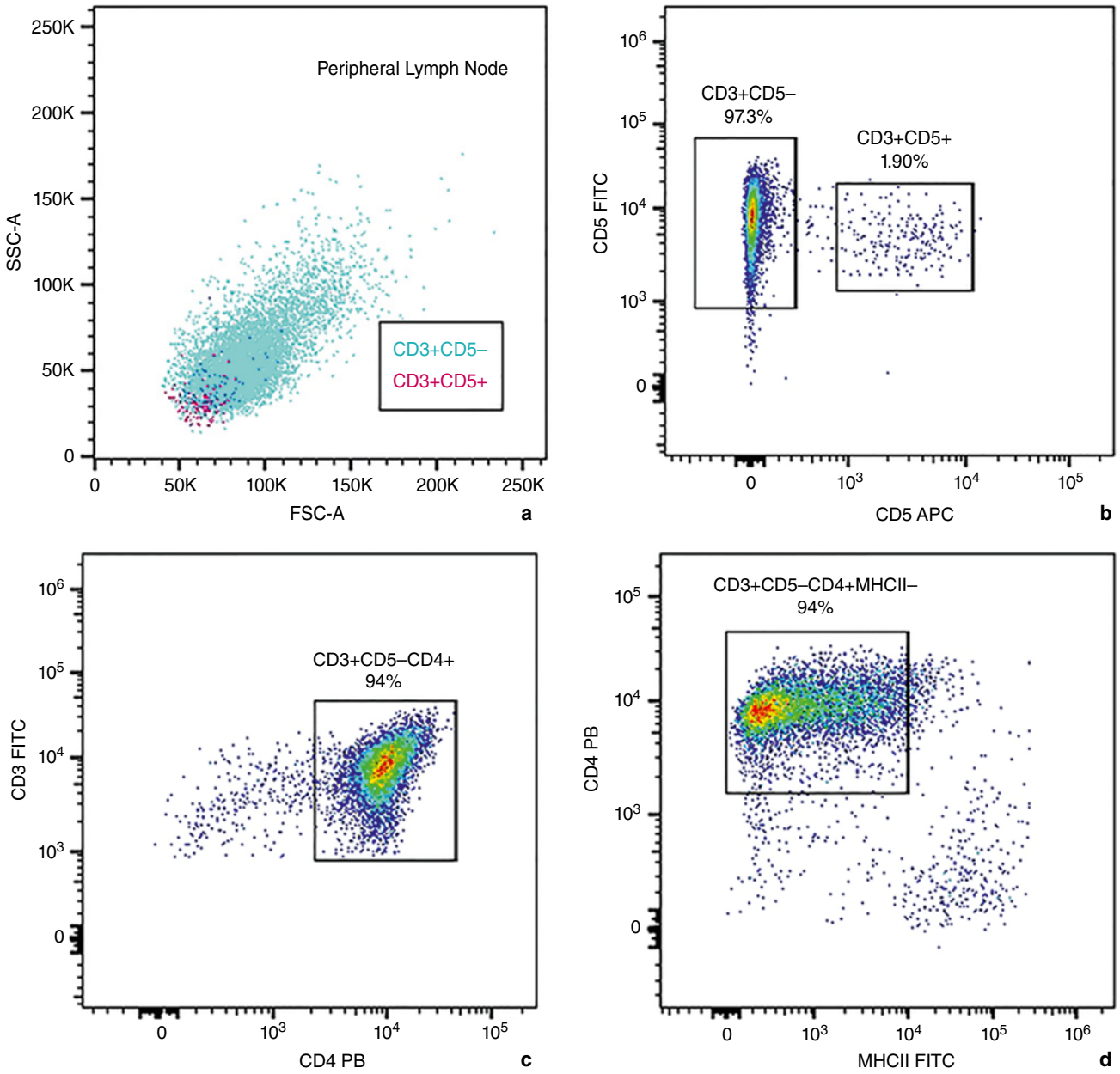


FIGURE 64.2 Flow cytometric analysis of a canine peripheral lymph node with multicentric T-cell lymphoma. (a) Increased light scatter shows an expansion of intermediate to large neoplastic T cells (aqua) with lower numbers of small non-neoplastic T cells (pink); (b) The neoplastic $CD3^+$ T cells comprise approximately 97% of the lymphocyte population and lack expression of CD5; (c) The population is $CD4^+$ and (d) lacks expression of major histocompatibility complex II.

CD11d expression in hepatosplenic types only.[37] Cutaneous epitheliotropic T-cell lymphoma (CETCL) is, according to both IHC and flow cytometry, a neoplasm of $CD4^-/CD8^+/MHCII^-$, $CD4^-/CD8^+/MHCII^+$, or $CD4^-/CD8^-/MHCII^-$ T cells.[22,27,51]

Feline Lymphoma

Little data are available characterizing the flow cytometric features of feline lymphoma. Intestinal lymphoma, which is the most common tissue type of feline lymphoma, is a low-grade, $CD3^+$ T-cell disease (low-grade alimentary lymphoma [LGAL]) and is more common than high-grade, large-cell, $CD21^+$ B-cell disease.[26,42,52,55,66] Differentiating LGAL from inflammatory bowel disease (IBD) can be a frustrating diagnostic dilemma. These diseases cannot be distinguished by cytology and may still be challenging with histopathology. Lymphoid clonality (PARR) testing is a sensitive means to support a diagnosis of feline LGAL, as well as being a predictor of biologic behavior and clinical outcome.[52,68] Flow cytometry may provide a useful adjunct to cytology and PARR, but it has not yet been thoroughly investigated. Large B-cell lymphoma can be diagnosed

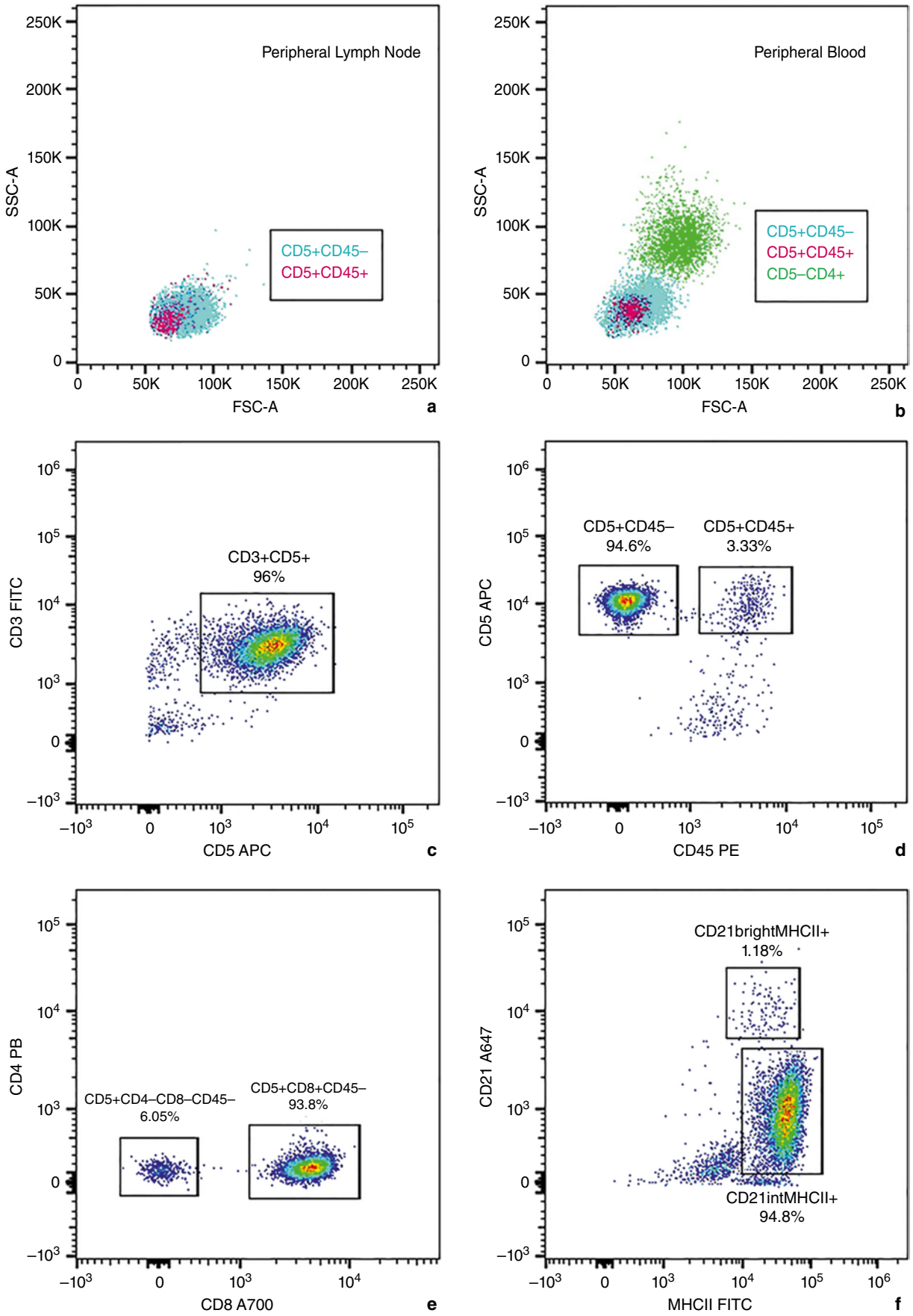


FIGURE 64.3 Flow cytometric analysis of a canine peripheral lymph node with T-zone lymphoma/leukemia (TZL). (a) Light scatter shows an expansion of intermediate neoplastic T cells (aqua) with lower numbers of small non-neoplastic T cells (pink); (b) A similar expansion of lymphocytes is seen in peripheral blood, $CD5^{-}CD4^{+}$ neutrophils are also shown; (c) The neoplastic $CD3^{+}CD5^{+}$ T cells comprise approximately 96% of the lymphocyte population and (d) lack expression of CD45; (e) the majority are $CD8^{+}$ with a lower percentage lacking expression of both CD4 and CD8; (f) The TZL population expresses a high level of major histocompatibility complex II and a lower level of CD21 than residual non-neoplastic B cells.

by cytology; however, immunophenotypic characterization may provide prognostic indicators. Feline intestinal large granular lymphoma is an aggressive, less-common form of intestinal lymphoma that may be diagnosed by cytology and is most often $CD3^+/CD8^+$.[61] As more reagents become available, the diagnostic and prognostic utility of flow cytometry for cats may increase.

LEUKEMIA

Chronic Leukemia

Canine B-Cell CLL

$CD21^+$ B-cell CLL (B-CLL) is one of the most common forms of canine CLL, occurring most often in older, small breed dogs. The disease is slowly progressive with a reported MST of 480 to >1000 days.[11,19,90] Although there are not widely agreed upon diagnostic criteria, one publication, which represents the largest study of canine B-cell CLL (491 cases), defined cases as dogs with: (1) an absolute peripheral blood lymphocyte count >5000 cells/μL and (2) >60% of lymphocytes comprising small $CD21^+$ B cells. Cells were categorized as small by flow cytometry if the ratio of the geometric mean of FSC of B cells to neutrophils was <0.55; cytomorphology was not included in this case definition.[11] Half of the cases in this study had lymphadenopathy and/or splenomegaly, which suggests frequent involvement of both solid tissue and peripheral blood. As noted in the earlier text, English Bulldogs are affected by a unique form of apparently reactive lymphocytosis that mimics B-cell CLL. However, in contrast to typical canine B-cell CLL, the B cells from affected English Bulldogs tended to have lower MHCII and CD25 expression as compared to non-English Bulldogs. Moreover, work by one of the authors has demonstrated that these dogs most likely have a non-neoplastic, nonclonal B-cell lymphocytosis (Rout et al., in review). Similar diagnostic criteria are recommended for flow cytometric evaluation of solid tissues (lymph nodes, tissues, or masses).

Although leukemic involvement of B-cell small lymphocytic lymphoma is a theoretical consideration in these patients, this is generally considered unlikely given the rarity of this lymphoma subtype in dogs.[33,82,83,85] In human medicine, the distinction between CLL and small lymphocytic lymphoma, in part, depends upon the distribution of neoplastic cells in the lymph node, but studies examining lymph node histology in cases of presumptive canine B-CLL are not available.

Canine T-Cell CLL

The entity historically referred to as "canine T-cell CLL (T-CLL)" likely represents at least three separate subtypes, namely, TZL, LGL CLL, and non-LGL CLL. The circulating neoplastic cells in canine TZL, which are reported present in up to 100% of nodal TZL cases, comprise a phenotypically aberrant population of $CD45^-CD5^+$ and/or $CD3^+$ T cells that demonstrate variable CD4 and CD8 expression.[70] LGL leukemia is characterized by an expansion of intermediate-sized $CD8^+$ T cells with fine intracytoplasmic granules, frequent splenomegaly, and minimal bone marrow involvement.[84,76] Canine LGL CLL has a higher prevalence in large breed dogs and has been shown to have an indolent course.[47] Other categories of T-CLL include non-LGL $CD8^+$ T-cell CLL and $CD4^-/CD8^-$ T-CLL.[71,77] The survival time of canine T-CLL is heterogeneous. In one study, dogs with $CD8^+$ T-CLL with absolute peripheral blood lymphocyte counts >30,000 lymphocytes/μL had a poor median survival time (131 days) compared with those presenting with <30,000 lymphocytes/μL (1098 days).[90] In another study, MST for 19 cases of T-cell CLL was 930 days and presence of anemia was associated with shorter survival time.[19] However, neither of these studies specifically excluded TZL.[19,90] A recently described form of aggressive T-cell leukemia that may be mistaken for T-cell CLL based on morphology and flow cytometry was identified in 118 dogs.[30] This disease is characterized by marked expansion of small, mature $CD5^+$ T cells that do not express CD4 or CD8 and express very low levels of MHCII. It is typically seen in young, primarily male dogs, and is most common in English Bulldogs; hepatic and splenic involvement is common and MST was 35 days.

It is important to note that the reactive $CD8^+$ lymphocytosis induced by *E. canis*, while typically <18,000 cells/μL, has been anecdotally reported to reach 30,000 cells/μL.[5,17,29,35,39,48] Notably, the $CD8^+$ T-cell response induced by *E. canis* infection may be clonal based on assessment using the PARR assay.[12]

Feline CLL

Most cases of feline CLL result from the expansion of T cells, namely, $CD4^+$, $CD4^+/CD8^+$, and $CD4^-/CD8^-$ cells.[15,65,92] The largest study of feline lymphocytosis (>6000 lymphocytes/μL) described five main flow cytometric immunophenotyes: (1) $CD21^+$ B cell, (2) $CD4^+$ T cell, (3) $CD4^-CD8^-$ T cell, (4) CD5 low T cells, and (5) heterogeneous (two or more lymphocyte lineages expanded).[65] The majority of B-cell and heterogeneous lymphocytoses demonstrated polyclonal PARR testing results and were considered non-neoplastic. Of the neoplastic lymphocytosis, $CD4^+$ T cell was most common and had a longer MST (752 days) than $CD4^-CD8^-$ (271 days) or CD5 low phenotypes (27.5 days). These results are similar to a prior study of 18 cats, in which the majority were $CD4^+$ T cell, with an overall MST of 14.4 months (range: 0.9–25.3 months), and a favorable response to treatment with oral chlorambucil and prednisolone.[15] By contrast, B-CLL appears to be uncommon in cats as clonality was identified in only two of 46 cases of $CD21^+$ lymphocytosis, including cats with B-cell counts as high as 26,000/μL.[65]

Acute Leukemia

Acute leukemia may be lymphoid (ALL), myeloid (AML, see Chapter 67), or undifferentiated (AUL). The flow cytometric detection of surface expression of the

stem cell marker CD34 and a corresponding lack of MHCII expression (i.e., $CD34^+/MHCII^-$) are characteristics of acute leukemia. Beyond this, lineage assignment of an acute leukemia into a myeloid or lymphoid origin can be challenging. Correspondingly, several studies assessing lineage of canine acute leukemia have reported variable frequencies of ALL, AML, and AUL likely due to variation in diagnostic methodology.[1,9,21,53,76,84] Further lineage assignment likely requires increase in usage of antibodies to cytoplasmic (intracellular) CD20 and CD3e[79,86]. Importantly, molecular clonality assessment (i.e., PARR testing) should not be used for lineage assignment in canine acute leukemia as T- and/or B-cell receptor rearrangements have been identified in up to 64% of canine AML cases.[12,73] The largest case series of canine $CD34^+/MHCII^-$ acute leukemia (194 cases) identified three immunophenotypes ($CD5^+$:ALL, $CD14^+$:AML, and AUL).[64] Golden Retriever and German Shepherd dogs were overrepresented in the ALL and AML groups, respectively.[64] Importantly, the clinical significance of lineage assignment in canine acute leukemia is uncertain, as all immunophenotypes shared a similarly poor clinical outcome. It is important to note that CD34 positivity should not be considered requisite in the diagnosis of acute leukemia as CD34-negative cases have been reported.[73,79,84,86,90] Moreover, CD34 expression should not be considered a specific marker of an acute neoplasm, as its expression has been described in a small subset of canine lymphomas.[31,58,89] In such cases, other flow cytometric, hematologic, and clinical data (e.g., MHCII expression, presence or absence of cytopenias, and clinical presentation) should be used to provide diagnostic context to the CD34 positivity. Cytochemistry can be useful for assigning a monocytic/myelomonocytic origin in acute leukemia and is discussed elsewhere (Chapters 61 and 67).[74] The flow cytometric detection of CD41, CD61, CD62P has been successfully used to diagnose acute megakaryocytic leuekmia.[3] Reports of acute leukemia in cats are rare and largely associated with FeLV infection.[71,78]

Histiocytic Neoplasia

There is limited published information on the role of flow cytometry in the diagnosis of canine histiocytic neoplasia. Flow cytometry has also been used to diagnose histiocytic neoplasia in dogs with peripheral blood involvement.[2,62] Dendritic cell leukemia was diagnosed in a Golden Retriever dog through the identification of a $CD1c^+/CD11c^+/CD18^+/MHCII^+/CD34^+/CD49d^+/CD54^+/CD45^+$ population that was negative for markers of macrophage (CD11d), granulocytic (CD4/11b), and of lymphoid (CD3/CD5/CD4/CD8/CD21/TCR) origin.[2] Disseminated histiocytic sarcoma with peripheral blood involvement was identified in a Bernese Mountain dog through the flow cytometric identification detection of a $CD1c^+/CD11c^+/CD18^+/CD45^+/CD14^+$ population.[62] In the authors' experience, histiocytic neoplasia of solid tissues can be diagnosed using flow cytometry from FNA samples based upon the identification of a large cell, $CD14^+$, $MHCII^+$, $CD45^+$ population.

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CHAPTER 65

Classification and General Features of Lymphoma and Leukemia

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Acronyms and Abbreviations

CLL, chronic lymphocytic leukemia; CNS, central nervous system; FeLV, feline leukemia virus; FC, flow cytometry; FIV, feline immunodeficiency virus; DLBCL, diffuse large B-cell lymphoma; ICC, immunocytochemistry; IHC, immunohistochemistry; LBL, lymphoblastic lymphoma; NCI, National Cancer Institute; NIH, National Institutes of Health; NMZL, nodal marginal zone lymphoma; MCL, mantle cell lymphoma; MI, mitotic index; MHC, major histocompatibility complex; PTCL NOS, peripheral T-cell lymphoma, not otherwise specified;; PTZL, peripheral T-zone lymphoma; REAL, revised European–American classification of lymphoid neoplasms; TCL, T-cell lymphoma; WHO, World Health Organization.

INTRODUCTION

As a complement to the preceding and following chapters, which provide subtype-specific clinical, diagnostic, to a limited degree, treatment information, this chapter will focus on: (1) the history and evolution of the classification of veterinary lymphoid neoplasms and (2) a general algorithmic approach toward the diagnosis of veterinary lymphoid neoplasms. For subtype-specific information, the reader is referred to Chapter 66 (Myeloproliferative Neoplasms), Chapter 68 (Acute Myeloid Leukemia), Chapter 69 (B-Cell Tumors), Chapter 70 (Plasma Cell Tumors), Chapter 71 (Hodgkin Lymphoma), and Chapter 72 (T-Cell Tumors).

CLASSIFICATION

Introduction to the Concepts of Classification

The development and implementation of lymphoid neoplasia classification schemes, both in human and veterinary pathology, have always been complex matters. This complexity results from a number of factors, including the inherently fluid nature of all classification schemes (which require iteration, as diagnostic and clinical knowledge expands), the persistent importance of cell morphology (which requires pathologist expertise), and the need to align geographically defined schemes (i.e., European vs. North American). One of the first

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mentioned systems in the human field is from 1934, i.e., the "Classification of Lymphatic and Reticular Tumors" from the American Registry of Pathology.[8] All lymphoid neoplasm classification schemes were initially developed for humans, but their subsequent application in veterinary medicine has revealed an astonishing level of variation in animal leukemia and lymphoma.[113] Their application has also revealed a number of differences, including a higher proportion of high-grade lymphomas, a lower proportion of follicular neoplasms, and the rarity of Hodgkin-like neoplasms in animals.[64] With advances in veterinary diagnostics, including clonality assays and flow cytometry (FC), the diagnosis of "lymphoma" is no longer considered adequate.[115]

History of Classification

Any discussion of lymphoma classification requires an understanding of the evolution of its basic language. The first mentioning of "leukemia" was by Rudolf Virchow in 1845, the term "lymphoma" was introduced in 1863, and Theodor Billroth introduced the term "malignant lymphoma" in 1871.[43] Notably, the term "lymphosarcoma" defined by the National Dictionary of Cancer Terms of the National Institutes of Health (NIH) is an "obsolete term for a malignant tumor of lymphatic tissue" (https://www.cancer.gov/publications/dictionaries/cancer-terms/def/lymphosarcoma). Additionally, the designation of lymphoma as "malignant" should also be abandoned as it is redundant (all forms of lymphoma are malignant) and it is no longer used in any currently used classification system.[20] The fact that there are indolent forms, such as follicular, mantle cell, and marginal zone lymphomas of B-cell type, and T-zone lymphoma of T-cell type,[114] should not justify this name.

One of the earliest classification systems, published in 1934 in the American Registry of Pathology, divided neoplastic cells into three groups according to cell size, namely: lymphocytic (small cells), lymphosarcoma (medium-sized cells), and reticulum cell (large cells).[8] Coincident with this (1938), an English classification system created by Robb-Smith defined benign and malignant lymphoid disorders using the reticuloendothelial system.[88] In 1942, Gall and Mallory created a new system based on morphology and clinical outcome that, among other features, was the first to define Hodgkin's and non-Hodgkin's lymphoma as it is still now discriminated.[30] This system was the first to be accepted in the USA. In 1944, Jackson and Parker presented another system focusing on Hodgkin's disease, which was used unchanged until 1966.[39] In 1956, based on the Gall and Mallory system, Rappaport proposed an eponymous classification system that divided lymphoid neoplasms into diffuse or follicular according to their architecture pattern.[83,84] Although this system became the standard classification in North America, as a result of its overly dogmatic embrace of architecture, some previously known lymphoma subtypes were lost and, in retrospect, the Rappaport system is considered a regressive step in classification history.

During the 1960s and 1970s, the classification landscape was transformed by research allowing for the routine discrimination of the three distinct lymphocyte cell lineages, namely, B, T, and natural killer lymphocytes.[47] Simultaneous with this work, two new classification systems were published, namely, the Kiel and the Lukes and Collins systems.[50,55] As the latter required neoplastic cell immunophenotyping (either B or T cell), which was still not widespread in availability, it was never widely used. The Kiel classification was based on a hypothetical scheme of leukocyte differentiation, and the lymphomas were grouped into low or high grade according to cytological features. The Kiel system was updated in 1988, but was primarily utilized in Europe and never in the USA.[101] During this time, various other classification systems were proposed, including the "working classification,"[21] the British national lymphoma investigation classification[6] used in the UK, and the World Health Organization (WHO) classification.

In an attempt to simplify the expanding classification landscape, the National Cancer Institute (NCI) developed the "working formulation."[90] The scheme, which was primarily based on the Rappaport classification, but also included terminology from Lukes and Collins, was directed at predicting outcome and clinical behavior of each subtype. Two features were new in this system, namely, grading based on "patient survival" (rather than tumor cell morphology) and the discrimination of tumors into "low", "intermediate," and "high grades" (based on morphology). Although this system allowed oncologists to tailor grade-specific treatment, tumor cell morphology and phenotype were somewhat neglected. This system was widely adopted in Northern America, but, in Europe, the Kiel classification persisted.

During the 1980s and 1990s, many new entities were characterized and monoclonal antibodies became increasingly available for immunophenotyping (including in veterinary medicine).[74] In 1991, the first attempt to develop a unified classification by the International Lymphoma Study Group (ILSG) resulted in the European–American classification of lymphoid neoplasms. This system, for the first time, combined morphology, phenotype, genetic features, and clinical aspects. The classification system was presented in 1993[43] and an updated version, the revised European–American classification of lymphoid neoplasms (REAL), was presented in 1994.[36,43] The current and most widely used classification system is the WHO classification. This classification (the WHO classification of hematologic malignancies), originated in 1995 and presented in 1999, is developed by the European Association of Pathologists and the Society for Hematopathology.[35] The WHO system updated and revised the REAL classification and stratified hematologic neoplasms according to lineage as either myeloid neoplasms, lymphoid neoplasms, mast cell disorders, or histiocytic neoplasms. Like the REAL scheme, it incorporates tumor cell morphology, immunophenotype, and genetic and clinical features. Importantly, the WHO scheme is flexible, allowing for

the identification of new entities and subtypes. The current WHO classification of tumors of hematopoietic and lymphoid tissues represents a revised 4th edition.[106]

Classification of Lymphoma and Leukemia in Veterinary Medicine

The classification of veterinary lymphoid neoplasms directly results from the adoption of human-developed schemes. Notable highlights in this history include the adaptation of the Kiel classification for canine, feline, and bovine lymphoma in 1981.[112] Using the scheme, canine lymphomas were classified according to the proportion of small cells as either large-cell (<50% of small cells), mixed-cell (50–70% of small cells), or small-cell (>70% of small cells) lymphomas.[25,26,49] A second study assigned tumor grade by combining cell size with mitotic index (MI).[81] Specifically, cases with a majority of small cells and a low or medium MI were classified as low-grade lymphomas, whereas those showing a majority of medium and large cells and a moderate to high MI were classified as high-grade lymphomas.[97] Similar to human medicine, there were geographic differences in the adoption of veterinary classification schemes. The "working formulation" was used in the United States, whereas the updated Kiel classification was used in Europe.[11,27,31,107,110,119]

As in human medicine, veterinary experts also wanted to employ a uniformly used classification with the goal to improve diagnostics, to compare larger patient cohorts in multicenter studies, and to enhance comparative collaboration with the human field. They aimed at strengthening the canine patient as a spontaneous disease model for canine lymphoma and to promote a classification system containing morphology, immunophenotype, and genetic and clinical information.

For this purpose, the first Histological Classification of Hematopoietic Tumors of Domestic Animals was published in 2002.[115] This scheme drew directly from the 2001 REAL/WHO system and attempted to harmonize different expert groups, continents, and philosophies. The most recent version of this classification (2011) describes the six most common diagnoses (representing 80% of all canine cases)—diffuse large B-cell lymphoma (DLBCL), marginal zone lymphoma (MZL), peripheral T-cell lymphomas not otherwise specified (PTCL, NOS), T-zone lymphoma (PTZL) T-cell lymphoblastic lymphoma (T-LBL), and follicular lymphoma.[113] Notably, the classification of veterinary lymphoid neoplasms is based exclusively on morphology and immunophenotype, as other features (cytogenetic and other molecular changes) are still under investigation and not routinely applied.[77] This system has been used in various publications for different species.[2,22,24,28,42,67,68,123,124]

A recent review of veterinary classification schemes identified the updated Kiel and the WHO systems as the most valuable.[98] In light of its inherent adaptability, its reproducibility, its diagnostic precision, its provision of prognostic information, and its ability to allow for the worldwide comparison through the creation of large patient cohorts, the authors encourage and embrace increasing adoption of the WHO system.

Cytologic Classification of Lymphoma

All lymphoma classification systems rely upon tumor and tumor cell morphology. Currently, the gold standard for the diagnosis and classification of veterinary lymphoid neoplasms is histopathology (with immunophenotyping). However, the role of cytologic morphology (i.e., cytomorphology) in the process of classification is less certain. One Kiel-based study using cytology was published in 1997; however, no similar work has evaluated cytology in the comparison with the WHO scheme.[25] In light of its ease and high pet owner compliance, incorporating cytology in the classification of lymphoma is an attractive proposition.[97] However, within the veterinary clinical pathology community, although fine needle aspirate cytology is considered useful in the diagnosis of lymphoma, its role in classification requires further study.[4,91,111,113]

Lymphoma

Basic Biologic Aspects of Lymphoma

Lymphoma, first described in 1863 as lymphosarcoma, is a heterogeneous group of diseases that encompasses a number of subtypes of varying malignancy that occur as the result of neoplastic transformation of B and T lymphocytes at different stages of development.[43] Although a review of the basic oncogenic features of lymphoma is beyond the scope of this chapter, like other cancers, lymphoma is characterized by chromosomal modifications and genetic mutations that contribute to tumor initiation and progression.[3] The WHO classification lists more than 30 subtypes of canine lymphoma and there is significant overlap in subtype frequency and biology between dogs and humans.[113]

Incidence and Manifestation

The majority of the published information on veterinary lymphoma describes canine and feline forms of the disease.[87,77,38,32,14] The age-adjusted overall incidence of canine lymphoma is 1.5 and 84 per 100,000 dogs younger than 1 year and greater than 10 years of age, respectively.[60] The most common subtype of canine lymphoma is diffuse large B-cell lymphoma, and the frequency of acute and chronic leukemias has not been estimated (Figure 65.1).[81,116] In the cat, lymphoma is the most common hematopoietic neoplasm and is the highest for any veterinary species.[73] The overall frequency of equine lymphoma is less studied, but represents 1–14% of all equine tumors.[44,22,54,63,109,86]

Lymphoma has been described in a number of species, including the cheetah, fossa, panther, lion, goat, rabbit, dolphin, harbor seal, and parrot.[52,51,41,37,42,120,2,1,34,56]

In the dog, there are worldwide studies demonstrating the impact of breed on the development of lymphoma. In one European study, Dobermans, Rottweilers, Boxers, and Bernese Mountain Dogs showed a significant predisposition to lymphoma, including an increased frequency of T-cell lymphoma, B-cell lymphoma, and

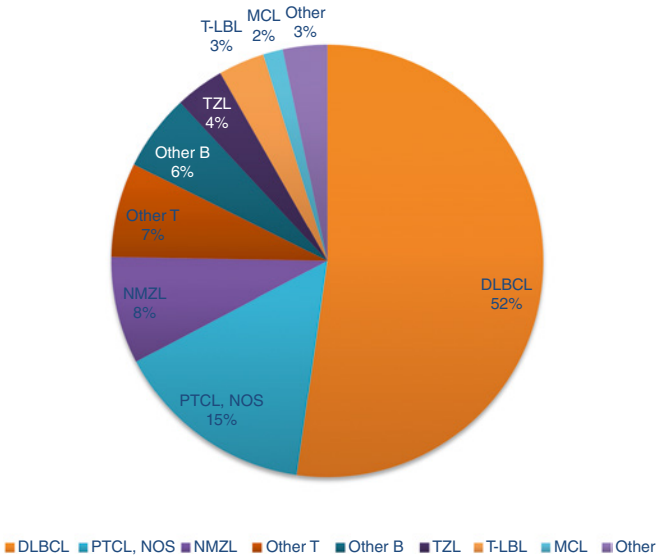


FIGURE 65.1 The frequency distribution of canine lymphoma as categorized by the WHO classification scheme.

high-grade T-cell lymphoma in Boxers, Rottweilers, and Labradors, respectively.[15] Similar results were reported in an Australian study while adding the Border Collie and Australian Cattle Dog.[125] In South Africa, the Labrador Retriever, Boerboel, Bull Terrier, Border Collie, Boxer, English Bulldog, Rhodesian Ridgeback, Basset Hound, Belgian Shepherd, Bullmastiff, Mastiff, Kerry Blue Terrier, and Schipperke were identified to be at an increased risk for lymphoma.[117] A US study identified geographic differences in the distribution of B-cell lymphoma and T-zone lymphoma in Golden Retrievers with T-zone lymphoma more likely to be diagnosed in dogs from the Northeast and East North Central regions as compared to dogs in the mountain region of the USA.[94] By contrast, European Golden Retrievers did not show any predisposition to lymphoma or T-zone lymphoma.[15] Limited information in the horse has shown that Quarter Horses almost exclusively develop T-cell-rich, large B-cell lymphoma and Thoroughbreds develop cutaneous lymphoma more commonly than other breeds.[67]

Causative Factors

There are limited studies defining the etiology of lymphoma in veterinary patients.[78,71] In humans, environmental agents, genetic predispositions, immune dysregulation, and infectious agents have been cited as potential causes.[117] In veterinary medicine, genetic abnormalities (e.g., chromosomal aberrations and epigenetic modifications) and environmental factors have been implicated.[17,71,78]

Twenty years ago, feline lymphoma was often affiliated with feline leukemia virus (FeLV) infection. Nowadays, FeLV is much less commonly associated with lymphoma. Cats with FeLV antigen and lymphoma are more likely to be younger and to have mediastinal lymphoma. A cat with FeLV antigenemia is less likely to have a long survival, possibly due to development of other diseases as well as lymphoma. However, FeLV is currently an uncommon disease, and the majority of cats with lymphoma are unaffected, so the clinical importance of this factor in contemporary veterinary oncology is comparatively low.[72]

Most forms of leukemia in veterinary medicine are believed to spontaneous in origin. Like lymphoma, given the association of specific leukemia subtypes with certain breeds of dogs, there is likely a heritable component to the development of canine lymphoma. In the cat, viral infection (e.g., FeLV and feline immunodeficiency virus (FIV), see Chapter 60) is well associated with both lymphoid and myeloid leukemias.

Clinical Presentations

The clinical presentation of lymphoid neoplasia is variable and related to location and extent of tumor involvement, tumor staging, and presence of paraneoplastic disease. In dogs, multicentric nodal lymphoma is most common (Figure 65.2a,b). The disease typically involves all peripheral lymph nodes and internal lymphoid tissues including tracheobronchial, sublumbar, and mesenteric lymph nodes. Tonsils, spleen, and liver are also frequently involved. Approximately 80–97% of dogs diagnosed show this form.[60] Less common anatomic variants include splenic,[96,102,104,118] hepatosplenic,[40] intestinal, cutaneous,[75,95,89] central nervous system (CNS) forms,[48] and ocular forms.[76,57] In the cat, alimentary lymphoma is most common.[79,73] By contrast, the multicentric form is hardly seen. In one study, alimentary lymphoma (47% of cases) was followed by cutaneous (17% of cases), nasal (13% of cases), laryngeal (10% of cases), nodal (7% of cases), ocular (3% of cases), and mixed (nodal and renal, 3% of cases).[124] Cats are affected by a unique form of nasal or nasopharyngeal lymphoma.[33,53]

In the horse, lymphoma is common and has a highly variable clinical presentation and course.[22] In the largest study (203 patients), affected patients had a mean age of 10.7 years and Quarter Horses, Thoroughbreds, and Standardbreds were the most commonly affected breeds. Multicentric lymphomas were common, as were skin and gastrointestinal tract. A total of 14 lymphoma subtypes were identified and T-cell-rich, large B-cell lymphoma, peripheral T-cell lymphoma, and diffuse large B-cell lymphoma were most frequent.[22] These findings mimic previous work.[54,67]

Like lymphoma, the leukemias are a heterogeneous group of diseases that are characterized by the proliferation of clonal lymphoid cells. In contrast to lymphoma, leukemia is defined by the accumulation of neoplastic cells in the peripheral blood and/or bone marrow. As exemplified by the most recent revision of the WHO classification of lymphoid tumors, it is important to note that the distinction between lymphoma and leukemia is based upon the tissue distribution of neoplastic cells rather than the biologic aspects of the neoplastic cells. For example, small cell lymphoma and chronic lymphocytic leukemia are considered tissue



FIGURE 65.2 Enlarged peripheral lymph nodes in two canine patients with multicentric lymphoma. (a) Mandibular lymph node; (b) Popliteal lymph node. (**Source:** Images courtesy of Birgitt Wolfesberger.)

variants of the same disease.[106] While this distinction is often less necessary in veterinary medicine, as there are fewer recognized subtypes and most patients present with advanced disease, there are specific diseases (e.g., T-zone lymphoma/leukemia) in which simultaneous significant tissue and peripheral blood involvement are common.[99]

Biological Behavior

Clinically, lymphoma and leukemia are heterogeneous diseases. The heterogeneity of lymphoma is best represented in the dog to include aggressive (e.g., diffuse large B-cell lymphoma and peripheral T-cell lymphoma) and indolent subtypes (e.g., T-zone lymphoma).[4,24,103] Specific lymphoma subtypes, and their clinical and pathologic findings, are discussed in the relevant B- and T-cell lymphoma chapters. Treatment and outcome differ widely between patients and it is beyond the scope of this chapter to detail subtype-specific biology. Broad clinical negative prognostic features include: illness at initial presentation, gastrointestinal or hepatosplenic lymphoma, and certain clinicopathologic features (e.g., thrombocytopenia, anemia, neutrophilia, increased neutrophil/lymphocyte ratio, cell size, hyperglobulinemia, hyperferritinemia, and increased serum lactate dehydrogenase activity).[13,61,18,12,16,4,59,66]

Considering the expanding diagnostic options, an increasing number of lymphoma prognostic features have been identified, including major histocompatibility complex (MHCII) status, CD25 expression, and Ki67 expression.[68,80,69,82] More importantly, it is increasingly evident that historical assertions, including that dogs with B-cell multicentric lymphoma have longer survival times than dogs with T-cell lymphoma, can no longer be claimed.[19,62,58,65,61] In canine leukemia, specific prognostic features include neoplastic cell size (in B-cell chronic lymphocytic leukemia, CLL) and neoplastic cell concentration (in T-cell CLL).[122] In feline leukemia, a number of different flow cytometric variants have been identified—each of which have distinct prognostic features. Based upon two studies, CD4+ T-cell leukemia, which is the most common feline immunophenotype, is a slowly progressive disease (medial survival time of 752 days) as compared to CD4−/CD8− T-cell leukemia (median survival time of 271 days) and $CD5^{low}$ T-cell leukemia (median survival time of 27.5 days).[93,9]

Regarding mutation analyses, overall survival time was significantly shorter in dogs with untreated high-grade lymphoma with p53 mutation than those with lymphoma retaining wild-type p53.[45] Another study demonstrated a higher Bcl-2/Bax ratio in dogs with T-cell lymphoma compared to B-cell lymphoma and normal dogs, respectively. This led to the theory that higher intrinsic resistance to apoptosis following cytotoxic treatment might contribute to the less favorable prognosis associated with multicentric T-cell lymphoma (TCL) in dogs. Nevertheless, genetic analyses are still less frequent and are so far most investigated in DLBCL.[3] One study revealed prognostically significant subtypes by molecular profiling.[29] Further discoveries are needed and will be key to clarifying subcellular pathologies that account for differences in clinical outcome.

PRESENTATION OF AN ALGORITHM FOR THE DIAGNOSIS/WORKUP OF LYMPHOMA AND LEUKEMIA

The diagnosis of lymphoid neoplasia begins with a thorough clinical history and physical examination followed by a clinicopathologic minimum database (CBC/Chem/Diff/UA) and medical imaging (Figure 65.3). Identification of peripheral or visceral lymphadenopathy, organomegaly, refractory cytopenias, unexplained hypercalcemia, and/or ambiguous clinical signs (i.e., inappetence,

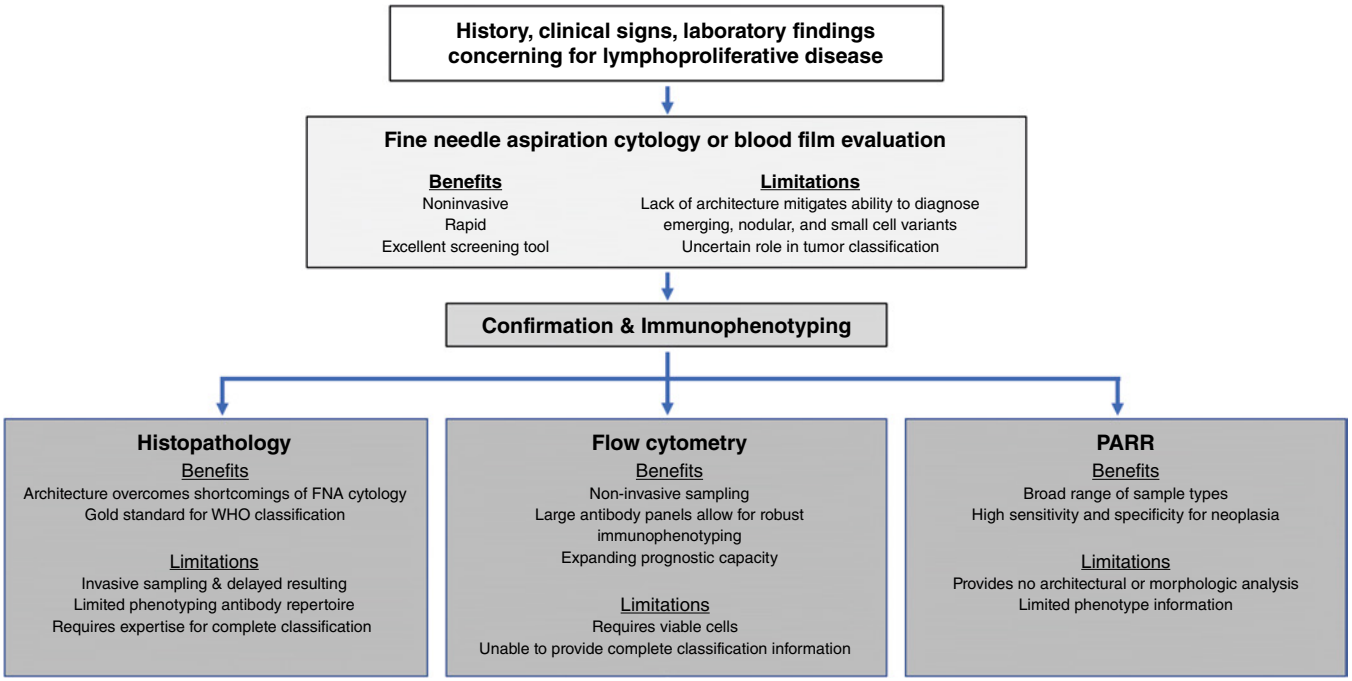


FIGURE 65.3 An algorithm for the diagnosis and classification of lymphoid neoplasms in veterinary medicine.

weight loss, gastrointestinal and respiratory signs) should prompt the diagnostician to consider more directed testing.[7]

Cytology and Hematology

Fine-needle aspirate cytology is a noninvasive, rapid, and frequently rewarding technique for the evaluation of lymphadenopathy and organomegaly.[7] Although there are tumor subtype-specific cytologic features (see T-cell and B-cell lymphoma chapters), the finding of a monomorphic population of large lymphocytes (1.5–2 times the diameter of a neutrophil) is consistent with the diagnosis of intermediate-to-large B-cell lymphoma. Neoplastic cells typically have a moderate amount of basophilic cytoplasm, a smooth chromatin pattern, and one or more visible nucleoli, although other morphologies (i.e., T-zone and large granular lymphomas) are also recognized.[92] Increased free cytoplasmic fragments, cytophagic (tingible-body) macrophages, and frequent mitotic figures are also common cytologic findings. Successful diagnosis is highly dependent on sample quality and location with decreased sensitivity reported for mesenteric lymph nodes and visceral organs.[46,100] Importantly, given the cytomorphologic and architectural heterogeneity of lymphoma (including nodular and small-/intermediate-cell-sized variants), additional diagnostics are often necessary.[7]

In leukemia, evaluation of the peripheral blood may reveal one or more cytopenias and the presence of circulating neoplastic cells. Diagnosis of leukemia may be made on evaluation of peripheral blood, but in cases where circulating neoplastic cells are rare or absent, bone marrow aspirate or core biopsy samples may be necessary. Assignment of lineage (i.e., lymphoid vs. myeloid) and maturation (i.e., acute vs. chronic) requires consideration of clinical and microscopic data as well as ancillary testing (cytochemical or immunocytochemical stains and/or flow cytometry).[92,105]

Confirmatory Tests and Immunophenotyping

Once a diagnosis of lymphoid neoplasia is established or if initial microscopy is equivocal, additional confirmatory and neoplastic cell phenotyping diagnostics are indicated. The used tools for this are immunocytochemistry (ICC), immunohistochemistry (IHC), and FC.[24,85,10] Phenotype, by facilitating subtyping and quantifying antigen expression, is considered one of the main prognostic indicators in lymphoma. Studies have highlighted the importance of immunophenotyping in treatment decisions with 31% of clinicians reportedly treating B- and T-cell lymphomas differently.[108] Furthermore, immunophenotyping leads to improved prognostication and a potentially more conservative and/or tailored treatment course.[23]

Histopathology and Immunohistochemistry

As noted in the earlier text, histology has been considered the gold standard for the diagnosis and classification of lymphoma. In addition to offering a standardized approach toward architectural and cytomorphologic assessment, histologic samples are readily repurposed for immunohistochemistry. Although histology is more invasive than cytology, requiring excisional, incisional, or tru-cut biopsy for diagnosis, it is superior to cytology in diagnosis of most cases of noneffacing, nonlarge cell

lymphoma. Importantly, as evident by evolving (and increasingly complex classification schemes), expertise in diagnostic hematopathology is increasingly important in establishing an accurate diagnosis.[116] Additional information on the histologic features of veterinary hematopathologic malignancies is available throughout this section.

Flow Cytometry

The role of flow cytometry in the diagnosis and immunophenotyping of lymphoid neoplasia is rapidly expanding. Indications for flow cytometry include peripheral blood or body fluid lymphocytosis, atypical circulating lymphocytes, and/or an expansion of monomorphic lymphocytes in lymph nodes, solid organs, or bone marrow.[7,108] There are limited studies examining the accuracy of hematologic malignancy diagnostics, but FC has a reported high sensitivity (98%) and specificity (100%) for the diagnosis of lymphoma in canine patients when compared to IHC.[108] Flow cytometry does not directly establish clonality; however, number, homogeneity, and/or phenotypic aberrancy of cells can be used to document a neoplastic population.[92] Specific information on flow cytometric methods and interpretation guidelines are available in Chapter 64.

Clonality Testing

PCR for antigen receptor rearrangement (PARR) is the most widely available and studied molecular assay used to detect clonality within lymphoid populations. PARR functions by amplifying the DNA encoding the variable regions of T- and B-cell receptors.[5] A more detailed discussion of clonality testing is presented in Chapter 62. In addition to defining the clonal nature of a cell population, clonality testing also provides limited phenotype information (i.e., B-cell vs. T-cell). From a practical perspective, samples for PARR testing can be derived from cytologic specimens (stained or unstained), fluids (including peripheral blood, cerebrospinal fluid, or body cavity effusion), formalin-fixed, or fresh frozen tissue. Importantly, owing to the phenomenon of "cross-lineage rearrangement", PARR may not accurately characterize phenotype and complete tumor classification requires additional morphologic and phenotyping diagnostics. Although PARR is highly specific (100% reported in one study), lower sensitivity was noted in identifying phenotype in contrast to flow cytometry in dogs (74% for PARR, 98% for flow cytometry) when compared to immunohistochemistry.[108,121] Finally, it is important to note that clonality is not equivalent to neoplastic, as benign clonal expansions have been described in some autoimmune disorders and certain infectious diseases (i.e., ehrlichiosis and leishmaniasis).[7] As a complement to PARR, additional clonality assays (i.e., X-linked inactivation pattern analysis) have yielded promising results and may become commonplace in the diagnosis and characterization of lymphoid neoplasia (see Chapter 62).[70]

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CHAPTER 66

Myeloproliferative Neoplasms

ERIC J. FISH

Introduction
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Molecular Pathogenesis
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Polycythemia Vera
Essential Thrombocythemia
Primary Myelofibrosis
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Summary and Future Directions

Acronyms and Abbreviations

AML, acute myeloid leukemia; ABL, Abelson Murine Leukemia; BCR, breakpoint cluster region; FGFR, fibroblast growth factor receptor; FSC, forward scatter; GTP, guanosine triphosphates; MAPK/ERK, mitogen-activated protein kinase/extracellular signal-related kinases; MDS, myelodysplastic syndromes; MPN, myeloproliferative neoplasms; PI3K, 3-Kinase/protein kinase B; PLT, platelet concentration; PTEN, phosphatase and tensin homolog; STAT, signal transducer and activator of transcription; WHO, World Health Organization.

INTRODUCTION

Myeloproliferative neoplasms (MPN), formerly termed *chronic leukemias* or *myeloproliferative disorders*, are rare clonal expansions of bone marrow precursor cells that lead to overproduction of well-differentiated blood cells, including erythrocytes, leukocytes, or platelets. While myelodysplastic syndromes (MDS) are also clonal disorders of hematopoietic tissue, there are key distinctions between MPN and MDS, namely that MPN tend to result in a profound increase in the number of circulating cells, versus one or more cytopenias with MDS, and the degree of dysplasia and proportion of blasts are usually much higher in MDS (see Chapter 67 for more information on MDS).

This heterogeneous group of disorders shares some characteristics. In general, MPN are indolent and slowly progressive, though they can transform into more aggressive acute myeloid leukemias (AML).[48] There is often a marked increase in the concentration of the affected cell lineage (>100,000/μL for leukocytes, >1,000,000/μL for platelets, and PCV >65% for erythrocytes). Patients with MPN tend to be older (though patients can be any age), and they may be asymptomatic or have mild, nonspecific clinical signs. Splenomegaly, with or without hepatomegaly, is common.

MPN in humans and domestic animals can usually be diagnosed with reasonable certainty based on history, clinical signs, and serial CBCs.[1] Given that the excess cells in circulation and the bone marrow often appear morphologically normal or minimally dysplastic, MPN must be discriminated from hyperplastic or reactive proliferations. Diagnosis of MPN in human patients and experimental settings are aided by a wider array of tools to prove a clonal etiology, including fluorescence in situ hybridization (FISH) cytogenetics, X-linked clonality assay (see Chapter 62), and specific genetic assays for driver mutations.

Classification System and Terminology

Myeloid neoplasms in domestic animals were initially classified by the 1991 Animal Leukemia Study Group based on the human French–American–British (FAB) terminology of National Cancer Institute (NCI).[23] This system divided myeloid neoplasms into MDS and seven subtypes of AML, M1–M7 (see Chapter 68). AML and MDS were discriminated based on >30% blasts in blood

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or bone marrow for acute leukemias and <30% blasts for MDS. At that time, chronic monocytic leukemia (CMoL) and chronic myelomonocytic leukemia (CMML) were considered subtypes of MDS, though the authors acknowledged several differences including the marked leukocytosis and minimal dysplasia. Veterinary medicine subsequently adopted the World Health Organization (WHO) human myeloid leukemia classification scheme. This scheme presents the parent category of MPN under which there are seven distinct entities, namely, chronic myeloid leukemia (CML), chronic neutrophilic leukemia (CNL), chronic eosinophilic leukemia (CEL), polycythemia vera (PV), chronic idiopathic myelofibrosis, essential thrombocythemia, and chronic myeloproliferative disease—unclassifiable.[31] Correlated with this adoption, the blast threshold between AML and MDS/MPN was lowered from 30% to 20%.[31]

The human WHO classification system was most recently revised in 2016 and recognizes the following subtypes of MPN: CML, CNL, PV, primary myelofibrosis (PMF), essential thrombocythemia (ET), chronic eosinophilic leukemia, and unclassifiable MPN (MPN-U).[1,3] The 2016 WHO criteria are complex and lay out specific diagnostic criteria for these entities based on clinical parameters, cell counts, and specific molecular lesions (Table 66.1).[1] Mastocytosis was previously included in this group, but is no longer considered to be a true MPN, and as such will not be discussed in this chapter. In human medicine, there is recognition of overlap between MDS and MPN, as well as some lymphoid neoplasms that have concurrent myeloid transformation that results in complex syndromes with similar characteristics.[3] In people, CMML and breakpoint cluster region–Abelson Murine Leukemia Viral Oncogene Homolog 1 (BCR–ABL)-negative atypical CML (aCML) are considered part of the MDS/MPN complex.[1] Juvenile myelomonocytic leukemia (JMML) is also included in this category and may be unique to people, as it has not been reported in domestic species.

The veterinary classification of MPN has not been independently updated since adoption of the 2016 WHO criteria and remains rudimentary and based on sporadic case reports rather than large studies. Furthermore, the latest WHO system increasingly relies on specific genetic markers, which are poorly defined in domestic species. However, in the spirit of comparative pathology, characteristics from the veterinary literature will be compared to the 2016 WHO classification system throughout this chapter.

Molecular Pathogenesis

The clonal nature of myeloid neoplasms has been known for over half a century. The first cytogenetic abnormality associated with a specific human cancer, the "Philadelphia chromosome" in patients with CML, was documented and characterized in the early 1960s, although its full significance would not be understood until decades later.[39] In the late 1980s, cats and dogs with myeloid leukemias were likewise shown to have karyotypic abnormalities such as aneuploidy, chromosomal deletions, and chromosome duplications.[16,17] However, it is only techniques such as whole genome sequencing, FISH, and chromosome immunoprecipitation (CHIP) that have allowed human oncologists and researchers to pinpoint the specific genes involved in MPN and other hematopoietic tumors.

MPN can be thought of as a warped version of normal hematopoiesis. In health, hematopoietic stem cells (HSCs) and subsequent lineage-specific progenitors and precursors are stimulated to survive and divide by cytokines such as erythropoietin (EPO), thrombopoietin (TPO), granulocyte colony-stimulating factor (G-CSF), interleukins, and many others (see Chapter 4 for more details on hematopoiesis).[25,49] The cytokines that normally regulate hematopoiesis bind to cell membrane receptors that activate one of the Janus kinases (JAK), which phosphorylate one or more transcription-regulating, signal transducer and activator of transcription (STAT) proteins (for more detail, see Chapter 4).[42] The JAK–STAT system can also interact with other key signaling pathways such as phosphoinositide 3-Kinase/protein kinase B (PI3K/AKT) and mitogen-activated protein kinase/extracellular signal-related kinases (MAPK/ERK). The downstream effects of JAK–STAT activation include cell survival and mitosis.

In MPN, HSCs acquire one or more somatic mutations that trigger constant proliferation even in the absence of growth-promoting cytokines or presence of cytostatic signals.[49] Eventually, these clonally transformed HSCs outcompete normal HSCs in the hematopoietic niche, replace a large portion of the bone marrow, and produce markedly increased peripheral blood concentrations of one or more blood cell lines. Though dozens of genetic mutations and epigenetic changes can promote clonal transformation of HSCs and early lineage-committed precursors, a relatively limited set of genes are involved in most cases of MPN.[32,33] Not surprisingly, most of the genes implicated in MPN are directly or indirectly involved in JAK–STAT signaling.[44] Some of these activating mutations affect cytokine receptors, such as colony-stimulating factor receptor 3 (CSFR3), which binds G-CSF, and myeloproliferative leukemia protein (MPL or CD110), which binds TPO. Other MPNs directly involve JAK mutations (such as the JAK2 V617F missense mutation in PV) or alterations that increase overall JAK–STAT signaling activity, such as the BCR–ABL1 fusion protein or calreticulin (CALR).

Nearly all cases of human CML, and some cases of acute lymphoid and myeloid leukemia, are caused by a chromosomal translocation resulting in fusion of the BCR and ABL1 genes (BCR–ABL1) and formation of the "Philadelphia chromosome."[1] The BCR gene encodes for a serine/threonine kinase that can activate guanosine triphosphates (GTP), and ABL encodes for a tyrosine kinase. The fusion product of this chromosomal translocation is constitutively active and results in constant cell signaling, particularly among hematopoietic cells. In addition to providing an explanation for the molecular pathogenesis, neoplasms with the BCR–ABL fusion gene may be targeted by specific tyrosine kinase inhibitors, such as imatinib mesylate, which has

TABLE 66.1 Comparative MPN classification in human and veterinary medicine.

Type of MPN	Human WHO criteria[1]	Reported characteristics in veterinary patients	Canonical genetic lesion(s)
1. Chronic myeloid leukemia (CML) (BCR–ABL$^+$)	Philadelphia chromosome-positive Accelerated phase (AP), one or more of the following: * Persistent or increasing leukocytosis (refractory to treatment) * Persistent or increasing splenomegaly (refractory to treatment) * Persistent thrombocytosis (refractory to treatment) * Persistent thrombocytopenia (refractory to treatment) * Basophilia (>20%) * 10–19% blasts (peripheral blood or marrow) * Additional genetic lesions besides BCR–ABL * New chromosomal abnormalities	BCR–ABL fusion gene documented in two dogs with CML and one with AML; marked monocytosis (>40,000/μL) with mature-appearing monocytes, blast percentage <20%, short survival times (4 weeks and 7 months)	BCR–ABL1 fusion gene‡
2. Chronic neutrophilic leukemia (CNL)	1. Leukocytosis >25,000/μL; >80% of WBCs are segmented neutrophils; myeloid precursors earlier than bands <10%; myeloblasts rare; monocytes <1000/μL; no dysplasia 2. Hypercellular marrow; myeloid hyperplasia; normal granulocyte maturation; <5% myeloblasts 3. Not compatible with CML or other MPN 4. Lacks PDGFRA, PDGFRB, or FGFR1, or PCM1–JAK2 rearrangement 5. Presence of canonical mutation OR neutrophilia >3 months with no obvious cause OR evidence of clonality	Dogs/cats: Neutrophilia 73,000–126,000/μL, variable presence of anemia, adequate platelets; Horse: Neutrophilia ranging from 8000–27,000/μL, moderate anemia, immature myeloid cells infiltrating and effacing lymph nodes, blasts <20% (single-case report)	CSF3R
3. Polycythemia vera (PV)	**Major criteria:** 1. Hgb > 16.5 g/dL (men) or 16 g/dL (women) OR Hct >49% (men) or 48% (women) 2. Hypercellular bone marrow, all lineages affected (panmyelosis), pleomorphic megakaryocytes 3. Documented canonical mutation **Minor criteria:** Low erythropoietin concentration *PV diagnosed by either all 3 major criteria OR major criteria 1+2 and minor criteria*	Hct >65%, may see reticulocytosis, rubricytosis; JAK2 (V617F) documented in one case report	JAK2‡

TABLE 66.1 (Continued)

Type of MPN	Human WHO criteria[1]	Reported characteristics in veterinary patients	Canonical genetic lesion(s)
4a. Primary myelofibrosis (PMF), early/ prefibrotic	**Major criteria:** 1. Megakaryocytic proliferation and atypia without reticulin fibrosis 2. Does not meet criteria for other MPN, MDS, MDS/MPN 3. Presence of canonical mutation OR demonstrated clonality **Minor criteria:** 1. Anemia without other cause 2. Palpable splenomegaly 3. Elevated LDH *Diagnosis requires 3 major criteria and ≥1 minor criteria*	Not clearly documented; may overlap with other case reports of animals with MPN/MDS	JAK2, CALR, MPL
4b. Primary myelofibrosis (PMF), overt	**Major criteria:** 1. Megakaryocytic proliferation and atypia with prominent reticulin and/or collagen fibrosis 2. Does not meet criteria for other MPN, MDS, MDS/MPN 3. Presence of canonical mutation OR demonstrated clonality **Minor criteria:** 1. Anemia without other cause 2. Leukocytosis >11,000/μL 3. Palpable splenomegaly 4. Elevated LDH 5. Leukoerythroblastosis *Diagnosis requires 3 major criteria and ≥1 minor criteria*	Marked thrombocytosis (>1,000,000/μL); splenomegaly; BM: megakaryocytic hyperplasia, increased dysplasia and blasts (single-case report)	JAK2, CALR, MPL
5. Essential thrombocythemia (ET)	**Major criteria:** 1. [PLT] > 450,000/μL 2. BM biopsy shows megakaryocytic hyperplasia and dysplasia with minimal erythroid/myeloid changes or fibrosis 3. Not compatible with BCR–ABL+ CML, MDS, or other MPN 4. Documented canonical mutation **Minor criteria:** Documented other clonal abnormality or exclusion of reactive cause *ET diagnosed by either all 4 major criteria OR major criteria 1–3 AND minor criteria*	Thrombocytosis ranging from 400,000/μL to 4,950,000/μL, moderate to marked nonregenerative anemia, leukocytosis with neutrophilia ± basophilia; increased PLT aggregation; megakaryocytic hyperplasia and myelofibrosis	JAK2, CALR, or MPL

(*Continued*)

TABLE 66.1 (Continued)

Type of MPN	Human WHO criteria[1]	Reported characteristics in veterinary patients	Canonical genetic lesion(s)
6. Chronic eosinophilic leukemia (CEL)-not otherwise specified[2]	1. Marked eosinophilia for >6 months (no cause identified) 2. Myeloblasts >2% blood OR <20% in bone marrow 3. Evidence of clonality 4. Does not meet criteria for MPN 1–5, MDS, MDS/MPN, 5. Does not have any of the following mutations: BCR–ABL fusion, PDGFRA, PDGFRB, or FGFR1; no PCM1/JAK2, ETV6/JAK2, or BCR/JAK2 fusion gene 6. Not compatible with AML	Marked eosinophilia (often >50,000–100,000/μL) without obvious cause, left-shift including blasts in blood and bone marrow; flow cytometry and cytochemistry compatible with normal eosinophils	N/a
7. Unclassifiable MPN	Does not meet WHO criteria for MPNs 1–6 mentioned above, MDS, MDS/MPN, or AML		N/a

[1]= Adapted from Arber et al.
[2]= Adapted from Bain et al.
[‡]= Mutation documented in veterinary patients.

transformed the prognosis for people with CML from grave to a manageable chronic long-term illness with a 10-year survival rate over 80%.[21]

Two case reports of dogs with CML documented a similar BCR–ABL fusion gene which researchers at North Carolina State University dubbed the "Raleigh chromosome."[7] In one report, the authors report a decline in the proportion of BCR–ABL-positive peripheral blood mononuclear cells from 17.3% to 3.3% after vincristine and prednisone treatment, suggesting that in addition to diagnostic uses, FISH could track cytogenetic response to treatment.[8] One of these dogs had simultaneous deletion of tumor suppressor gene phosphatase and tensin homolog (PTEN), illustrating that even in patients with a classic driver mutation, multiple genetic defects likely contribute to hematopoietic tumors.[7] An additional case report documented a dog with AML that had 34% circulating atypical CD34+ blast cells, and 32% of the peripheral blood mononuclear cells were positive for the BCR–ABL translocation.[12]

Nearly four in five people with CNL have activating mutations in the gene coding for CSFR3.[28] This mutation has not been identified in animals, although one report of a dog with CNL described a marked increase in phagocytic activity among leukemic neutrophils on an order of magnitude seen with exogenous G-CSF administration.[51] Since CSFR3 is the receptor for G-CSF, this could provide circumstantial evidence for a possible link.

Approximately 95% of cases of human MPN other than CML and CNL are caused by activating mutations in JAK2, CALR, or the thrombopoietin receptor (also known as MPL or CD110).[28,31] Multiple genetic lesions leading to clonal transformation of erythroid precursors have been documented in humans with PV. Approximately 90% of people with PV have mutations in the receptor tyrosine kinase JAK2 (primarily V617F) that leads to constitutive cell signaling and excessive erythropoiesis.[5]

PMF is primarily thought to arise from clonal transformation of HSCs rather than the fibroblasts themselves. However, experimental evidence from mice suggests that a nearly identical syndrome can be reproduced by deleting Rb or retinoic acid receptor gamma genes in myeloid-derived cells from the microenvironment and transplanting them into wild-type mice.[52,53]

Chronic Myeloid Leukemia (CML)

MPNs include several variants that overproduce leukocytes, including CNL, CML, and CEL. Cases of CNL and CML must be differentiated from mimics such as extreme, physiologic neutrophilic leukocytosis (aka "leukemoid reaction") secondary to marked inflammatory or paraneoplastic stimuli or marked leukocytosis because of the rare genetic disease leukocyte adhesion deficiency (LAD).[4,13,56]

CNL/CML has been uncommonly reported in dogs, and rarely in cats and horses.[24,26,32,35,48,51] The veterinary literature on CML is confounded by a lack of uniform terminology, with authors using varied labels such as "chronic granulocytic leukemia," "chronic myelogenous leukemia," "chronic myelomonocytic leukemia," and "chronic neutrophilic leukemia" to describe patients with similar leukogram patterns.

The leukocyte concentration of patients with CNL/CML often exceeds 100,000 cells/μL (Table 66.1 and Figure 66.1). Neutrophils in CNL and monocytes in CML often appear morphologically normal, but can show mild hypersegmentation or dysplasia (Figures 66.1 and 66.2). Leukocytes in CNL/CML do not appear to have impaired functional properties. The neutrophils in a dog with CNL were shown to have normal to increased

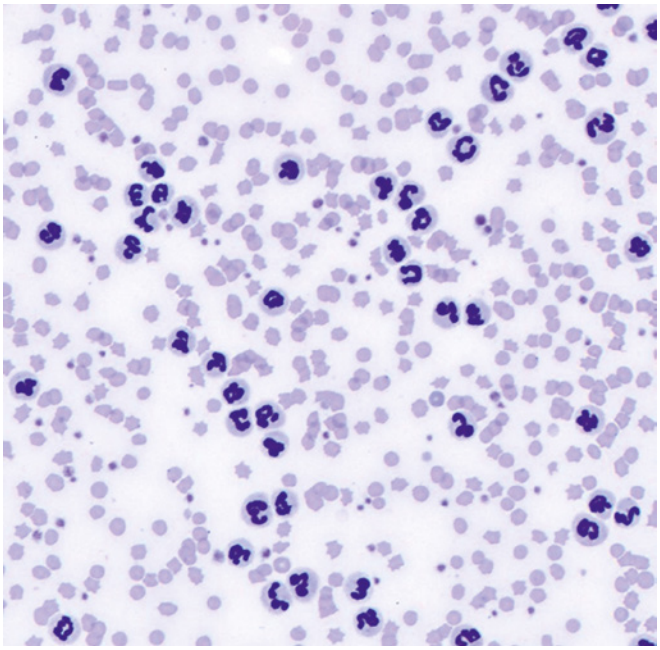


FIGURE 66.1 Chronic neutrophilic leukemia in a 19-year-old cat. The total leukocyte concentration was 342,870/μL. Virtually, all the leukocytes in this field are segmented neutrophils. They appear normal apart from a few hypersegmented types. Previous CBCs from this cat going back 4 years were unremarkable, excluding leukocyte adhesion deficiency (LAD), and the magnitude of this leukocytosis was considered far beyond the range for extreme neutrophilic leukocytosis ("leukemoid reaction"). Wright–Giemsa stain; original magnification 200×. (**Source:** Image courtesy of Laura Snyder, DVM, DACVP, IDEXX Laboratories)

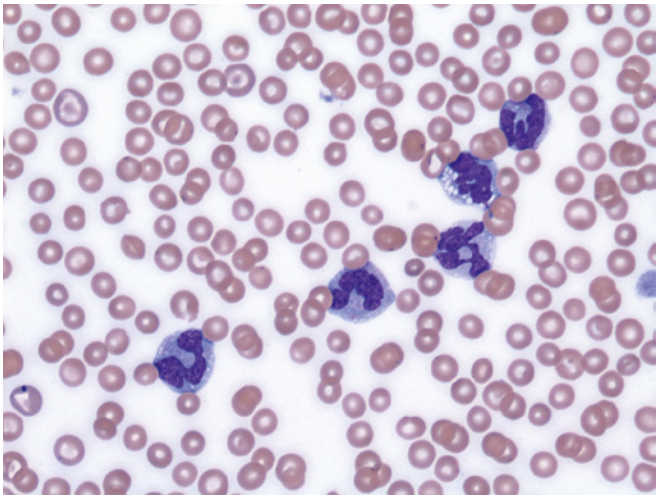


FIGURE 66.2 Chronic monocytic leukemia from an 8-year-old male castrated American Staffordshire Terrier. This patient presented with a leukocytosis of 36,700/μL, of which 80% were monocytes and 9% were immature blasts. All the leukocytes in this field are mature-appearing monocytes with medium blue cytoplasm, occasional clear vacuoles, and mildly pleomorphic nuclei with fine chromatin and absent nucleoli. One week later, the patient presented with a higher leukocytosis characterized by 67% circulating blasts and generalized lymphadenopathy with infiltration by these cells, indicating rapid transformation to acute monocytic leukemia (AML-M5). Wright–Giemsa stain; original magnification 200×.

phagocytic activity and normal migration and aggregation responses compared to healthy controls.[51] Anemia is a variable feature of CML/CNL and platelet concentrations are typically normal. CNL in the horse may have substantially lower peripheral neutrophil concentrations than in dogs or cats (Table 66.1).[24] One cat with CNL had concurrent eosinophilia and basophilia, illustrating that multiple cell lines can be stimulated to overproduce cells in MPN.[35] The variant aCML characterized by precursor tissue infiltrates, bone marrow dysplasia in erythroid, myeloid, and megakaryocytic cells, and negative BCR–ABL status has been diagnosed in one dog.[29]

Flow cytometry lacks many markers for myeloid neoplasms and there is no consensus on its role in the diagnosis of veterinary MPNs. However, certain antibodies (e.g., CD4 on canine neutrophils and CD14 on monocytes) can provide limited utility in some cases of MPN and AML (see Chapter 64 for more information on diagnostic flow cytometry).[36] One study identified increased neutrophil activation based on higher forward scatter (FSC) and decreased CD18 expression in immature granulocytes as subtle findings that differentiate CML from reactive neutrophilia.[6] Alkaline phosphatase cytochemistry has also been used to identify monocytic origin.[45]

Some cases in the literature document prolonged survival of 21 months (CML, dog) to 63 months (CNL, cat).[18,32] However, prognosis for these cases remains guarded, as they have been reported to progress to a blast crisis or acute myeloid leukemia in weeks to months after diagnosis.[8,48]

CEL is extremely rare, with two cases reported in the cat and one report of presumptive CEL in a dog.[14,37,43] These cases were characterized by marked eosinophilia (>50,000 cells/μL), dysplasia and/or immature eosinophils (including >1% to <20% blasts), and tissue infiltration. The marked eosinophilia with a left-shift can overlap with idiopathic hypereosinophilic syndrome (IHES), though IHES should not present with dysplastic changes or blasts/precursors. In one case of feline CEL, lineage assignment of poorly granulated and immature-appearing granulocytes was aided by cytochemistry, as the neoplastic cells were positive for alkaline phosphatase, but negative for myeloperoxidase, chloroacetate esterase, alpha-naphthyl acetate esterase, and toluidine blue (expected pattern for feline eosinophils; see Chapter 61 for more information on cytochemistry).[14] Increased serum IgE concentrations have been used to support a diagnosis of IHES as opposed to CEL in dogs.[46] Even in human oncology, it is considered quite difficult to definitively exclude CEL from IHES, myeloid/lymphoid neoplasms with fibroblast growth factor receptor 1 (FGFR1) mutations, or paraneoplastic hypereosinophilia.[2] For discussion of chronic basophilic leukemia, see the section entitled Unclassifiable Myeloproliferative Neoplasms.

Polycythemia Vera

PV is an uncommon MPN in dogs, cats, and calves characterized by overproduction of mature erythrocytes.[9,15,20,27,50]

These patients frequently have a chronic, progressive increase in measures of circulating red blood cell mass such as hematocrit, red blood cell concentration, and hemoglobin concentration. The magnitude of these changes in PV is often moderate to marked, with Hct values ranging from 65% to 80% (or more). Inappropriate reticulocytosis and/or rubricytosis may also be noted. Moderate to marked leukocytosis and/or thrombocytosis can also be present, as the transformed stem cell may be a pluripotent erythroid–myeloid precursor.

PV is a diagnosis of exclusion that must be differentiated from other causes of increased circulating red blood cell mass (erythrocytosis). Erythrocytosis can be subcategorized into relative and absolute causes. In relative erythrocytosis, the patient has a normal circulating red blood cell concentration, but splenic contraction or fluid shifting from the intravascular space to extravascular space due to severe dehydration, heatstroke, or hemorrhagic gastroenteritis increases the fraction of RBCs in proportion to the plasma volume. Relative erythrocytosis usually, but not always, results in less severe Hct changes than PV, and should resolve with correcting the underlying fluid deficits.

Absolute erythrocytosis is a true increase in circulating red blood cell mass. It may be a physiologically appropriate response secondary to increased erythropoietin production by renal interstitial sensing hypoxia as an adaptation to high-altitude climates or in pathologic conditions such as right-to-left cardiac shunts, chronic pulmonary disease, or renal infarcts. Inappropriate absolute erythrocytosis can be produced by PV and rarely by EPO-secreting renal tumors.[20] Erythropoietin levels may help differentiate between causes of erythrocytosis, although this assay is available on limited basis from commercial laboratories and there can be an overlap between presumed primary and secondary causes.[9]

Bone marrow evaluation in patients with PV reveals prominent erythroid hyperplasia, usually with a significantly decreased M:E ratio, and a relatively orderly maturation without dysplasia or an expanded population of rubriblasts.[54] Notably, marked erythrocytosis can, because red blood cells rely on glucose, result in artifactual hypoglycemia.[30]

PV may be asymptomatic and discovered incidentally on routine labwork. For patients with advanced disease, clinical signs are related to increased blood viscosity, including cardiac insufficiency, uveitis, seizures, or abnormal coagulation (including thromboemboli).[15,27] Treatment of PV patients is symptomatic and aimed at reducing signs of hyperviscosity. Clinicians have used a variety of approaches, including serial therapeutic phlebotomy and drugs such as hydroxyurea to decrease excess marrow production of red blood cells.

Essential Thrombocythemia

ET is an MPN characterized by megakaryocytic hyperplasia and marked thrombocytosis. The most recent WHO classification system for ET identifies several major criteria, including moderate to marked thrombocytosis with platelet concentration ([PLT] >450,000/μL), bone marrow biopsy showing marked fibrosis and megakaryocytic proliferation, not meeting criteria for other MPN, and identifying clonal genetic lesions or classic driver mutations (such as JAK2, CALR, or MPL) (see Table 66.1).[3] These mutations have not been documented in domestic animals and ET remains a diagnosis of exclusion in veterinary medicine. Differential diagnoses include other conditions resulting in a moderate to marked reactive thrombocytosis, particularly inflammatory/autoimmune disorders, hyperadrenocorticism, iron deficiency, administration of corticosteroids or vincristine, and a paraneoplastic response to nonmyeloid tumors.[38,57]

Probable ET has been reported in four dogs and one cat (Figure 66.3).[11,19,22,47] Features of these cases (see Table 66.1) include moderate to marked thrombocytosis, nonregenerative anemia, leukocytosis characterized by neutrophilia with or without basophilia, megakaryocytic hyperplasia, and myelofibrosis. Ultrastructural platelet and megakaryocyte abnormalities reported in one dog include platelets with large, asymmetric dilated open canalicular systems, megakaryoblasts with alpha granules, and dysplastic megakaryocytes that had poor cytoplasmic organization.[47] Humans with ET are hypercoagulable and prone to thromboembolic complications. This has not been conclusively demonstrated in animals, although in one case report, platelets from a dog with ET demonstrated increased aggregation responses.[11]

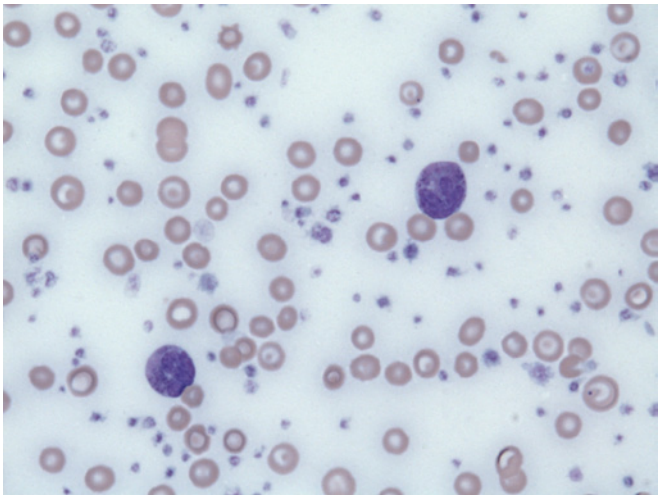


FIGURE 66.3 Marked thrombocytosis (1,552,000/μL) and basophilia (2577/μL) in a 6-year-old intact female Bulldog. The patient had a prior history of marked splenomegaly treated with splenectomy. This photomicrograph shows many hypergranulated platelets and two mature basophils. Core bone marrow biopsy was hypercellular with megakaryocytic hyperplasia, a markedly left-shifted myeloid series, and multifocal fibrosis. The differential diagnosis was ET with basophilia or unclassified MPN. Wright–Giemsa stain; original magnification 500×. (**Source:** Image courtesy of Cheryl A. Lawson, DVM, MS, DACVP, Iowa State University)

Primary Myelofibrosis

Myelofibrosis is the proliferation of stromal fibroblasts that produce reticulin and collagen fibrils. Myelofibrosis is classified as primary (i.e., neoplastic) or secondary (i.e., reactive). Secondary myelofibrosis may occur as a response to a wide variety of bone marrow insults such as myelitis, ischemia, necrosis, chronic overproduction of stimulating cytokines (such as with chronic hemolysis from pyruvate kinase deficiency or immune-mediated hemolytic anemia), or infiltration by nonmyeloid neoplastic cells.[40,55] Bone marrow aspirate cytology from patients with myelofibrosis is often unrewarding and results in poorly cellular "dry taps." Bone marrow core histopathology often reveals thin eosinophilic fibers that cause unusual cell bunching on H&E sections. Reticulin fibers seen in early myelofibrosis are visualized with silver stains, while the larger collagen fibrils seen later are identified with Masson's trichrome stain (Figure 66.4). Whether primary or secondary, myelofibrosis can contribute to one or more cytopenias.

PMF is an uncommon but well-defined entity in human medicine caused by clonal transformation of marrow HSCs. The major WHO criteria for PMF in people includes: megakaryocytic proliferation and atypia, presence of JAK2/CALR/MPL mutation or other clonal marker, and does not meet criteria for another MPN or MDS (Table 66.1). The degree of reticulin and collagen fibrosis can subdivide cases into prefibrotic and overt PMF.

In one retrospective study of 19 dogs with myelofibrosis, the vast majority (18/19) were classified as secondary.[55] In the one case of presumed PMF, the dog shared many characteristics of human PMF, including anemia, splenomegaly, marked thrombocytosis, and a bone marrow biopsy with prominent fibrosis, megakaryocytic hyperplasia, and multilineage dysplasia. It bears mentioning that while thrombocytosis is common with PMF, it is not a required diagnostic criterion and differentiating people with ET and early PMF can present a diagnostic dilemma, as many of the clinical and hematologic features overlap.[3,41]

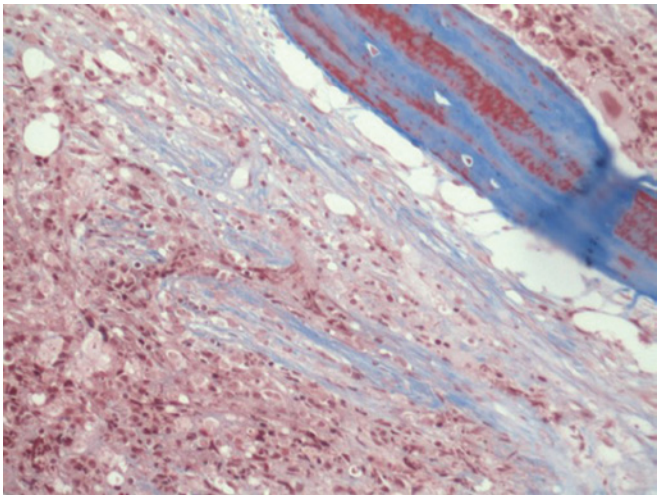


FIGURE 66.4 Myelofibrosis in a patient with presumptive ET or MPN-U (same patient as Figure 66.3). Bands of collagen fibrosis stain bright blue. Masson's trichrome; original magnification 200×. (**Source:** Image courtesy of Cheryl A. Lawson, DVM, MS, DACVP, Iowa State University)

Unclassifiable Myeloproliferative Neoplasms

Cases that do not fulfill the classification criteria for any of the specific MPNs are labeled MPN-U. In the veterinary literature, MPN-U includes several reports of "basophilic leukemia."[34] In addition to a marked basophilia, affected patients present with a marked thrombocytosis (sometimes between 2,000,000 and 3,000,000/μL) which can overlap with the case definition of ET. Notably, chronic basophilic leukemia is not currently recognized in the latest WHO classification of MPN, and could represent an entity unique to domestic animals. However, basophilia can accompany multiple other MPN in people and domestic animals, and may represent a negative prognostic marker of progression into AML.[10] For best concordance with human literature, this diagnostic entity should either be labeled MPN-U or considered a variable finding that can coexist with several of the MPN described in this chapter. Further research is needed in this area.

Summary and Future Directions

The classification, molecular understanding, and treatment of human MPN have advanced remarkably in the past 20 years. People with these disorders now benefit from targeted therapies that greatly increase life span with minimal adverse effects compared to traditional cytotoxic chemotherapy agents. By contrast, MPN in domestic species are less characterized, with much of what veterinary pathologists and oncologists "know" based on anecdotal opinion and personal experience. Larger studies and comparative research into driver mutations in veterinary patients with MPN are essential to align the classification system with the human scheme and provide revised diagnostic approaches and tailored molecular therapies that may improve the prognosis for these patients.

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CHAPTER 67

Myelodysplastic Syndromes

DOUGLAS J. WEISS and RANCE K. SELLON

Introduction
Classification of Myelodysplastic Syndromes
Myelodysplastic Syndromes in Dogs
  MDS with Refractory Cytopenia
  Refractory Cytopenia with Multilineage Dysplasia
  MDS with Excessive Blasts
  Myelodysplastic Syndrome Unclassifiable
  Secondary Myelodysplastic Syndromes
Secondary Dysmyelopoiesis in Dogs
  Diseases/Drugs/Toxins
Myelodysplastic Syndromes in Cats
  Refractory Cytopenia with Multilineage Dysplasia
  Myelodysplastic Syndrome with Excessive Blasts
Secondary Dysmyelopoiesis in Cats
  Diseases/Drugs/Toxins
Myelodysplastic Syndrome in Horses

ACRONYMS AND ABBREVIATIONS

BM< bone marrow; DNA, deoxyribonucleic acid; IMHA, immune mediated hemolytic anemia; PRCA, pure red cell aplasia.

INTRODUCTION

Myelodysplastic syndromes (MDS) are a group of acquired clonal hematologic disorders, described in dogs, cats, horses, and humans, that originate from acquired genetic mutations in hematopoietic stem cells.[6,8,12,22,51] MDS are considered the most common reason for bone marrow (BM) failure syndromes in adult humans[5] and represent a diverse array of syndromes reflecting the underlying mutations. As in other neoplasms, clonal expansion is usually the result of one or more gene abnormalities including karyotypic abnormalities and mutations in genes with a variety of functions including tumor suppression, DNA repair, modification of histones, regulation of epigenetic events and apoptosis, intracellular signaling, and others.[2,16,32,35] A clonal origin has been documented in cats infected with feline leukemia virus (FeLV).[21] Myelodysplastic syndromes in dogs have been assumed to be clonal because of an absence of concurrent diseases or drug or toxin exposure, and because of the similarity of the MDS to those described in humans.[28,51] Compared to people, the genetic and molecular basis of MDS in veterinary species is poorly described and understood.

While the MDS are heterogeneous disorders, they are consistently characterized by peripheral blood (PB) cytopenias and dysplastic changes in one or more hematopoietic cell lineages. Some variants of MDS are associated with an increased risk of progression to acute myeloid leukemia. Cytopenias result from a combination of defects in growth and differentiation and increased apoptosis of hematopoietic cells. In people, some evidence supports defective immune system deletion of clones as contributing to the MDS, while other lines of evidence support a potential role of chronic inflammation and innate immune system signaling in the emergence of clonal expansion of hematopoietic cells.[2,4,35] These defects result in ineffective hematopoiesis.[9,29,37]

Major subtypes of MDS must be differentiated to better define appropriate treatment and establish a prognosis. While differentiation commonly relies on morphologic abnormalities noted on a blood smear or in a bone marrow sample, molecular testing plays an increasingly important role in the diagnosis of specific

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types of MDS in people.[2] Myelodysplastic syndromes must also be differentiated from secondary dysmyelopoiesis due to diseases, drug and toxin exposure, robust hyperplasia, or congenital disorders.[1,20,42,43,45,49] The most reliable features that help differentiate MDS from secondary dysmyelopoiesis include increased numbers of myeloblasts or rubriblasts in bone marrow, a high percentage of dysplastic cells in bone marrow, and prominent megaloblastic changes in erythroid precursor cells.[9,45] However, clinical history and repeat peripheral blood and/or bone marrow analysis may be needed. Congenital dysmyelopoiesis is generally seen in juvenile animals and may be breed-restricted.

CLASSIFICATION OF MYELODYSPLASTIC SYNDROMES

Although veterinary medicine utilizes similar terminology, the approach toward the diagnosis and classification of MDS has been defined by human medicine. Myelodysplastic syndromes were initially classified by the French–American–British (FAB) cooperative group (Table 67.1).[7] This system has been applied to both dogs and cats.[9,25,30] The FAB system categorized MDS into five categories primarily based on the distribution and type of dysplastic features and the percentage of myeloblasts present. These five categories included refractory anemia (RA), refractory anemia with ring sideroblasts (RARS), refractory anemia with excess blasts (RAEB), refractory anemia with excess blasts in transition (i.e., 20–30% myeloblasts in bone marrow), and chronic myelomonocytic leukemia. Acute leukemia was defined as greater than 30% myeloblasts in bone marrow.[7]

The World Health Organization (WHO) modified the FAB classification system incorporating diagnostic information such as cytogenetics, immunologic markers, and molecular genetics.[18] The WHO classification system itself has undergone several revisions, most recently in 2016, with the current MDS classifications based not on specific cytopenias but the number of dysplastic cell lineages, the proportion of ring sideroblasts (RS) in bone marrow, and the proportion of blasts in BM and PB.[3] Sideroblasts are nucleated erythroid cells that contain many large iron deposits within mitochondria. Because mitochondria are distributed around the nucleus, iron deposits frequently form a perinuclear collar, and the cells are termed *ring sideroblasts* (Figure 67.1). Additional classification systems, notably an International Prognostic Scoring System (IPSS), a revised IPSS, and MD Anderson Global Prognostic Scoring System, exist; each was developed with the goal of improving clinical decision-making and prognosis, but with each having limitations.[26]

In the current WHO classification system of human MDS, the syndrome formerly referred to as RA is now MDS with single-lineage dysplasia (MDS-SLD) or multilineage dysplasia (MDS-MLD). MDS-SLD is characterized by one or two cytopenias (anemia, neutropenia, or thrombocytopenia) with a single dysplastic lineage, less than 15% ring sideroblasts in the bone marrow, less than 5% blasts in the BM, and less than 1% blasts in PB. MDS-MLD has the same BM and PB blast features as MDS-SLD, but MDS-MLD has two or three dysplastic lineages and is associated with one to three cytopenias (Figures 67.2 and 67.3). Refractory anemia with ring sideroblasts is now classified as MDS with RS and either SLD or MLD. Dysplastic lineages, cytopenias, and proportions of blasts in BM and PB in MDS-RS-SLD or MDS-RS-MLD are the same as for MDS-SLD or MDS-MLD, but the two MDS-RS entities have ≥15% RS in the BM. Refractory anemia with excess blasts in the FAB classification system is, in the WHO system, MDS with excessive blasts (EB)-1 or -2 depending on the number of blasts in PB and BM. MDS-EB-1 is characterized by cytopenias, SLD or

TABLE 67.1 Classification systems for myelodysplastic syndromes (MDS).

French–American–British	World Health Organization[3]	Dog/cat system[48]
Refractory anemia	MDS single-lineage or multilineage dysplasia (SLD or MLD)	MDS with refractory cytopenia
Refractory anemia with ring sideroblasts	MDS (either SLD or MLD) with ring sideroblasts	Refractory cytopenia with multilineage dysplasia with or without ring sideroblasts
Refractory anemia with excess blasts	MDS with excessive blasts-1	MDS with excess blasts (5–30% blasts)
Refractory anemia with excess blasts in transition	MDS with excessive blasts-2	
	MDS with isolated del(5q) chromosome abnormality	
	MDS unclassifiable	MDS unclassifiable
Chronic myelomonocytic leukemia		
Acute myeloid leukemia (>30% blasts)		Acute myeloid leukemia (>30% blasts)

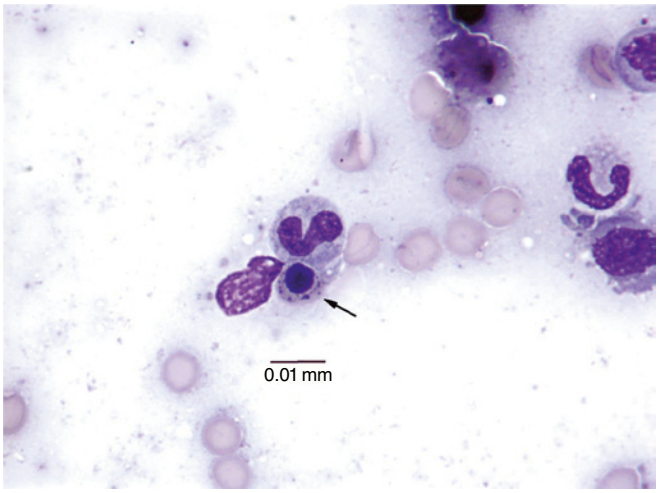


FIGURE 67.1 Ring sideroblast (arrow) in a bone marrow aspirate from a dog with sideroblastic-type refractory cytopenias with multilineage dysplasia. Wright–Giemsa stain.

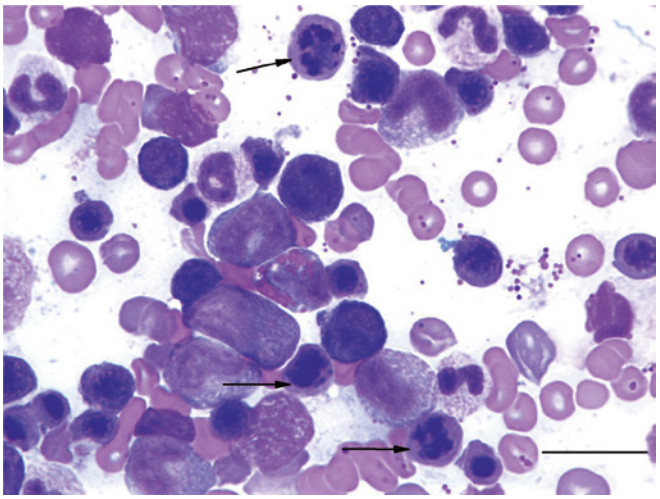


FIGURE 67.2 Dyserythropoiesis in a bone marrow aspiration smear from a dog with MDS with refractory cytopenia. Note the presence of fragmented nuclei in rubricytes (arrows). Wright–Giemsa stain; bar = 20 µm.

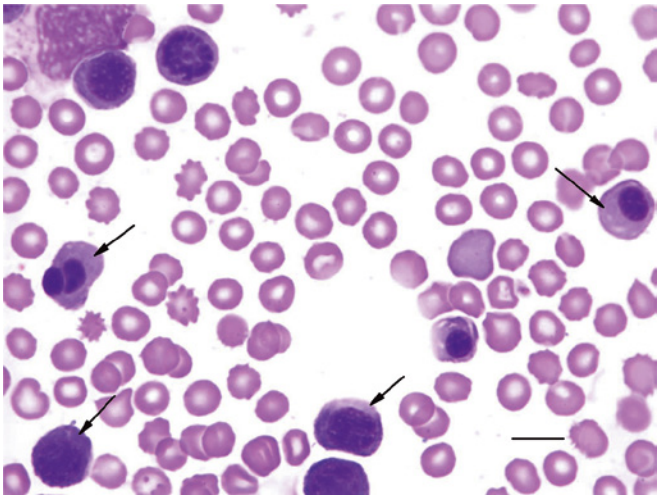


FIGURE 67.3 Mild megaloblastic dyserythropoiesis in a bone marrow aspirate from a dog with MDS with refractory cytopenia. Note the large size of nuclei in rubricytes relative to their maturity (arrows). Wright–Giemsa stain; bar = 10 µm.

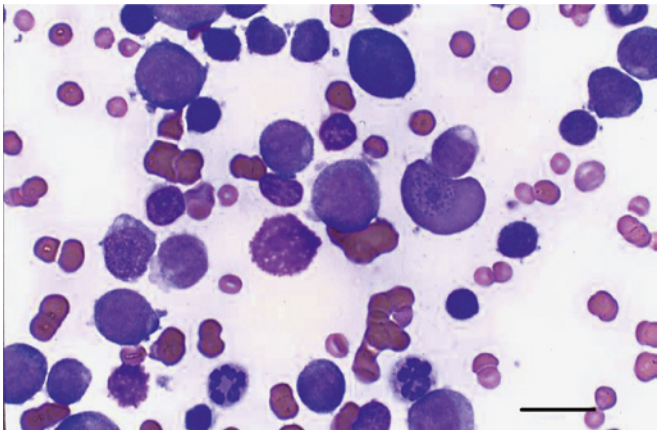


FIGURE 67.4 Increased numbers of myeloblasts and multilineage dysplasia in a bone marrow aspirate from a cat with MDS excess blasts. Note a large megaloblastic rubricyte and hypersegmented neutrophils. Wright–Giemsa stain; bar = 20 µm.

MLD, 2–4% blasts in PB, and/or 5–9% blasts in BM; MDS-EB-2 has 5–19% PB blasts and 10–19% BM blasts (Figure 67.4). In humans, MDS associated with isolated del(5q) chromosome abnormality has been shown to be sufficiently homogeneous to keep its own categorical classification. Acute myeloid leukemia is defined as greater than 20% myeloblasts in bone marrow. Neither acute nor chronic myelomonocytic leukemia is included in current WHO classifications of MDS.

A modified classification system (Table 67.1) has been described for dogs and cats,[48] but the proposed system has yet to be widely evaluated. Unfortunately, inconsistent use of a single system and variations in diagnostic criteria make it difficult to rely upon the published scientific literature for guidance. Nevertheless, in the system proposed in this chapter, major categories include: (1) MDS with refractory cytopenias, (2) refractory cytopenias with multilineage dysplasia, and (3) MDS with excess blasts. Myelodysplastic syndrome with refractory cytopenia is characterized by nonregenerative anemia, or occasionally multiple cytopenias, dysplastic features usually most prominent in the erythroid lineage, and less than 5% myeloblasts in bone marrow. Refractory cytopenias with multilineage dysplasia may occur without or with sideroblastic differentiation, the latter characterized in dogs by microcytic or hypochromic anemia and sideroblasts and ring sideroblasts in bone marrow.[46] MDS with excess blasts is typically characterized by bicytopenia or pancytopenia, bone marrow dysplasia in two or more cell lines, and greater than 5% bone marrow myeloblasts.

MYELODYSPLASTIC SYNDROMES IN DOGS

Retrospective studies have provided some insight into canine MDS. In a study of clinical bone marrow reports from a single veterinary teaching hospital, primary MDS was reported in 3.7% of sampled dogs.[46] MDS-EB was the most common, with MDS with refractory cytopenias and MDS-SD seen in fewer numbers. In another study, 12 dogs with primary MDS had features consistent with MDS with refractory cytopenia or MDS-EB based on the presence of pancytopenia and greater than 5% myeloblasts in bone marrow (Table 67.2).[52] Finally, another study found 13 of 267 dogs with bone marrow examinations had primary MDS with 4/13 having features of MDS with refractory cytopenias (n = 4) and the largest group having MDS-EB.[49] Collectively, these studies suggest that MDS-EB is the most common form of MDS observed in dogs.

MDS with Refractory Cytopenia

Dogs with MDS with refractory cytopenia (refractory anemia in older classification schemes) are typically older than 7 years with no breed predilection (Table 67.2).[49,52] Affected dogs have an insidious onset of lethargy and exercise intolerance. Frequent physical examination findings include pale mucous membranes, increased heart and respiratory rates, and anemia-related heart murmur. Hematologic alterations consist of moderate to severe, nonregenerative normocytic normochromic anemia. Total leukocyte and platelet concentrations are usually within reference ranges. Bone marrow is normocellular or hypercellular with a normal or decreased myeloid-to-erythroid ratio due to erythroid hyperplasia. Rubriblasts are frequently increased in number (5–30% of all nucleated bone marrow cells) and myeloblasts are consistently less than 5% of all nucleated bone marrow cells.[49,52] Dysplastic features, which are usually limited to the erythroid series, consist of binucleation, megaloblastosis, nuclear fragmentation, and asynchronous maturation.[49,52]

Treatment of dogs with MDS with refractory cytopenia has included prednisolone, cyclophosphamide, cytosine arabinoside, and erythropoietin.[10,40,52] Most dogs for which follow-up was available survived for months to years with no reports of progression to acute leukemia.[10,52] Of three dogs treated with recombinant erythropoietin, all had significant improvement in hematocrit and were nonanemic at last contact.[10,40,52] In the limited number of cases reported, response to treatment or survival did not appear to be dependent on the percentage of rubriblasts in bone marrow.

Refractory Cytopenia with Multilineage Dysplasia

Refractory cytopenia with multilineage dysplasia (RCMD) is characterized by prominent dysplastic features in two or more hematopoietic cell lineages, but no increase in bone marrow myeloblasts.[28,48] Dogs with RCMD include dogs with sideroblastic- and non-sideroblastic-types of anemia (Table 67.2).[15,28,43,46,48,50] Several dogs with non-sideroblastic anemia had normocytic normochromic nonregenerative anemia, thrombocytopenia, and dysplastic features in all hematopoietic cell lines.[15,28] Treatment of affected dogs has included low-dose aclarubicin, cytosine arabinoside, prednisolone, cyclosporine, and cytarabine ocfosfate, and has resulted in varied survival times.[15,23,28] Several cases of MDS with sideroblastic differentiation have been described.[46,49,50] However, because these conditions consistently had prominent dysplastic features in all cell lineages, it has been suggested that these are better categorized as RCMD.[46,48]

Dogs with sideroblastic-type RCMD demonstrate similar historical and physical exam findings as those with MDS with refractory cytopenias. Hematologic alterations consist of a microcytic or hypochromic nonregenerative anemia with many siderocytes in blood smears. The presence of siderocytes can be confirmed by staining the iron-containing granules with Prussian blue stain. Bone marrow is normocellular or hypercellular and usually contains increased amounts of hemosiderin. A characteristic feature is the presence of five or more

TABLE 67.2 Characteristics of myelodysplastic syndromes in dogs.[48]

Condition	Signalment	Clinical signs	Peripheral blood findings	Bone marrow findings
MDS with refractory cytopenias	Middle-aged to old	Lethargy, exercise intolerance	Normocytic normochromic nonregenerative anemia	Dyserythropoiesis, variable increase in rubriblasts, <5% myeloblasts
RCMD (without sideroblasts) RCMD (with sideroblasts)	Middle-aged to old	Uncertain Lethargy, exercise intolerance	Normocytic normochromic nonregenerative anemia Microcytic, hypochromic nonregenerative anemia	Dysplasia in multiple cell lineages, <5% myeloblasts
MDS with excessive blasts	Middle-aged to old	Lethargy, anorexia, fever, petechial hemorrhage	Bicytopenia or pancytopenia	Dyserythropoiesis, dysgranulopoiesis, dysmegakaryopoiesis, 5–20% myeloblasts

large iron deposits in rubricytes and metarubricytes (in contrast to the normal one to three small siderotic granules seen in the same cells from normal adult dogs).[13,43] Variable numbers of ring sideroblasts are also present. Although rubriblasts are frequently increased, myeloblasts are usually less than 5% of all nucleated cells. Too few cases of sideroblastic-type RCMD have been reported to establish treatment protocols or to evaluate outcome. However, among the few cases observed by one of the authors (DJW), both response to treatment and survival time appear to be less than that for MDS with refractory cytopenias. Cases of RCMD must be differentiated from sideroblastic anemia. Sideroblastic anemia has been associated with inflammatory diseases, myelofibrosis, and chloramphenicol therapy.[20,43]

MDS with Excessive Blasts

MDS-EB (refractory anemia with excess blasts in older classifications) is the most common form of primary myelodysplasia in dogs.[51,52] MDS-EB is a disease of middle-aged to old dogs (range 4–13 years) with no apparent breed or sex predilection (Table 67.2).[52] Dogs with MDS-EB usually appear more ill than those with MDS with refractory cytopenia. Signs of illness, which may appear acutely or progressively worsen over weeks to months, include lethargy, depression, anorexia, diarrhea, and fever.[52] Hematologic alterations include moderate to severe, normocytic normochromic nonregenerative anemia, neutropenia, and thrombocytopenia. Bone marrow is usually hypercellular with an increased myeloid-to-erythroid ratio. A hallmark feature of MDS-EB is increased numbers of myeloblasts (6–20% of all nucleated bone marrow cells).[52] Dysplastic features are typically present in all bone marrow cell lineages. Features of dysgranulopoiesis include small myeloblasts and promyelocytes, giant metamyelocytes and band cells, asynchronous maturation, and hypersegmentation.[6,49] Features of dyserythropoiesis include megaloblastic changes, asynchronous maturation, binucleation or trinucleation, nuclear fragmentation, and micrometarubricytes.[6,49] Features of dysmegakaryopoiesis include asynchronous maturation, large hypolobulated forms, dwarf megakaryocytes, and dispersed nuclei.[6,49]

Treatment of MDS-EB in dogs has been mostly supportive.[15,28,52] Broad spectrum antibiotics are indicated when severe neutropenia, fever, or sepsis is present. Red blood cell or whole blood transfusions are indicated to treat severe anemia. Erythropoietin and other hematopoietic growth factors have been given. Although these treatments may improve the cytopenias, they do not appear to significantly prolong survival. Administration of chemotherapeutic agents, including hydroxyurea, low-dose cytosine arabinoside, and low-dose aclarubicin, has been tried in individual cases; hydroxyurea has been used most frequently.[40] Survival times of dogs with MDS-EB are short, ranging from days to months.[15,52] Death or euthanasia is frequently associated with sepsis, organ failure, bleeding, or disseminated intravascular coagulopathy. Progression to acute myeloid leukemia has been documented in some cases.[52] In general, survival and progression to acute myeloid leukemia can be predicted by the percentage of myeloblasts present in bone marrow.

Myelodysplastic Syndrome Unclassifiable

Variant forms of MDS are occasionally seen. These probably result from different combinations of genetic mutations in hematopoietic stem cells. Primary dysmegakaryopoiesis is one variant form.[41] Affected dogs have a history of chronic moderate to severe thrombocytopenia unresponsive to immunosuppressive therapy. In bone marrow, dysplastic features of megakaryocytes include large hypolobulated or dwarf forms, asynchronous maturation, and discrete rather than lobulated nuclei. Causes of secondary dysmegakaryopoiesis, including immune-mediated hemolytic anemia, immune-mediated thrombocytopenia, monocytic ehrlichiosis, and lymphosarcoma, should be ruled out before a diagnosis of MDS is made.[41] Affected dogs appear to have prolonged survival.

Secondary Myelodysplastic Syndromes

Secondary MDS is a clonal disorder of hematopoietic stem cells associated with drug or radiation treatment or toxin exposure.[34] In humans, secondary MDS has been associated with benzene exposure and treatment with alkylating agents and radiation. Secondary MDS frequently occurs months to years after exposure. Drug-associated MDS has not been reported in dogs, but dysmegakaryopoiesis has been reported as a preleukemic change in dogs after exposure to gamma radiation.[36] With ever increasing use of chemotherapeutic agents and radiation therapy in veterinary medicine, secondary MDS may become more frequently recognized.

SECONDARY DYSMYELOPOIESIS IN DOGS

Diseases/Drugs/Toxins

Secondary dysmyelopoiesis includes nonclonal dysplastic conditions that should not be classified as MDS. Secondary dysmyelopoiesis is associated with a variety of conditions including immune-mediated hemolytic anemia/immune-mediated thrombocytopenia, lymphosarcoma, myelofibrosis, administration of certain drugs, exposure to heavy metals, iron deficiency, and some congenital conditions.[19,41,43,49] Implicated drugs include antimetabolites (e.g., azathioprine, cytosine arabinoside, hydroxyurea), folate antagonists (e.g., methotrexate), plant alkaloids (e.g., vincristine), alkylating agents (e.g., cyclophosphamide), phenobarbital, estrogen, cephalosporins, chloramphenicol, and colchicine.[1,9,20,24,41] In the erythroid lineage, dysplastic findings include binucleation, fragmented nuclei, giant forms, atypical mitotic figures, and megaloblasts. Phenobarbital therapy has been associated with a severe normocytic

normochromic nonregenerative anemia and dyserythropoiesis.[24,27] Sideroblastic anemia was documented in a dog treated with chloramphenicol.[20]

Secondary dysmyelopoiesis has also been associated with iron deficiency, lead, zinc, and arsenic poisoning, and infection with *Leishmania infantum*, *Anaplasma platys*, and *Hepatozoon canis*.[9,11,14,17,19,38] Iron deficiency is characterized in its later stages by a microcytic hypochromic anemia.[39] Dysplastic features are restricted to the erythroid lineage and are characterized by rubricytes and metarubricytes with a ragged rim of cytoplasm and patchy areas of basophilia within the cytoplasm.[19] A late-stage maturation arrest in the erythroid series may be present in some dogs. Lead inhibits several enzymes in the heme synthesis pathway leading to iron accumulation in erythroid precursors and sideroblastic anemia.[17] Lead also inhibits a pyridine-specific 5′-nucleotidase involved in degradation of ribosomal ribonucleic acids. Retention of ribosomes may result in basophilic stippling. Arsenic poisoning causes marked dyserythropoiesis characterized by karyorrhexis, megaloblastosis, and multinuclearity. Infection with *Leishmania* and other vector-borne agents in dogs has been associated with dysgranulopoiesis, dysmegakaryopoiesis, and dyserythropoiesis.[11,14]

Other forms of secondary dysmyelopoiesis include selective dysmegakaryopoiesis and sideroblastic anemia.[41,43] Dysmegakaryopoiesis is associated with the same spectrum of disease conditions as other forms of secondary dysmyelopoiesis. Secondary dysmegakaryopoiesis must be differentiated from megakaryoblastic leukemia. Little or no increase in megakaryoblasts is seen in secondary dysmegakaryopoiesis, whereas megakaryoblastic leukemia is characterized by a moderate to marked increase in megakaryoblasts in blood or bone marrow.[41] Unlike other types of secondary dysmyelopoiesis, sideroblastic anemias are associated with inflammatory diseases and myelofibrosis.[43] Affected dogs have a moderate to severe microcytic or hypochromic anemia that may be confused with iron deficiency anemia. Dysplastic features are similar to those associated with RCMD with ring sideroblasts except that the percentage of rubriblasts in bone marrow is not increased. Dysplastic features are restricted to, or are most prominent in, the erythroid lineage. The role of pyridoxine deficiency as a cause of the sideroblastic changes has not been investigated.

Differentiating MDS from secondary dysmyelopoiesis on morphology alone can be challenging.[9,42] Generally, in secondary dysmyelopoiesis, the percentage of dysplastic cells is lower and the percentages of rubriblasts and myeloblasts are not increased. Exceptions include sepsis and bone marrow recovering from toxic injury. In the case of sepsis, a combination of increased myelopoiesis and rapid mobilization of mature myeloid cells into the blood results in a shift toward immaturity in the myeloid series. The presence of toxic changes in the myeloid cells is helpful in identifying sepsis-associated dysmyelopoiesis. In marrow recovering from toxic injury, a wave of immature cells may be seen, resulting in a high percentage of blasts cells.[31] Dysplastic features are usually more numerous in MDS compared to secondary dysmyelopoiesis and megaloblastic features are usually more prominent, but in individual cases, these differences may be insufficient to make a conclusive diagnosis. Therefore, diagnosis necessitates exclusion of diseases, drug treatments, or toxin exposures that are known to cause secondary dysmyelopoiesis, and, occasionally, serial data collection and assessment of response to therapy.[42,49]

MYELODYSPLASTIC SYNDROMES IN CATS

In a retrospective study of clinical bone marrow reports at a veterinary teaching hospital, 11.6% of sampled cats had a diagnosis of MDS. Of these, 4.3% had a diagnosis of RCMD, and 6.8% had a diagnosis of MDS-EB (Table 67.3).[45] Cytopenias in cats with MDS have been associated with decreased numbers of granulocyte–monocyte colony-forming units and erythroid colony-forming units in four of seven cats tested, and decreased size of granulocyte–monocyte colony-forming units in all seven cats tested.[29]

Refractory Cytopenia with Multilineage Dysplasia

Cats with RCMD include cats previously diagnosed with refractory anemia and MDS with refractory cytopenia.[48] Cats diagnosed with refractory anemia with ring sideroblasts also fit into this category because of the presence of multiple cytopenias, blood and bone marrow sideroctyes, variable numbers of ring sideroblasts, and multilineage dysplasia (Table 67.3).[22,33,47]

The age and FeLV status of cats with RCMD varied between studies. In some, the median age was approximately 3 years and 70–90% of the cats were FeLV-positive.[22,33] In another, 25% of cats were FeLV-positive and the median age was 8.7 years.[45] In

TABLE 67.3 Classification and characteristics of myelodysplastic syndromes in cats.[48]

Condition	Signalment	Clinical signs	Peripheral blood findings	Bone marrow findings
RCMD	Variable	Anorexia, depression, weight loss, weakness	Macrocytic normochromic nonregenerative anemia, bicytopenia or pancytopenia, metarubricytosis	Dysplasia in multiple cell lineages, <5% myeloblasts
MDS-EB	Variable	Lethargy, anorexia, fever, petechial hemorrhage	Normocytic or macrocytic nonregenerative anemia, bicytopenia or pancytopenia	Dyserythropoiesis, dysgranulopoiesis, dysmegakaryopoiesis, 5–20% myeloblasts

general, cats with RCMD are less severely ill than cats with MDS-EB. Frequently encountered clinical signs include anorexia, depression, weakness, and loss of body mass. Affected cats have a moderate to severe macrocytic normochromic nonregenerative anemia and frequently have metarubricytosis and autoagglutination. Unlike dogs, cats with RCMD with sideroblasts have a macrocytic normochromic anemia. Most cats are thrombocytopenic and some are leukopenic. Bone marrow is normocellular or hypercellular, myeloblasts are <5% of all nucleated cells, and rubriblasts are typically increased. Dysplastic features are present in the erythroid line of all cats and frequently present in granulocyte and megakaryocyte lineages. Features of dysgranulopoiesis include maturation arrest, hypersegmentation, hyposegmentation, giant band and segmented neutrophils, and asynchronous maturation of nucleus and cytoplasm. Features of dyserythropoiesis included asynchronous maturation of nucleus and cytoplasm, binucleation, fragmented or lobulated nuclei, and megaloblastosis. Features of dysmegakaryopoiesis included large megakaryocytes with mature-appearing granulated cytoplasm but poorly lobulated or multiple discrete nuclei, megakaryocytes with mature hyperlobulated nuclei but immature-appearing homogeneous blue cytoplasm, and many small megakaryocytes with single or multiple discrete round nuclei. Evaluation of bone marrow core biopsy specimens revealed myelonecrosis and myelofibrosis in one-third of cats with RCMD.[45]

Although prognosis is guarded, some cats with RCMD respond to supportive therapy and have prolonged survival.[45] Cats with RCMD appear to have less of a tendency to progress to acute myeloid leukemia and have a longer survival time compared to those with MDS-EB. However, this difference is difficult to prove statistically when small groups are compared.[22] Therapy has consisted of prednisolone, prednisolone and cytarabine, and cyclosporine A.[22]

Myelodysplastic Syndrome with Excessive Blasts

Cats with MDS-EB, alternatively known as refractory anemia with excessive blasts, are characterized by multiple cytopenias, multilineage dysplasia, and increased myeloblasts in bone marrow (Table 67.3). In studies in which most affected cats were FeLV-positive, the age of the cats was often less than 4 years, whereas in studies in which more affected cats were FeLV-negative, the age of affected cats skewed older.[22,33,45] Cats with MDS-EB tended to be severely ill with clinical signs including anorexia, weakness, depression, loss of body mass, fever, vomiting, diarrhea, and platelet-related bleeding. Affected cats have a moderate to severe nonregenerative anemia and some have metarubricytosis and autoagglutination. Most cats are thrombocytopenic, and some are leukopenic. Bone marrow is usually normocellular or hypercellular, but may be hypocellular. Erythroid hypoplasia is a relatively frequent finding in FeLV-positive cats. All cats had 6–20% myeloblasts in bone marrow with variable increases in rubriblasts. Dysplastic features are present in all cell lines. Features of dysgranulopoiesis, dyserythropoiesis, and dysmegakaryopoiesis are similar to those associated with RCMD. Myelonecrosis and myelofibrosis are present in bone marrow of some affected cats.[45] Presently, survival for cats with MDS-EB varies from days to a few months with progression to acute myeloid leukemia possible in some cats.[22,45]

SECONDARY DYSMYELOPOIESIS IN CATS

Diseases/Drugs/Toxins

In a retrospective study of clinical bone marrow reports in cats, 6.3% were categorized as secondary dysmyelopoiesis.[45] Diseases associated with secondary dysmyelopoiesis included immune-mediated hemolytic anemia, immune-mediated thrombocytopenia, pure red cell aplasia, lymphosarcoma, glomerulonephritis, and feline infectious peritonitis.[45,47] Bone marrow had normal or increased cellularity, and dysplastic features were most prominent in the erythroid series, but could be found in granulocytes and megakaryocytes. In general, these changes were similar to those seen in MDS except that blast cells were not increased, the percentage of dysplastic changes was usually not as high, and megaloblasts were infrequently seen.

As in dogs, MDS can be difficult to differentiate from secondary dysmyelopoiesis using morphology alone, particularly in presumed immune-mediated anemia. Across all conditions, patients demonstrate a severe nonregenerative anemia, metarubricytosis, and possible agglutination.[45] Several factors are useful in differentiating these conditions. Marked macrocytosis (i.e., mean corpuscular volume (MCV) >65 fL) is a useful indicator RCMD. Additionally, >10% dysplastic changes in bone marrow are more consistent with MDS. Megaloblastic changes are prominent in MDS, but rare in secondary dysmyelopoiesis, and cats with MDS typically have increased rubriblasts or myeloblasts, while cats with secondary dysmyelopoiesis do not. An increased number of small lymphocytes in bone marrow is a consistent finding in immune mediated hemolytic anemia (IMHA) and pure red cell aplasia (PRCA).[44]

MYELODYSPLASTIC SYNDROME IN HORSES

Myelodysplastic syndrome, most consistent with MDS-EB, has been described in a Quarter Horse.[12] Peripheral blood findings consisted of a macrocytic nonregenerative anemia, leukopenia, and thrombocytopenia. Bone marrow evaluation revealed 9% myeloblasts, marked dyserythropoiesis, and increased plasma cells and eosinophils. Dyserythropoiesis was characterized by megaloblastic changes.

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CHAPTER 68

Acute Myeloid Leukemia

TRACY STOKOL

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Species Summary
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Acronyms and Abbreviations

ALL, acute lymphoid leukemia; ALP, alkaline phosphatase; ANBE, alpha-naphthyl-butyrate esterase; AML, acute myeloid leukemia; AML-NOS, acute myeloid leukemia-not otherwise specified; CAE, chloroacetate esterase; CHOP, cyclophosphamide, doxorubicin, vincristine, and prednisone; DNMT, DNA methyltransferase; FAB, French–American–British; FeLV, feline leukemia virus; FLT3, FMS-like tyrosine kinase 3; FMS, feline McDonough Sarcoma MDS, myelodysplastic syndrome; MHC, major histocompatibility complex; MPx, myeloperoxidase; NPM, nucleophosmin SBB, Sudan Black B; WHO, World Health Organization.

Acute myeloid leukemia (AML) is an aggressive hematopoietic neoplasm that encompasses a myriad of subtypes, which differ in phenotypic and genetic characteristics and biologic behavior, including progression, response to therapy, and prognosis.[56] It is the most common type of leukemia in adult human beings,[72] and, even with advances in genetic testing and genetically tailored therapy, the prognosis remains poor.[19] Classification schemes in human medicine continue to evolve with the application of advanced technological tools, such as next-generation sequencing for genetic and epigenetic defects.[3,20,64] In veterinary medicine, we are behind our human counterparts in the tools available for diagnosis and classification of leukemia and treatment options. Our biggest challenge remains the initial diagnosis of AML, particularly distinguishing it from lymphoid tumors such as the leukemic phase of lymphoma or acute lymphoblastic leukemia. Challenges with AML facing human medicine, such as standardizing treatment protocols or outcomes, optimizing treatment strategies, prolonging life with individualized therapy, and identification and discrimination of minimal residual disease from clonal hematopoiesis of indeterminate potential,[7,20,52] are not even at play in animals. For this review, only selected individual case reports are referenced, with the summary of AML in certain species largely restricted to larger case series.

PATHOGENESIS

Acute myeloid leukemia involves the clonal expansion of myeloid stem cells, which can either minimally or partially differentiate down a particular myeloid lineage. The term myeloid can have several connotations, but in this case it means all nonlymphoid lineage cells, including granulocytes (neutrophil, eosinophil, basophil), megakaryocytes, erythrocytes, mast cells, and monocytes.[64] In rare cases of acute leukemia, there is evidence of mixed lineage differentiation (e.g., myeloid + B cell, myeloid + T cell) on the same or different cell populations,[36] but these mixed phenotype acute leukemias are not categorized as AML but rather as leukemia of ambiguous lineage

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(similar to acute undifferentiated leukemia).[3,56,64] In acute leukemia, the neoplastic cells have a proliferative advantage, resulting in their accumulation in bone marrow, usually at the expense of normal hematopoiesis. They can spill over into blood or infiltrate extramedullary tissues. Rather than consisting of one neoplastic clone, AML is thought to consist of multiple subclones or leukemia stem cells, which are thought to arise from accumulating heterogeneous genetic mutations in a founding clone.[37,68] Leukemic stem cells have been identified in dogs with AML,[27] and these quiescent chemoresistant cells are likely responsible for disease recurrence or refractoriness to treatment. Partly differentiated myeloid cells can also revert to having "stem-like" properties.[38]

In human patients, AML is usually a de novo neoplasm, but it can emerge from a pre-existing myelodysplastic syndrome (MDS), mixed myelodysplastic/myeloproliferative disorder or myeloproliferative neoplasm (termed a blast crisis), or as a consequence of therapy for an unrelated hematopoietic or nonhematopoietic neoplasm (with alkylating agents, topoisomerase II inhibitors, antimetabolites, antitubulin agents, or ionizing radiation).[6,64] In animals, AML is usually a de novo neoplasm in most species except for domestic cats, in which MDS progression to AML occurs secondary to feline leukemia virus (FeLV) infection.[30,31,62] There have been rare individual reports of MDS-[41] and radiation-associated AML in dogs.[49,60]

Genetic defects, such as internal duplications, gene fusions, and point mutations, underlie the pathogenesis of AML. A large and diverse range of defects have been discovered (>5000 in one study),[46] with the most common involving cellular pathways of proliferation (e.g., activating mutations in feline McDonough sarcoma (FMS)-like tyrosine kinase 3 [FLT3]), differentiation (e.g., nucleophosmin or NPM1), and epigenetic control (e.g., the DNA methyltransferase [DNMT3A]) (Table 68.1).[24,46] The current consensus is that, similar to other cancers, leukemogenesis is a multistep pathway with more than one genetic defect being required for manifestation.[24,46] Although most human patients with AML have more than one abnormality, single-gene defects in signaling proteins (e.g., FLT3 or RAS), transcription factors (e.g., RUNX1), spliceosome proteins (which remove introns, e.g., U2AF1), and cohesins (protein complexes that control chromatid separation during mitosis or meiosis, e.g., STAG2) have been identified, some of which can initiate leukemia in murine models.[12] A study of 1524 human patients with AML provided genetic evidence for structured clonal evolution of AML. Defects in epigenetic (DNMT3A, TET2, and IDH) and chromatin (ASXL1) modifiers appeared to be early events and usually coexisted with other mutations, suggesting these mutations may "set the stage" for leukemogenesis, but do not result in AML alone.[46] However, mutations in these genes also occur in older patients with clonal hematopoiesis of indeterminate potential,[53] and may just reflect clonal hematopoiesis versus a preleukemic process. Defects in signaling genes, such as RAS, were later events and mutations in NPM1 were usually secondary. Some mutations frequently coexisted,[46] suggesting synergistic interactions between genes that promote the leukemia.[46,68]

TABLE 68.1 General categories (distinguished by cellular function) of identified genetic abnormalities, with example defects, in human patients with AML.[12]

Cellular function	Example defects
Transcription factors	
Fusions	PML–RARA, CBFB–MYH11, RUNX1–RUNX1T1
Mutations	RUNX1, CEBPA
Signaling molecules (gain of function)	
Fusions	BCR–ABL (rare)
Mutations/internal random duplications	FLT3, KIT*, KRAS*, NRAS, NF1
Tumor suppressor mutations (loss)	TP53, WT1
Epigenetic modifiers	
Fusions	MLL-multiple genes
Mutations	DNMT3*, TET, IDH
Chromatin modulator mutations	ASXL1, EZH2, NPM1**
Cohesin mutations	STAG2, RAD1
Spliceosome mutations	U2AF1, SF3B1

*Identified in dogs.[11,22,63]
**This nucleolar protein has multiple roles in cellular function, including chromatin remodeling, mRNA transport, and genetic stability.

Genetic defects are being used in newer classification schemes, to tailor individualized therapy and for prognostication.[3,19,20,46,55,64] In contrast to human medicine, little genetic analysis has been done in animals with AML and the few studies are restricted to dogs (Table 68.1).[11,22,63]

Aside from the leukemic cells themselves, there is growing awareness of the role of the microenvironment in tumor initiation, persistence, progression, and chemoresistance.[28] Less is known about how the microenvironment influences AML. The bone marrow microenvironment is complex, consisting of vascular, endosteal, and megakaryocyte niches and an array of cell types, including immune cells, fibroblasts, pluripotent mesenchymal cells, nerve cells, adipocytes, endothelial cells, osteoblasts, and osteoclasts. Through structural support, cell-to-cell adhesive interactions and release of soluble or extracellular vesicle-associated growth factors, cytokines (e.g., CXCL12, transforming growth factor-β), and microRNA, stromal cells play a vital role in normal hematopoiesis. The microenvironment provides homing signals to hematopoietic stem cells (e.g., CXCL12–CXCR4 interactions) and helps maintain stem cell quiescence and self-renewal, while inhibiting differentiation and apoptosis.[42,51] Studies are just beginning to elucidate the mechanisms by which the microenvironment promotes leukemogenesis, not only through the provision of a supportive and protective locale via tumor–stromal cross-talk, but also through genetic defects that may drive leukemogenesis.[42,51] For example,

deletion of the ribonuclease, Dicer1, in osteoprogenitor cells in mice results in MDS and secondary AML.[48] Targeting the microenvironment may prove to be a promising new strategy to treat patients with AML or reduce the high rate of relapse, which is attributed to the persistence of leukemic clones in bone marrow niches.[51]

DIAGNOSIS

Since AML usually arises in the marrow and the leukemia effaces the marrow or inhibits normal hematopoiesis, the hallmark laboratory findings are bicytopenia or pancytopenia with or without circulating blasts.[1,4,9,17,18,33,45,57,66] The neoplastic cells can also infiltrate extramedullary sites, often in a vascular-oriented distribution, including the liver, spleen, and lymph nodes and infrequently other organs, including the skin, gastrointestinal system, and brain.[4,17] In some human patients, AML can manifest as a solid extramedullary tumor, myeloid sarcoma, which can precede, occur concurrently with, or follow after a diagnosis of AML. Myeloid sarcomas are differentiated from standard leukemic organ infiltration patterns by the presence of a solid mass that effaces normal architecture.[56] Myeloid sarcomas have been reported rarely in animals.[21,43] The most common manifestation of myeloid sarcoma in dogs in the author's experience is a mediastinal mass, which can be large and mimic mediastinal lymphoma.[21] A diagnosis of acute leukemia (not categorized as lymphoid or myeloid) can be readily made when there are large numbers of circulating blasts and concurrent cytopenias. In the absence of circulating blasts, the diagnosis of AML requires a bone marrow aspirate to confirm an acute leukemia. However, lymphoma can infiltrate the bone marrow, and morphologic features alone are unreliable for determining the leukemic lineage (whether confined to marrow or in peripheral blood). Thus, the diagnosis of AML in animals relies upon combining cytomorphology and phenotype to ascribe a myeloid lineage to the tumor cells. The results of this testing may not be entirely declarative and often necessitates using a preponderance of evidence (i.e., do the tumor cells express more myeloid than lymphoid morphologic and phenotyping features?) for lineage assignment. In human medicine, cytomorphologic assessment and phenotyping are still essential components of diagnosis; however, genetic analysis has become commonplace and a required part of the diagnostic evaluation of AML.[3,20,56,64]

Cytomorphology

The basic requirement for the diagnosis of AML in humans is the presence of ≥20% myeloid blasts in blood or bone marrow. The World Health Organization (WHO) applies three exceptions to this rule: (1) Myeloid sarcoma, a solid proliferation of myeloid cells in extramedullary tissues, which can be diagnosed as a solitary entity; (2) Patients with specific gene fusions (RUNX1–RUNX1T1, CBFB–MYH11, PML–RARA); and (3) Pure erythroid leukemia, in which a cutoff of ≥30% proerythroblasts is used.[3] It is recommended to determine the blast percentage by morphologic assessment of a 200 or 500 count of nucleated cells on Romanowsky-stained smears of blood and bone marrow, respectively, versus expression of stem cell markers, like CD34, on flow cytometric analysis.[64] The rationale behind the latter recommendation is that some cases of AML may not express CD34 in human[64] and veterinary patients,[54,65,66] and methodologic issues, such as hemodilution and the clone of antibody used, can affect percentages. However, in cases where there are insufficient circulating blasts or a fibrotic marrow precludes aspiration, immunostaining for stem cell markers on histologic sections of bone marrow from human patients may be helpful. Notably, anti-CD34 antibodies have not been validated for the diagnosis of AML using bone marrow histologic specimens in animals.

The blast percentage encompasses myeloblasts, megakaryoblasts, and monoblasts (with promonocytes being included in the blast count for monocytic variants of AML).[56,64] Proerythroblasts (rubriblasts) are quantified for pure erythroid leukemia.[3] In contrast to humans, most publications in veterinary medicine have used a cutoff of ≥30% blasts for diagnosing AML,[1,9,33,34,57,66] with ≥30% rubriblasts in erythroid-dominant marrows.[33] More recently, the author and others have adopted the WHO cutoff of ≥20% myeloblasts for the diagnosis of AML.[4,17,54] In the author's experience, the diagnostic choice of 20% or 30% blasts does not seem to matter in most cases of AML because, by the time the disease is diagnosed, the blast percentage is much higher than either cutoff (often >60%). More commonly, at the time of presentation, the main diagnostic distinction in veterinary medicine is differentiating AML from acute lymphoid leukemia (ALL). In this author's experience, there are only infrequent patients that present with a lower blast percentage. In such cases, the diagnostic conundrum is principally differentiating AML from marrow-infiltrating lymphoma.

Since the blast percentage is key in diagnosing AML and distinguishing AML from MDS,[34,56,64] it is important to define what constitutes a blast. In human hematopathology, the WHO recommends criteria established by the International Working Group on Morphology of Myelodysplastic Syndrome[44] to define blasts.[64] Agranular blasts have variably shaped nuclei (round, oval to indented to rarely reniform) with usually fine chromatin and one or more nucleoli. Agranular blasts contain variably basophilic cytoplasm with a high nuclear to cytoplasmic ratio and no Golgi zone (with rare exceptions). By contrast, granular blasts contain azurophilic cytoplasmic granules and are distinguished from promyelocytes by the lack of a Golgi zone. Auer rods may be present in both types of blasts (but have not been reported in animals).[44] Monoblasts may have more cytoplasm than myeloblasts, with or without low numbers of cytoplasmic vacuoles or granules. By contrast, promonocytes have more irregular to deeply folded nuclei with fine chromatin. Nucleoli may or may not be apparent and the cytoplasm may contain fine or coarse granules.[56]

In 1991, the Animal Leukemia Study Group identified three types of blasts for classifying AML and MDS

in dogs and cats. Type 1 corresponded to an agranular blast (which could have a Golgi zone), whereas type II and III blasts were distinguished by the presence and number of cytoplasmic granules (<15 tiny granules in type II and many granules in type III).[34] The latter are now incorporated as granular blasts. The study group also defined monoblasts as cells containing round to irregular to folded nuclei (with fine chromatin and one or more nucleoli) and a moderate amount of blue cytoplasm with a prominent Golgi zone. They also defined promonocytes as cells containing cerebriform nuclei with prominent nuclear folds and stippled or lacy chromatin with no nucleoli. These cells are agranular with more abundant and less-blue cytoplasm than monoblasts. The definition of blasts or promonocytes has not been modified since in veterinary medicine, although it is likely that individual authors use their own criteria.

Even with these defined criteria, the morphologic identification of blasts in veterinary medicine remains subjective with high interindividual variability. It is also difficult to distinguish promyelocytes from granular blasts, promonocytes from more mature or dysplastic monocytes, and dysplastic megakaryocytes from megakaryoblasts (Figure 68.1), and blast identification ultimately ends up being a "matter of opinion." In addition to the challenges in distinguishing myeloid blasts from their slightly more mature progeny, such blasts may be erroneously assigned a lymphoid origin (i.e., called "lymphoblasts"). Based on the pitfalls of morphology-based lineage assignment, this author recommends that immature cells (fine chromatin, indicating mostly euchromatin, with or without nucleoli) in blood and bone marrow be given the generic term of "blasts" (when confident, such cells are not reactive lymphocytes), until further phenotyping or classification studies are performed. Morphologic features alone cannot be used to differentiate blasts of myeloid or lymphoid (including lymphoma or ALL) origin. Although features of myeloid differentiation are helpful in supporting a diagnosis of AML (Figures 68.1 and 68.2 and Table 68.2), these features can be subtle or subjective and there are many cases in which such features are lacking and the diagnosis relies on phenotyping to assign a myeloid lineage to the neoplastic cells.

Immunophenotyping

Immunophenotyping is the procedure by which cell lineage is ascertained using antibody-based staining on liquid (flow cytometry), cytologic (immunocytochemistry), or histologic (immunohistochemistry) specimens. The applied antibodies can detect cell surface proteins (e.g., CD45, CD3), intracytoplasmic proteins (e.g., MPx, CD79a, CD3-ε), or intranuclear proteins (e.g., Pax-5). The primary antibodies can be conjugated with a fluorophore (immunofluorescent microscopy or flow cytometry) or a chromogenic enzyme (immunocytochemical or histochemical staining). Alternately, antibodies can be identified using similarly conjugated-secondary antibodies raised against the host species in which the primary antibody was made. Some antigens are normally expressed in multiple cell types, whereas others are more lineage-restricted, which helps identify both normal cells and lineages of leukemic cells (Table 68.3). This section will concentrate on flow cytometry, the most commonly used method for phenotyping leukemias.

Flow cytometry is used on blood and aspirates of bone marrow or tissues and usually employs antibodies raised against surface antigens, which avoids the requirement for cell permeabilization. The latter can alter forward and side scatter features that are characteristic of different cell populations, making it difficult to

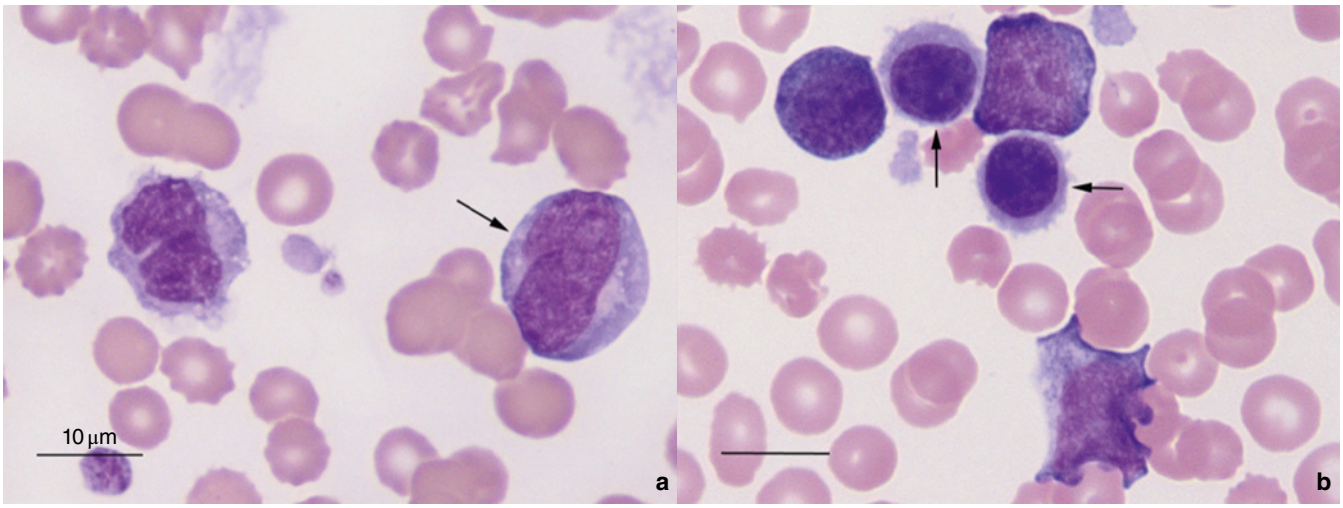


FIGURE 68.1 Distinguishing blasts in AML in modified Wright-stained smears. (a) A promonocyte (arrow) and monocyte in blood from a dog with AML-not otherwise specified (AML-NOS), subtype acute monoblastic/monocytic leukemia. The promonocyte has a deeply folded nucleus with fine chromatin. The promonocyte would have been included in the blast count; (b) Three blasts (presumptive, megakaryoblasts) and dwarf dysplastic megakaryocytes with light pink cytoplasm and ruffled borders (arrows) in blood from a dog with AML-NOS, acute megakaryoblastic leukemia (bar = 10 µm).

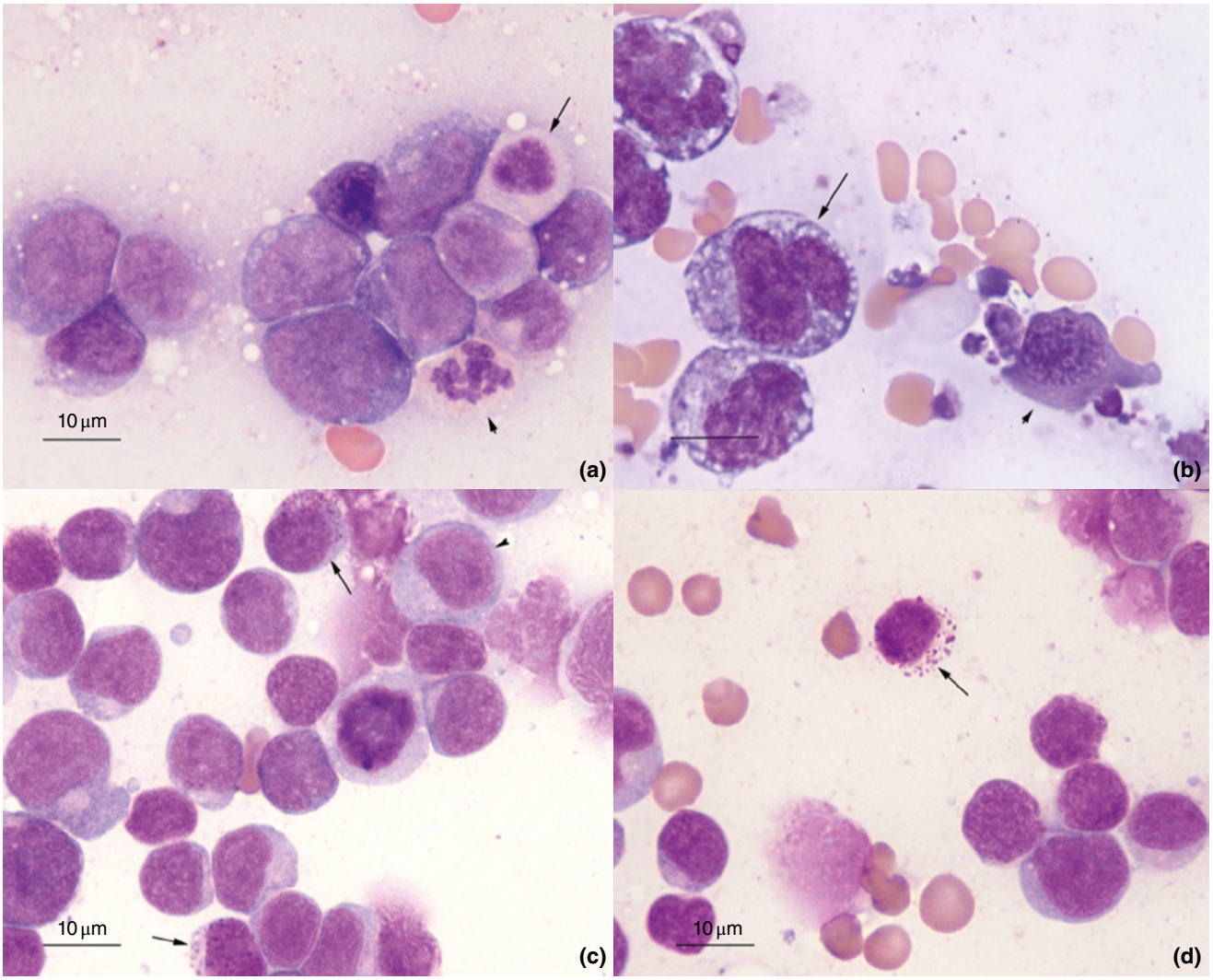


FIGURE 68.2 Features of myeloid differentiation in modified Wright-stained bone marrow smears from animals with AML. (a) A continuum between differentiated myeloid cells with moderate amounts of light blue to clear cytoplasm (arrow) and blasts is seen in the bone marrow of a dog with AML-NOS, acute myelomonocytic leukemia. A hypersegmented (dysplastic) neutrophil is also evident (arrowhead); (b) Dysplastic monocytes (arrow) and erythroid progenitors (arrowhead) are evident in a bone marrow aspirate from a horse with AML-NOS, acute monocytic leukemia (bar = 10 µm); (c) Granular blasts (arrows) and cells with increased amounts of light blue cytoplasm (arrowhead) are features of myeloid differentiation in the bone marrow of a dog with AML-NOS, acute monoblastic leukemia; (d) Blast with "mast-cell-like" chunky purple granules (arrow) in the bone marrow from the same dog as in image (c).

distinguish between cell types with certainty. In addition, with permeabilization, false positive reactions (e.g., nuclear staining with CD79a, which is normally expressed in the cytoplasm) cannot be avoided, unless additional steps are taken to confirm such positive reactions with immunocytochemical or histochemical staining, where the cellular location of the staining pattern can be identified. There are more commercially available flow cytometry antibodies directed against dog antigens (as compared to horses and cats) and more directed at T cell antigens, neutrophils, and monocytes (as compared to antigens of less-differentiated or stem cell antigens or B cells). Different laboratories use different staining methods, antibody clones, and analytical methods. As such, published results are not always comparable between labs (particularly if different clones are used).

Flow cytometry, when combined with cytomorphology, can often yield a definitive diagnosis of AML when there is expression of myeloid-associated antigens with concurrent lack of T and B cell markers. However, in both human and animal patients, cross-lineage expression of B and T cell markers occurs in AML[54,56] and subpopulations of monocytes in dogs can express antigens more typically associated with T cells (such as CD1c, CD11d, and CD90) (personal observations). This

TABLE 68.2 Morphologic, immunophenotypic, and cytochemical features of myeloid differentiation in acute leukemia.

Test	Supporting features*
Morphologic assessment of Romanowsky-stained blood or bone marrow smears	Differentiation (gradation between blasts and more mature identifiable myeloid cells), monocytoid nuclei, multinucleation or megakaryoblastoid nuclei, pink to red cytoplasmic granulation within a light to medium blue cytoplasm, chunky purple cytoplasmic granules ("mast-cell-like," Figure 68.2), dysplasia
Immunophenotypic testing modalities (flow cytometry, immunocytochemistry, immunohistochemistry)	Expression of myeloid antigens such as: myeloperoxidase (MPx), CD11b, CD11c, CD4 (with negative T cell markers), CD11d or CD1c (usually with negative T cell markers), CD14, unspecified neutrophil antigens, CD61 Expression of stem cell markers: CD34, CD90 with lack of MCHII expression Usually negative expression for T and B cell markers (cross-lineage expression can and does occur)
Cytochemical staining reactions	Positive for MPx, alkaline phosphatase (ALP), chloroacetate esterase (CAE), α-naphthyl butyrate esterase (ANBE, diffuse or chunky pattern), Sudan Black B (SBB)

*None of these features alone are diagnostic for AML, but should be used in combination with all the available findings.

complicates interpretation and makes it difficult to definitively distinguish T cell neoplasms from AML using flow cytometry. In the author's experience, the most useful myeloid differentiation markers are CD11b, CD11c, and CD14 (often with dual expression of CD4) for myelomonocytic, neutrophilic, and monocytic variants of AML. CD11b is rarely expressed on lymphoid neoplasms, whereas CD11c can be expressed on T cell neoplasms, particularly those of granular morphology. Double staining for MHCII major histocompatibility complex type II (MHCII) and CD34 is also useful, because CD34-expressing AMLs usually lack MHCII expression[54] in contrast to CD34-expressing lymphoid neoplasms (more commonly B[69] than T cell tumors), which co-express CD34 and MHCII (personal observations). CD34 expression, however, may not be detected in some dogs with AML,[54,65,66] particularly more-differentiated variants, such as AML-NOS, acute myelomonocytic leukemia, in dogs,[54,66] and CD34 is not reliably detected in horses with AML (personal observations). Similarly, CD11b, CD11c, and CD14 may not be detected on less-differentiated variants of AML, which may only express stem cell markers, such as CD34 and CD90.[54] If only flow-cytometric-based immunophenotyping (particularly with limited antibodies) is done on the latter cases, they could be diagnosed as acute undifferentiated leukemia. In this author's experience, many CD34-positive MHCII-negative acute leukemias have positive cytochemical staining reactions (particularly to ALP), which supports their myeloid origin.[54] In general, this author recommends a multiparameter approach for the flow cytometric diagnosis of AML and that flow cytometry data be interpreted against known patterns of marker expression in AML.[54] It also cannot be overemphasized that flow cytometry alone should not be used to diagnose AML, but should always be interpreted along with neoplastic cell morphology (in blood, bone marrow, or tissues), imaging studies, patient history, clinical signs, and physical examination findings.

In the absence of liquid specimens, immunophenotyping can be done on cytologic and histologic samples. Slides for immunocytochemistry can be acetone-fixed and stored at −70 °C for over a year with antigen retention.[47] Limitations of diagnostic immunocytochemistry include the often low numbers of available slides and a lack of preservation of cell morphology, which makes it difficult to conclusively identify blasts. Immunohistochemical staining is substantially limited by the number of antibodies that work in formalin-fixed tissue (Table 68.3). As such, the diagnosis of AML on such tissues is usually one of exclusion or based on lack of staining for standard lymphoid markers with morphologic features of myeloid differentation.[17] Of the available markers, staining for MPx (or megakaryocyte markers in the case of acute megakaryoblastic leukemia) may be helpful to confirm AML in dogs. However, in the author's experience, monocytic variants of AML may be negative for MPx and other monocytic/macrophage markers, including lysozyme and MAC387. Ionized calcium-binding adaptor molecule I (Iba-1) is considered a highly specific marker for macrophages and dendritic cells;[58] however, this marker has only been tested in two case series of equine AML and there is conflicting data on its specificity.[4,17] Many antibodies successfully used in flow cytometry can be applied to snap-frozen tissues (which is not routinely done) or to hematologic or cytologic slides. However, immunostaining of smears is labor-intensive and time-consuming process, limiting its routine application. Similar to flow cytometry, there are no standardized cutoffs for positive staining on cytologic or histologic smears.

Cytochemical Staining

Cytochemical staining was the traditional mainstay for determining lineage of leukemias and was the basis of the French–American–British (FAB) classification scheme,[5] that is still used in veterinary medicine[17,45,54,57,66] and, in part, in human medicine (see in the following text). Cytochemical stains are useful for confirming myeloid lineage, with the exception of erythroid cells, because granulocytes and monocytes express enzymes

TABLE 68.3 Antigens used for immunophenotyping of hematopoietic neoplasms by flow cytometry in humans and dogs, cats, and horses.*

Cell lineage	Human[20,56]	Dogs[c], cats[f], and horses[e1,2,4,45,54,57,65,66]
Stem cell	CD34, CD117, CD133	CD34[c,e], CD90[c]
Early myeloid cell	CD13, CD33, CD38, CD123	None
Differentiating or mature neutrophil	CD15, CD16, CD36, CD65, myeloperoxidase	CD4[c], CD11b[c,f], CD11c[c], CD90[e], CD172a[e], MPx[c], unspecified (e.g., neutrophil-specific antigen)[c,e,f]
Differentiating or mature monocyte	CD11c, CD14, CD36, CD64	CD1c[c], CD11b[c,f], CD11c[c], CD11d[c], CD14[c,e,f], CD90[c], CD163[e], CD172a[e], MHCII[c], MAC387[c]
B cell	CD5, CD19, CD22, CD10	CD5[c,f,e] (subset), CD19-like[e], CD20[c], CD21[c,e,f], CD22[c], CD79a[c,e,f], MHCII[c]
T cell	CD2, CD3 (cytoplasmic against epsilon not zeta chain), CD4, CD5, CD7, CD8	CD2[e], CD3[c,e,f], CD5[c,e,f], CD4[c,e,f], CD8[c,e,f], TCRαβ[c], TCRγδ[c], CD1c[c] (subset), CD11d[c] (subset), CD90[c] (subset), MHCII[c]
Common leukocyte		CD45,[c] CD18,[c] MHCII[e]
Megakaryocyte	CD41, CD42, CD61	CD41,[c] CD61[c]
Erythroid	CD36, CD235a	None

*This list is not exhaustive, including known cross-reactive antibodies or species-specific clones that have been used to phenotype leukemia in the selected species, specifically by flow cytometry. Underlined antibodies can be used on formalin-fixed paraffin-embedded section with immunohistochemical staining and some can be used across species (e.g., CD20, CD61). There are no standardized cutoffs for interpretation of positive cells with different authors stating various cutoffs (≥20% with ≥5% for CD34[54] and ≥20–30% with ≥10% for MPx[1,66]).

or proteins that react with the stains. By contrast, lymphocytes are generally negative for the stains, with some exceptions (Table 68.4). Since the advent of flow-cytometric-based immunophenotyping, cytochemical staining is rarely applied in veterinary medicine for diagnosis of AML. However, when flow cytometric results are inconclusive or nondiagnostic (e.g., concurrent expression of lymphocyte and myeloid markers, leukemias that are only CD34-positive and MHCII-negative), cytochemical staining can help confirm a diagnosis of AML in such cases. Indeed, cytochemical staining is still recommended for phenotyping leukemias, particularly AML-NOS, in human patients.[20,56,64] It is important to note that, similar to immunophenotyping, aberrant cytochemical staining can be seen in some cases of AML.[25]

Clonality Testing

Clonality testing in dogs and cats is usually done via polymerase chain reaction for antigen receptor rearrangements in T and B cell receptor genes (PARR).[35] However, clonal rearrangements in B and T cells have been reported in AML in human patients[13,39,50] and dogs,[54] and clonality testing should not be used to differentiate AML from lymphoid neoplasms.

CLASSIFICATION

Based on the 2008 updated and 2016 revised WHO criteria,[3,56,64] AML is classified into six different categories in human patients: (1) AML with recurrent genetic abnormalities, (2) AML with myelodysplasia-related changes, (3) therapy-associated AML, (4) AML-NOS, (5) myeloid sarcoma, and (6) myeloid proliferations related to Down's syndrome. Given the lack of comparable veterinary genetic testing and acknowledgment that there are no veterinary correlates to some human conditions, AML in animals is usually categorized as AML-NOS and assigned a morphologic subtype based on human criteria (Table 68.5). The AML-NOS category is primarily based on the FAB criteria established in 1976[5] and was used by the Animal Leukemia Study Group in 1991 to originally classify AML and MDS in dogs and cats.[34] It is still the most commonly used classification scheme

TABLE 68.4 Expected cytochemical reactions of commonly used stains for different leukocytes in dogs[c], cats[f], and horses[e].[4,25,26,34,54]

Leukocyte	Staining reaction
Neutrophil (mature, immature)	MPx[c,e,f], CAE[c,e,f], ALP[e], SBB[c,e,f]
Eosinophils	MPX[c,e], ALP[c,f], SBB[e]
Basophils	CAE[c,e,f], ALP[c]
Monoblasts, promonocytes	ALP[c], ANBE[e,f] (diffuse, chunky, ±fluoride inhibited**), ±SBB[c,e,f]
Monocytes	ANBE[c,e,f] (±fluoride inhibited), ±SBB[c,e,f]
T cells (subsets)	ANBE[c,e,f] (focal to multifocal, granular, ±fluoride resistant)
Megakaryocyte	ANBE[c,f]

ALP: alkaline phosphatase, ANBE: alpha-naphthyl butyrate esterase, CAE: chloroacetate esterase, MPx: myeloperoxidase, SBB: Sudan Black B.
*Not all stains are shown. A positive reaction is when ≥3% of blasts are positive.[54,56]
** Fluoride sensitivity or resistance is not routinely done, rather the overall pattern of staining in blasts is ascertained.

TABLE 68.5 Morphologic subtypees of AML-NOS in human patients, with classification features.[3,56,64]

Acute myeloid leukemia with minimal differentiation (AML-M0)
No evidence of myeloid differentiation morphologically or with cytochemical stains. Blasts are agranular. Myeloid origin confirmed by immunophenotyping (expression of stem cell markers and early myeloid antigens, e.g., CD13, CD33) or ultrastructure. Rarely express lymphoid antigens. Differential diagnoses include precursor lymphoid neoplasms (lymphoma or leukemia), acute megakaryoblastic, monoblastic, or erythroid leukemia, and mixed phenotype acute leukemia.

Acute myeloid leukemia without maturation (AML-M1)
Blasts (≥3%) are positive on cytochemical staining (e.g., MPx, SBB) or contain Auer rods (not seen in animals); blasts can be granular. Express early myeloid-associated antigens, but no or minimal differentiating antigens on immunophenotyping (Table 68.3). Can express lymphoid antigens (e.g., CD7, CD2, CD4). Differential diagnoses include precursor lymphoid neoplasms, AML with maturation or acute monoblastic leukemia.

Acute myeloid leukemia with maturation (AML-M2)
Evidence of neutrophilic differentiation (progranulocytes, myelocytes, and mature cells comprise ≥10% of marrow cells) with differentiating monocytes being <20% of marrow cells and blasts being positive for myeloid markers on cytochemical staining. Express early myeloid and differentiating neutrophil antigens (e.g., CD11b, CD15), with some cells expressing stem cell markers. Can express lymphoid antigens. Differential diagnoses include MDS with excess blasts, AML without maturation, and acute myelomonocytic leukemia.

Acute basophilic leukemia (AML-M2B)
Blasts contain coarse basophilic granules (which can resemble mast cell or basophil granules ultrastructurally) with few mature basophils. Blasts are positive for toluidine blue and negative for other cytochemical stains (MPx, SBB, CAE, ALP, ANBE). Blasts express early myeloid and differentiating myeloid antigens (e.g., CD11b), and may express stem cell markers. Usually lack CD25 (mast cells) and lymphoid antigens. Differential diagnoses include other AML genetic subtypes with basophilia, mast cell leukemia, and granular versions of lymphoid leukemia (rare in humans).

Acute myelomonocytic leukemia (AML-M4)
Consists of differentiating neutrophils (≥20% of marrow cells) and monocytes (≥20% of marrow cells) with promonocytes included in the blast count. Blasts may express both granulocyte and monocyte cytochemical stains, with some cases having dual positive cells (Figure 68.3a). Cells express differentiating granulocyte and monocyte antigens and there may be a subpopulation of blasts expressing stem cell antigens. Lymphoid antigen expression is rare. Differential diagnoses are AML with maturation, acute monocytic leukemia, and chronic myelomonocytic leukemia.

Acute monoblastic or monocytic leukemia (AML-M5)
Consists of differentiating monocytes (≥80% of marrow cells) with most cells being monoblasts or promonocytes in acute monoblastic or monocytic leukemia, respectively. Can have a neutrophilic component (<20%). Blasts express both monocyte-related cytochemical reactions and early myeloid and monocyte markers on immunophenotyping (macrophage antigens like CD163 are more often seen in monocytic leukemia). There may be a subpopulation of blasts expressing stem cell antigens. Lymphoid antigen expression can occur. Differential diagnoses for the monoblastic form include AML with minimal differentiation or without maturation, acute megakaryoblastic leukemia, precursor lymphoid neoplasms, and for the monocytic form are acute and chronic myelomonocytic leukemia.

Acute erythroid leukemia, pure erythroid type (AML-M6)
Consists of ≥80% nucleated erythrocyte precursors, with ≥30% proerythroblasts (rubriblasts) and <20% nonerythroid myeloblasts (of total marrow cells; those with ≥20% myeloblasts are put in other AML categories). Have immunophenotyping features of AML without maturation or with minimal differentiation (may not express erythroid-associated antigens), so diagnosis appears to be based largely on morphologic features. Do not usually express lymphoid antigens. Differential diagnoses include AML without maturation or minimal differentiation, precursor lymphoid neoplasms, and acute megakaryoblastic leukemia.

Acute megakaryoblastic leukemia (AML-M7)
Consists of ≥50% cells of megakaryocyte lineage. Blasts may have pseudopodia or cytoplasmic blebs and may see dysplasia in megakaryocytes (and other lineages). The marrow may be fibrotic. Blasts may express ANBE, but are negative for other myeloid stains (SBB, MPx, CAE). Cells can express early myeloid antigens and megakaryocyte markers (particularly cytoplasmic, because platelet-derived microparticles may bind to blasts of other lineages, giving false positive reactions). Blasts often lack stem cell markers and do not usually express lymphoid antigens. Differential diagnoses include AML without maturation or with minimal differentiation, acute monoblastic leukemia, acute erythroid leukemia, acute panmyelosis with myelofibrosis, precursor lymphoid neoplasms, and metastatic tumors.

Acute panmyelosis with myelofibrosis
Marrow is fibrotic (reticulin), but with hypercellular areas containing increased precursors of all lineages. Most difficult variant to identify blast percentage (most are 20–25%). Differential diagnoses include acute megakaryoblastic leukemia, chronic myeloproliferative disorders, and desmoplasia with metastatic tumors.

for leukemia in animals.[17,45,54,57,66] As indicated previously, the Animal Leukemia Study Group and many authors use a blast cutoff of ≥30% for diagnosis of AML.[1,25,26,34,57,66] However, many authors have adopted a 20% blast cutoff. This study group also used a complicated scheme to distinguish myelodysplastic syndrome from AML in cases with erythroid predominance, which mostly occurs in cats.[34]

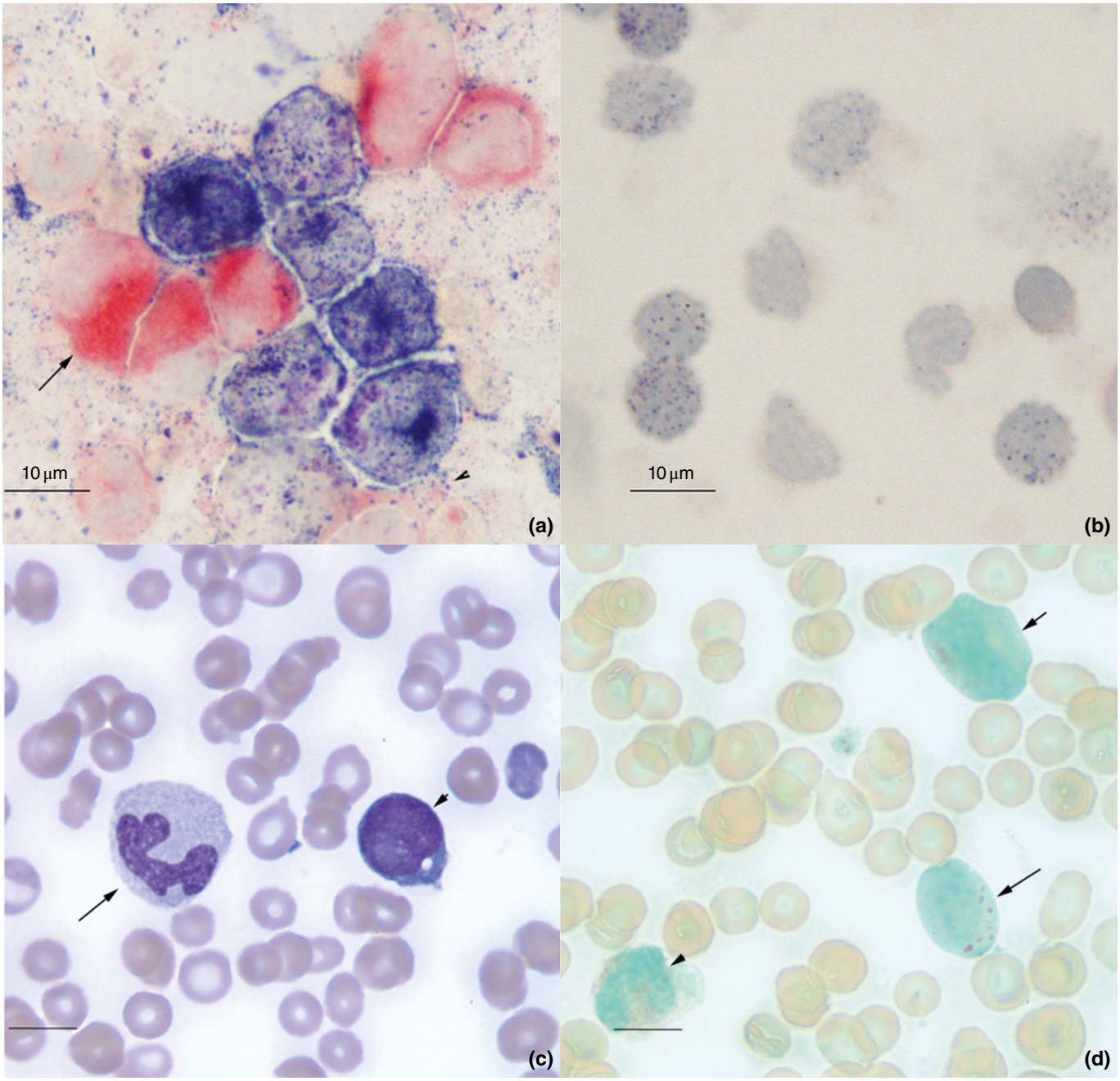


FIGURE 68.3 Cytochemical staining reactions in dogs with AML. (a) Blasts are positive for ALP (blue) and CAE (red) in the bone marrow of a dog with AML-NOS, acute myelomonocytic leukemia (same case as in Figure 68.2a). More-differentiated cells (myelocytes) are CAE-positive (arrow) and some blasts are double positive for ALP and CAE (arrowhead); (b) Blasts are only positive for ALP (blue) in the bone marrow of a dog with AML-NOS, acute monoblastic leukemia (same case as in Figure 68.2c,d); (c) A dysplastic neutrophil (arrow) and blast (arrowhead) in the blood from a dog with AML-NOS, acute myelomonocytic leukemia (modified Wright's stain) (bar = 10 µm); (d) Some blasts in the blood from the same dog have granular multifocal staining for ANBE (brown, long arrow; a pattern more typical of T cells), with more diffuse positivity in a promonocyte (short arrow) and monocyte (arrowhead) (bar = 10 µm). Other blasts had more diffuse ANBE reactivity (not shown). This case shows the overlap in staining patterns, which can complicate leukemia classification.

SPECIES SUMMARY

Acute Myeloid Leukemia in Dogs

Acute myeloid leukemia is most frequently recognized in dogs as compared to other species. Dogs usually present with nonspecific clinical signs of anorexia or inappetence, lethargy, and gastrointestinal signs such as diarrhea and vomiting.[1,18,26,54,65,66] Rare cases have presented for neurologic symptoms.[14,29] Some affected dogs can present with signs more commonly associated with lymphoma, including solitary or multiple peripheral lymphadenopathy, abdominal organomegaly (spleen, liver), and mediastinal masses.[1,18,21,26,54,66] Several dogs

have been referred to the author's institution with primary diagnoses of multicentric or mediastinal lymphoma, but have AML on phenotypic testing, illustrating the overlap in clinical presentation between these neoplasms. Dogs are mostly middle-aged, but AML can occur in dogs at or below 1 year of age.[1,18,26,45,54,66] There are no breed or gender predilections. Hematologic findings are usually the first clue as to an acute leukemia, because most affected dogs have bicytopenia or pancytopenia and circulating blasts.[1,18,26,45,54,66] Anemias are usually nonregenerative, but regenerative anemias can be seen.[18,54] Blast counts can be quite high and blasts can dominate in blood.[1,18,54,66] Some dogs lack circulating blasts[18,26,54] and can have normal hemograms.[1,54] Dysplasia can be evident in blood, marrow, and tissue aspirates, particularly in the myelomonocytic and megakaryoblastic variants.[16,54] Aspirates of enlarged organs or body cavity effusions may mimic lymphoma or reveal an infiltrative population among a residual lymphoid population (Figure 68.4). In published cases, the diagnosis of AML has been made on the percentage (using varying percentages) and morphology of blasts in the blood or bone marrow, on cytochemical staining reactions,[26,34] or on the basis of flow-cytometric-based immunophenotyping (varied expression of myeloid antigens and/or lack of lymphoid antigen expression).[1,45,57,65,66]

At the author's institution, blast morphology and flow cytometric immunophenotyping are first-line tests, and, as needed, are followed by cytochemical staining. The collective results of these tests are considered to distinguish AML from leukemic phase of lymphoma and ALL.[18,54] Using FAB criteria, the most common types of AML-NOS in dogs are acute monoblastic/monocytic or myelomonocytic leukemia,[1,18,54,66] with megakaryoblastic,[16,18,45,70] basophilic, and erythroid variants being quite rare.[45,61] Most dogs with AML are treated with chemotherapeutic protocols based on cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) or anthracycline–cytosine arabinoside combinations,[18,45,66] the first-line drug choice in human patients with AML.[19] At the author's institution, discrimination between AML and ALL is desired because anthracycline–cytosine arabinoside-based and CHOP-based protocols are the treatment of choice for AML and lymphoid neoplasms, respectively. Unfortunately, the prognosis remains dismal, with most dogs being euthanized shortly after diagnosis or within 1–4 months after chemotherapy,[18,45,66] with rare dogs surviving longer.[70]

Acute Myeloid Leukemia in Cats

Acute myeloid leukemia is less common in cats versus dogs[34] and is usually secondary to infection with FeLV,[31,62] although cats with AML and negative FeLV tests have been reported.[23,67] There have been three case series of AML in cats, all of which used a ≥30% blast cutoff.[9,25,33] One of these studies focused on FAB subtypes, clinicopathologic data, and neoplastic cell morphology.[33] Affected cats were generally young, with a median age of 4 years (range of 6 months to 16 years), and most were domestic short or longhair cats, with both males and females affected.[9,25] Similar to dogs, presenting signs consisted of inappetence, lethargy, weakness, and weight loss.[9] Unlike dogs, lymphadenopathy was not a common finding but hepatomegaly/splenomegaly was identified in one-third of the cats in one study.[9] As for dogs, cats were often cytopenic (one or more lineages) and 50–68% had circulating myeloblasts.[9,25] Anemia was typically severe[9,25,33] and frequently macrocytic,[9] despite the lack of a regenerative response. The macrocytosis is likely due to concurrent FeLV-induced myelodysplasia. Unlike dogs, a normoblastosis was seen in many cats. Dysplasia in single or multiple lineages was evident in blood.[9,25,33] Rare cats can have normal hemograms.[25] In one study, most cats with bone marrow evaluation had megakaryocytic and erythroid hypoplasia, except for cats with erythroleukemia (defined as >50% erythroid precursors, with ≥30% myeloblasts of nonerythroid cells).[9] All cats had evidence of erythroid dysplasia and the majority of cats had megakaryocytic dysplasia and stainable iron in marrow.[9] The presence of sideroblasts, siderocytes, and myelofibrosis is a supportive finding for hematopoietic neoplasia in cats, either MDS or AML.[8,9] On postmortem examination, neoplastic infiltrates can be found in spleen (red pulp), liver (primarily in the portal region), lymph nodes (medullary cords, medullary and subcapsular sinuses, and paracortical regions), and kidneys (corticomedullary). Intravascular tumor cells and tissue infiltrates may be seen in other tissues, including the central nervous system.[9] In the one study which categorized AML in 107 cats, the most frequent types were M1, M2, and M6,[33] with rare cases of acute basophilic and megakaryoblastic leukemia.[10,15] Testing for FeLV by ELISA was positive in most cats.[9,25] Most cats died or were euthanized within 1 week of diagnosis. Three cats treated supportively lived for 2 weeks to just over 2 months.[9,25]

Acute Myeloid Leukemia in Horses

Horses rarely suffer from AML, with only six and three cases being diagnosed over a period of 16 and 22 years in two separate studies,[4,17] versus 25–35 dogs over 4–8 years in three canine studies.[18,45,57] In the two equine case series, horses ranged from 2–26 years of age and clinical signs were nonspecific, including inappetence or anorexia, edema, lethargy, fever, and poor performance. Some horses had mild to moderate lymphadenopathy, affecting single or multiple peripheral lymph nodes. Unlike dogs and cats, where blasts can dominate the hematologic picture, horses with AML typically have bicytopenia or pancytopenia with low numbers of circulating blasts. The latter cells may not be included in a differential cell count in all horses, but can usually be identified on smear examination and are a clue as to the underlying cause of the cytopenias. Several horses had concurrent evidence of inflammation (left shift and toxic change in neutrophils, hyperglobulinemia, high serum amyloid A concentrations), likely from concurrent infections secondary to immunosuppression. Bone marrow aspirates are usually higher cellular with decreased megakaryocytes and erythroid precursors. Precise blast

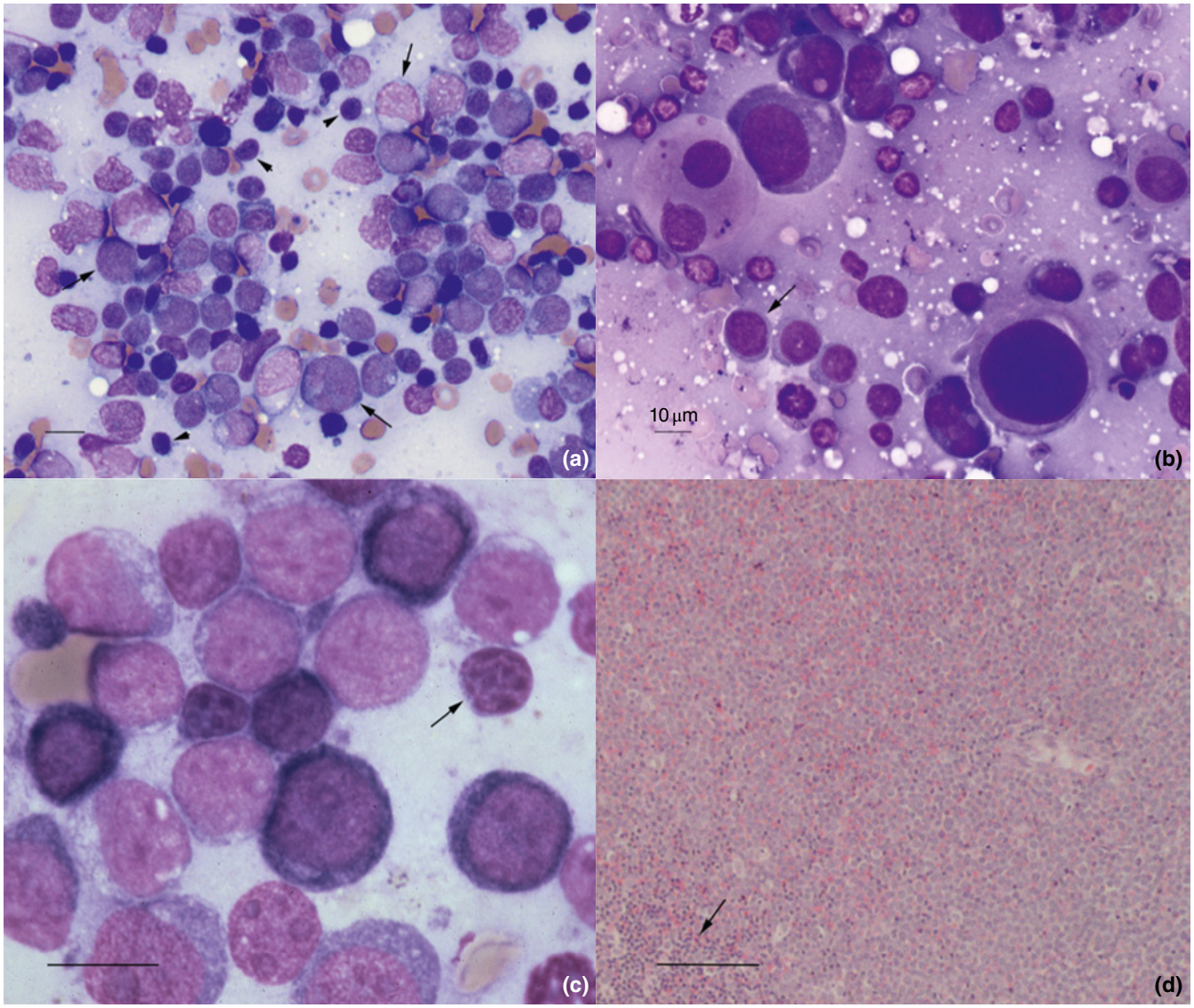


FIGURE 68.4 Lymph node infiltrates in dogs with AML. (a) Variably sized blasts (arrows), some of which have pleomorphic nuclei, are observed among a residual population of small lymphocytes (arrowheads) and plasma cells in a dog with AML-NOS, acute monoblastic leukemia (bar = 10 µm, modified Wright's stain). The main differential diagnosis would be an early lymphoma, an infiltrative lymphoma from another site, an infiltrative acute leukemia (AML or ALL), or a lymphoma emerging from remission. The nuclei of the blasts are monocytoid, raising suspicion for AML. The cytologic diagnosis in this case would have been nonspecific hematopoietic neoplasia, with a recommendation to review a blood smear, aspirate the bone marrow, or perform phenotyping for lineage determination; (b) Dysplastic megakaryocytes and presumptive megakaryoblasts (arrow) are present in this lymph node aspirate from a dog with AML-NOS, acute megakaryoblastic leukemia (modified Wright's stain); (c) Two populations of blasts, one with dark blue cytoplasm and the other with lighter blue cytoplasm, with low numbers of small lymphocytes (arrow) in a lymph node aspirate from a dog with AML-NOS, acute myelomonocytic leukemia (bar = 10 µm, modified Wright's stain). These two morphologic blast variants can be a useful finding to raise suspicion of AML in tissue aspirates; (d) The lymph node has been effaced by uniform blasts, with a residual population of small lymphocytes (arrow), in this histologic section of a lymph node from a dog with AML-NOS, acute monocytic leukemia (bar = 10 µm, H&E stain). On histologic examination, AML is frequently misdiagnosed as lymphoma (non-B or non-T, if phenotyping is done) on histologic examination of tissues, particularly if the tumor has effaced the tissue, is forming solid masses, and lacks vascular orientation or features of myeloid differentiation.

enumeration in horses can be more challenging, as they may contain fewer blasts than in dogs and cats and there may be a continuum between blasts and differentiated myeloid cells.[4,17] Some horses had concurrent dysplasia in one or more lineages.[4] On postmortem examination, tumor infiltrates can occur in multifocal perivascular to interstitial patterns in multiple tissues, particularly the lymph nodes, kidney, spleen, and liver, but also skin,

lungs, gastrointestinal tract, brain, heart, mediastinum, and mesentery.[4] Nodular lesions can be seen on visceral surfaces of thoracic and abdominal cavities and mesentery,[17] and lymph nodes can be effaced by the tumor.[4] In the study in which the leukemia was subclassified, myelomonocytic was the most common subtype, with one case each of eosinophilic and basophilic leukemia.[17] As with dogs and cats, the prognosis is poor, with most horses dying or being euthanized within 1 month of diagnosis.[4,17]

Acute Myeloid Leukemia in Other Species

Individual case reports of AML have been documented in ruminants,[40,71] reptiles,[32,59] and amphibians.[32] This author has identified cases of presumptive AML in bearded dragons and hedgehogs. In exotic species, it can be difficult to confirm a diagnosis of AML, because of aberrant cytochemical staining patterns and lack of immunologic markers to confirm AML or exclude lymphoid neoplasia.

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CHAPTER 69

B-Cell Tumors

LUCA ARESU, STEFANO COMAZZI, LAURA MARCONATO, and FRANCESCO BERTONI

Introduction
Precursor B-Cell Neoplasms
  Acute Lymphoblastic Leukemia and Lymphoblastic Lymphoma
    Clinical Features
    Pathologic Features
Mature B-Cell Neoplasms
  B-Cell Chronic Lymphocytic leukemia/small lymphocytic Lymphoma
    Clinical Features
    Pathologic Features
  B-Cell Prolymphocytic Leukemias
  Lymphoplasmacytic Lymphoma
    Pathologic Features
  Marginal Zone Lymphoma
    Clinical Features
    Pathologic Features
    Comparative Features
  Mantle Cell Lymphoma
    Clinical Features
    Pathologic Features
    Comparative Features
  Follicular Lymphoma
    Clinical Features
    Pathologic Features
    Comparative Features
  Diffuse Large B-Cell Lymphoma
    Clinical Features
    Pathologic Features
    Comparative Features
  Burkitt Lymphoma
    Clinical Features
    Pathologic Features
    Comparative Features

Acronyms and Abbreviations

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; B-CLL, B-cell chronic lymphocytic leukemia; BL, Burkitt lymphoma; BLL, Burkitt-like lymphoma; BCR, breakpoint cluster region; BTK, Bruton tyrosine kinase; CFA, canine familiaris; CCND1, cyclin D1; CD, cluster of differentiation; CHOP, cyclophosphamide, doxorubicin hydrochloride, vincristine sulfate, and prednisone; CNV, copy number variation; DLBCL, diffuse large B-cell lymphoma; EMZL, extranodal marginal zone lymphoma; FL, follicular lymphoma; FAB, French–American–British; HPF, high powered field; KLF2, Kruppel-like factor; IGHV, immunoglobulin heavy chain variable; LDH, lactate dehydrogenase; LBL, lymphoblastic lymphoma; LPL, lymphoplasmacytic lymphoma; MHC, major histocompatibility complex; MCL, mantle cell lymphoma; MUM, mutated melanoma-associated antigen; MCL, myeloid cell leukemia; MZL, marginal zone lymphoma; MALT, mucosa-associated lymphoid tissue; NMZL, nodal marginal zone lymphoma; PLL, prolymphocytic leukemia. PARR, PCR for antigen receptor rearrangement; PAX, paired box; SLL, small lymphocytic lymphoma; SMZL, splenic marginal zone lymphoma; IRF, interferon regulatory factor; ALK, anaplastic lymphoma kinase; HHV, human herpesvirus; TP53, tumor protein 53 (TP53), CDKN2A, cyclin dependent kinase inhibitor 2A, ATM, ataxia-telangiectasia mutated,

INTRODUCTION

Leukemias and lymphomas of B-cell phenotype are relatively common in dogs, infrequent in cats, affect all breeds, ages, and sexes, and exhibit extraordinary biologic, morphologic, and clinical heterogeneity. In the last decade, investigations of the molecular and genetic drivers of lymphomagenesis and advances in monoclonal antibody technology have had significant clinical and biologic implications and help to explain the

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complexity of these diseases. As a result, many subtypes of leukemia and lymphoma have been described, each apparently representing the neoplastic counterpart of a specific compartment of the hematopoietic–lymphoid system and having a distinct biologic behavior and treatment response (Table 69.1). In general, the diagnosis of leukemia or lymphoma requires a close integration between the oncologist and pathologist. All patients suspected of having a hematologic or lymphoid malignancy should undergo complete staging, which includes sampling of all possibly involved tissues and organs. The accurate diagnosis and complete classification of B-cell neoplasms requires a combination of morphologic examination and phenotypic or molecular studies. While the list of genetic aberrations in canine B-cell tumors has been growing, these molecular techniques are not yet incorporated into daily diagnostic practice. This chapter describes the clinical and pathological features of veterinary B-cell tumors and highlights the most significant differences and analogies with their human counterparts (Table 69.2).

TABLE 69.1 Revised WHO classification of the mature B-cell, T-cell, and NK-cell neoplasms.

B-cell neoplasms
- Precursor B-lymphoblastic leukemia/lymphoma
- Mature (peripheral) B-cell neoplasms
- B-cell chronic lymphocytic leukemia/prolymphocytic leukemia/small lymphocytic lymphoma
- Lymphoplasmacytic lymphoma
- Mantle cell lymphoma
- Follicular lymphoma
- Marginal zone B-cell lymphoma:
- Extranodal mucosa-associated lymphoid tissue type (± monocytoid B-cells)
- Splenic marginal zone lymphoma (± villous lymphocytes)
- Nodal marginal zone lymphoma
- Plasmacytoma/plasma cell myeloma
- Diffuse large B-cell lymphoma
- Burkitt lymphoma
- Burkitt and Burkitt-like lymphomas

T-cell and putative natural killer-cell neoplasms
- Precursor T-lymphoblastic lymphoma/leukemia
- Mature (peripheral) T-cell neoplasm
- T-cell chronic lymphocytic leukemia/prolymphocytic leukemia
- Large granular lymphocyte leukemia (LGL)
- Mycosis fungoides/Sezary syndrome
- Peripheral T-cell lymphomas, unspecified
- Hepatosplenic γδ T-cell lymphoma
- Subcutaneous panniculitis T-cell lymphoma
- Angiocentric lymphoma
- Intestinal T-cell lymphoma (± enteropathy-associated)
- Anaplastic large cell lymphoma, CD30+

Hodgkin lymphoma

TABLE 69.2 Subtypes of human large B-cell lymphoma. Bold denotes distinct entities here.

Diffuse large B-cell lymphoma, not otherwise specified (NOS)
- Morphological variants (centroblastic, immunoblastic, anaplastic, and other rare variants)
- Molecular subtypes (germinal center B-cell subtype; activated B-cell subtype)

Other large B-cell lymphomas
- T-cell/histiocyte-rich large B-cell lymphoma
- Primary diffuse large B-cell lymphoma of the CNS
- Primary cutaneous diffuse large B-cell lymphoma, leg type
- EBV-positive diffuse large B-cell lymphoma, NOS
- Diffuse large B-cell lymphoma associated with chronic inflammation
- Lymphomatoid granulomatosis
- Large B-cell lymphoma with interferon regulatory factor (IRF4) rearrangement
- Primary mediastinal (thymic) large B-cell lymphoma
- Intravascular large B-cell lymphoma
- Anaplastic lymphoma kinase (ALK)-positive large B-cell lymphoma
- Plasmablastic lymphoma
- Human herpesvirus (HHV)8-positive diffuse large B-cell lymphoma
- Primary effusion lymphoma

High-grade B-cell lymphoma
- High-grade B-cell lymphoma with MYC and BCL2 and/or BCL6 rearrangements
- High-grade B-cell lymphoma, NOS

B-cell lymphoma, unclassifiable
- B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and classic Hodgkin lymphoma

PRECURSOR B-CELL NEOPLASMS

Acute Lymphoblastic Leukemia and Lymphoblastic Lymphoma

Clinical Features

Acute leukemias, either myeloid or lymphoid in origin, are rare in domestic animals, although reported in dogs, cats, cattle, and horses.[100] Acute leukemias in dogs occur with a bimodal distribution showing frequency peaks at 3–4 and 8–10 years, respectively.[66] Clinical features of acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) overlap, and definitive lineage identification requires ancillary techniques. Clinical signs in patients with ALL are usually acute in onset and nonspecific in nature. Anorexia, lethargy, and weakness are the most reported, but vomiting, fever, weight loss, diarrhea, polyuria/polydipsia, and epistaxis may occur. Splenomegaly is a frequent finding, while severe lymphadenomegaly is rarer.[114,100] The prevalent hematological presentation in ALL (i.e., severe infiltration of bone marrow (BM) and/or bone marrow and modest lymph node enlargement) may help to differentiate ALL from its nodal counterpart (lymphoblastic lymphoma (LBL)).

In ALL, according to several studies, anemia occurs in 60–90% of canine cases and thrombocytopenia is even more frequent (about 90% of cases). Leukocytosis is evident in more than 70% of cases, but circulating blasts are found in more than 90% of dogs.[8,66,95] Leukopenia and neutropenia may also rarely occur. In two different studies, leukocytosis was described as variable with median values of 60 and 73 × 10^3/μL, respectively.[8,66] Bone marrow cellularity is normal to hypercellular with presence of more than 20% of blasts among all nucleated cells in bone marrow or peripheral blood.[100] Myelophthisis may be a common finding and mitotic figures may be found in peripheral blood and bone marrow samples. Finally, tumor cell morphology cannot be used to definitively differentiate ALL and AML, and this distinction requires additional diagnostics. Blast cell appearance may be variable among cases. In human medicine, the French–American–British (FAB) classification attempted to classify blasts according to morphology in three different classes (namely, ALL-1, ALL-2, and ALL-3). With the advent of immunophenotyping using specific monoclonal antibodies, this classification has been replaced on that based on immunotype. In veterinary medicine, no extensive studies using FAB classification for ALL are reported and this has been supplanted by immunophenotypic, cytogenetic, and molecular subclassification. Neoplastic blasts may show variable size, with nuclei ranging from less than two RBC to more than two RBC in diameter, scant to moderately abundant basophilic cytoplasm, often with perinuclear clear halo, and round to indented nucleus with decondensed chromatin (Figure 69.1). Nucleoli may be inconspicuous to visible and cytoplasmic clear vacuoles may be found.[83]

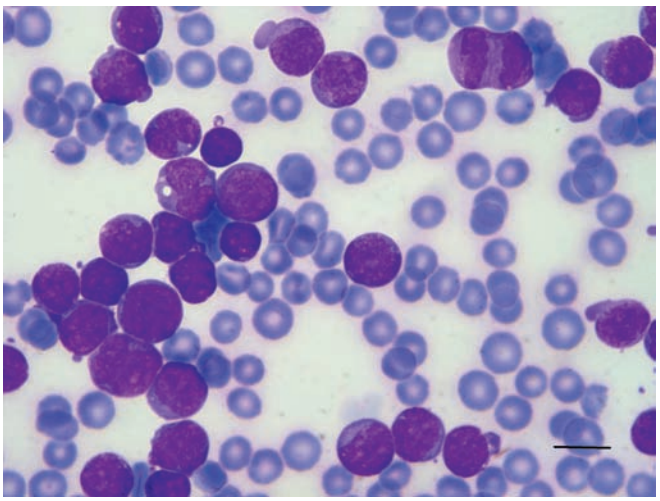


FIGURE 69.1 Peripheral blood from a dog with acute lymphoblastic leukemia (ALL). Neoplastic blasts are medium-sized (nuclei about two erythrocytes in diameter) with scant, basophilic cytoplasm, occasional vacuolization and cytoplasmic blebs, and oval to indented nuclei with smooth chromatin and visible nucleoli. Peripheral anemia and thrombocytopenia are frequent features. May–Grünwald–Giemsa.

ALL is considered a rapidly fatal disease in animals with overall survival times ranging from few days to (rarely) weeks.[26,43,66] Although extensive studies with standardized diagnostic and therapeutic approaches are lacking, some retrospective case collections reported that a normal neutrophil count, lack of anemia, and severe leukocytosis are positive prognostic factors.[66] Treatment is largely unsatisfactory, with most cases showing a poor response to chemotherapy or experiencing rapid recurrence. Although most veterinary oncologists recommend a cyclophosphamide, doxorubicin hydrochloride, vincristine sulfate, and prednisone (CHOP)-based chemotherapy with or without the addition of cytosine arabinoside, efficacious protocols have not been developed so far.[29,66]

Pathologic Features

The tissue distribution, phenotype, cell lineage, and maturation stages of neoplastic cells in blood and/or bone marrow are crucial to differentiate ALL from AML and stage V lymphoma with severe blood involvement, and to further define cell lineage in ALL. As noted previously, the definitive phenotype of lymphoid blasts is not possible based on cytology and CBC alone, and immunophenotyping and/or cytochemistry is necessary. Flow cytometry is a powerful immunophenotyping technique, but its application for the diagnosis of veterinary ALL is often unrewarding. This is partly due to a paucity of antibodies recognizing precursor cells in dogs and cats and the realization that neoplastic cells may lack expression of lineage-specific antigens. Neoplastic cells in ALL are uncommonly positive to common leukocyte markers cluster of differentiation (CD45) and CD44 and weakly positive to CD18 integrin.[22,39] As a marker of precursor cells, CD34 positivity has been reported in 37% and 76% of canine ALL.[2,8] CD34 is a membrane glycoprotein expressed by precursor cells of both lymphoid and myeloid lineage, and cross-reactive antibodies to CD34 are available for the dog. Expression of CD34 on leukemic cells in human ALL ranges from 44% to 70% of cases and correlates with a poorer prognosis. Also, in dogs, CD34 positivity is strongly suggestive of acute leukemia, although aberrant CD34 positivity has been described in some lymphoma subtypes.[40,108] A B-cell origin of ALL is assigned by positivity to antibodies against CD79a, CD20, and/or CD21. However, most cases of ALL fail to express such mature antigens and the final diagnosis is often acute undifferentiated leukemia. Neoplastic cells demonstrate variable expression of major histocompatibility complex (MHC) II.[93]

By definition, bone marrow or peripheral blood shows more than 20% blasts, but blasts in many ALL may strongly exceed this percentage, accounting for more than 90% of bone marrow nucleated cells. Cellularity is increased and few fat cells may persist around the large vessels of the central cavity. Focal foci of residual hematopoiesis may also be found.

Splenomegaly is a common feature in ALL and microscopic evaluation often reveals uniform infiltration by

neoplastic cells with focal areas of hemorrhagic infarction and atrophy of lymphoid follicles. The liver and other organs may be also involved. Interestingly, cell morphology in cytology may vary in different organs or tissues. Based on the experience of the authors, a slight variation in immunoreactivity may be found occasionally among neoplastic cells found in different tissues. The reason of this finding is unknown, but immunophenotypical aspects should be interpreted with care in samples obtained from different organs. Although severe lymphadenomegaly is rare in ALL, moderate lymph node enlargement may occur. Infiltrated lymph nodes contain a population of neoplastic blast cells resembling those in peripheral blood with residual small lymphocytes and plasma cells. It is important to note that it is not possible to differentiate ALL from LBL, particularly if few residual lymphocytes are present there. Like ALL, lymphoblastic lymphoma is a neoplasm of precursor cells. The predominant solid tissue presentation and the lack of severe blood and bone marrow infiltration are parameters used to differentiate LBL from ALL. However, some authors and the most recent revised WHO scheme[3] suggest that ALL and lymphoblastic lymphoma might represent two different presentations of the same disease. Cytologically, LBL is characterized by a population of lymphoblasts, small cells with mild variably basophilic cytoplasm, and a unipolar round or often indented nucleus with a perinuclear halo. Chromatin is finely dispersed, and nucleoli are generally inapparent. Many mitotic figures are found. Like ALL, canine LBL may be of B or T lineage, but mature antigens are generally poorly expressed.

MATURE B-CELL NEOPLASMS

B-Cell Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma

Clinical Features

Chronic lymphocytic leukemia is the most frequent leukemia in dogs and cats and may be of either T- (more frequently) or B-cell origin. In dogs, B-cell chronic lymphocytic leukemia (B-CLL) is characterized by bone marrow involvement and peripheral lymphocytosis with no or mild peripheral cytopenia.[105] Neoplastic cells show prolonged life span, with decreased apoptosis and proliferation rate, thus accumulating in the bone marrow.[99] The slow accumulation of clonal cells in bone marrow might be the cause of mild clinical symptoms and the frequent indolent clinical behavior. A recent retrospective showed a predisposition of B-CLL in 10 small canine breeds and in two large canine breeds (English Bulldog and Pit Bull).[11] Affected dogs showed a median age at onset of 11 years explaining the high frequencies of small breeds affected. Splenomegaly is common, whereas lymphadenomegaly is usually reported as mild.[99] However, the frequency of lymphadenomegaly is probably underestimated and was recently identified in 46% of cases with B-CLL.[11]

A consensus criteria classification for canine B-CLL is lacking, but in the study of Bromberek et al.,[11] B-CLLs were diagnosed if more than 5000 lymphocytes/μL and more than 60% of lymphocytes were small B cells. Median lymphocyte count was about 25,000/μL, but extreme lymphocytosis (>50,000/μL) was also frequent and leukocyte counts often exceeded those of ALL. Anemia was reported in 26% of cases and it was generally mild. Thrombocytopenia and neutropenia were rare.[11] Some larger cells may be found suggesting a possible evolution of the disease in a more accelerated phase, but a real blast crisis is a very rare event. Based on the authors' experience, canine B-CLL is a slowly progressive disease that can culminate in multiorgan failure. However, Richter syndrome may also occur. Richter syndrome is a quite rare, peculiar evolution reported in about 10% of human B-CLL, characterized by the transformation of B-CLL into a solid lymphoma, mainly diffuse large B-cell lymphoma (DLBCL).[48] A similar evolution and incidence were reported in dog.[23] Clinically, the disease is accelerated with a fatal course. The demonstration of the same clonal rearrangement is mandatory to differentiate Richter syndrome from a second malignancy.[23]

In human medicine, two B-CLL classification schemes are used: The Rai system and the Binet system. Both schemes are based on the number of affected lymphoid tissue groups and the presence of anemia or thrombocytopenia and classify cases as low, intermediate, or high risk. To date, a similar staging system is not available for domestic animals, but it is reasonable to consider the presence of anemia, thrombocytopenia, or organ involvement as features for an advanced stage. Treatment of B-CLL is generally conservative unless clinical signs are present. Treatment appears to have a limited effect on prolonging survival and B-CLL has a median survival of more than 1 year from the diagnosis.[21] In cats, B-CLL appears to be uncommon and, as such, extensive studies on feline B-CLL are lacking. In a recent study on cats with lymphocytosis (total lymphocyte higher than 6000 lymphocytes/μL), B cell expansion was found in 16–19% of cases, but in 94% of cases PCR for antigen receptor rearrangement (PARR) analysis showed polyclonal results.[82] This result is in accordance with those obtained in another study on feline CLL in which just one out of 18 cases was B-phenotype,[12] supporting the extreme rarity of B-CLL in cats.

Pathologic Features

Lymphocytosis composed of small B cells is the most relevant clinicopathologic feature of B-CLL (Figure 69.2). The three published case series in which immunophenotype was available reported median concentrations of lymphocytes of 137,489 ± 103,833 (n = 7; range not reported),[95] 35,980 lymphocytes/μL (n = 17; range, 8000–201,000)[21] and 24,600 lymphocytes/μL (n = 191; range, 5000–812,544).[11] A nuclear diameter less than two times the volume of RBC or smaller than a neutrophil is suggestive of small cells. Alternatively, forward scatter

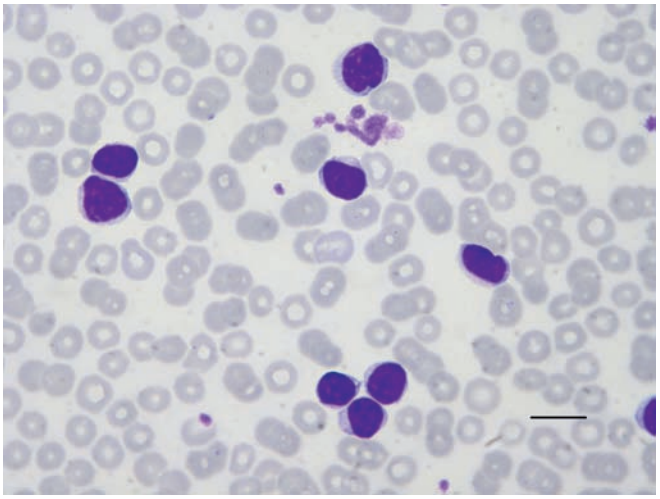


FIGURE 69.2 Peripheral blood from a dog with B-chronic lymphocytic leukemia (B-CLL). Neoplastic lymphocytes are small (nuclei less than two erythrocytes in diameter) with scant, clear, or slightly basophilic cytoplasm, round to oval nuclei with condensed chromatin, and inconspicuous nucleoli. May–Grünwald–Giemsa.

(FSC)-determined flow cytometrically may be used to estimate cell size. A lymphocyte/neutrophil FSC ratio <0.55 confirms the small size of cells. If lymphocyte counts do not exceed 10,000/μL, bone marrow fine-needle aspiration may be used to identify infiltration (more than 30% of total nucleated cells) of small lymphocytes. PARR and/or flow cytometry may be also useful to exclude reactive lymphocytosis. Neoplastic cells are positive to B-cell markers CD21, CD22, CD20, and CD79a and to cytoplasmic immunoglobulin. MHC II and CD25 are generally positive, thus suggesting a mature phenotype, but some variable expression is reported in the quantitative expression from case to case and in different breeds.

B-Cell Prolymphocytic Leukemias

Prolymphocytic leukemia (PLL) is a rare entity. To date, neither extensive studies on B-cell PLL nor flow cytometry data are available in dogs or cats. Thus, there is no consensus on the diagnosis of PLL. As proposed, PLL is characterized by a proliferation of small- to medium-sized cells with a moderately abundant light cytoplasm extended in a so-called hand-mirror shape. Neoplastic cell nuclei are round or slightly indented, multiple chromocenters are often visible, while nucleoli are rare. Typical presentation resembles CLL with leukocytosis often exceeding 50,000 cell/μL and severe infiltration of bone marrow. Prolymphocytic leukemia is rarely described in cattle, while in cats and dogs it is generally interpreted as an accelerated phase of CLL. Severe infiltration of spleen and less marked infiltration of liver and lymph node can be found. Both T- and B-cell PLL are described. Differentiation of PLL from CLL is mainly based on cellular size and morphological appearance of more immature cells.[99]

Lymphoplasmacytic Lymphoma

Lymphoplasmacytic lymphoma (LPL) is a neoplasm of small and intermediate B-lymphocytes usually involving lymph nodes and spleen, which generally does not fulfill the criteria for any of the other small B-cell lymphoid neoplasms.[103] A peculiar morphological feature of LPL is represented by the presence of neoplastic cells with plasmacytic morphology. LPL occurs in all domestic animals, but is seen most frequently in older cats, involving the intestine and mediastinum in almost 50% of cases. Also, it is described preferentially occurring in the intestine of the horses. The disease is classified as indolent and rare, even if the morphology is very similar to CLL/small lymphocytic lymphoma (SLL) leading to potential misdiagnosis. Moreover, no relevant clinical features have been described both in dogs and cats.[56,71,76,99]

Pathologic Features

In LPL, affected lymph nodes retain the normal architectural features with dilated sinuses and residual germinal centers (Figure 69.3a). There is a monotonous proliferation of small lymphocytes with a plasmacytic morphology. Few cases show severe architectural alteration and have follicular growth pattern with more prominent residual germinal centers. The presence of prominent large B cells should raise the possibility of either disease progression or an alternate diagnosis. LPL cells express CD20 and CD79a.[103] No data are reported for (paired box) PAX-5 immunoassaying. Conversely, CD5 and CD3 are most typically negative. Interestingly, one study identified a weak expression of transferrin receptor 1 (TfR1) in LPL compared to aggressive lymphoma histotypes.[75] TfR1 is a type II transmembrane glycoprotein that binds and internalizes transferrin–iron complexes. Cytologically, LPL is characterized by a dominant population of small lymphocytes, often with moderately abundant cytoplasm, eccentric nuclei, and a visible Golgi zone, suggestive of plasma cells (Figure 69.3b,c). Mitotic figures are generally few. Cytology is of limited use to differentiate LPL from other small cell lymphomas, even when associated with flow cytometry. No pathognomonic flow cytometric patterns have been reported in veterinary medicine and neoplastic cells express common B-cell markers such as CD21, CD20, and CD79.

Marginal Zone Lymphoma

Marginal zone lymphoma (MZL) refers to a group of indolent B-cell lymphomas that originate from the marginal zone of the follicles. MZL occurs in all animals, but is most frequently recognized in dogs and occasionally in cats. MZLs account for approximately 15% of lymphomas in dogs.[47] Historically, canine MZL has been described as having an indolent clinical course, a low mitotic index, and slow progression. However, a recent work has described a more aggressive disease course for dogs developing the nodal form.[27]

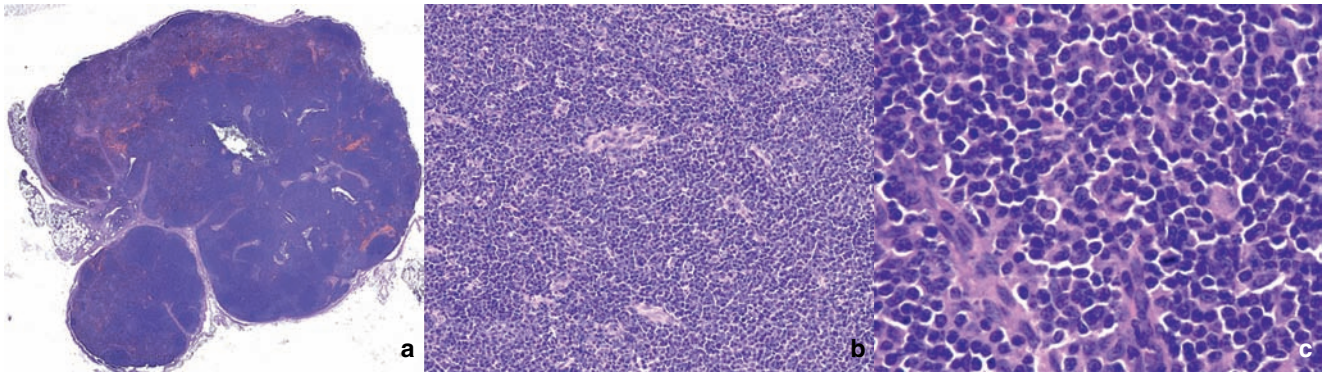


FIGURE 69.3 Lymph node from dog with lymphoplasmacytic lymphoma. (a) Histologically, the lesion is diffuse and involves the entire node. Node is enlarged in volume and shows multifocal hemorrhages and edema. The capsule is taut and diffusely reduced in thickness; (b) The growth pattern is diffuse and composed of tumor cells without evidence of residual germinal centers. Mitoses are infrequent and cell boundaries are irregularly distinct; (c) Diffuse population of small lymphocytes associated with larger plasmacytic forms can be observed. H&E.

In both humans and dogs, three anatomic types of MZL are described: (1) extranodal marginal zone lymphoma (EMZL) of mucosa-associated lymphoid tissue (MALT) lymphoma, (2) nodal marginal zone lymphoma (NMZL), and (3) splenic marginal zone lymphoma (SMZL).[99] MZLs are characterized by a characteristic growth pattern of medium-sized cells (1.5–2 times the red blood cell), with scant eosinophilic cytoplasm, a round nucleus, and a single prominent central nucleolus (medium macronucleolated cells).

Clinical Features

The clinical data related to canine NMZL are sparse and have been largely drawn from pathologic rather than clinical series. NMZL occurs predominantly in adult medium-sized breed dogs and causes localized or generalized lymphadenopathy. In more advanced cases, dogs tend to remain asymptomatic, but stage V disease and splenic involvement are common.[27] The liver is less frequently infiltrated, but cytologic interpretation is always needed. Unlike SMZL, bone marrow and blood are frequently involved in NMZL. In a recent case series, peripheral blood infiltration was detected via flow cytometry in 97% of dogs with advanced disease and the median percentage of neoplastic cells was 6.4%, while BM was infiltrated in 57% of dogs.[27] In another study, dogs with peripheral blood infiltration ≥30% demonstrated a shorter time to progression compared with dogs showing blood infiltration <30%. In the same study, it was also shown that dogs with bone marrow infiltration <1% lived significantly longer than those having infiltration of 1–20% or ≥20%.[58]

Regarding SMZL, it has been described mainly in dogs and is the most frequent lymphoma affecting the spleen.[67,90,91] SMZL represents clinically a distinct form and is likely unrelated to NMZL and MALT. SMZLs generally have a favorable prognosis and most of the cases are incidentally identified on routine abdominal ultrasound examination. However, nonspecific clinical signs can be observed, such as hemoabdomen, lethargy, anorexia, vomiting, and weight loss. Bone marrow and peripheral blood are typically not infiltrated, even in advanced clinical stages. Splenectomy is curative in most cases, although symptomatic disease may prompt the need for chemotherapy. A large case series of SMZLs in dogs reported an overall median survival time after splenectomy of 383 days; if only cases that survived >1 week after splenectomy were analyzed, the median survival time increased to 571 days.[67] Negative prognostic factors include postoperative sepsis and dehiscence. Interestingly, in this study, three dogs lived more than 1000 days confirming the good outcome in dogs affected by SMZL.

Pathologic Features

There are two patterns of infiltration in NMZL: nodular and diffuse. The nodular growth pattern is evident in the early phase and is characterized by well-formed nodules surrounding and colonizing reactive germinal centers. The node focally retains its normal architecture, in which the follicles are surrounded by cells with clear cytoplasm. In the diffuse pattern, which is evident in advanced disease, sheets of neoplastic cells efface the nodal architecture (Figure 69.4). Regardless of their distribution, neoplastic cells are small- to medium-sized with a variable number of scattered blasts. Neoplastic lymphocytes have a prominent single, central nucleolus and abundant lightly stained cytoplasm. The number of mitoses is low (three to five per high powered field [HPF]) except in advanced cases.[103] MZL cells strongly express CD79 and CD20 and lack CD3. No data are reported for PAX-5 immunostaining.[7] A large cell component, usually <30% of the infiltrate, is generally identified. NMZLs with a higher density of large cells (30%–50%) are described, but no evidence of more aggressive behavior is reported. The presence of sheets of large cells is generally an indication of transformation to a diffuse large B-cell lymphoma.[20]

Cytologic smears show a dominant population of medium-sized lymphoid cells with round nucleus and a

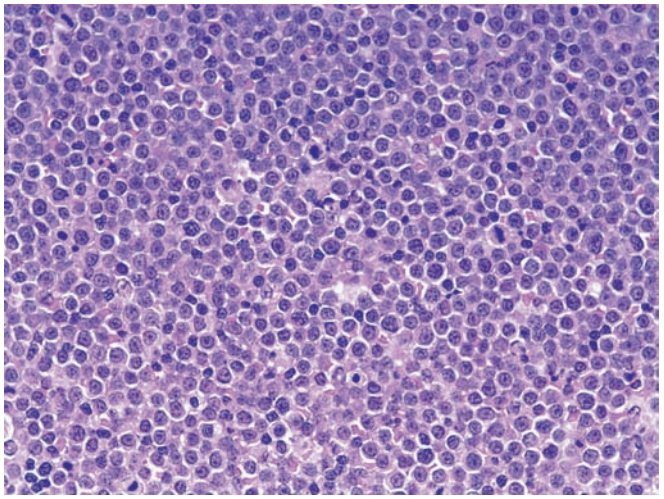


FIGURE 69.4 Lymph node from dog with marginal zone lymphoma (MZL). Histologically, the neoplastic lymphocytes are diffusely homogeneous and originate from the marginal zone area, with most cells having a prominent single central nucleolus. There are few tingible body macrophages and mitoses. H&E.

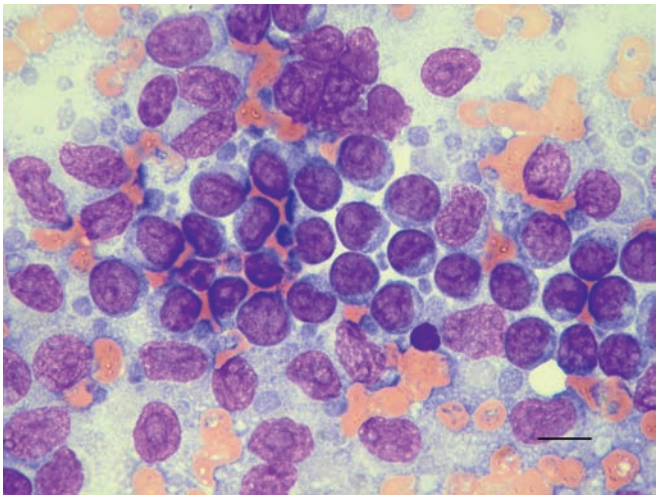


FIGURE 69.5 Cytologic smear from a dog with nodal marginal zone lymphoma (NMZL). A homogeneous population of medium-sized blasts (nuclei about two erythrocytes in diameter) with scarce blue cytoplasm, round/oval nuclei with decondensed chromatin, and single central prominent nucleolus is present. May–Grünwald–Giemsa.

prominent central macronucleolus (Figure 69.5). Centroblasts and immunoblasts are rare, but tend to increase during progression. Mitotic figures are rare to intermediate. Flow cytometric immunophenotype confirm the B phenotype (CD21, CD20, and CD79), and Ki67 value is intermediate or low. Differently from human medicine, immunophenotypic markers are currently not available to discriminate NMZL, SMZL, and MALT in dogs.

An important morphologic and biological consideration in the diagnosis of canine NMZL is its relationship with DLBCL.[41] This distinction can be particularly problematic in nodal lymphomas with a consistent histological or cytological infiltration of large B cells. In 2013, Frantz et al., evidenced by DNA microarray transcriptomic similarities, suggested that DLBCL and MZL might represent a continuum of the same disease. One year later, investigators compared transcriptome of nine NMZL with 32 DLBCL using a similar approach and the results showed a complete overlapping of the two tumors at the transcriptomic level.[35] Similarly, genomic copy number aberrations investigated by array comparative genomic hybridization reported no significant differences in the genomic profiles between NMZL and DLBCL, confirming the high similarity of these two histotypes also at the genetic level.[41] These findings align with the recent work in humans indicating that there is a continuum between NMZL and DLBCL.[22]

Grossly, splenic MZL is characterized by a marked nodular or multinodular expansion of white pulp with frequent merging and coalescence of the follicles. Microscopically, germinal centers are atrophic and unrecognizable (Figure 69.6a). There is a prominent expansion of the marginal zone due to a population of intermediate-sized lymphoid cells. These cells contain a moderate amount of cytoplasm and a round to oval to irregular nucleus with clumped chromatin and a single, small central nucleolus. Occasional interspersed larger cells (centroblast-like) are also identified.

The neoplastic cells in SMZL usually show immunoreactivity for CD20, CD45, CD79a, PAX-5, and Bcl-2 and are negative for CD3, CD10, Bcl-6, and cyclin D (Figure 69.6b). Also, SMZL may demonstrate scattered neoplastic cell positivity for CD5, mutated melanoma-associated antigen 1 (MUM-1), myeloid cell leukemia-1 (MCL-1), and cyclin D3.[92] Conversely, cytological and flow cytometric aspects do not differ from those of NMZL.[27]

Comparative Features

In humans, MALT lymphoma, SMZL, and NMZ represent three distinct types of mature B-cell neoplasms.[94] While MALT lymphomas account for 7–8% of all lymphomas, SMZL and NMZL are much less common with each one representing less than 2% of lymphomas. The MZLs could be considered a continuum of disease which makes a precise diagnosis often impossible in the case of disseminated disease.[10,15] All MZL types present trisomies of chromosomes 3 and 18 and deletions at 6q23 (TNFAIP3 gene), somatic mutations of genes coding for proteins involved in chromatin remodeling, and nuclear factor kappa B (NF-κB).[10,80] Mutations affecting the NOTCH pathway and the transcription factor KLF2 (Kruppel-like factor) are more common in SMZL and NMZL than MALT lymphomas.[14,81,89] While SMZL is specifically characterized by deletions of chromosome 7q, NMZL shows inactivation of protein tyrosine phosphatase receptor type D (PTPRD) and a much higher prevalence of mutations affecting lysine-specific methyltransferase 2D (KMT2D).[14,49,69,81,88] SMZL and NMZL do not present specific recurrent chromosomal translocations, instead observed in MALT lymphomas, in which, at least three of them activate the NF-κB pathway.[10,112]

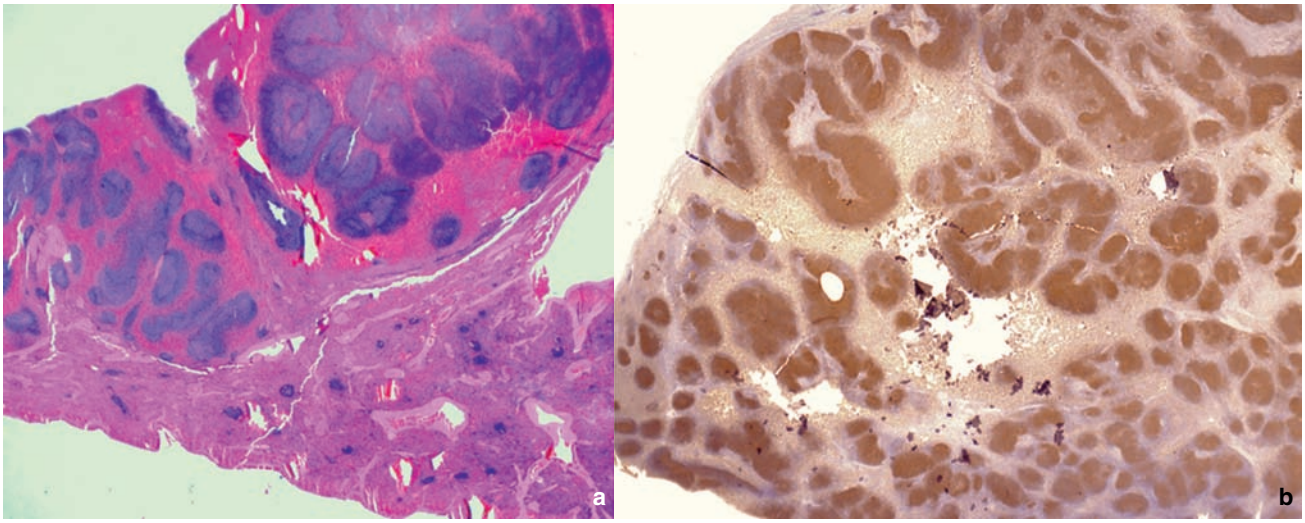


FIGURE 69.6 Spleen from dog with marginal zone lymphoma. (a) The spleen is characterized by diffuse and irregular coalescence of follicular areas. The germinal centers are faded and obliterated, multifocally they tend to coalesce. Sinus blood flow is altered by compression and results into hemorrhagic infarction. A small portion of normal spleen is visible. H&E; (b) CD20 immunostaining shows multiple follicular areas positive and a clear distinction between follicular centers and peripheral marginal zone is lost.

In humans, MALT lymphoma clearly arises from B cells in the context of chronic autoimmune or infectious inflammations.[112,113] It is well established that gastric MALT lymphoma, the most common in humans, is associated with *Helicobacter pylori* chronic gastritis.[112,113] Similarly, MALT lymphomas in other anatomical sites have been associated with other infectious agents: orbital adnexal MALT lymphoma with *Chlamydia psittaci*, cutaneous MALT lymphoma with *Borrelia burgdorferi*, immunoproliferative small intestine disease (IPSID) with *Campylobacter jejuni*, and pulmonary MALT lymphoma with *Achromobacter xylosoxidans*.[112,113] Finally, hepatitis C virus is associated with all the MZLs.[25,33,73,74,104] The importance of the chronic inflammation is further supported by the pattern of immunoglobulin heavy chain variable (IGHV) rearrangements in MZL, which strongly indicates the lymphoma cells have undergone an antigen-driven process. MZLs usually present somatically mutated IGHV genes with intraclonal variation due to ongoing mutations and a biased usage of some IGHV segments.[10,24,98,110–112] Similar to humans, one study of five canine NMZL cases showed variable ongoing somatic IGHV mutations.[78]

Mantle Cell Lymphoma

Mantle cell lymphoma (MCL) is a distinct subtype of lymphoma characterized by follicular proliferation of mature B-lymphocytes. MCL is derived from antigen-naïve pre germinal center B cells (GCB) located in the mantle zone and surrounding normal germinal centers.[103] Neoplastic lymphocytes are small- to medium-sized with irregular and indented nuclei. Most nuclei lack visible nucleoli, and the tumor shows a low mitotic rate. MCL in humans is characterized by cyclin D1 expression. Human MCLs have a distinct immunophenotypic profile characterized by expression of CD5, CD20, and other phenotypical markers.[68] This is completely unknown in veterinary medicine.

Clinical Features

In animals, MCL is rare. Unlike MZL, which tends to evolve after a long period of marginal zone hyperplasia, MCL appears not to be detected at a stage of hyperplasia. This is contrast with human MCL, in which the presence of cyclin-D1-positive lymphoid cells restricted to the mantle zones and bearing the cyclin D1 (CCND1) rearrangements is recognized as "in situ mantle cell neoplasia" according to the WHO classification.[94] MCL tends to present as a multinodular and multifollicular proliferation that then undergoes coalescence. Associated changes in the spleen include atypical plasmacytosis with fibrohistiocytic nodules and occasionally focal hemorrhagic infarction.[99] In a study of 388 cases of B-cell lymphoma, only four were suggestive of MCL.[72] However, these cases were characterized by a high mitotic rate and a high Ki-67 index, which contrasts with human MCL. Eleven cases of canine MCL were identified in a collection of 461 cases with an incidence in the dog of about 2%. The age of affected dogs ranged from 1.5 to 15 years with a mean of 8 years. MCL appears to be similarly rare in cats in which one case was identified in a study of 150 cases of lymphoma.[99] Another peculiar feature of the canine MCL is the relative higher frequency as primary lymphoma in the spleen and rectum than in the nodes.[99]

Pathologic Features

In the lymph node, MCL usually effaces the normal architecture showing one of two growth patterns:

mantle zone growth (follicular) or diffuse. MCLs with a mantle zone growth pattern can be difficult to diagnose, especially when only a moderate lymphadenopathy is reported. These cases require complete lymph node extirpation and tru-cut samples should be always avoided. This form of MCL is characterized by an expansion of the follicle mantle area by tumor cells surrounding a reactive, "naked" germinal center. However, differentiating follicular MCL from follicular or mantle cell hyperplasia can be difficult. With disease progression, neoplastic cells proliferate to cause paracortical atrophy and fading of hyalinized germinal centers.[107] Given the difficulties in diagnosing follicular MCL, lymphoid clonality testing should be considered in suspect cases. Diffuse MCL, which is the most common variant, is characterized by the involvement of the entire node (Figure 69.7). Based upon neoplastic cell morphology, two main variants of MCL are reported in the human WHO classification, classical and blastoid. In dogs, MCL resembles the classical variant, as it is characterized by a monotonous proliferation of small- to medium-sized lymphoid cells with scant cytoplasm, variably irregular nuclei, evenly distributed condensed chromatin, and inconspicuous nucleoli. Nuclear morphology is best visualized in cytologic smears. Histologically, differentiating diffuse MCL from SLL or CLL may be difficult.[99] By immunohistochemistry (IHC), the neoplastic cells in MCL express CD20 and CD79. Specific description on cytological and flow cytometric aspects in domestic animals is currently lacking. Considering the indolent clinical course, bone marrow and blood are usually not involved.[27]

Macroscopically, splenic MCL is characterized by a generalized nodular-pattern-like SMZL. Hemorrhages and organized hematomas are also reported as secondary lesions. Histologically, white pulp nodules are enlarged with a variable involvement of the red pulp. Some tumor foci surround hyalinized and hypocellular germinal centers, which indicates their relationship to fading follicles. Mantle cells in the spleen generally have strong immune reaction to antibodies for CD45, CD79a, and Bcl-2 and are negative to CD3, CD10, Bcl-6, and cyclin D1.[92]

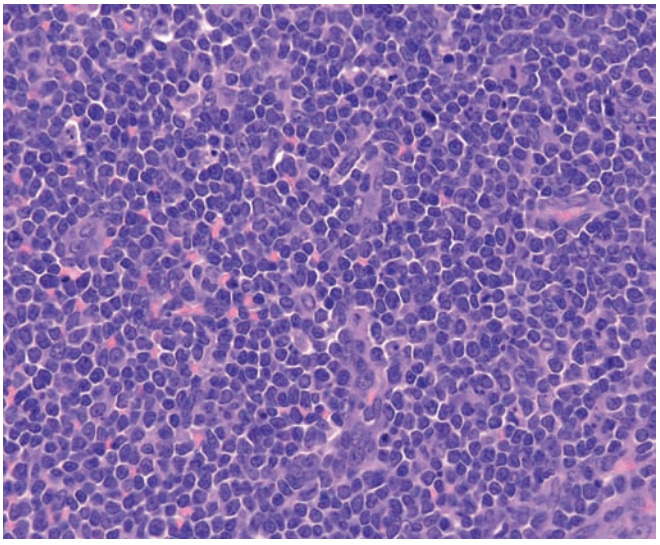


FIGURE 69.7 Lymph node from dog with mantle zone lymphoma. Histologically, a diffuse growth of neoplastic lymphocytes is observed causing an architectural effacement. The population is homogeneous and cells resemble centrocytes of the germinal center associated with few large and pleomorphic cells. H&E.

Comparative Features

Human MCL accounts for 5–10% of all lymphomas.[13,31,55,94] As mentioned, the genetic hallmark of the human disease is the chromosomal translocation juxtaposing the CCND1 gene to the immunoglobulin heavy-chain genes. Other frequent genetic lesions include inactivation of the tumor suppressor genes tumor protein 53 (TP53), cyclin dependent kinase inhibitor 2A (CDKN2A), and ataxia-telangiectasia mutated (ATM), and somatic mutations affecting genes coding chromatin-remodeling genes and the NF-κB pathway.

Follicular Lymphoma

In both humans and veterinary species, follicular lymphoma (FL) is a B-cell neoplasm composed of small centrocytes and large centroblasts. In a retrospective study of 502 canine lymphoma cases, 20 cases of FL were identified. In one review of 176 cases from the New York City area, there were 19 cases of FL for a total incidence of 5.7%. In a review of 602 feline cases of lymphoma, only six (1%) were diagnosed as FL. A review of 1198 cases of bovine lymphoma, identified by inspection at Canadian slaughter plants, documented four cases (0.3%) of FL. Follicular lymphoma appears to be rare in the horse, where the incidence appears to be less than 1%. Given the challenges of diagnosing FL, its true incidence in veterinary species is unknown, but it is likely somewhere between 2% and 4% of all canine lymphoma cases.[41]

Clinical Features

Dogs with FL tend to have a multicentric disease, involving both peripheral and intracavitary lymph nodes, with no sex, age, and breed predisposition. In most cases, FL is discovered at an advanced disease stage, highlighting its capacity to progress unnoticed. There is no consensus treatment for canine FL.[27] Given the low prevalence of FL in animals, no studies identifying prognostic factors, or the impact of blood and bone marrow infiltration, are available. Peripheral blood and bone marrow involvement would likely be difficult to identify via cytology or flow cytometry alone, given the small size of the neoplastic cells. PARR might play a possible role, but no specific studies are available to date.

Pathologic Features

FL is a neoplasm composed of B cells originating from the follicle center (germinal center). The size of node is moderately increased, and the capsule is characteristically

thin and taut, with the peripheral sinus compressed and compromised in areas where extranodal tissues infiltrated by neoplastic cells are present. The neoplastic population is composed of both centrocytes and centroblasts or large transformed cells. FL produces a nodular infiltrate in lymph nodes that eventually effaces normal germinal centers. Characteristically, the lymphoid follicles in FL lack a well-defined mantle zone and polarization. Early cases of FL may have a starry-sky pattern resembling a reactive process complicating the morphological evaluation (Figure 69.8). FL also exhibits atrophy of the paracortical and medullary areas. The pre-existing reticulin network is usually compressed to the periphery of the neoplastic follicle, resulting in the formation of variably prominent interfollicular reticular trabeculae. This modification is observed by Manuel's silver impregnation stain.

The major differential diagnosis for FL is benign follicular hyperplasia. Differentiating FL from follicular hyperplasia can be difficult, as FL recapitulates the architecture, growth pattern, cytology, and immunophenotype of normal/hyperplastic germinal centers.[99] However, there are several clues that can help differentiate the two. In FL, the lymphoid nodules are usually closely packed with a back-to-back arrangement and have comparable size and shape, which differ from florid follicular hyperplasia. In follicular hyperplasia, many of the follicles within the tissue

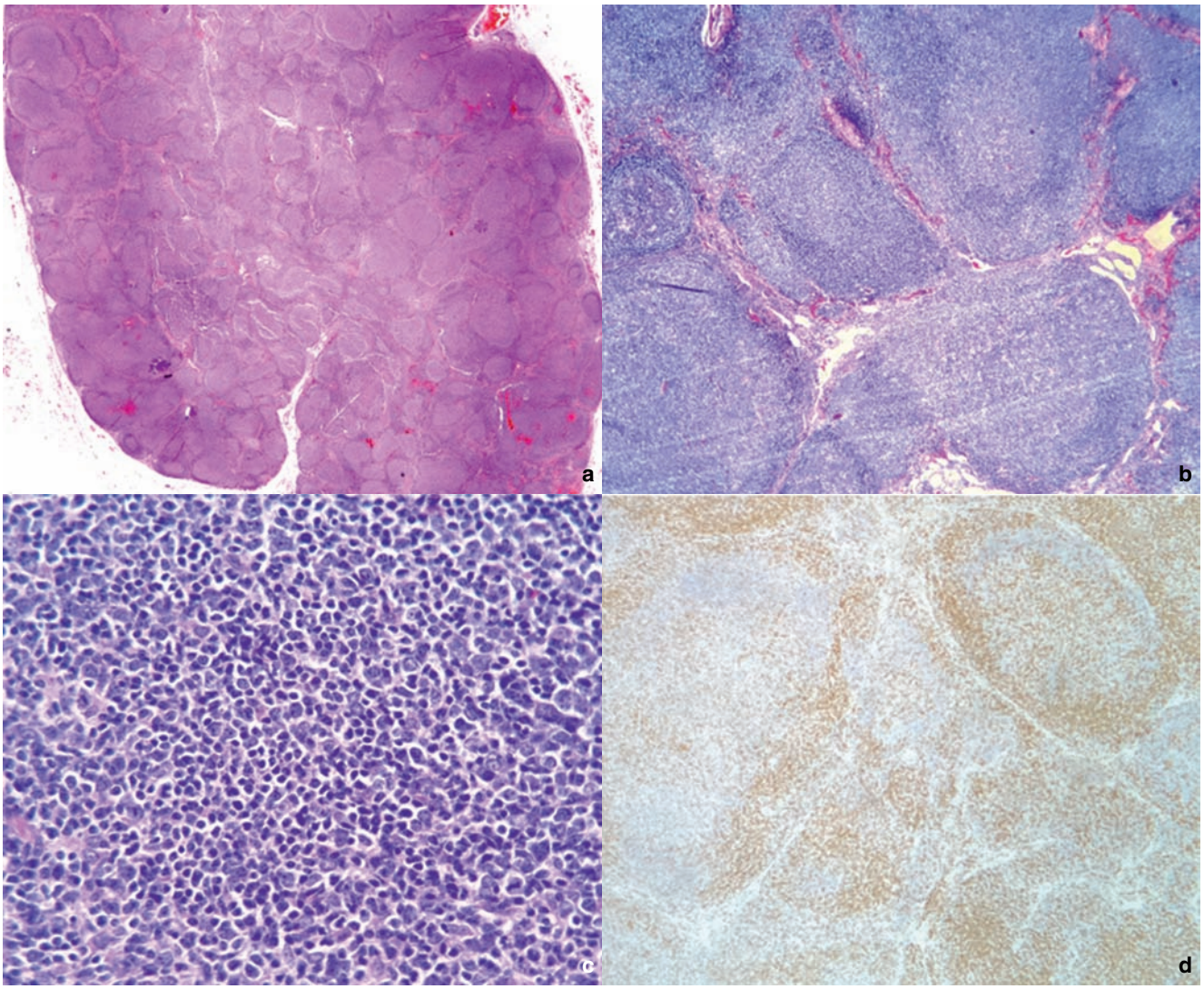


FIGURE 69.8 Lymph node from dog with follicular lymphoma. (a) Lymph node architecture is effaced by enlarged follicles with a starry-sky pattern and a lack of mantle zones. Conversely, marginal zone differentiation is still present multifocally at the periphery of the neoplastic follicles; (b) The entire lymph node is replaced by a nodular proliferation characterized by neoplastic follicles composed of mostly small lymphoid cells with a rim of germinal center cells at the periphery; (c) The cellular composition is typically heterogenous and atypical cells are intermediate in size, showing a blastoid appearance and lacking of nucleoli. A moderate number of centroblasts are present; (d) CD3 immunostaining identifies few T cells in the neoplastic follicles.

have antigen-related polarity and there is usually some level of residual mantle cell cuff. These features are lost in the neoplastic follicles of FL. In follicular hyperplasia, the involvement of perinodal tissue is frequently present, but is almost never nodular or follicular, as usually occurs in FL. Given this morphologic overlap, clonality testing is essential in accurately differentiating FL from hyperplastic lesions. Considering the importance of tissue architecture, small biopsies (tru-cut) of affected lymph nodes are not recommended.

Considering the importance of tissue architecture and the multifocal distribution of the neoplastic follicles, cytology is of limited use in the diagnosis of FL. When adequate, cytology may show a mixed population of centrocytes and small lymphocytes with percentage of centroblasts variable according to the grade. Mitotic figures are few (less than one to two per five HPF), as well as positivity to Ki67. The presence of a population composed by follicular cells and centroblasts without an increased number of plasma cells and the absence of a progressive differentiation may suggest FL, but a definitive diagnosis is impossible using cytology alone. Similarly, although flow cytometry can confirm the B-cell origin of the neoplasm, it cannot definitively diagnose FL. In human medicine, CD10 is a valuable marker of FL, but no antibodies have been reported in veterinary medicine. In dog, a histological grading scheme for FL has been proposed; however, this histotype is so rare that the utility of such a scheme is uncertain.

In the authors' experience, transformation of FL to DLBCL occurs in the dog. The comparison of biopsy material from the same dog revealed an evolution of follicular morphology to a diffuse architecture with a mixture of medium-to large lymphoid cells and a higher number of mitotic figures. However, an unequivocal demonstration of transformation would require the demonstration of a clonal relationship between the original FL and subsequent neoplasm. Given that a true follicular architecture may be very subtle in most of FL cases, immunohistochemistry should be always performed to achieve a final diagnosis. FLs are strongly positive with CD79a and CD20 and are negative with CD3. A meshwork of dendritic cells and T cells is identified in follicular areas, but is usually sparser and more irregularly distributed than in follicles.

Splenic FL has not been reported in animals, whereas three cases of primary colorectal FL have been described in dog.[79] Histologically, all cases were described as well-circumscribed, nodular masses confined to the submucosa. The masses were diagnosed as FL based on the presence of prominent, often confluent, lymphoid follicles with expanded germinal centers that had lost the morphologically distinct light and dark zones consistent with normal polarity. A mixed population of intermediate-sized centrocytes with characteristic indented, heterochromatic nuclei and indistinct nucleoli and centroblasts with large euchromatic nuclei and multiple large amphophilic nucleoli that often marginalized along the nucleolemma were found.

Comparative Features

In humans, FL is the second most common type of lymphoma, accounting for approximately 20% of all lymphomas with a higher incidence in Western Europe and USA than in the other places.[32,37,94] The FL genetic hallmark is the presence of the t(14;18)(q32;q21) chromosomal translocation, which relocates the BCL2 gene (18q) adjacent to the immunoglobulin heavy-chain genes locus (14q) resulting in the overexpression and accumulation of the anti-apoptotic protein Bcl-2. Many other genetic lesions are also frequent, and, importantly, the lymphoma cells have a very strict relationship with nontumoral cells present in the microenvironment.[28,37,50]

Diffuse Large B-Cell Lymphoma

DLBCL is the most frequent type of lymphoma in dogs, accounting for about 60% of lymphomas diagnosed in this species, but it is described in all domestic animals.[47] DLBCL may occur in nodal and extranodal sites and encompasses marked morphologic, genetic, epigenetic, transcriptomic, and clinical heterogeneity.[6] Involved lymph nodes are typically enlarged due to the diffuse proliferation of medium or large B cells. Two cytomorphological variants are described in dogs (i.e., centroblastic or immunoblastic forms), but no clinical benefit of this subtyping has been identified. Centroblasts are large noncleaved lymphoid cells resembling the proliferating cells of the germinal center with oval to round vesicular nuclei, with fine chromatin and multiple membrane-bound nucleoli, and scanty amphophilic to basophilic cytoplasm. Immunoblasts have an oval or round nucleus with opened chromatin and a large central solitary nucleolus, and abundant deeply basophilic cytoplasm. DLBCL has been associated in cats and cattle to retroviral infections, but lymphomas also occur in the absence of viral involvement.[101,106]

In dogs, emerging data both from omics platforms and clinical trials indicate the importance of breakpoint cluster region (BCR) signaling in the pathogenesis of DLBCL.[6,86] The interruption of BCR signaling by target drugs has substantial antitumor activity in an in vitro model and dogs with DLBCL.[38] A similar mechanism is described in human activated B-cell (ABC)-DLBCL, characterized by a constitutive activation of NF-κB. These data are unknown in the canine species and the integration of viral genomes into neoplastic cells should be also investigated to completely exclude the viral pathogenesis.[6]

Clinical Features

Dogs with DLBCL typically demonstrate painless peripheral lymphadenopathy with variable clinical signs secondary to the effects of lymphoma on infiltrated organs, such as spleen, liver, gastrointestinal tract, or bone marrow. Due to the nonspecific nature of these findings, weeks to months may elapse before the diagnosis of lymphoma is established.

Peripheral lymphadenopathy is moderate to severe, whereas extranodal involvement occurs in less than 20% of cases at initial presentation.[57] Anemia and thrombocytopenia may be present and represent negative prognostic factors, as does an increased serum lactate dehydrogenase (LDH) level.[1,17,59,62]

Neoplastic cells are found in the peripheral blood and bone marrow in about half of dogs with DLBCL.[57] However, this percentage is highly variable depending upon analytic approach (cytology, flow cytometry, or PARR) or cutoff values.[51,59] The prognostic value of low-level peripheral blood infiltration is unknown, but a recent study has shown that the percentage of infiltration in bone marrow is inversely correlated with lymphoma-specific survival and time to progression.[59] Furthermore, an infiltration cutoff value of 3% to discriminate prognosis has been proposed. Dogs with lymphoma surviving more than 2 years had an absence of bone marrow infiltration in another study.[60] Importantly, bone marrow infiltration can be documented in dogs lacking either cytopenias or circulating neoplastic cells.[61] In cats with high-grade lymphoma or large granular lymphoma, up to 15% are leukemic, but studies aimed at correlating this feature with outcome are lacking.[34,53]

Circulating neoplastic cells may be medium- to large-sized with prominent nucleoli resembling those of lymph node aspirates, but frequently they do not reflect the morphology of neoplastic cells in lymph nodes. Flow cytometry can easily quantify the percentage of large B cells (CD21+/CD22+) in peripheral and marrow blood with a higher sensitivity and specificity than cytology. PARR may be more sensitive and specific to confirm infiltration, but its prognostic role has not been elucidated.[5] DLBCL must be distinguished from large T-cell lymphoma, late-stage MZL, and intermediate-sized Burkitt-like lymphoma (BLL). In certain forms of Burkitt-like lymphoma, the histology (intermediate-sized cells, numerous mitotic figures, and numerous tingible body macrophages) overlaps with DLBCL.

First-line treatment consists of traditional dose-intense chemotherapy; however, it is rarely curative. The goal of treatment is palliative, aiming at improving quality of life by ameliorating symptoms of disease, and possibly prolonging survival. The backbone of current therapy is represented by systemic multiagent chemotherapy, incorporating drugs such as vincristine, cyclophosphamide, and doxorubicin, with or without L-asparaginase.[18,36,87] Shorter treatment cycles are the goal of ongoing research to minimize treatment-related toxicity. In recent years, immunotherapy in combination with chemotherapy has been established as a powerful way to treat DLBCL.[57] In human cases, the treatment of choice for newly diagnosed patients with advanced DLBCL is R-CHOP (rituximab—a monoclonal antibody targeting CD20−, cyclophosphamide, doxorubicin, vincristine, and prednisolone) chemo-immunotherapy, which results in 5-year survival in the range of 60%.[19]

Pathologic Features

In dogs, three morphologic variants of DLBCL have been reported, namely, centroblastic, immunoblastic, and anaplastic variant. The first two types have been described in the earlier text. The anaplastic variant is characterized by marked pleomorphism of tumor cells that may resemble Reed–Sternberg cells or carcinoma cells. Notably, given the lack of clinical outcome or treatment data differentiating these morphologic variants, sub-classification is of limited value in veterinary medicine.[4] Affected lymph nodes are enlarged with a thin capsule and neoplastic colonization of perinodal tissues. The neoplastic growth is diffuse with fading or

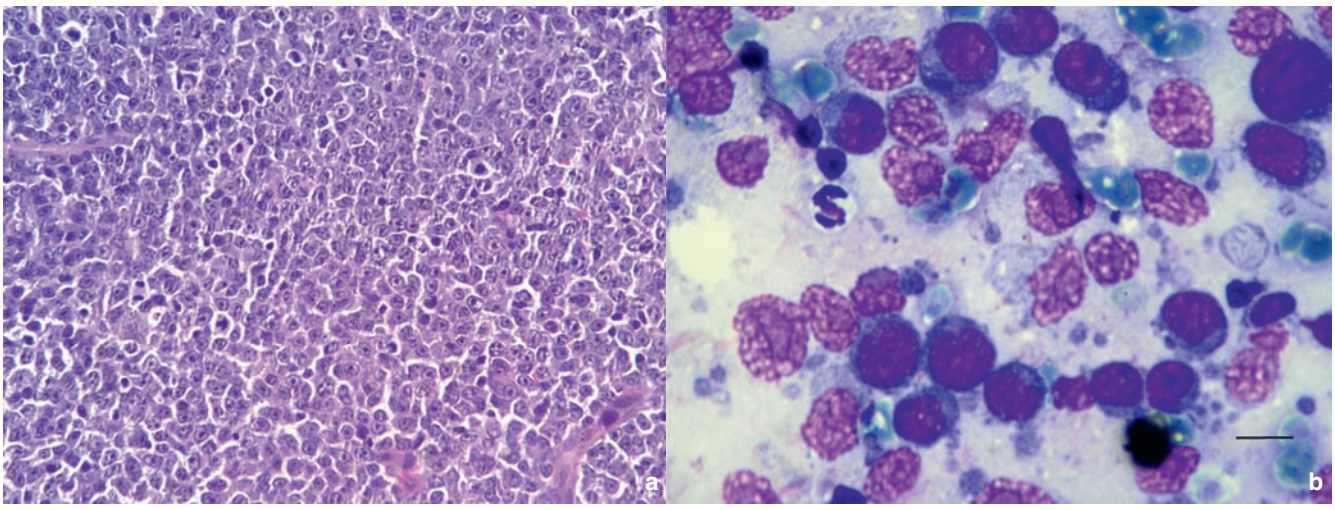


FIGURE 69.9 Lymph node from dog with diffuse large B-cell lymphoma immunoblastic type. (a) Diffuse effacement of node architecture is observed. The neoplastic population is characterized by large nuclei with a fine chromatin and a single, centrally located prominent macronucleolus with variable amounts of eosinophilic cytoplasm; (b) Cytologically, neoplastic cells comprise a homogeneous population of large (nuclei larger than two erythrocytes in diameter) blasts with deeply basophilic cytoplasm, round nucleus with decondensed chromatin, and single, central prominent nucleolus. May–Grünwald–Giemsa.

compression of residual germinal centers. Compression of the peripheral sinuses and complete effacement of medullary structures are common. Necrosis may be seen in dogs undergoing chemotherapy treatment. DLBCL tends to be monomorphic and the immunoblastic variant is defined when the population is almost entirely composed of immunoblasts (>90%) (Figure 69.9a). Centroblastic DLBCL is more pleomorphic having both centroblasts and immunoblasts (Figure 69.10a). Many cases, however, are difficult to subclassify into one of the previous categories, having features intermediate between variants. The anaplastic variant of DLBCL, which is uncommon in dogs, is characterized by large neoplastic B cells with bizarre nuclei, and abundant amounts of cytoplasm. The differential diagnoses are aggressive Burkitt-like lymphomas, peripheral T-cell lymphomas, and histiocytic sarcomas. The final diagnosis is always performed with immunohistochemistry.

Mitotic rate is variable in DLBCL. In a recent study, survival of dogs with DLBCL was correlated to the mitotic index. Median survival in dogs with fewer than 20 mitoses per 400× field was 188 days, whereas the median survival of dogs with 21 or more mitoses per 400× field was much shorter (31 days).[102] However, this study has lacked clinical staging data and standardized therapeutic protocols. Affected tissues in canine DLBCL often contain variable number of tingible body macrophages, which appears to be associated with the apoptotic rate of the lymphoma. In affected tissues, small foci of small-sized cells with densely staining nuclei that demonstrate sharp shallow nuclear indentations and inapparent nucleoli may be seen. These cells are CD3+ and CD5+ tumor infiltrating T cells that were recently found to express PD-1 marker and may be associated with prognosis.[5]

The cytology of DLBCL is variable and may reveal a dominant population of large immunoblasts, centroblasts, or, more frequently, a heterogeneous population of centroblasts, immunoblasts, and medium-sized macronucleolated cells (Figures 69.9b and 69.10b). In cats, the immunoblastic variant tends to be prevalent over centroblastic. Several macrophages, often containing tingible bodies, are found and cytoplasmic fragments are often seen in the background. Mitotic figures may vary from three to more than five per HPF. The neoplastic cells typically express pan-B-cell markers such as CD20, CD79a, and PAX-5.

Flow cytometry reveals a population of cells with high FSC that express CD45, CD44, CD18, CD21, CD20, CD22, CD79a, MHC II, and surface immunoglobulin (Figure 69.10c). Low expression of MHC II may be associated with a poorer outcome in B-cell lymphoma.[77] Occasionally, CD34 may be aberrantly expressed, but no association with outcome has been demonstrated. To date, no flow cytometric markers have been shown capable of differentiating DLBCL from other B-cell lymphoma and a definitive diagnosis must combine immunophenotyping and histopathology.

DLBCL can also be classified based on the anatomic location, including intravascular, mediastinal, splenic, and primary effusion types of lymphoma. One variant of large B-cell lymphoma, which has an affinity for vessels, localizes in any tissue, but it has a major affinity for the central nervous system (CNS).[30] Macroscopic findings at postmortem examination are characterized by multifocal hemorrhagic areas, particularly in the forebrain with a bilateral distribution of tumor cells. Histologically, both neuroparenchymal and meningeal vessels are infiltrated by neoplastic aggregates that occupy the vascular lumen with no apparent cohesion with the endothelium. Neoplastic cells have marked anisokaryosis with multinucleated and binucleated cells, round to oval voluminous nuclei, markedly pleomorphic, round to polyhedral, with distinct cell borders and variable nucleus/cytoplasm ratios. Multiple prominent nucleoli are visible, and several atypical mitotic figures are evident. Immunohistochemistry is always needed to confirm the diagnosis and the phenotype. CD20 and PAX-5 are usually positive.

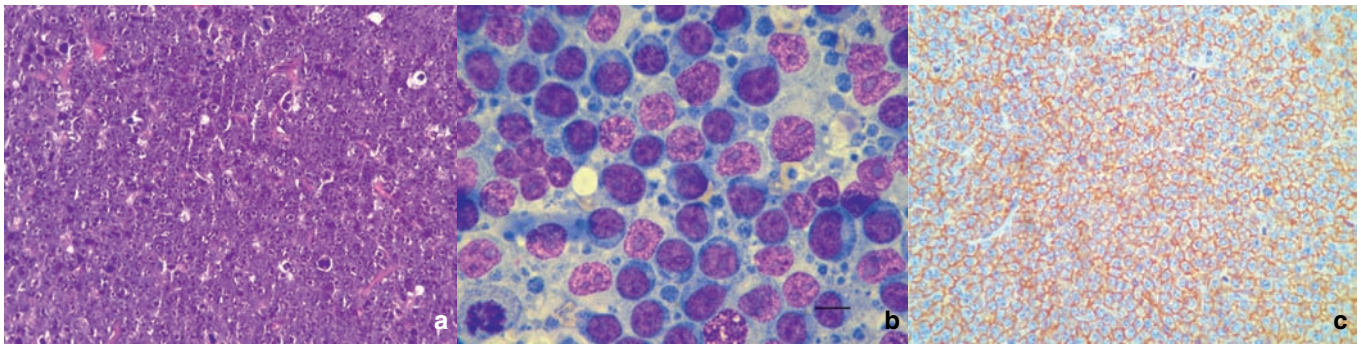


FIGURE 69.10 Lymph node from dog with diffuse large B-cell lymphoma centroblastic type. (a) A diffuse effacement of node architecture is observed. The neoplastic population is characterized by medium- to large-sized cells with vesicular nuclei, irregular (clumped) chromatin, and several nucleoli located at the periphery close to the nuclear membrane. H&E; (b) Cytologically, neoplastic cells are composed by a variable population of medium to large (nuclei equal or larger than two erythrocytes in diameter) blasts with deeply basophilic cytoplasm, often with evident perinuclear clear halo, round to oval nucleus with smooth chromatin, and multiple prominent nucleoli. Several lymphoglandular bodies are visible in the background and mitotic figures are often visible. May–Grünwald–Giemsa stain; (c) CD20 immunostaining shows diffuse membrane-positive staining of centroblasts.

Hemorrhagic infarction due to multifocal partial to complete venous thrombosis is common. In a case series of cerebral intravascular lymphoma, pulmonary involvement was also observed.[30] The pulmonary involvement was limited to small-caliber pulmonary veins and interstitial capillaries, whereas arteries and larger-caliber vessels were spared. Animals with this form of lymphoma usually show a rapid onset of depression, disorientation, tetraparesis, recurrent seizures, and ataxia. Because of poor prognosis and progressive worsening of the neurological condition, euthanasia is performed in most cases.

Mediastinal large B-cell lymphoma is rare in dogs, but common in cats.[64] This disease is usually diagnosed by ultrasound-guided fine-needle aspiration or tru-cut biopsy.[9] In a series of 602 cases of feline lymphoproliferative diseases, mediastinal lymphomas showed an aggressive biological behavior and were graded as high-grade.[101] These lymphomas show a high rate of necrosis and a high degree of mitotic activity. The most common presenting signs include lethargy, anorexia, polyuria/polydipsia, coughing, dyspnea, and vomiting. Splenic DLBCL is reported, but the frequency is low, and it is less common than marginal zone and mantle cell lymphoma. The growth is initially multifocal and becomes rapidly coalescing.[99] The tumor expands within the white pulp, compressing the red pulp, which leads to grossly visible, coalescing nodules adjacent to associated hemorrhage and infarction.

Comparative Features

Gene expression studies of canine DLBCL have identified patterns associated with prognosis and tumor biology. In two different studies, dogs with DLBCL showed a molecular profile resembling the ABC-DLBCL in humans. The signature is characterized by the expression of genes involved in the activation of B cells, as well as genes normally expressed by plasma cells, thus suggesting a post-GCB origin. Constitutive nuclear factor (NF)-κB, MYC, and PI3K signaling pathways were demonstrated to promote malignant cell survival in these lymphomas.[65,78,86] Two Bruton tyrosine kinase (BTK) inhibitors (ibrutinib and acalabrutinib) underwent early preclinical evaluation in dogs with B-cell lymphoma further confirming the role of NF-κB activation in the pathogenesis of this tumor and highlighting a possible crucial upstream effect of BTK in driving NF-κB pathway.[44,46] Additionally, RNA-seq of 50 dogs with DLBCL revealed two groups showing different survivals; dogs with enrichment of immune system signatures had an unfavorable prognosis.[6]

A small number of copy number variations (CNVs) have been identified in canine DLBCL. The most extensive and highly recurrent CNVs were gains of canine familiaris (CFA) 13 and 31, but recurrent gain of the MYC oncogene and absence of deletion of the CDKN2A/B tumor suppressor gene locus were also identified. The clinical impact of this data is not wholly certain, but the gain of CFA 13 was found more frequently in dogs demonstrating a complete response after treatment compared with dogs that relapsed during treatment and in dogs initially diagnosed with stages III and IV disease. Moreover, the gain of CFA 31 was more frequent in dogs with a worse prognosis. Additional epigenetic modifications, such as methylation aberrations, were found to be associated with a more aggressive disease, worse clinical outcome, and unique patterns of gene expression.[5]

In humans, DLBCL is the most common type of lymphoma, encompassing 25–35% of all cases in Western countries and even higher in other countries.[94,97] DLBCL has always been considered a heterogenous disease and genomic technologies have identified multiple subtypes, including 16 distinct entities in the latest WHO classification.[94] The main variant is diffuse large B-cell lymphoma, not otherwise specified (DLBCL-NOS), which encompasses multiple subgroups with specific genetic, biologic, and clinical features. The two main subtypes are the GCB and ABC.[70,94] Clinical outcome is worse for ABC-DLBCL than for GCB-DLBCL patients and the distinction between them is considered mandatory.[52,94,96] Between 2018 and 2020, additional subgroups based on genetic lesions have been identified.[16,85,109]

Burkitt Lymphoma

In humans, Burkitt lymphoma (BL) and BLL are well-characterized diseases. However, in veterinary medicine, both entities are highly controversial, reported in dogs, and rarely in cats and pigs.

Clinical Features

Burkitt lymphoma is an aggressive B-cell malignancy reported to account for 4% of canine lymphomas.[44] Given its rarity, there is limited knowledge about its clinical behavior and response to therapy. It is described as having a rapid clinical course and affected dogs demonstrate generalized lymphadenopathy with variable extranodal disease at the time of diagnosis. One retrospective study reported a good response to chemotherapy with 26% of dogs surviving more than 2 years; however, dogs were not uniformly staged.[45] A recent work by the authors describes a series of dogs with BLL that demonstrate generalized lymphadenopathy present for a median of 20 days (range 7–90) with rare extranodal involvement. Despite treatment with CHOP-based chemotherapy or chemo-immunotherapy, overall prognosis was poor, with a median overall survival of 228 days and a median time to progression of 130 days (Front Vet Sci, 2021 Mar 17;8:647009. doi: 10.3389/fvets.2021.647009. eCollection 2021).

Pathologic Features

Histologically, both BL and BLL are challenging diagnoses. In 2014, a blinded group of veterinary pathologists with an expertise in hematopathology failed to consistently differentiate BL and BLL from DLBCL.[103] The architecture of affected lymph nodes is characterized by a diffuse proliferation of neoplastic cells that compresses

normal cells (Figure 69.11). Neoplastic cells are intermediate-sized (1.5–2 RBCs in diameter) with round to oval nuclei and a vesicular appearance. Cytoplasm is moderately abundant and with formalin fixation there may be slight cytoplasmic retraction, leading to squared-off edges between neighboring cells. Although three to five nucleoli have been reported in human BL, there are usually fewer in canine BLL with mild anisokaryosis. BL and BLL are highly proliferative and the mitotic rate is 10/HPF or greater. There are numerous tingible body macrophages (usually two to three times the number of mitoses) creating a starry-sky pattern. Liver, mesenteric lymph nodes, intestinal wall, and spleen involvement have been reported in the dog, but the histopathology of these lesions has never been described. Histologic differential diagnoses for Burkitt tumors include anaplastic forms of DLBCL, ALL, and LBL. The latter two can be differentiated based on nuclear features, namely, aggregated chromatin, parachromatin clearing, and small nucleoli in Burkitt tumors versus uniformly dispersed chromatin and obscured nucleoli in ALL and LBL. Cytologically, canine BLL is described as a monomorphic population of small (less than two RBCs in diameter) cells with a round, noncleaved nucleus, and deeply blue cytoplasm often with clear vacuoles. Nucleoli are often visible and multiple. Macrophages with tingible bodies are frequent causing the peculiar "starry-sky" appearance to the cytological smear. Mitotic figures are frequent. Using IHC, both BL and BLL stain strongly with CD20, CD79a, and PAX-5. Flow cytometry supports the B-cell origin with positivity to CD20, CD21, and CD79a, but, given the rarity of this lymphoma subtype, specific studies on cytological and flow cytometric are lacking.

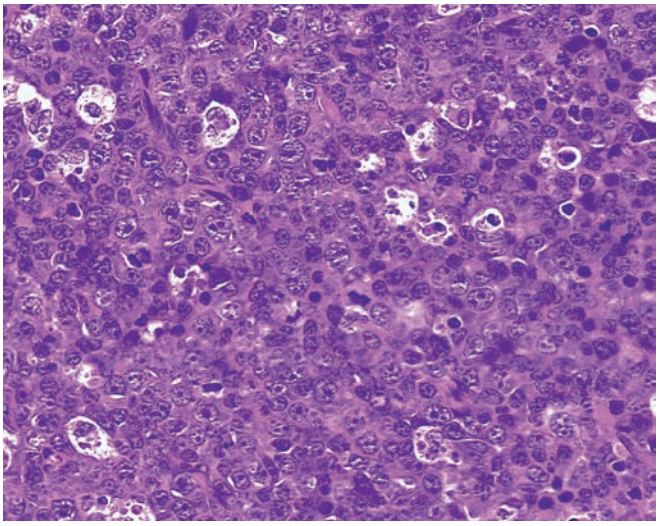


FIGURE 69.11 Lymph node from dog with Burkitt lymphoma. The node is characterized by intermediate to large neoplastic lymphoid cells with distinct cell borders, moderate anisokaryosis with cleaved or oval-shaped nuclei, and vesicular appearance. Numerous tingible body macrophages phagocytizing apoptotic debris and starry-sky appearance are present.

Comparative Features

Specific information on molecular or chromosomal changes in BLL in animals is lacking. In humans, BL comprises three epidemiological variants: endemic BL, sporadic BL, and immunodeficiency-associated BL.[94] The endemic BL is the most common form in equatorial Africa and in Papua New Guinea, where it is the common pediatric cancer. The geographical distribution of endemic BL overlaps with regions endemic for malaria, and, in almost all the cases, the lymphoma cells contain Epstein–Barr virus (EBV) genome. Although seropositivity for EBV has been reported in dogs and cats, the demonstration of viral DNA in lymphoma cells is missing. Sporadic BL is the most common variant in Western countries, but it accounts for only 1–2% of all lymphomas. Only 20–30% of sporadic cases are EBV-positive. Immunodeficiency-associated BL mainly occurs in patients with a severely compromised immune system, such as patients infected with human immunodeficiency virus (HIV), patients after organ transplantation, or patients with congenital immunodeficiency. Human BL is characterized by the presence of the t(8;14)(q24;q32) chromosomal translocation, juxtaposing the MYC oncogene into the immunoglobulin heavy-chain locus. Rare variants involve MYC and the immunoglobulin kappa or lambda loci.[42,84]

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CHAPTER 70

Plasma Cell Tumors

ANTONELLA BORGATTI

Multiple Myeloma
- Epidemiology
- Etiology
- Pathogenesis and Disease Evolution
- Clinical Manifestations of Disease
- Diagnosis
- Treatment
- Prognosis

Solitary and Extramedullary Plasmacytic Tumors
- Clinical Manifestations of Disease
- Diagnosis
- Treatment
- Prognosis

Cutaneous Plasmacytosis
Waldenström's Macroglobulinemia

Acronyms and Abbreviations

BM, bone marrow; BMSC, bone marrow stromal cell; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (lomustine); CD, cluster of differentiation; CEMP, cutaneous extramedullary plasmacytoma; CT, computed tomography; CP, cutaneous plasmacytosis; EMP, extramedullary plasmacytoma; FeLV, feline leukemia virus; FIV, feline immunodeficiency virus; HVS, hyperviscosity syndrome; IFE, immunofixation electrophoresis; IFN, interferon; Ig, immunoglobulin; IL, interleukin; IRF, interferon regulatory factor; IRF4, interferon regulatory factor 4; MGUS, monoclonal gammopathy of unknown significance; MM, multiple myeloma; MRD, myeloma-related disorders; MRI, magnetic resonance imaging; NCEMP, noncutaneous extramedullary plasmacytoma; NFκB, nuclear factor kappa B; OAF, osteoclast-activating factor; PBSC, peripheral-blood-derived stem cell; PET, positron emission tomography; PFI, progression-free interval; PT, prothrombin time; PTT, partial thromboplastin time; RANK, receptor activator of the nuclear factor kappa B; RANKL, receptor activator of the nuclear factor kappa B ligand; REAL, revised European American Lymphoma classification; SOP, solitary osseous plasmacytoma; SPB, solitary plasmacytoma of bone; SSA, sulfosalicylic acid; TNF-α, tumor necrosis factor-α VAD, vincristine adriamycin (doxorubicin) prednisone; VBAP, vincristine BCNU adriamycin (doxorubicin) prednisone; VBMCP, vincristine BCNU melphalan cyclophosphamide prednisone; VEGF, vascular endothelial growth factor; VEGF-R1, vascular endothelial growth factor receptor 1; WM, Waldenström's macroglobulinemia; MHC, major histocompatibility complex.

Plasma cell tumors represent a spectrum of diseases characterized by clonal (predominantly monoclonal) proliferation of homogeneous immunoglobulin (Ig)-producing plasma cells or B cells.[41] They encompass clinically indolent conditions such as monoclonal gammopathy of unknown significance (MGUS) and Waldenström's macroglobulinemia (WM), as well as malignant processes including multiple myeloma (MM) and solitary plasmacytoma (solitary osseous plasmacytoma (SOP) and extramedullary plasmacytoma (EMP)). All these disorders share common cytomorphologic features, Ig production patterns, and immune dysfunction.

Five major classes of Ig—IgG, IgA, IgM, IgD, and IgE—are synthesized by normal B cells and plasma cells, whereas neoplastic plasma cells secrete one of these molecules or, occasionally, only light-chain molecules. When the production of heavy- and light-chain molecules is unbalanced, the excess of light-chain proteins is excreted in the urine (i.e., Bence Jones proteinuria). In non-secretory-type myeloma, plasma cells do not secrete these proteins.

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MULTIPLE MYELOMA

Epidemiology

MM is estimated to account for <1% of all malignant tumors occurring in domestic animals and approximately 8% of all hematopoietic malignancies in dogs.[40,53] The true incidence of MM in cats is unknown, but it appears to be less common in this species, and it is extremely rare in other domestic animals.[20,62] In humans, more than 50% of MM patients are older than 70 years. Likewise, older dogs (average 8–9 years)[53,62] and cats (average 12–14 years)[31,62] are most affected.

Etiology

Factors associated with the development of MM in companion animals have not been clearly identified. In humans, exposure to ionizing radiation is consistently linked to MM development. Genetic and hereditary factors may also play a role. Recurrent infections or antigenic stimulation have been proposed as predisposing factors, although epidemiologic studies have not confirmed this association.

A link between MM and feline leukemia virus (FeLV) or feline immunodeficiency virus (FIV) infection has not been identified, but the diagnosis of disease among siblings suggests a familial association.[31] The role of oncogenes, tumor-suppressor genes, and cytokines and their interaction with the bone marrow (BM) microenvironment in the etiopathogenesis of the disease are being investigated in animal models. Overexpression of cell cycle regulators such as cyclin D1 and dysregulation of receptor tyrosine kinase have been implicated in the pathogenesis of plasma cell tumors and MM.[10] Progression of B-cell lymphoma to MM in one dog and of solitary plasma cell tumors to MM in both dogs and cats has been reported.[66] Furthermore, rapid, aggressive progression of a cutaneous plasma cell tumor to plasma cell leukemia has been reported in a Bernese Mountain Dog.[70] MGUS, described as a premalignant condition, rarely progresses to MM.

Pathogenesis and Disease Evolution

Multiple myeloma is characterized by the proliferation of malignant plasma cells or their precursors, arising as a clone from a single cell. It is a germinal center-derived tumor with a B-cell phenotype and Ig gene hypermutation and rearrangement. MM is associated with a dysregulation of genes responsible for controlling cell growth and survival, resulting in a multistep process that involves alterations in oncogenes and tumor suppressor genes.[9] Alterations in molecular mechanisms of cell control have been implicated in the disease etiology.[10,42]

Like other lymphocytes, B cells do not have multilineage potential, but they can self-renew at multiple steps of development. These properties may be coopted by malignant plasma cells to maintain a "stem cell" phenotype. It has been demonstrated that memory B and T cells share common gene signatures with hematopoietic stem cells involved in the self-renewal process.[44] Multiple myeloma stem cells also may possess or acquire drug resistance: (1) by maintaining quiescence; (2) by increasing intracellular detoxifying enzymes; or (3) by expression of drug transporters on the surface membrane, as has been demonstrated for brain tumors and leukemic stem cells.[4,29] Stem cell signaling pathways also may support cancer stem cells; e.g., aberrant activation of the Hedgehog signaling pathway has been associated with the development of MM, and its inhibition induces plasma cell proliferation.

It has been hypothesized that a subset of MM may derive from MGUS. Furthermore, monosomy 13 seems to be implicated in the transition of MGUS to MM.[3] Interactions between cancer cells and microenvironment (bone marrow stromal cells (BMSCs) and extracellular matrix) are mediated by myeloma-cell-derived adhesion molecules, which are essential for tumor cell localization, growth, and survival. Moreover, increased microvessel density has been demonstrated in human patients with MM compared to patients with MGUS.[67,78] Immunohistochemical studies also have shown vascular endothelial growth factor (VEGF) expression by myeloma cells, thus implying the potential role and clinical relevance of angiogenesis in MM. While VEGF does not appear to have considerable proliferative effects on myeloma cells, it plays an important role in cancer cell migration and angiogenesis.[64,65] Indeed, myeloma cells have been shown to express the vascular endothelial growth factor receptor 1 (VEGF-R1), which may provide an interesting therapeutic target for emerging anti-angiogenic strategies.

The secretion of tumor necrosis factor-α (TNF-α) by myeloma cells not only stimulates the production by BMSCs of interleukin-6 (IL-6), an essential growth and survival factor for these cancer cells, but also induces nuclear factor kappa B (NFκB) activation leading to the upregulation of adhesion molecules.

Clinical Manifestations of Disease

The infiltration of various organ systems by neoplastic cells, the production of cytokines by the tumor or the bone marrow microenvironment, and the high circulating level of a single type of immunoglobulin (M-component) lead to a wide array of clinical manifestations. Multiple myeloma patients are usually immunocompromised and thus highly susceptible to infections. This occurs due to suppression of normal Ig concentrations secondary to a variety of factors, including a direct effect of the monoclonal Ig, bone marrow crowding, decreased T helper cell function, and macrophage-related factors influencing the progression of B cells to plasma cells.[59]

A variety of pathologic conditions are associated with MM, including bleeding diathesis, hyperviscosity syndrome (HVS), renal disease, cardiac disease, skeletal lesions, hypercalcemia, bone marrow hypoplasia, and hypercholesterolemia. The pathogenesis of bleeding diathesis is likely multifactorial. The M-component may interfere with normal coagulation

and lead to hemostatic defects by various mechanisms that include: (1) inhibition of platelet aggregation and release of platelet factor 3; (2) adsorption of minor clotting factors; (3) induction of abnormal fibrin polymerization; and (4) functional decrease in calcium.[45,73] Clinically, hemorrhages occur in approximately one-third of dogs and one-fourth of cats affected by MM.[31,53,62] In instances where myelophthisis is present due to profound bone marrow infiltration, thrombocytopenia may develop and contribute to hemorrhagic events. Approximately 50% of dogs affected by MM have abnormal prothrombin time (PT) and partial thromboplastin time (PTT). In a retrospective study of MM in 16 cats, thrombocytopenia was identified in just over 50% of the cats and one had evidence of a prolonged PT.[62] In another study of feline myeloma-related disorders (MRD), thrombocytopenia was identified in one of 15 cats. In the remaining cats, platelet counts were either within the normal reference range or deemed adequate on blood smears. PT and PTT were evaluated in two cats and were normal in both cases. None of the cats in this study had clinical evidence of bleeding tendencies, although subclinical coagulopathies may have been undetected.[11]

Hyperviscosity syndrome is characterized by clinicopathologic abnormalities that occur secondarily to increased serum viscosity, which is associated with the M-component. Hyperviscosity syndrome is most commonly associated with IgM macroglobulinemia due to the high molecular weight of IgM.[26] However, it also can occur in presence of IgA and rarely with IgG.[53] Hyperviscosity syndrome leads to bleeding diathesis, neurologic signs (such as seizures, depression, coma), congestive heart failure, renal failure, and ophthalmic abnormalities, including tortuous and dilated retinal vessels, retinal hemorrhages, and retinal detachment, sludging of blood within small vessels, and impaired delivery of nutrients and oxygen to tissues. Approximately 20% of dogs with MM develop HVS and it has been reported in a few cats.[11,62]

Renal disease occurs in approximately 30–50% of dogs and about 30% of cats with MM.[53,62] The pathogenesis of renal disease is likely multifactorial and implicated mechanisms include: (1) tumor infiltration within the renal parenchyma; (2) hypercalcemia; (3) amyloidosis; (4) decreased renal perfusion due to HVS; (5) dehydration; (6) ascending urinary tract infections; (7) Bence Jones proteinuria; and (8) development of light-chain casts causing interstitial nephritis.[53] Light-chain proteins have a low relative molecular mass and are normally filtered through the renal glomerulus. In MM, however, an imbalance between heavy- and light-chain proteins occurs, which leads to an excess of light chains that can precipitate and cause renal tubular obstruction and injury. In the absence of a concurrent monoclonal spike, light-chain proteinuria is most likely indicative of light-chain disease. Bence Jones proteinuria has been estimated to occur in approximately 25–40% of dogs and approximately 65% of cats with MM.[53,62] Urine protein electrophoresis remains the preferred diagnostic modality to detect monoclonal light-chain proteinuria.

Heart failure may occur because of hyperviscosity-related myocardial hypoxia and increased cardiac workload. In addition, amyloid deposition in the myocardium and anemia may exacerbate the severity of this condition. Approximately 50% of cats with MM in one study had an idiopathic heart murmur.[62] In another study, two cats exhibited signs consistent with heart failure prior to their MRD diagnosis.[11]

Skeletal lesions can be either solitary (well-circumscribed, "punched-out" area of osteolysis) or multiple (diffuse osteopenias) and occur in approximately 50–56% of dogs with MM.[53] Rarely, pathologic fractures are seen. Skeletal lesions are typically identified in bones involved in active hematopoiesis (e.g., ribs, vertebrae, pelvis, and proximal and distal aspects of long bones). In cats, the reported incidence of lameness or bone lesions ranges from approximately 7% to 67%.[31,57,62] In a study of MRD in cats, a pathologic humeral fracture was noted in one cat, although complete survey radiographs were not performed in all cases.[11]

Hypercalcemia has been reported in approximately 15–20% of dogs and 20–25% of cats affected by MM.[31,62,72] It is thought to result from the release of an osteoclast-activating factor (OAF) by either the bone marrow microenvironment or by cancer cells locally in bone.[53] Parathyroid-hormone-related peptide also may contribute to the pathogenesis of MM-related hypercalcemia.[69] In humans, IL-1, IL-6, TNF-α, and the receptor activator of the nuclear factor kappa B ligand (RANKL) all modulate osteoclast activity and may contribute to hypercalcemia. As the M-component may bind calcium, the functional consequences of an increased total calcium concentration should be evaluated using an ionized calcium determination.

Approximately 30% of dogs and 75% of cats have normocytic normochromic nonregenerative anemia associated with anemia of inflammatory disease, myelophthisis, hemostatic abnormalities resulting in blood loss, or increased RBC destruction induced by HVS.[53] In humans with MM, decreased erythropoietin responsiveness has been documented. Multiple myeloma-related thrombocytopenia and neutropenia can also be observed in cats and dogs.[31,62]

Hypocholesterolemia was noted in approximately 69% of affected cats in one study.[62] This finding was inversely correlated with globulin concentration and was suspected to be related to downregulation of hepatic cholesterol production in the attempt to maintain adequate oncotic pressure despite the presence of hyperglobulinemia.

Diagnosis

Diagnosis of multiple myeloma in dogs typically follows documentation of bone marrow plasmacytosis, presence of osteolytic bone lesions, and identification of M-component in serum or urine. In addition, demonstration of plasma cell clonality or progressive increase in the M-component can inform a diagnosis when lytic bone lesions are absent.[79] In cats, plasma cell morphology and visceral organ involvement are

considered for diagnostic purposes, as bone marrow plasmacytosis is not as common or marked as it is in dogs.[57,62] Symptoms suggestive of MM include the onset of bone pain or back pain, renal failure, anemia, and a history of recurrent infections. Patients exhibiting these signs should be evaluated with a complete blood count, serum biochemical profile, and urinalysis. Serum and urine protein electrophoresis should be performed in the presence of hyperglobulinemia and proteinuria, respectively, to confirm the presence of a monoclonal gammopathy and identify the class of Ig involved in the disease process. In patients lacking a hyperglobulinemia, serum protein electrophoresis may be insensitive for the detection of M-proteins.[70] In patients experiencing bleeding, a hemostatic profile should also be performed, and serum viscosity measurements considered. In dogs affected by MM, the incidence of IgA and IgG is comparable, whereas in cats, IgG is most commonly involved.[53,69,72] Rarely, occurrences of biclonal gammopathies have been reported and, on occasions, cryoglobulinemia has been described in dogs.[20]

Screening for Bence Jones proteinuria should not be based solely upon urine dipstick because this modality primarily detects albuminuria. The sulfosalicylic acid (SSA) test provides greater sensitivity for globulin detection, but specificity is low due to the concomitant detection of albumin, globulin, Bence Jones proteins, proteases, and polypeptides. In people, false positive results for Bence Jones proteins detected by means of heat precipitation can occur due to excessive amount of polyclonal light-chain proteins in patients affected by a variety of conditions, including non-plasmacytic tumors and chronic renal failure. Therefore, urine protein electrophoresis remains the preferred diagnostic modality for the detection of monoclonal light-chain proteinuria.

Bone marrow infiltration by MM can be quite variable, with many patients having an amount of plasma cells greater than 5%.[79] The appearance of malignant plasma cells can range from normal to mildly pleomorphic to bizarre and atypical forms. Plasma cell morphology can be an important predictor of severity of disease (Figure 70.1).[23] In humans, histopathologic grade is correlated with disease progression, with grade I disease being slow-growing in contrast to grade III disease (plasmablastic-type) having an aggressive behavior.[6]

Survey radiographs are typically recommended to screen for skeletal lesions. These vary from areas of osteopenia observed in early stages of diseases to lytic, "punched-out" lesions typical of later stages. Biopsy and histopathology of a lytic lesion may sometimes be necessary for a definitive diagnosis. Skeletal lesions are rare in patients with Waldenström macroglobulinemia where cancer cells more commonly infiltrate other organs, including liver, spleen, and lymph nodes.[26,34]

In cats, the frequent identification of abdominal organ neoplasia (85% of organs with an imaging abnormality) underscores the need to incorporate abdominal imaging as part of the routine staging (Figure 70.2).[57] In one study where liver cytology was performed in 10 of 15 feline MRD cases, plasma cell neoplasia was confirmed or suspected in 8 cases in the absence of liver enzyme elevation. In the same study, splenic cytology was positive or suspicious for plasma cell neoplasia in 10 out of 12 cases. One cat with confirmed splenic and hepatic involvement had no obvious abnormalities on abdominal ultrasonography.[11]

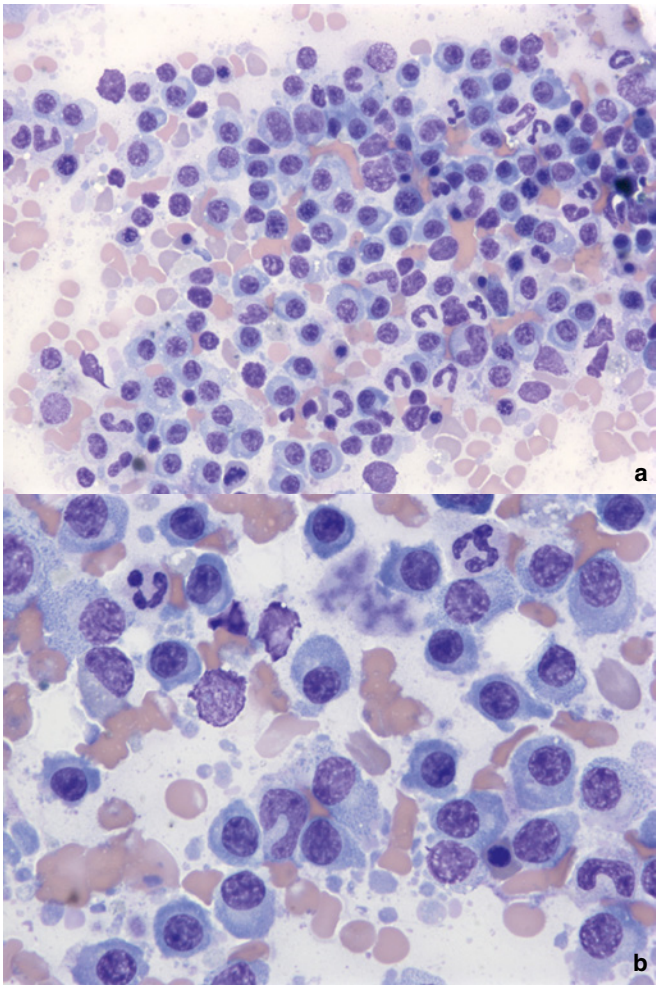


FIGURE 70.1 (a) Canine bone marrow aspirate (500×); numerous well-differentiated plasma cells admixed with late-stage granulocyte precursors and metarubricytes; (b) Canine bone marrow aspirate (1000×); well-differentiated plasma cells. (**Source:** Photomicrographs courtesy of Dr. Laura Snyder, Marshfield Labs.)

A funduscopic examination should be performed to rule out ocular lesions, such as retinal hemorrhages, retinal detachment, venous tortuosity, dilation, sacculation, and blindness. Other conditions associated with monoclonal gammopathies that should be considered as differential diagnoses include lymphoproliferative disorders, such as lymphoma and acute or chronic lymphocytic leukemia, chronic infections (e.g., ehrlichiosis and leishmaniasis in dogs, and feline infectious peritonitis in cats), immune-mediated disorders, and other neoplastic conditions.[62]

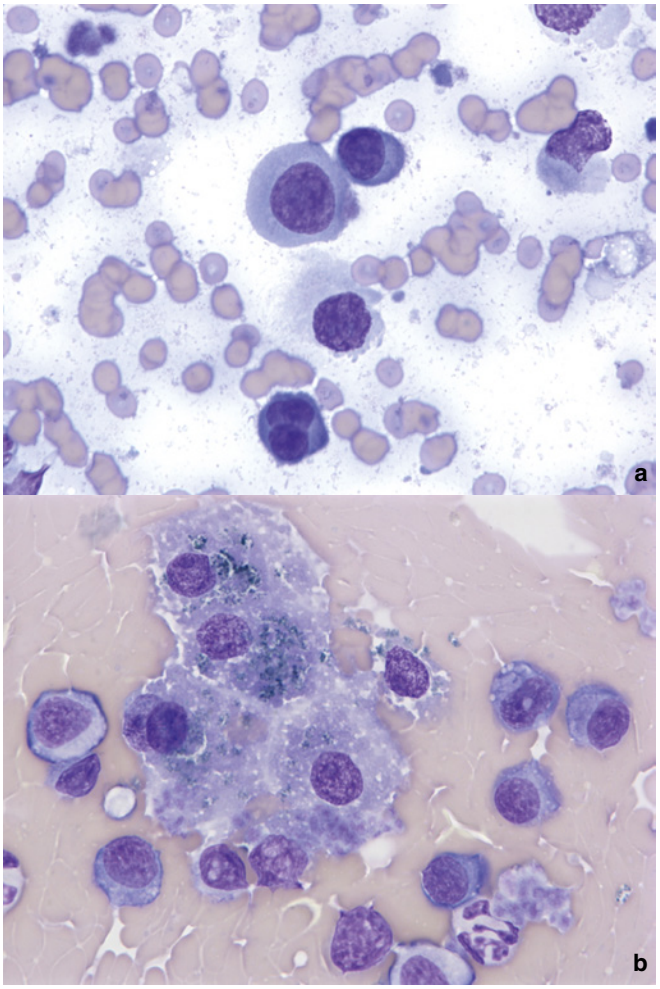


FIGURE 70.2 (a) Feline splenic aspirate (1000×); well-differentiated plasma cell (top center right), large poorly differentiated plasma cell (top center left), plasma cell with bilobed nucleus (bottom); (b) Feline hepatic aspirate (1000×); hepatocytes surrounded by RBCs and plasma cells. (**Source:** Photomicrographs courtesy of Dr. Laura Snyder, Marshfield Labs.)

Treatment

Treatment of MM with oral melphalan and prednisone is considered standard-of-care therapy in dogs. In cats, this combination appears to be more myelosuppressive than in dogs, potentially leading to treatment discontinuation. A recent study comparing the efficacy and toxicity of different chemotherapy protocols for feline MRD indicated that a combination of cyclophosphamide and corticosteroids was well-tolerated and could be considered as first-line treatment for cats with systemic disease.[11] In general, the goal of the treatment in any species is to reduce the bulk of the tumor and the symptoms of disease. Although complete eradication of disease is only rarely achieved, chemotherapy is often effective in decreasing tumor burden, reducing serum Ig levels, promoting bone remodeling, and providing symptomatic relief. Overall, this leads to improved quality and possibly duration of life.[53,54]

Melphalan is an alkylating agent whose oral absorption is unpredictable, requiring administration to be made preferably on an empty stomach. In dogs, melphalan is initially administered at 0.1 mg/kg orally once a day for 10 days, and then at 0.05 mg/kg orally every other day. Prednisone is generally administered at 1 mg/kg for the first 10 days of therapy, and then decreased to 0.5 mg/kg every other day. Treatment is continued indefinitely, until relapse or myelosuppression occurs. Possible myelosuppression is generally monitored via serial complete blood counts performed biweekly for 2 months and monthly thereafter. A pulse-dosing regimen, where melphalan is administered at 7 mg/m^2 orally for 5 consecutive days every 3 weeks, has been used successfully in patients experiencing dose-limiting thrombocytopenia with the more conventional low-dose treatment described in the earlier text. In cats, a dosing schedule similar to the one used in the dog has been reported.[17,31] An alternative protocol uses melphalan at 2 mg/m^2 every 4 days continuously.[57] In a study of feline MRD, melphalan was used at 2 mg/cat every 2–7 days, resulting in discontinuation of therapy in five cases due to grade 3–4 myelosuppression. In the same study, cyclophosphamide was used in some cases as first-line treatment at a starting dose of 25 mg/cat orally twice weekly. There were no cases where treatment was discontinued due to hematologic toxicity.[11]

Cyclophosphamide is an alkylating agent prodrug, which is activated in the liver into 4-hydroxycyclophosphamide and aldophosphamide. It is supplied as a sterile powder for injection and in tablet form for oral use. Due to its platelet-sparing effect, cyclophosphamide may be used in place of melphalan in thrombocytopenic patients, under careful monitoring.

Other alkylating agents including chlorambucil (leukeran) and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU or lomustine) also have been used to treat MM in dogs and cats.[21] Chlorambucil, administered at 0.2 mg/kg orally once daily, has been used successfully for the treatment of IgM macroglobulinemia in dogs. CCNU has been used in cats at 50 mg/m^2 orally every 21 days, resulting in one partial response.[81] Combination chemotherapy protocols incorporating vincristine, carmustine (bis-chloroethylnitrosourea, BCNU), melphalan, cyclophosphamide, and prednisone (VBMCP) or vincristine, melphalan, cyclophosphamide, and prednisone/vincristine, BCNU, doxorubicin, and prednisone (VMCP/VBAP) have been extensively utilized in people, but outcomes are essentially comparable to those of patients treated with prednisone and melphalan alone.

Glucocorticoids have been shown to induce apoptosis in vitro via inhibition of IκB activation and decreased NFκB activity. The administration of high-dose dexamethasone in conjunction with vincristine and doxorubicin (VAD) was investigated in humans with refractory myeloma and resulted in a response rate greater than 50%. Rapid tumor response, alleviation of bone pain, resolution of hypercalcemia, and absence of cumulative damage to bone marrow stem cells were remarkable advantages of this treatment combination.[1,5]

Anecdotally, in dogs, responses of a few months duration have been achieved with a combination of doxorubicin (30 mg/m^2 intravenously every 21 days), vincristine (0.7 mg/m^2 intravenously on days 8 and 15), and prednisone (1 mg/kg orally daily).[79] Rescue approaches have been taken in dogs with refractory myeloma with high-dose cyclophosphamide (limited success) and liposome-encapsulated doxorubicin, which resulted in a durable remission in one dog.[37] A partial response to the tyrosine kinase inhibitor toceranib has been reported in a dog with refractory MM.[42]

The efficacy of interferon (IFN) for the treatment of MM is controversial. While a response rate of approximately 20% was reported in humans with relapsed myeloma, the addition of IFN to standard chemotherapy approaches failed to provide a significant benefit to the overall survival time in a meta-analysis of 2286 patients.[50] A monoclonal antibody-mediated delivery of IFNα has been developed to enhance tumor cell killing in MM and B-cell malignancies and has shown some promise in preliminary studies.[83]

High-dose chemotherapy in association with autologous transplantation using BM or peripheral-blood-derived stem cells (PBSCs) is now widely used for the treatment of hematological malignancies including MM.[22,32] This approach yielded complete remissions in refractory human patients, but mortality rate due to bone marrow suppression was high. Contamination of most bone marrow and blood stem cell samples with neoplastic cells has been shown in MM even after high-dose chemotherapy.[15,16,18,51] The presence of tumor cells within the autograft has resulted in recurrence of disease, emphasizing the need to optimize purging techniques.

In humans with indolent myeloma, treatment is generally withheld until disease progression, symptomatic manifestations, or new lytic bone lesions develop. However, bisphosphonates such as zoledronic acid have been used to prevent or delay the onset of bone lesions and associated bone pain. Denosumab is a human, monoclonal antibody of IgG2 class that binds to RANKL with high specificity and has been recently proposed as an alternative to bisphosphonates for myeloma bone disease.[76]

Treatment of human patients affected by indolent myeloma with the anti-angiogenic agent thalidomide resulted in a 66% response rate and the drug appeared to have potential to delay the onset of clinical signs associated with the disease. The efficacy of thalidomide for the treatment of refractory/relapsed MM has also been confirmed. The combination of thalidomide and dexamethasone has yielded response rates greater than 50% in patients with relapsed myeloma and 70% in newly diagnosed patients.[61,67,80] Studies evaluating the role and possible efficacy of thalidomide for the treatment of MM in pet animals are lacking.

Bortezomib, a proteasome inhibitor, induces apoptosis of MM cells and inhibits their binding to BMSCs, which otherwise would trigger the transcription of IL-6 via an NFκB-dependent pathway.[33] In a multicenter phase II trial of bortezomib, a 35% response rate was achieved with a median duration of remission of 12 months and an overall survival time of 16 months. Addition of dexamethasone to the treatment regimen improved responses in 19% of treated patients.

Response to therapy is assessed based on amelioration of clinical signs as well as improvement in the radiographic appearance of skeletal lesions and clinicopathologic abnormalities.[53] Because complete resolution of MM is extremely rare, a ≥50% reduction in the pretreatment of M-component (Bence Jones proteins or Ig) is considered a good response to therapy.[45]

Patients experiencing severe clinical signs secondary to hypercalcemia and renal impairment, HVS, or pathologic fractures may require additional therapy specifically directed to the clinical complications of disease. For example, fluid therapy and diuretics may need to be instituted for patients with severe hypercalcemia. Similarly, plasmapheresis may be beneficial in minimizing clinical signs of hyperviscosity, while aggressive fluid therapy may be necessary for patients with renal failure.

Prognosis

In humans with MM, the course of disease is extremely variable, with survival times ranging from less than 1 year with aggressive forms to more than a decade in indolent forms. Prognostic variables in people include: (1) tumor burden (serum calcium, immunoglobulin, albumin, α_2-microglobulin, number of lytic bone lesions, percentage of bone marrow plasmacytosis); (2) tumor biology (mitotic rate, IgA myeloma, plasma cell labeling index); (3) tumor microenvironment (bone marrow microvessel density and matrix metalloproteinase 9 level, among others); (4) treatment (12 months or less of prior treatment, achievement of complete remission, tandem transplantation, second transplantation within 6 months); and (5) patient (performance status, albumin level, other unrelated conditions). Beta-2 microglobulin has been consistently identified as a predictor of survival in MM. It is a light-chain protein belonging to the class I of histocompatibility antigens expressed on the surface of nucleated cells and released into the blood. Because beta-2 microglobulin is excreted by the kidneys, its level reflects tumor burden as well as renal function.[13,25]

In humans, a nodular pattern of bone marrow infiltration appears to be associated with a worse prognosis compared to a diffuse pattern.[5] In addition, severity of disease has been correlated with cell morphology. For example, plasmablastic disease has been associated with a more aggressive behavior of the cancer.

In dogs with MM, the short-term prognosis is good in respect to initial tumor control and improvement of quality of life. However, the long-term prognosis is generally poor due to high likelihood of tumor relapse and recurrence of clinical signs as well as development of resistance to chemotherapeutic agents. In a study of 60 dogs with MM treated with melphalan/prednisone combination chemotherapy, 8% did not respond to

therapy; 43% achieved complete remission (defined as normalization of serum Ig); and 49% achieved a partial remission (defined as less than 50% decrease in the pretreatment Ig level). The median survival time was 540 days. Negative prognostic factors identified in this study include presence of hypercalcemia, extensive bone lysis, and evidence of Bence Jones proteinuria.[53]

The reported median survival of cats with MM and other MRDs ranges from 4 months to greater than 1 year. [11,17,31,53,72] In a recent study of feline MRD, median survival was 252 days and 394 days for cats initially treated with melphalan and cyclophosphamide, respectively.[11]

IgM macroglobulinemia has been identified and treated only in a small number of dogs. In nine dogs treated with chlorambucil, 77% achieved remission and the reported median survival time was 11 months.[45]

SOLITARY AND EXTRAMEDULLARY PLASMACYTIC TUMORS

EMP is a neoplastic plasma cell tumor originating from soft tissues in the absence of primary bone marrow disease and both cutaneous (CEMP) and noncutaneous (NCEMP) forms have been described.[40,50] SOP or solitary plasmacytoma of bone (SPB) is an isolated lesion of bone origin with potential to progress to systemic MM.[46,56] Plasma cell tumors account for approximately 2.4% of all canine tumors and most commonly occur at cutaneous sites (86% of cases), followed by mucous membranes of lips and oral cavity (9%), colon, and rectum (8%). Approximately 22–28% of EMPs in dogs are located within the oral cavity.[14] Feline EMPs have been reported to occur in skin, upper lip, gingiva, orbit, gastrointestinal tract, brain, and retroperitoneal space, and their incidence appears to be low.[12,28,43,48,49,58]

The median age of dogs affected by plasmacytomas is 9–10 years and breed predispositions include American Cocker Spaniel, English Cocker Spaniel, and West Highland White Terrier. The median age of affected cats is 10.6 years, and the European Shorthaired Cat is overrepresented.[43,63] Individual cases have been reported in Abyssinian, Persian, and Burmese breeds.

Historically, EMP carries a better prognosis than MM. However, the biological behavior of EMP highly depends upon its anatomic location. For example, EMPs in cutaneous and oral cavity locations are generally associated with a low metastatic rate, and thus may be curable by means of surgery alone. Multicentric oral EMPs have been reported in a small number of dogs, both within the same location and a different location in the mouth.[74] Surgery remains the treatment of choice in these cases, if feasible. Intravascular neoplastic cells have been described in examined sections of canine cutaneous plasmacytomas in one study, more commonly in association with tumors of the distal limbs (36%) compared to other locations (11%), but this finding did not affect prognosis.[19] Progression of cutaneous EMPs to systemic disease has been documented in two cats, one of which developed MM, the other nodal and distant metastatic disease.[12,66] Noncutaneous and nonoral forms of EMPs typically have a more aggressive behavior. Gastrointestinal EMPs, affecting esophagus,[30] stomach,[47] small intestine,[36] and large intestine,[36,39,47] are frequently associated with lymph node metastases, although monoclonal gammopathies and bone marrow involvement are rare. Penile EMP has been reported in two dogs. One dog was treated with surgery and radiation therapy and remained free of disease for >1600 days.[82]

Solitary osseous plasmacytoma has been reported to occur in ribs and zygomatic arch of dogs. Solitary osseous plasmacytoma frequently progresses to MM, although this may take months to years to occur.[47,56]

Clinical Manifestations of Disease

Clinical manifestations are dependent on the anatomic location of the tumor. Cutaneous forms generally manifest as a solitary, raised, alopecic, smooth nodule 1–10 cm in diameter, and clinical signs of illness are typically absent. Bleeding from the mouth was the chief complaint in canine oral EMP. Gastrointestinal EMP is associated with nonspecific signs, whereas colorectal EMP often results in tenesmus, rectal prolapse, rectal bleeding, and hematochezia.[39] Pain, lameness, and neurologic signs may be observed in dogs with SOP affecting the appendicular or the axial skeleton.

Diagnosis

Fine-needle aspiration or biopsy is needed to confirm a diagnosis of SOP or EMP. Reclassification of reticulum-cell sarcomas as plasmacytomas has resulted in an increased incidence of this type of tumor in veterinary medicine.[14]

Plasmacytomas are generally characterized by a monomorphic population of plasma cells with occasional binucleate or multinucleate cells present. A polymorphic blastic plasma cell that appears to be associated with a more aggressive behavior in the dog has also been described.[10,39] Immunohistochemical detection of cytoplasmic Igs with predominant clonality for κ- or λ-chains is a common finding and 97–100% of EMPs are positive for λ-chain reactivity.[2,47] Moreover, monoclonal light-chain reactivity is helpful in differentiating plasmacytomas from other round cell tumors. However, Ig heavy chains (IgG and IgA chains) have been confirmed in other feline tumor cells, which often contain amyloid deposits. [12,43,49,63] Canine plasmacytomas stain positively for IgG, A, or M as well as cluster of differentiation (CD)45, CD18, CD45RA, and CD79a (Mb-1) leukocyte antigens. Immunohistochemical labels utilized in veterinary medicine also include multiple myeloma oncogene 1/interferon regulatory factor 4 (MUM1/IRF4) and CD20. However, the limited availability of antibodies for use in veterinary medicine makes characterization of normal and neoplastic plasma cells difficult. Using a standard flow cytometry panel, normal canine plasma cells typically have low to absent CD21 antigen

expression, and high CD45 and major histocompatibility complex (MHC) class II expressions.[70]

Cutaneous plasmacytoma cells frequently express monoclonal immunoglobulins and vimentin.[19] A divergent pseudoglandular configuration of cutaneous plasmacytoma has been described in dogs where pseudoglandular structures resemble neoplastic acini seen in epithelial tumors. In one study, these neoplastic cells were strongly immunopositive to MUM1 and immunonegative to pancytokeratin AE1/AE3.[55] While the cyclin D1 marker has been identified in MM, no association has been found that relates cyclin D1 to plasmacytomas. Interestingly, the identification of this marker in EMPs has been postulated to be a possible indicator of a highly aggressive malignancy or a metastatic lesion from MM.[10] Lymphocyte clonality testing is often used as an adjunctive tool to characterize lymphoid neoplasms.[77] It has also been utilized to provide evidence in support of a diagnosis of EMP.[70,75] Specifically, clonality testing based on the immunoglobulin heavy chain (IGH) gene has been described in canine and feline plasmacytomas. The sensitivity of detecting IGH clonality was examined in canine cutaneous plasmacytomas and frequency of detection was low, ranging from 0 to 34.5% in one study, possibly due to somatic hypermutation of the IGH locus.[75]

A complete staging should be performed before initiation of therapy to rule out systemic involvement, which, if present, would alter treatment recommendations and prognosis. Diagnostic modalities should include serum protein electrophoresis, skeletal survey radiographs, bone marrow aspiration, and cytology, particularly for SOPs and gastrointestinal EMPs due to their higher metastatic potential.

Treatment

Surgery is considered the treatment of choice for cutaneous plasmacytomas, and, on rare occasions, melphalan and prednisone therapy has been successfully utilized in canine and feline cases where surgery resulted in incomplete tumor margins or local tumor recurrence. Surgery is also recommended in patients with SOPs associated with a pathologic fracture or with neurologic compromise. Spinal cord decompression combined with removal of the primary tumor and spinal cord stabilization may need to be performed in selected patients. The efficacy of radiation therapy in palliating bone pain associated with the tumor itself and/or the presence of pathologic fractures has been reported; however, most patients eventually developed MM.[46,56]

In patients with SOPs and no evidence of systemic involvement, it is debatable as to whether chemotherapy should be instituted at the same time as local therapy. Likewise, gastrointestinal EMPs are managed solely with surgery, unless systemic disease is documented, in which case the incorporation of chemotherapy would be warranted. Mandibulectomy or maxillectomy is recommended for treatment of oral plasmacytomas accompanied by boney infiltration.[14] All dogs with oral plasmacytomas excised with incomplete surgical margins experienced local tumor regrowth in one study.[81] Periodic monitoring and restaging are pivotal in allowing early detection of tumor regrowth and metastatic disease.

Prognosis

Oral, cutaneous, and mucocutaneous plasmacytomas are associated with a good prognosis and can be cured by means of surgery alone, provided complete surgical margins are achieved.[10,81] Anecdotally, multiple plasmacytomas that eventually resulted in death of affected dogs have been reported. Tumor cell proliferation rate (based on Ki-67 staining) and histopathologic grade have not been found to hold prognostic significance in dogs or cats, although a more aggressive behavior has been attributed to a polymorphic blastic type of canine plasmacytoma.[10,48] Cyclin D1 expression and presence of amyloid also do not seem to have clinical significance.[10]

Radiation therapy is an effective treatment option for solitary plasmacytomas. This is most likely due to the high-grade nature of round cell tumors that makes them susceptible to therapy. Long-term survival times have been reported for dogs with gastrointestinal EMPs or EMPs affecting liver, uterus, or other abdominal organs, undergoing surgery and/or chemotherapy.[39,46,47] The median survival time of nine dogs with colorectal plasmacytomas was 15 months;[39] two dogs treated with surgery alone experienced tumor regrowth 5 and 8 months postoperatively. DNA aneuploidy and c-myc oncoprotein content of canine plasmacytomas were analyzed by means of flow cytometry and found to be predictive for survival; however, findings strongly correlated with noncutaneous forms of disease, suggesting that location of the tumor is a prognostic indicator of equivalent importance.[24]

In cats, prognostic information is limited mostly due to the rarity of these tumors and the related paucity of cases reported in the literature. In general, principles of therapy that apply to dogs should be also applied to feline patients.

CUTANEOUS PLASMACYTOSIS

Cutaneous plasmacytosis (CP) is a rare syndrome occurring in humans and recently described in dogs.[8] In people, CP is characterized by multiple cutaneous plasma cell tumors appearing as raised lesions or red-brown plaques without bone marrow, liver, spleen, and lymph node infiltration. Polyclonal hypergammaglobulinemia is a typical finding.[38] CP is generally considered a benign process with indolent behavior; therefore, therapy is usually reserved for patients with clinical signs (such as fever or lymphadenopathy) or compromised quality of life. Both topical and systemic treatments have been utilized for more aggressive cases with limited efficacy.[35]

In a recent study of canine CP, more than 50% of patients had 10 or more lesions, described as pink to red, raised, alopecic or ulcerated. Most patients were asymptomatic.[8] While the degree of staging varied among patients, confirmed or suspected regional lymph node or abdominal visceral involvement were reported in some cases. A polyclonal gammopathy was identified in two of three dogs in which total protein concentrations were elevated.[8] Histologically, tumors were characterized by dense sheets of anaplastic round cells with eccentric nuclei and marked anisocytosis and/or anisokaryosis. This is in contrast with CP in people, which typically consists of an expansion of mature plasma cells. Neoplastic cells were positive to plasma cell immunoreactivity (MUM1, lambda light chain, and CD79).[8] The most common first-line treatment consisted of melphalan chemotherapy with an overall response rate of 74% and a median progression-free interval (PFI) of 143 days. Median PFI after the first treatment for all treatments combined was 153 days. Median survival was 542 days, suggesting a fair to good prognosis with treatment despite a relatively short PFI. Treatment with a combination of an alkylating agent and corticosteroids seems appropriate first-line systemic treatment for cases in which locoregional treatment is inadequate or not feasible.[8]

WALDENSTRÖM'S MACROGLOBULINEMIA

WM is a rare lymphoproliferative, immunosecretory disorder occurring in dogs, cats, and humans. According to the WHO classification scheme, it is considered a form of lymphoplasmacytic lymphoma that involves spleen, bone marrow, and lymph nodes and can manifest with IgM-related signs of cryoglobulinemia or hyperviscosity.[7] This description corresponds to the lymphoplasmacytic type of immunocytoma incorporated into the Kiel classification and to the revised European American Lymphoma classification (REAL) lymphoplasmacytic lymphoma/immunocytoma. A consensus has not yet been reached among pathologists regarding the new classification, most likely due to the subjectivity involved in the distinction between small or plasmacytoid lymphocytes and plasma cells.

Waldenström's macroglobulinemia is characterized by bleeding tendencies, anemia, lymphadenopathy, and high-serum viscosity with a high-molecular-mass M-component, and is associated with a monoclonal expansion of B cells infiltrating the bone marrow or other lymphoid organs producing an IgM serum paraprotein. IgM consists of high relative molecular mass and positively charged proteins that can bind to RBCs leading to rouleaux formation. Waldenström's macroglobulinemia differs from MM in that bone lysis is generally absent and hepatosplenomegaly, lymphadenopathy, or both are frequently encountered clinical findings. Peripheral neuropathy, HVS, and HVS-associated complications, including retinal hemorrhage, epistaxis, cardiac and renal damage, and central nervous system abnormalities, can occur. In addition, IgM can cause immune-mediated disorders such as cryoglobulinemia, cold agglutinin hemolytic anemia, and peripheral neuropathies secondarily to autoantibody activity.[27,60] Bleeding diathesis has been reported to occur in approximately 33% of affected dogs and it is suspected to derive from the interference of IgM with clotting factors and/or platelet aggregation.[45] Possible laboratory findings in both humans and dogs include anemia, pancytopenia, leukocytosis or leukopenia, hypercalcemia, IgM hypergammaglobulinemia, and Bence Jones proteinuria.[27] In addition to WM, the production of IgM paraproteins has been reported in association with MM, chronic lymphocytic leukemia, splenic marginal zone lymphoma, extranodal marginal zone lymphoma, plasmacytoid lymphoma, and non-neoplastic conditions such as leishmaniosis.[7,27,45]

In human patients, findings that may aid in differentiating WM from other non-neoplastic monoclonal gammopathies or MGUS include the degree of bone marrow infiltration and the amount of M-component, and it has been suggested that the minimum threshold of IgM level is 30 g/L and the percentage of plasma cells in bone marrow is 20%.[40] Diagnostic features of this disease in dogs are still a matter of debate. Immunophenotyping and serum immunofixation electrophoresis (IFE) are often necessary to diagnose lymphoproliferative disorders more definitively in pet animals.

Treatment of WM is unlikely to provide a cure; rather, it is directed at diminishing tumor burden and alleviating clinical signs associated with the disease. Approximately 77% of dogs with IgM macroglobulinemia respond favorably to prednisone therapy combined with alkylating-agent-based chemotherapy, and median survival times range from 11 months to 14 months.[52] Chlorambucil is most frequently used in conjunction with prednisone, but melphalan and cyclophosphamide have also been shown to have activity against IgM macroglobulinemia.[27,40]

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CHAPTER 71

Hodgkin and Hodgkin-Like Lymphoma

DANIEL A. HEINRICH and ERIN N. BURTON

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Acronyms and Abbreviations

HRS, Hodgkin/Reed–Sternberg; LP, lymphocyte predominant; WHO, World Health Organization; HL, Hodgkin lymphoma; CHL, classic Hodgkin lymphoma; NLPHL, nodular lymphocyte predominant Hodgkin lymphoma; HLL, Hodgkin-like lymphoma; HIV, human immunodeficiency virus; RS cell, Reed/Sternberg cell.

INTRODUCTION

Hodgkin lymphoma (HL) is a human lymphoid neoplasm most commonly affecting cervical, axillary, and inguinal lymph nodes. HL encompasses a number of subtypes, which are all characterized by variable numbers of a morphologically unique population of large and often multinucleated neoplastic cells. The nomenclature of these cells can be cumbersome and requires clarification. These cells, based upon their morphology, are termed lymphocyte predominant (LP) cells, Hodgkin cells, or Reed–Sternberg (RS) cells. Based upon morphologic similarities, the latter two are often combined and denoted as Hodgkin/Reed–Sternberg (HRS) cells. Similarly, LP cells may be referred to as lymphocytic and histiocytic (L&H) cells. Importantly, these cells are often surrounded by robust inflammation and represent a small component of the overall cell population.[6,7,21,22] In humans, viral disease such as Epstein–Barr virus and human immunodeficiency virus (HIV) have been implicated as causative agents for classical Hodgkin lymphoma (CHL).[7,21]

HISTOLOGY OF HUMAN HODGKIN LYMPHOMA

There are multiple classification schemes and thus subtypes of Hodgkin lymphoma. Like non-Hodgkin lymphoma, these subtypes differ in histomorphology, clinical characteristics, and immunophenotype. However, in all cases, the neoplastic HRS or LP cells are germinal center B-cells.[21,22] HRS cells and their lacunar variants (see in the following text) are found in CHL, whereas LP cells are found in nodular lymphocyte predominant Hodgkin lymphoma (NLPHL). On histologic preparations, RS cells are large cells with a moderate to often high nuclear to cytoplasmic ratio, blue-pink cytoplasm, and one or more lobulated nuclei with a single large nucleolus (Figure 71.1). Some binucleated cells appear to have mirror-image nuclei. Hodgkin cells are a variant of RS cells, but have a single, nonlobulated nucleus. Lacunar variants of HRS cells are also described and are an artifactual subtype related to tissue processing leaving thin, filamentous cytoplasmic projections to

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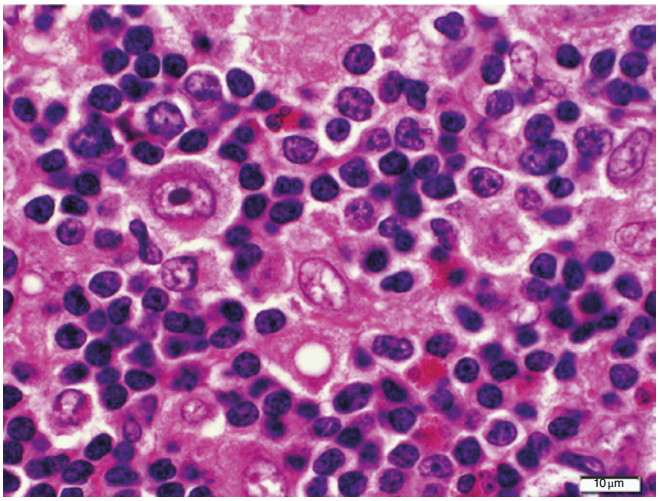


FIGURE 71.1 Human lymph node with CHL. HRS cells are noted among an inflammatory background of small lymphocytes and eosinophils. HRS cells are large and uninucleate to binucleate with large nuclei with open chromatin and often a prominent nucleolus. (**Source:** Slides from Cornell tutorial.)

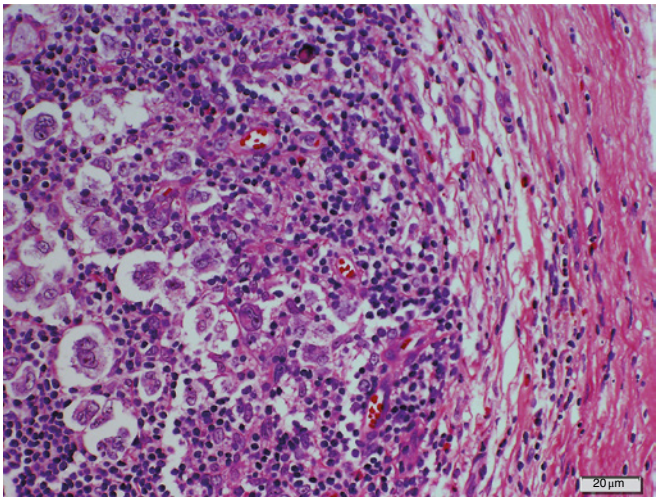


FIGURE 71.2 Human lymph node with nodular sclerosis CHL. Foci of lymphoid proliferation are accompanied by large numbers of lacunar-type Reed–Sternberg cells (left center; note areas of peripheral clearing). Note the prominent nucleoli in several of the Reed–Sternberg cells. The right side of the image is made up of broad bands of dense mature collagen separating the lymphoid nodules. H&E stain. (**Source:** Slides from Cornell tutorial.)

the cell margin and a clear space reminiscent of osteocytes in lacunae (Figure 71.2).[7,21] LP cells are smaller than HRS cells, have a moderate to high nuclear to cytoplasmic ratio, and have single lobulated nucleus. The lobulated nucleus of the LP cell explains why these cells are often termed "popcorn cells" (Figure 71.3).[6,22] HRS and LP cells can be misidentified as macrophages or histiocytic cells and, thus, immunophenotyping may be required for characterization.

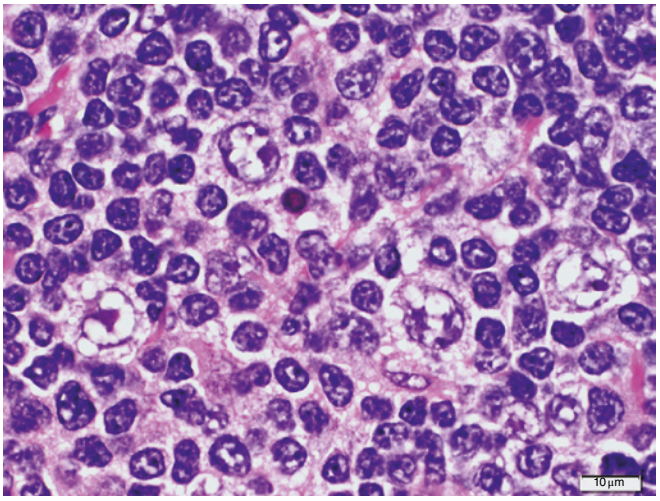


FIGURE 71.3 Human lymph node with NLPHL. Note many LP cells with a "popcorn cell" appearance among small to intermediate lymphocytes. IHC is necessary to differentiate lobulated RS cells and LP cells in some instances. H&E stain. (**Source:** Slides from Cornell tutorial.)

CLASSIFICATION OF HUMAN HODGKIN LYMPHOMA

The World Health Organization (WHO) classification of Hodgkin lymphoma includes two unique types: (1) CHL and (2) NLPHL. CHL contains four subtypes: (1) nodular sclerosis CHL; (2) mixed-cellularity CHL; (3) lymphocyte-rich CHL; and (4) lymphocyte-depleted CHL.[21] Each of these types and subtypes requires coordination of clinical and histological findings for diagnosis. Sometimes immunohistochemical (IHC) and other molecular techniques may be needed for an accurate diagnosis. A brief description of each type and subtype is provided in the following text; however, in-depth detail of the human disease is beyond the scope of this chapter and the reader is referred elsewhere for more information.[7,21]

Classic Hodgkin Lymphoma

CHL is characterized by low numbers of HRS cells within a background of mixed inflammatory cells.[1,21] Affected patients frequently demonstrate enlargement of cervical lymph nodes, although other peripheral lymph nodes can be involved. The disease is often slow growing and biologically indolent, but multiorgan metastasis (with infrequent bone marrow involvement) is described. Patients uncommonly present with primarily extranodal disease.[7]

Nodular Sclerosis CHL

This is the most common subtype of CHL, comprising more than 60% of cases. Affected patients present with enlargement of lymph nodes located above the diaphragm.[7] Histologically, this subtype is characterized by broad bands of collagenous connective tissue

separating nodular areas of lymphoid proliferation. The neoplastic RS cells are relatively numerous and are of the lacunar-type. These cells frequently have eosinophilic cytoplasm as the cells undergo necrosis. The nonneoplastic population consists of variable percentages of small and medium lymphocytes, numerous eosinophils, and neutrophils dispersed in a fine supporting fibrovascular network. In the most recent WHO classification, nodular sclerosis CHL was subdivided into two grades based upon the magnitude of lymphocyte depletion and percentage of anaplastic lacunar cells.[7,22]

Mixed-Cellularity CHL

This is the second most common subtype of CHL. Affected patients often present with systemic signs and disease.[7] Histologically, the minority population of RS cells tend to be of the classic type with binucleation and prominent nucleoli and the inflammatory infiltrate comprises a mixture of small lymphocytes with variable numbers of neutrophils, eosinophils, plasma cells, and macrophages. There is only fine fibrosis, but foci of necrosis are frequently present.[7]

Lymphocyte-Rich CHL

This is a recently described subtype of CHL, but shares some similarities with NLPHL.[7] It is characterized by a diffuse or nodular lymphoid proliferation with infrequent classical RS cells. In some instances, cells similar in morphology to LP cells can be present, necessitating IHC to differentiate lymphocyte-rich CHL from NLPHL in these cases.[7]

Lymphocyte-Depleted CHL

This is the least common subtype of CHL, representing approximately 1% of cases. This subtype affects older individuals that present with systemic signs and extensive, below-the-diaphragm, organ involvement, involving the liver, spleen, retroperitoneal lymph nodes, and bone marrow. Architecturally, the tissues are characterized by diffuse fibrosis of nonmature collagenous connective tissue with cell depletion mainly involving small lymphocytes. The RS may occur in clusters and in some cases display anaplastic features.[7,21]

Nodular Lymphocyte Predominant Hodgkin Lymphoma

NLPHL is a recently characterized subtype of Hodgkin lymphoma that demonstrates unique epidemiological, histological, immunological, and clinical characteristics. According to previous classification schemes, NLPHL may have been classified as lymphocyte-rich CHL. Patients generally present with persistent, slowly progressive peripheral lymph node enlargement, commonly affecting the axillary and cervical lymph nodes. Histologically, there is widespread disruption of the lymph node architecture by a diffuse, nodular, or a mixed infiltrate of small lymphocytes, histiocytes (some with epithelioid characteristics), and LP cells.[6,7,21,22] Six immunoarchitectural patterns of NLPHL are recognized and the reader is referred elsewhere for their description.[5,6,22] The LP cells are often present in easily identifiable numbers, but can be rare in some instances. Although typical LP cells are morphologically distinct from HRS cells, immunohistochemistry may be necessary for definitive differentiation.[6,7,21]

IMMUNOPHENOTYPE AND IMMUNODIAGNOSTICS

CD15 and CD30 are often useful and the most recognizable antigens used in the diagnosis of Hodgkin lymphoma.[7] The IHC detection of both antigens on HRS cells is expected in most cases of CHL. It is important to note that CD30 expression can be found in other lymphoid neoplasms and coexpression of CD15 can help improve specificity. Despite their B-cell origin, CD20 and CD79 positivity is infrequent in HRS cells, although PAX-5 is expressed in most cases of CHL. CD45 expression is generally absent in HRS cells. In NLPHL, the LP cells may demonstrate low to absent expression of CD30 and CD15 with consistent expression of CD20, CD79, and CD45.[6,7] Numerous additional IHC markers have been evaluated and the reader is referred elsewhere for this information.[6,7,10] The neoplastic B cells from both CHL and NLPHL demonstrate clonal somatic immunoglobulin gene rearrangement.[7,14,21]

TREATMENT AND PROGNOSIS

Prognosis is excellent for patients with CHL, with cure rates over 80%. Radiation therapy and surgical debulking are highly effective, although adjuvant chemotherapy may be needed. For NLPHL, there is disagreement regarding its optimal treatment and survival rates are quite variable, depending on stage and age at diagnosis. However, overall prognosis is considered good.[6] For more detailed treatment and prognostic information, the reader is referred elsewhere.[21]

DIFFERENTIAL DIAGNOSES

Histological mimics of CHL include subtypes of non-Hodgkin lymphoma, inflammatory/reactive diseases (such as infectious mononucleosis), and certain nonlymphoid neoplasms (e.g., metastatic carcinoma or melanoma). Specific neoplastic histologic differentials for CHL include B-cell lymphoma unclassifiable with features consistent with gray-zone lymphoma, primary mediastinal large B-cell lymphoma, diffuse large B-cell lymphoma, NLPHL, and anaplastic large-cell lymphoma. Extensive information is available on differentiating these from CHL.[6,7,21]

For NLPHL, histologic differentials include follicular lymphoma, peripheral T-cell lymphoma—not otherwise specified, lymphocyte-rich CHL, and follicular hyperplasia with progressively transformed germinal centers.

T-cell-rich/histiocyte-rich large B-cell lymphoma (TCRBCL) is an additional histologic differential for both CHL and NLPHL. Genotyping can be useful to differentiate it from CHL, but a distinction from NLPHL can be more difficult. Some have proposed that T-cell-rich/histiocyte-rich large B-cell lymphoma may exist on a continuum with, or represents an aggressive transformation of, diffuse NLPHL.[6,7] Notably, a similar dilemma exists in animals, in which it is unclear if T-cell-rich large B-cell lymphoma and Hodgkin-like lymphoma (HLL) are identical or distinct entities. Unfortunately, many antibodies used to distinguish these neoplasms in humans are not readily available in animals.

HODGKIN-LIKE LYMPHOMA IN ANIMALS

In animals, there is no definitive evidence for the existence of Hodgkin lymphoma as a distinct lymphoma subtype. Importantly, this absence of evidence, which in part results from a lack of a specific repertoire of diagnostic antibodies and the recognition that animal cases do not align with the human Hodgkin lymphoma scheme, should not be considered evidence of absence.[24] There are a number of case series and case reports in which affected animals demonstrate disease characteristics similar to Hodgkin lymphoma. Given this diagnostic uncertainty, affected animals are appropriately diagnosed with HLL. With only limited studies on species-specific reagents, the diagnosis of HLL in animals requires the constellation of clinical presentation, cytology, histology, and immunohistochemistry.[3,17]

Further muddying the diagnostic waters, the distinction between HLL and TCRBCL in animals is unclear. In humans, immunophenotyping and other molecular diagnostics are important tools used to make this distinction, particularly as TCRBCL can have large multinucleated cells resembling HRS and LP/L&H cells.[17,23] CD15 and CD30 expression patterns, among other IHC markers and genotypic evaluation, are helpful for this differentiation, but these are not all available in veterinary medicine.[23] In cats, one study has evaluated the preliminary use of CD30 in feline lymphoma, including its potential for the differentiation of HLL and NLPHL or TCRBCL; however, this is a preliminary study with only seven of 60 total lymphoma cases being diagnosed as either HLL, NLPHL, or TCRBCL and lacking clinical follow-up.[3] A single study identified CD15 and CD30 positive Reed–Sternberg-like cells in a dog with pulmonary lymphoid granulomatosis, concluding these atypical lymphoid cells were immunohistochemically similar to those in human primary pulmonary HL.[17] At the time of this chapter's preparation, a clear distinction between these two entities has not been realized and, as such, this review will focus on HLL. Among veterinary species, HLL is best described in cats, although partial and single animal reports have described HLL in dogs, horses, and ferrets.[2,4,9,11,12,15,16,20,23,25,27,28,30] There are rare case reports of HLL in nondomestic species.[13,19,29]

Cats

In published reports, the distinction between feline HLL and TCRBCL is not always clear and it is uncertain whether these truly represent two distinct lymphoma subtypes.[23,24,27] Notably, the authors of the largest (20 cases) review of feline HLL highlight that previous publications of feline TCRBCL report cases that are similar to human CHL and NLPHL.[4,20,27] Cats with HLL, similar to humans with HL, typically present with solitary, slowly evolving lymph node enlargement (often cervical) that may extend to contiguous lymph nodes (commonly prescapular or mediastinal lymph nodes).[27]

Single case reports of cats with an atypical clinical presentation of HLL based on the identification of RS-like cells outside the cervical or contiguous nodes on cytological or histological evaluation are reported. Specific examples include conjunctival, subcutaneous with spontaneous regression of disease, and primary cerebellar HLL.[9,16,30] It is challenging to determine from these single case reports if these are separate subtypes or later-stage presentations of HLL.

Cytological and Histological Findings

The lymph node cytology of feline HLL is characterized by a heterogeneous lymphocyte population that consists primarily of small- and intermediate-sized lymphocytes with scattered plasma cells, vacuolated macrophages, and small numbers of large RS-like cells. The RS-like cells are discrete, large (20–40 μm diameter), and contain a moderate amount of pale amphophilic to basophilic cytoplasm. These cells are binucleated or multinucleated with fine to coarsely granular chromatin and multiple prominent nucleoli (Figure 71.4).[23] Importantly, the lack of RS-like cells on cytologic preparations should not exclude a diagnosis of HLL, as they are a minor component of the lymphoid population and may be missed on sampling.[27] Accordingly, HLL can be challenging to diagnose by cytology alone. In these cases, the aspirates are often interpreted as reactive lymphoid hyperplasia with occasional histiocytes or "histiocytic" cells.[23,27]

Histology is necessary to definitively diagnose HLL (in cats and other species). Across all morphologic subtypes, the histology of feline HLL is analogous to human HL. Specifically, four histologic subtypes of feline HLL have been described that are similar to their human HL subtypes, namely: NLPHL, mixed-cellularity CHL, lymphocyte-rich CHL, and nodular sclerosis CHL.[23,27] However, these subtypes did not perfectly align with current or previous human histomorphologic classification systems.[23,27]

The neoplastic RS-like and LP/L&H-like cells of feline HLL comprise a minor component of the overall background inflammatory population. In feline HLL, the RS-like cells are morphologically similar to human HRS cells

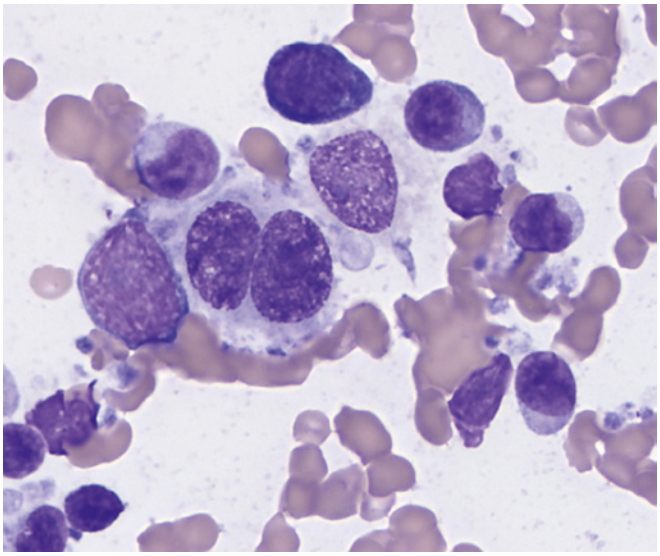


FIGURE 71.4 Fine-needle aspirate cytology sample from a cervical mass in a cat. Binucleate and large RS-like cell among small, intermediate, and large lymphocytes. Diff-Quik stain, 100× objective. (**Source:** Provided by Dr. Jennifer Steinberg.)

and the LP/L&H-like cells are similar to human LP cells. RS-like cells are large discrete cells (25 µm diameter) with a single large, pale, vesicular nucleus or larger (30–40 µm diameter) binucleated and multinucleated cells (up to five to six nuclei occasionally in rings) with sparse cytoplasm and rare mitosis (Figure 71.5).[23] LP/L&H like-cells display a delicate, multilobed nucleus resembling popped or exploded popcorn kernels (i.e., "popcorn cells").[23,27]

Immunohistochemistry is vital to the diagnosis of HLL.[23] In veterinary medicine, given the lack of general availability of CD15 and CD30, the detection of the RS-like cells, and thus the diagnosis of HLL, is based upon the identification of cells with HRS-like morphology that lack expression of CD18, CD3, CD20, and CD79a. One study evaluated the potential use of CD30 in the identification of RS-like cells and in the differentiation of classical HLL, NLPHL and TCRCBL in cats with initial positive results for the identification of RS-like cells and HLL, although further evaluation is needed.[3] LP/L&H-like cells are documented based on their morphologic characteristics and expression of CD20, CD45, and CD79a. The latter immunophenotype distinguishes them from HRS-like cells.[23,27]

Transformation and Prognosis

HLL in cats is largely considered an indolent disease. In one study, evaluating 20 cats with HLL, survival times in untreated cats ranged from months to 4 years.[27] However, additional studies and large case sets are needed to better define disease progression.

Dogs

In dogs, owing to the sparse published literature, there is limited understanding of the natural biology of canine HLL.[12,28] In one case report, a 3-year-old dog presented with cervical lymphadenopathy, vomiting, and diarrhea. Two months after presentation, the dog was euthanized, and necropsy identified numerous masses. Histologically, the masses were composed of a minority population of HRS-like cells among a majority background of small lymphocytes, globular leukocytes, eosinophils, and neutrophils. The HRS cells had inclusion-like and prominent nucleoli and mirror cells were identified. The HRS cells were negative for CD30 and CD15, but despite this the dog was diagnosed with HLL based on the appearance of the HRS cells.[12] In comparison to cats, dogs tend to have systemic involvement, including liver, spleen, lungs, and skin. Although additional cases are needed to better understand this disease process in dogs.[26]

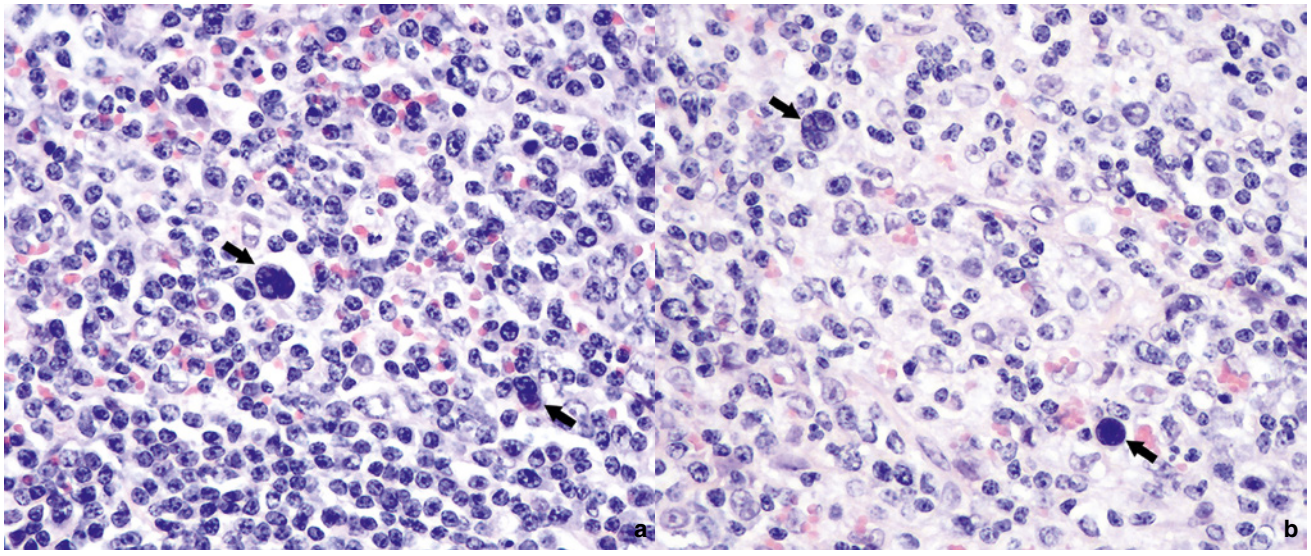


FIGURE 71.5 (a) and (b) Cervical mass histopathology from the same cat as Figure 71.4. Note large, binucleated, or multinucleated Reed–Sternberg-like cells (arrows) scattered among intermediate lymphocytes. H&E stain, 40× objective. (**Source:** Provided by Dr. Jennifer Steinberg.)

Horses

No explicitly diagnosed cases of HLL have been published in the horse. However, there are two publications of equine TCRBCL which have been erroneously cited as HLL.[8,11,16] Across both reports, affected horses commonly demonstrated subcutaneous disease with rare peripheral lymph node involvement. Histologically, the neoplastic infiltrate was composed of a minor population of large, pleomorphic B cells with large and often multiple nuclei with a vesicular chromatin pattern and multiple and prominent nucleoli. The more numerous background lymphocyte population was predominated by small T cells.[8,11]

Other Species

Other species with in which HLL has been reported comprise ferrets, skunks, a killer whale, transgenic mice, and rats.[2,13,18,19,29] Most of the affected animals had multifocal lymphadenopathy with HRS-like cells. The transgenic mice, interestingly, had HRS-like cells that were CD30 positive, had B-cell-derived IgH gene rearrangements, and somatic hypermutations that occur in germinal center B cells, similar to human HRS cells.[18]

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CHAPTER 72

T-Cell Tumors

NARIMAN DERAVI, STEFAN KELLER, and DOROTHEE BIENZLE

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  Classification of T-Cell Neoplasms in Dogs
  Precursor T-Cell Neoplasms
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  Lymphoblastic T-Cell Lymphoma
  Mature T-Cell Neoplasms
    Chronic Lymphocytic Leukemia
    Peripheral T-Cell Lymphoma, Not Otherwise Specified
    T-Zone Lymphoma
    Cutaneous T-Cell Lymphoma
    Enteropathy-Associated T-Cell Lymphoma
    Subcutaneous Panniculitis-Like Lymphoma
    Anaplastic Large Cell Lymphoma
    Intravascular Large T-Cell Lymphoma
    Extranodal T-Cell Lymphoma, Nasal Type
    Hepatosplenic T-Cell Lymphoma
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Feline T-Cell Neoplasms
  Precursor T-Cell Neoplasms
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    T-Cell Chronic Lymphocytic Leukemia
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    Cutaneous T-Cell Lymphoma
    Angioimmunoblastic T-Cell Lymphoma
    Extranodal T-Cell Lymphoma, Nasal Type
    Feline Intravascular Large T-Cell Lymphoma
Equine T-Cell Neoplasms

Acronyms and Abbreviations

ALL, acute lymphocytic leukemia; CI, confidence interval; CLL, chronic lymphocytic leukemia, CTCL; cutaneous T-cell lymphoma, DLBCL, diffuse large B-cell lymphoma; EATL, enteropathy-associated T-cell lymphoma; FeLV, feline leukemia virus; HC-TCL, hepatocytotropic T-cell lymphoma; HS-TCL, hepatosplenic T-cell lymphoma; IHC, immunohistochemistry; LBBCL, lymphoblastic B-cell lymphoma; LBTCL, lymphoblastic T-cell lymphoma; MHCII, major histocompatibility class II; MF, mycosis fungoides; MUM, multiple myeloma; NLPHL, nodular lymphocyte predominant Hodgkin lymphoma; PR, pagetoid reticulosis; PTCL, peripheral T-cell lymphoma; PTCL-NOS, peripheral T-cell lymphoma, not otherwise specified; THRLBCL, T-cell/histiocyte-rich large B-cell lymphoma; LGL, T-cell large granular lymphocytic lymphoma; TCR, T-cell receptor; TCRBCL, T-cell-rich B-cell lymphoma; TZL, T-zone lymphoma; WHO, World Health Organization.

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INTRODUCTION

Compared to B cells, T cells are more diverse and have a multitude of functions including killing of infected/neoplastic cells, cytokine production, and immunoregulation. T cells are highly heterogeneous, and more than 100 antigens characterize different types with unique functions and anatomic locations. Each individual lymphocyte is antigen-specific and undergoes clonal expansion after responding to cognate antigen. Considering enormous clonal expansion and concomitant changes in gene expression during differentiation, lymphocytes may be particularly prone to acquire chromosomal translocations and oncogenic mutations that result in inappropriately regulated proliferation manifesting in neoplasms such as lymphoma or lymphocytic leukemia.[76]

Proliferations of B and T cells can be differentiated using flow cytometry and/or immunohistochemistry (IHC) to detect lineage-specific surface and/or cytoplasmic antigens. With IHC, T-cell neoplasms are defined by detection of CD3 (the T-cell signal transducer) and absence of B-cell antigens such as CD20, CD21, CD79a, and PAX5, and B-cell neoplasms by the converse constellation of antigens.[158,172] Formalin exposure alters epitope accessibility and conformation; and most antibodies directed to canine or feline lymphocyte epitopes recognize only native antigens on nonfixed cells such as detected in flow cytometry or frozen sections. Therefore, molecules characteristic of T-cell subtypes, such as CD4, CD8, and the T-cell receptor (TCR), are currently only identifiable on unfixed specimens. Major histocompatibility class II (MHCII) is expressed on lymphocytes but not neutrophils, while CD45, the common leukocyte antigen, is expressed on all leukocytes, and CD34 on leukocyte precursors (and non-leukocytes).[36,71,111,169]

Neoplastic T cells are characterized by clonal rearrangement of genes coding for the TCR, which can be detected by high-resolution analysis of DNA amplified from specific segments of the V, D, and J genes comprising the TCR. By itself, detection of clonally rearranged TCR (or B-cell receptor) genes may be insufficient to diagnose T-cell neoplasia, since cross-lineage rearrangement of TCR genes may occur in lymphocytes phenotypically of B-cell type, non-neoplastic T-cells in older dogs, and acute myeloid leukemia cells in dogs.[67,68,72,144,153] The fidelity of lineage-specific antigen detection on neoplastic T cells may be variable. For example, loss of cell surface CD3 may be observed in T-cell neoplasms positive for CD4, CD5, or CD8.[36,71] These findings emphasize the importance of diagnosing lymphoid neoplasms by appropriate combinations of microscopic assessment, immunophenotyping via a panel of antibodies, and assessment of clonality.

CANINE T-CELL NEOPLASMS

Compared to B-cell lymphoma, T-cell lymphoma is less common in dogs, accounting for 20–40% of all lymphomas.[105,116,126,153,158,163] Acute lymphocytic leukemia (ALL) is a rare diagnosis in dogs, and current information is based on individual case reports or retrospective small case series that lack assessment with a uniform set of diagnostic tests. Therefore, although T-acute lymphoblastic leukemia (T-ALL) was more commonly reported than B-acute lymphoblastic leukemia (B-ALL), evidence for higher frequency of either type is limited.[119,145,151,161,165] Among chronic lymphocytic leukemia (CLL) types, T-cell lineage is more common than B-cell lineage.[28,151,177]

There is genetic variation in the predisposition to B- versus T-cell lymphoma among breeds. For example, Basset Hounds and Cocker Spaniels are more likely to have B-cell lymphoma, while Boxers and Irish Wolfhounds are more likely to have T-cell lymphoma.[88,105,123]

Classification of T-Cell Neoplasms in Dogs

In 1994, the International Lymphoma Study Group proposed a revised World Health Organization (WHO) classification for human lymphoid neoplasms that combined morphologic, immunologic, and genetic assessment.[64] Revisions in 2001, 2008, and most recently 2016 have incorporated clinical features and new genetic and molecular knowledge.[149,150]

This WHO classification of human lymphoid neoplasms was adapted for dogs, and a similar classification that considers tissue architecture, location, and a limited array of IHC findings arose from a consensus study.[49,157,158,163] Accordingly, canine lymphoid neoplasms are also categorized broadly into precursor lymphoid, mature B-cell, mature T-cell, and natural killer (NK) cell neoplasms, and further classified into subtypes (Table 72.1).[150] Multicentric lymphoma is the most common form of T-cell lymphoma in dogs, and includes peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS), T-zone lymphoma (TZL), and T-cell lymphoblastic lymphoma (TLBL). Other relatively common T-cell neoplasms in dogs are T-chronic lymphocytic leukemia (T-CLL), epitheliotropic cutaneous T-cell lymphoma (CTCL), and enteropathy-associated T-cell lymphoma (EATL).

Precursor T-Cell Neoplasms

T-ALL and TLBL are precursor T-cell neoplasms with tumor burden predominantly in bone marrow, or lymph nodes and thymus, respectively. According to the WHO description, in humans a T lymphoblast is a small to medium cell with one or several nucleoli, scant cytoplasm that sometimes contains vacuoles, commitment to T-cell lineage as indicated by expression of CD3 and rearrangement of the TCR, and absence of differentiating antigens such as CD4 and CD8.[150,158] In veterinary medicine, the term lymphoblast is sometimes used to describe large lymphocytes with nucleoli.[12] This is inconsistent with the WHO nomenclature, and also inconsistent with the morphologic appearance of cells in cases of T-ALL (Figure 72.1a) in dogs. Therefore, it is recommended that lymphocytes be described according to morphologic appearance, and that the term "lymphoblast" be reserved

TABLE 72.1 The main T-cell lymphoma types in dogs according to the WHO classification.

Entity	Clinical features	Cytopathology	Histopathology	Immunophenotype[b]	Prognosis
Precursor cell neoplasms[a]					
T-ALL	• Usually bicytopenia or pancytopenia • Usually marked lymphocytosis	• Small to medium cells with one or several small nucleoli • Scant amount of cytoplasm	• Extensive bone marrow involvement • Myelophthisis	$CD34^+$, $CD3^+$, $CD4^\pm$, $CD5^\pm$, $CD8^\pm$, $MHCII^-$	• Limited data • Likely guarded
LBTCL	• Most often nodal followed by mediastinum or intestine • Frequent hypercalcemia	• Irregular-shaped nuclei, inconspicuous nucleoli • Frequent mitoses	• Diffuse effacement of lymph nodes • Irregular nuclei, frequent mitoses	$CD3^+$, $CD4^+$, $CD5^\pm$, $CD8^\pm$, $MHCII^{-/low}$ TCR αβ	• 1–4 weeks without therapy • 3–5 months with combination chemotherapy
Mature cell neoplasms					
CLL	• Mild to marked lymphocytosis • Cytopenias are rare • Often incidental diagnosis	• Small to medium cells • Nucleoli inapparent • May have cytoplasmic granules	• Splenomegaly due to hypercellular and expanding red pulp	$CD3^+$, $CD4^\pm$, $CD5^\pm$, $CD8^\pm$, $MHCII^+$, TCR αβ > γδ	• Survival typically years after diagnosis without therapy • Anemia and/or marked lymphocytosis at diagnosis are negative prognostic factors
PTCL-NOS	• Most often peripheral node enlargement • Variable duration of enlargement • May have hypercalcemia	• Pleomorphic cell appearance • Mostly medium cell size • Often pale cytoplasm • May have variable nuclei	• Diffuse lymph node effacement with variable extracapsular extension	$CD3^+$, $CD4^\pm$, $CD5^\pm$, $CD8^\pm$, $MHCII^{low/+}$	• Survival highly variable • CD4–/CD8+/MHCII+ and CD4–/CD8–/MHCII+ had better prognosis than other types
TZL	• Most often single enlarged node • Slowly progressive enlargement • Mild lymphocytosis	• Small to medium cells with dense nuclei • Characteristic unilateral cytoplasmic extension	• Paracortical expansion • Remnant small follicles at periphery of node • Cleaved or indented nuclei	$CD3^+$, $CD4^\pm$, $CD5^\pm$, $CD8^\pm$, $MHCII^+$, $CD45^-$, $CD21^{low/mod}$	• Survival typically >1 year without therapy • Survival may be >3 years
CTCL	• Skin or mucocutaneous junction nodules • Ulceration, erythema, hypopigmentation	• Pleomorphic cells • Frequent nuclear lobation, indentation	• Epitheliotropism in MF and PR • MF involves dermis • Epidermal changes • Non-epitheliotropic form is less common	$CD3^+$, $CD4^-$, $CD5^\pm$, $CD8^+$, $MHCII^+$, TCR αβ or γδ	• Limited data • 5–7 months with chemotherapy • 3–5 months without chemotherapy
HS-TCL	• Clinically ill patient • Peripheral lymph nodes not enlarged • Fever, anorexia • Anemia ± thrombocytopenia	• Medium to large cells • Cytoplasmic granules common • May see inflammation and hemic cell phagocytosis	• Neoplastic cells centered on hepatic sinusoids • Splenic red pulp effacement	$CD3^+$, $CD4^-$, $CD8^+$, $MHCII^+$, $CD11b^+$, TCR γδ, granzyme B^+	• Limited data • Most cases survival days to weeks
HC-TCL	• Jaundice, hepatic enzymopathy • Peripheral lymph nodes not enlarged	• Medium-size cells proximal to hepatocytes	• Invasion of hepatic cords • Cells may be intrahepatocytic	$CD3^+$, $CD4^-$, $CD8^+$, $MHCII^+$, $CD11b^-$, TCR γδ	• Limited data • Suggested survival weeks to months

[a] ALL = acute lymphoblastic leukemia; LBTCL = lymphoblastic T-cell lymphoma; CLL = chronic lymphocytic leukemia; PCTL-NOS = peripheral T-cell lymphoma, not otherwise specified; TZL = T-zone lymphoma; CTCL = cutaneous T-cell lymphoma; HS-TCL = hepatosplenic T-cell lymphoma; HC-TCL = hepatocytotropic T-cell lymphoma.
[b] The most common immunophenotype is indicated. CD3 may be present only intracellular and not on the cell surface.

for small to medium cells that have nucleoli and are positive for CD34, and either positive or negative for CD4, CD5, or CD8. Neoplastic cells in T-ALL and LBTCL are morphologically similar, and differentiation between T-ALL and LBTCL is based on predominance of neoplastic cells in blood and bone marrow versus in nodal and extranodal sites, respectively.[150]

T-Acute Lymphoblastic Leukemia

The diagnosis of ALL is based on presence of abnormal circulating lymphocytes, bone marrow effacement, cytopenia, and absence of nodal enlargement. While there is no agreed lower limit for the proportion of blasts in the bone marrow to confirm a diagnosis of ALL in humans or dogs, the threshold of >20% as recommended for acute myeloid leukemia in both species is likely appropriate.[96,150] Common clinicopathological abnormalities in ALL include thrombocytopenia, anemia, neutropenia, increased serum alkaline phosphatase activity, and hypercalcemia.[12] Once a diagnosis of ALL has been made, categorization as T-ALL depends on immunotyping and/or detection of a clonal TCR. Similar as in human T-ALL, membranous or cytoplasmic expression of CD3 is the hallmark of T-ALL, and if CD4, CD5, or CD8 is present (Figure 72.1b), PTCL with secondary leukemia should be considered. Presence of CD34 characterizes most canine T-ALL, while all are negative for the B-cell markers CD20, CD21, CD22, and CD79a.[12,146,150]

There is limited information on prognostic factors, response to therapy, and outcome for dogs with T-ALL. Lymphoblast phenotype plus CD34 detection was associated with worse prognosis than lack of CD34 detection in human ALL,[30,34,154] which may also apply to T-ALL in dogs.[171] Similarly, marked lymphocytosis is a negative prognostic indicator in humans, and was also reported to be an adverse predictor in dogs.[12,38,66,] However, T-ALL in dogs requires more precise definition, and analysis of outcome in relation to consistent assessment and treatment.

Lymphoblastic T-Cell Lymphoma

Lymphoblastic T-cell lymphoma (LBTCL) originates in either the lymph nodes or extranodal tissue, and accounts for approximately 4% of all canine lymphomas.[158] There is a higher prevalence of this lymphoma type in the Boxer breed.[88,89] Lymphocytosis may occur with LBTCL necessitating distinction from ALL. Primary tumors are most often in peripheral lymph nodes, but the mediastinum and gastrointestinal (GI) tract are also often affected.[89,106] Among dogs with nodal or extranodal lymphoma with concurrent lymphocytosis, lymphoblastic B-cell lymphoma (LBBCL) was slightly more common than LBTCL.[126,151,158] Hypercalcemia has been reported in 59–68% of patients with LBTCL compared to 12% with T-ALL.[12,89,106]

Cytologically, LBTCL consists of intermediate-sized cells with blue cytoplasm, irregular-shaped nuclei, inconspicuous nucleoli, and frequent mitoses (Figure 72.2). Histopathological findings in lymph nodes consist of a paracortical expansion of intermediate-sized lymphocytes that have indented nuclei with dense nuclear chromatin and indistinct nucleoli, and a high mitotic rate. Mitotic nuclei may be subtle due to limited staining of separated chromosomes, and there is complete effacement of the node with capsular thinning and focal perinodal extension.[20,158] Early in the disease process, LBTCL may be difficult to distinguish from the PTCL-NOS subtype, but the higher frequency of mitotic activity identified with an IHC stain such as Ki-67 is helpful. Immunohistochemistry is necessary to distinguish LBTCL from LBBCL due to their similar histological appearance.[158]

Lymphoblastic T-cell lymphoma cells are precursor cells, and most often are positive for CD3, TCR αβ, and CD4.[49] Phenotypes characterized by CD3, TCR αβ, and $CD4^+/CD8^+$ or $CD4^-/CD8^-$ or $CD4^-/CD8^+$ have also been described.[49] Distinction of LBBCL from PTCL-NOS may be challenging based on cytologic appearance, and histomorphology may be required.[89] Mediastinal LBTCL is most likely $CD3^+$, TCR $\alpha\beta^+$, and $CD4^+/CD8^+$.[81] In one study, low expression of MHCII in $CD4^+$ LBTCL was reported.[6] Immunophenotyping by flow cytometry is useful to differentiate mediastinal LBTCL from thymoma or thymic hyperplasia.[81]

A recurrent mutation deleting the CDKN2 locus that encodes the cyclin kinase inhibitors p16, p15, and the p53 regulator p14/ARF has been identified in LBTCL and other high-grade T-cell lymphomas; by contrast, this abnormality was not present in any B-cell or low-grade T-cell lymphomas.[47]

Lymphoblastic T-cell lymphoma is an aggressive form of lymphoma associated with a shorter survival than other forms of multicentric lymphoma. Boxer dogs diagnosed with LBTCL had a median survival time of 109 days (range 8–214 days; 34 patients). Most patients in that study were treated with combination chemotherapy, but a few received only prednisone monotherapy.[89] In another study, Boxers with T-cell lymphoma treated with combination chemotherapy had a median survival of 141 days (95% confidence interval [CI]) 115–167 days; nine patients).[58] Although histological classification was not reported, since Boxers primarily develop LBTCL, the results nevertheless support a relatively poor response to therapy.[88,89] In another study of lymphoblastic lymphomas (including LBTCL, LBBCL, and Burkitt lymphoma), a median survival time of 160 days (40 patients; range not provided) was reported; interestingly, in that study, survival time with only prednisone therapy was similar to no therapy, but combination chemotherapy prolonged survival time.[157]

Mature T-Cell Neoplasms

Chronic Lymphocytic Leukemia

CLL is a mature lymphocyte neoplasm primarily of adult dogs (typically 7–13 years old).[135,151,161] Across several studies, T-cell type accounted for 60–90% of all CLL, which usually had an indolent disease course.[135,151,161] In recent WHO classifications, the entity T-CLL in humans was reclassified as T-cell prolymphocytic leukemia,

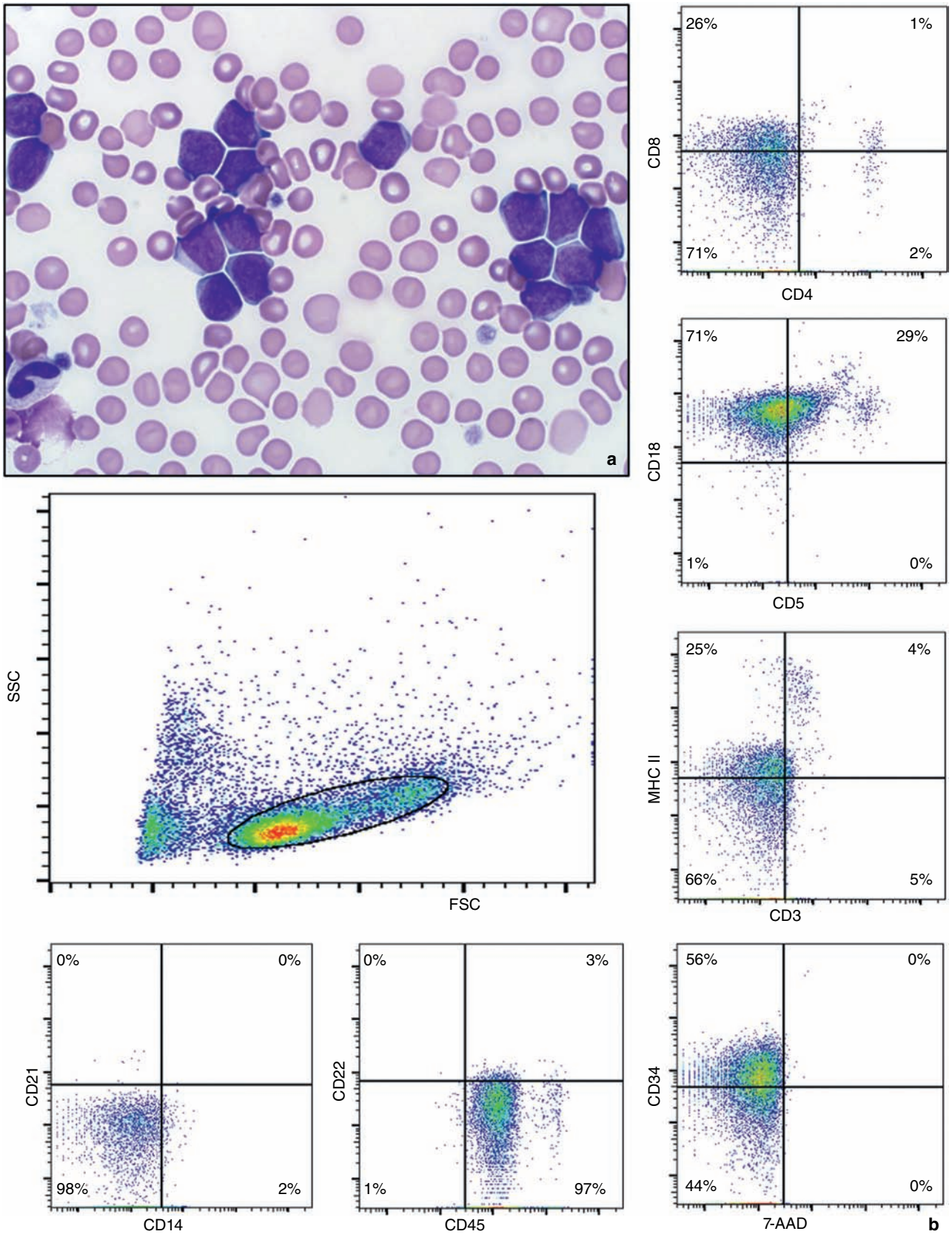


FIGURE 72.1 (a) Blood film from a 10-year-old Portuguese Water Dog with anemia and thrombocytopenia, and WBC count of 140.4 × 10^9/L. Lymphocytes predominate and are of medium size with a small amount of blue cytoplasm, irregular to angular-shaped nuclei with dense chromatin, and a single nucleolus; (b) On flow cytometric analysis, forward and side light scatter identify a population of cells with low side scatter. Cells are $CD3^{low}$/$CD5^{low}$/$CD8^{mod}$/$CD18^{hi}$/$CD34^{hi}$/$CD45^{hi}$/$MHCII^{mod}$. Interpretation: T-acute lymphoblastic leukemia (T-ALL). The dog was treated with combination chemotherapy and euthanized after 65 days due to disease progression.

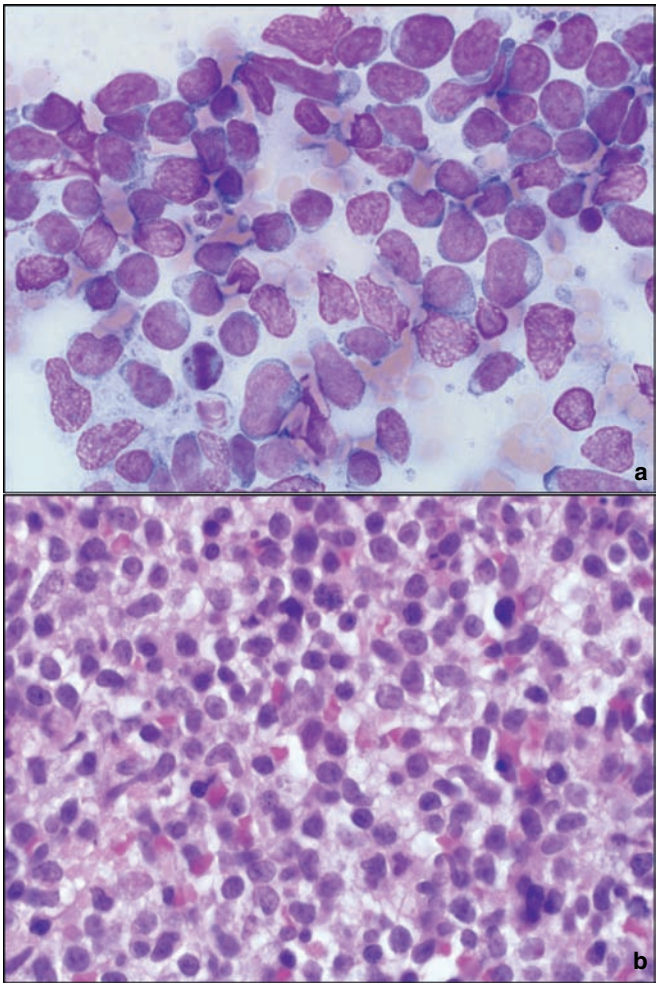


FIGURE 72.2 Lymph node aspirate and biopsy sections from a 7-year-old Boxer with hypercalcemia. The dog had multiple markedly enlarged lymph nodes. (a) Cytologically, the sample consists of medium-size lymphocytes with a small amount of dark blue cytoplasm, pleomorphic, including indented, nuclei and prominent small nucleoli; (b) Histologically, cells are also pleomorphic with round to angular to reniform nuclei, and small discrete nucleoli. On flow cytometric analysis, the cells were $CD3^{mod}/CD4^{hi}/CD5^{hi}/CD18^{hi}/MHCII^{low}$. Interpretation: Lymphoblastic T-cell lymphoma. The dog was treated with combination chemotherapy, did not achieve remission, and was euthanized 3 months after diagnosis.

which is a neoplasm with an aggressive disease course. Hence, despite similar presentation of an increased number of circulating small cells, canine T-CLL is biologically different than human T-cell prolymphocytic leukemia.[27,150,170]

Canine T-CLL is often an incidental diagnosis on a CBC obtained for preventative geriatric medical care. Absolute lymphocytosis typically consists of 10×10^9 cells/L to 50×10^9 cells/L, but numbers $>500 \times 10^9$ cells/L have also been reported.[27,146] The cells can vary in size from small to medium, and often contain subtle to prominent azurophilic cytoplasmic granules. Nucleoli are typically inapparent.[151] Most dogs present without cytopenia, or may have mild to moderate anemia or rarely thrombocytopenia.[151] Neither hypercalcemia nor hyperphosphatemia is a feature of T-CLL.[74,79] Splenic involvement is extensive in most cases and may result in splenomegaly, while bone marrow infiltration is sparse and likely occurs late in disease.[94]

Despite being distinguishable by cell and granule size, granular CLL is sometimes referred to as T-cell large granular lymphocytic (LGL) leukemia. This nomenclature is confusing. The originally described T-LGL is an aggressive type of lymphoma in dogs (and cats), usually of intestinal or peri-intestinal, and sometimes of mediastinal origin, which is often accompanied by low-grade leukemia.[94,120,142] The granules in these neoplastic lymphocytes are usually fewer in number and large, and the cells themselves are also large. Reasons for this mistaken nomenclature being applied to canine CLL may have resulted from its similarities with human LGL leukemia.[94,150]

Most T-CLLs consist of $CD3^+$ lymphocytes, although absence of cell surface of CD3 has also been reported.[27,151,161,170] Loss of cell surface CD3 expression occurs in several T-cell neoplasms and indicates the need to assess for other T-cell markers (i.e., CD4, CD5, CD8) or for intracellular CD3.[27] Markers that characterize most T-CLL are CD8 (consistent with the granular cytomorphology) and MHCII.[28,135,146,151,161] T-CLL variants without cytoplasmic granules appear to be inconsistently positive for CD4 or CD8.[161] Presence of the TCR αβ is more common than that of the TCR γδ.[161] Consistent CD11d (integrin $\alpha_d\beta_2$) expression by granular T-CLL cells aligns with origin of the neoplasm from splenic red pulp T-cells.[94]

T-CLL is an indolent neoplasm with a favorable prognosis characterized by median survival times of 930–1098 days.[27,170] Negative prognostic indicators include anemia and lymphocytosis $>30 \times 10^9$ cells/L at the time of presentation.[27,170] Malignant transformation of CLL to a more aggressive lymphoma is referred to as Richter's syndrome, which has been reported in humans and dogs.[29,150] In both species, this appears to occur more often with B-CLL but it has also been reported for canine T-CLL.[29,150]

Peripheral T-Cell Lymphoma, Not Otherwise Specified

PTCL-NOS is an umbrella category that consists of lymphomas that do not fit into other T-cell categories. "Peripheral" refers to any extrathymic location; however, multiple lymph nodes are most commonly affected in this form of lymphoma.[158] This subtype accounts for approximately 15% of all canine lymphomas.[157,158]

The cytopathology of lymphomas in this subtype varies. Medium-size cells with round to angular, pleomorphic nuclei and pale cytoplasm describe the most common manifestation (Figure 72.3), but types with cytoplasmic basophilia and granules, single or multiple nucleoli of variable prominence, and other features, have also been noted (Figure 72.4).[36] Accordingly, the histopathologic features of this subtype are also varia-

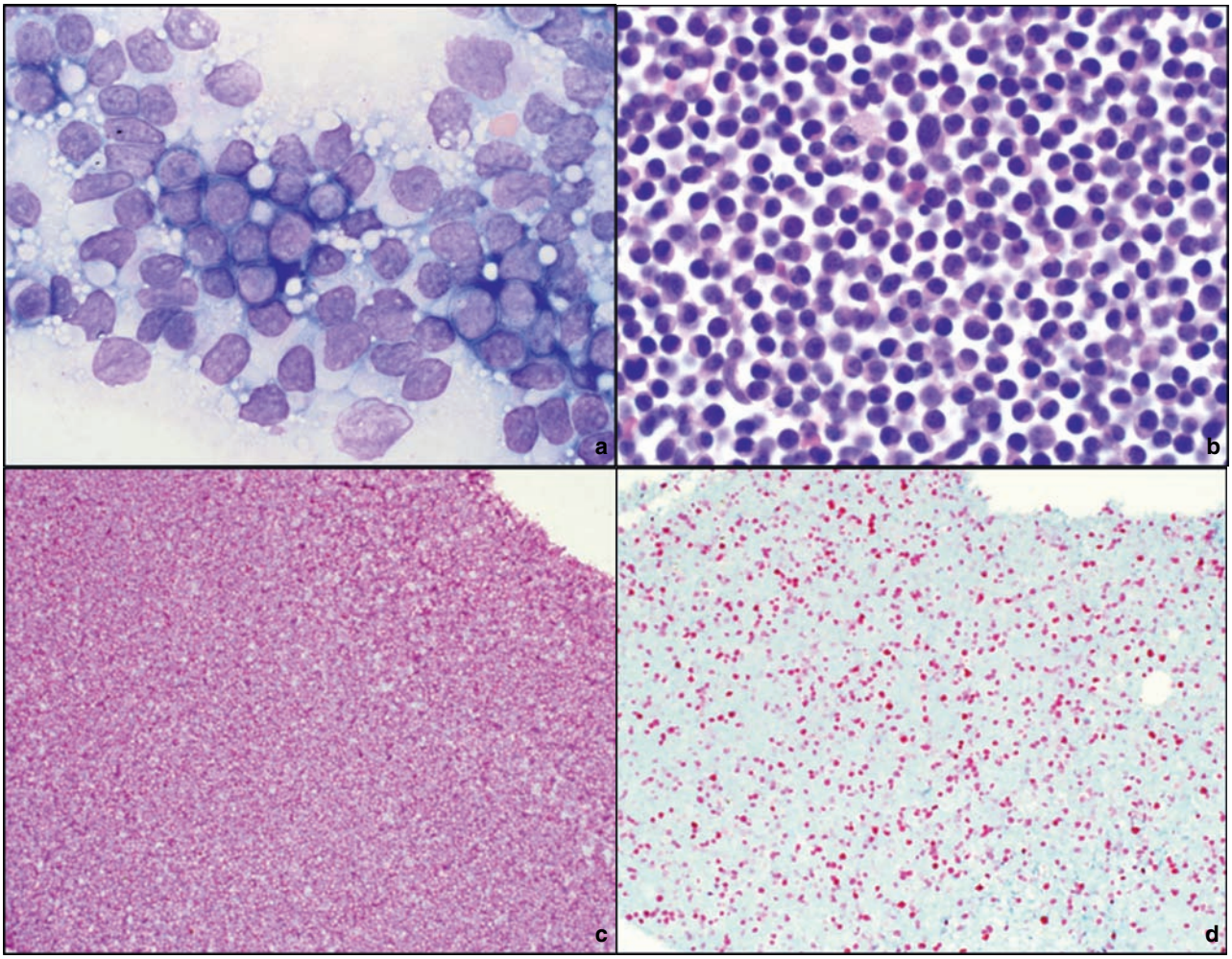


FIGURE 72.3 Lymph node aspirate and biopsy sections from a 13-year-old Poodle cross dog. The dog had a single enlarged prescapular lymph node for approximately 6 months. The lymph node continued to gradually enlarge to approximately 5 × 6 × 4 cm^3. (a) Cytologically, the sample consists of small- to medium-size lymphocytes with abundant watery blue cytoplasm, round to oval nuclei, and inapparent nucleoli; (b) Histologically, cells are uniformly round with round to slightly oval, often eccentrically located nuclei; (c) Cells are uniformly and strongly positive for CD3, and (d) 20–30% of cells are positive for Ki-67. On flow cytometric analysis, the cells were $CD3^{hi}/CD5^{hi}/CD8^{hi}/MHCII^{mod}$, consistent with PTCL-NOS. The dog was treated with lomustine and prednisone for 3 months, but the size of the lymph node continued to increase. Development of a hepatopathy 7 months after diagnosis prompted euthanasia.

ble. Most PTCL-NOS lymphomas consist of intermediate to large lymphocytes that diffusely efface lymph nodes, sometimes extending past the capsule and into the perinodal tissue.[63] The mitotic index is low to intermediate, but characteristic of this subtype is the relative absence of tingible body macrophages. Flow cytometrically, PTCL-NOS is characterized by positivity for CD3 and CD4, low to moderate MHCII, and frequent loss of CD5.[63] Some but not all dogs with this type of PTCL-NOS have hypercalcemia.[63] Variants with cytomorphologic features similar to late-stage/aggressive TZL or early/indolent LBTCL may be within this subtype.[158] Human PTCL-NOS has been further divided into distinct categories by immunophenotype and molecular features, and similar effort has characterized a few canine PTCL-NOS subtypes.[71,117,141,126,134] Histopathologically, $CD4^+/8^-$, $CD4^-/8^+$, and $CD4^-/CD8^-$ PTCL-NOS subtypes were not reliably distinguishable.[63] Potential cytopathological differences remain to be explored. The same recurrent mutation causing deletion of the CDKN2 locus in LBTCL has also been identified in high-grade PTCL-NOS.[47] Analysis of gene expression patterns in a limited number of $CD4^+/8^-$ cases of PTCL-NOS identified enrichment in a broad range of pathways.[63]

Although prognostic data including survival and response to therapy are of limited utility given the heterogeneous nature of PTCL-NOS, in one study a median survival of 162 days for high-grade PTCL-NOS was reported.[157] Subtypes with longer progression-free

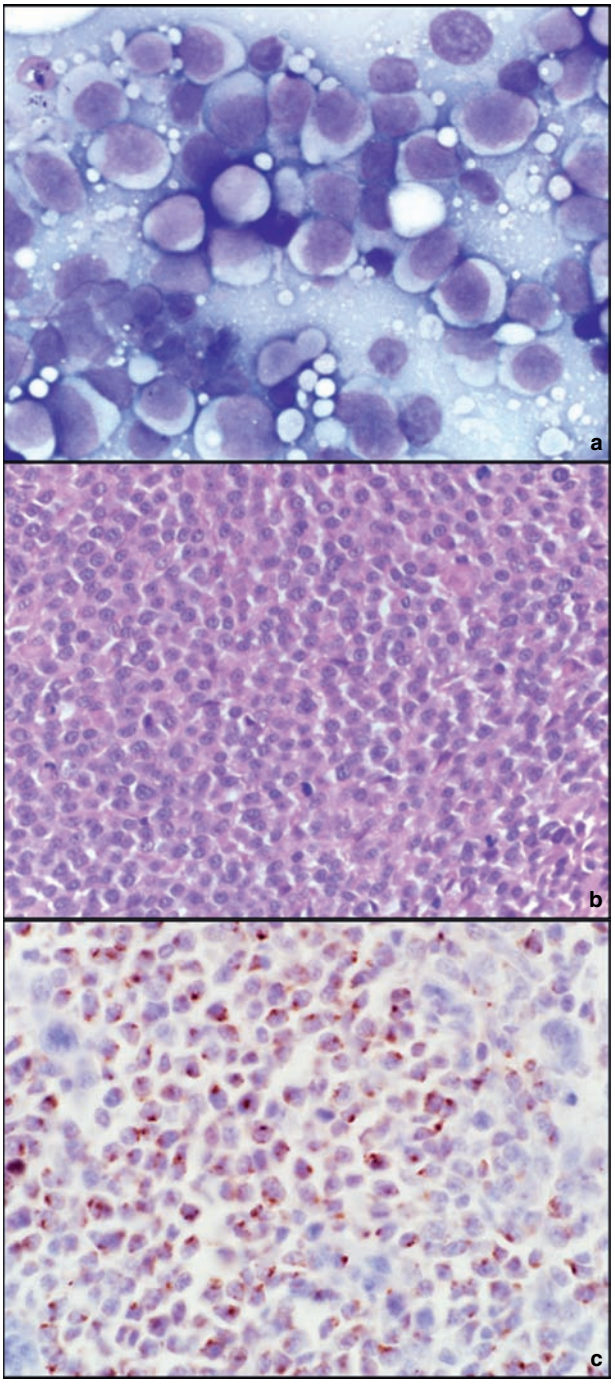


FIGURE 72.4 Lymph node aspirate and sections from a 9.3-year-old Mastiff with multiple moderately enlarged peripheral lymph nodes, lymphocytosis (12×10^9/L), and no clinical illness. (a) Cytologically, the lymphocytes are large with abundant pale blue cytoplasm, irregularly shaped nuclei, and a single small nucleolus; (b) On histopathology, lymphocytes are tightly packed, have a moderate amount of eosinophilic cytoplasm, uniform size, and three to five mitoses per 40× field; (c) On IHC, most cells have punctate positivity for granzyme B. By flow cytometry, cells were $CD3^{low}$/$CD5^{low}$/$CD8^{hi}$/$MHCII^{hi}$. Interpretation: PTCL-NOS. Two doses of doxorubicin induced no change in lymph node size or the magnitude of lymphocytosis. Treatment with lomustine resulted in remission. The dog died 20 months after diagnosis due to heart failure. Postmortem, there was no evidence of lymphoma.

interval and survival among PTCL-NOS were $CD4^-$/$CD8^+$/$MHCII^+$ and $CD4^-$/$CD8^-$/$MHCII^+$.[36]

Unclassifiable, High-Grade Plasmacytoid-Type Lymphoma Unclassifiable, high-grade plasmacytoid-type lymphoma is a rare form of PTCL-NOS in dogs and does not have an analog in the WHO classification of human neoplasms. Although these tumors cytologically resemble plasma cells, they express T-cell markers. Clinical presentation includes regional or generalized lymphadenopathy with hepatic, splenic, mediastinal, cardiac, bone marrow, and peripheral blood involvement. Hypercalcemia may be present. Survival appears to be short, even with chemotherapy.[10,49,125] Histologically, there are a large number of tingible body macrophages creating a characteristic starry-sky appearance.[125] Detection of CD3 and CD8 is characteristic, though coexpression of CD4 is also possible, but these tumors are multiple myeloma oncogene-1 (MUM-1)-negative.[10,49,125]

Renal T-Cell Lymphoma Renal PTCL-NOS is a rare but anatomically unique subtype of T-cell lymphoma that usually involves both kidneys. Neoplastic cells may be shed in sufficient quantity into urine to form the basis for diagnosis.[53,173] Several dogs with renal PTCL-NOS were polycythemic, which was attributed to tumor or increased renal interstitial cell erythropoietin production.[40] The disease course of renal PTCL-NOS appears rapid with typically extensive extrarenal involvement at the time of diagnosis.

T-Zone Lymphoma

TZL, formerly termed small clear cell lymphoma, is an indolent form of lymphoma that is common in dogs but not humans.[150,158] In dogs, it accounts for 3–12% of all lymphomas.[126,157,158] A predisposition has been suspected in Golden Retrievers and Shih Tzu dogs;[62,104,141] however, this may be linked to geographic location.[93] The prevalence of TZL in Golden Retrievers varies regionally within the United States, with dogs being diagnosed with TZL more often in the Northeast and East North Central regions than in other regions.[134] A postulated non-neoplastic counterpart of TZL cells (T-zone-like cells of undetermined significance; TZUS) was detected more often and in larger number in nontumor-bearing older Golden Retrievers (30.6% of individuals) than in dogs of other breeds (14% of individuals).[68] Furthermore, genetic analysis of TZL in Golden Retrievers identified single-nucleotide polymorphisms (SNP) on chromosomes 8 and 14.[80] The SNPs on chromosome 8 were in a region proximal to thyroid-regulating genes, and were suggested to contribute to a reduced risk for development of TZL in Golden Retrievers with hypothyroidism.[80]

Typically, a single peripheral lymph node is enlarged, although extranodal (lingual) TZL has been reported.[62] Cytologically, neoplastic cells typically have small round nuclei with dense chromatin and absent nucleoli. They have abundant pale blue cytoplasm that frequently extends asymmetrically into one direction producing a so-called "hand-mirror" appearance (Figure 72.5a).[178] It is

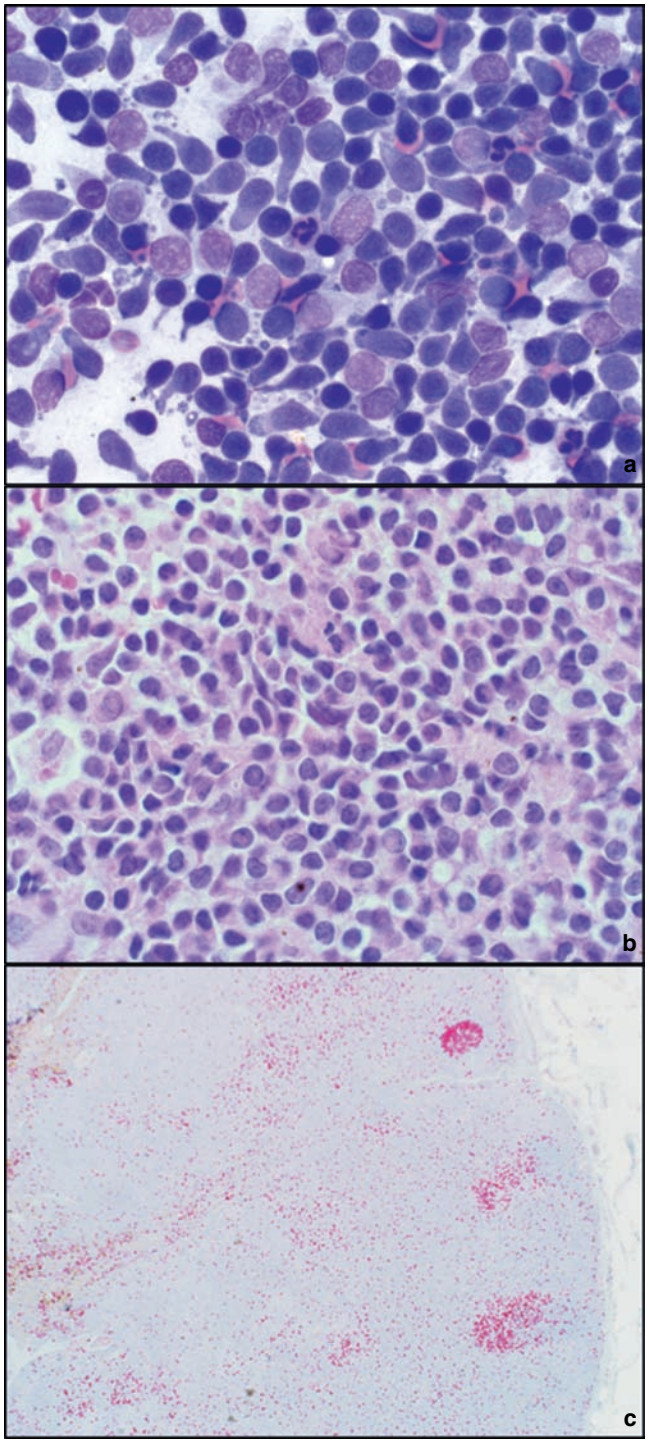


FIGURE 72.5 Samples from a 13-year-old Shih Tzu with incidentally identified bilateral mild mandibular lymph node enlargement and lymphocytosis (6×10^9/L). (a) The lymph node aspirate consists predominantly of round or elongate cells with dense, darkly stained chromatin. The cytoplasm is light blue, and many cells have a single cytoplasmic extension. Small centrally placed nucleoli are apparent in some cells. There are also larger cells with less darkly stained nuclei; (b) Moderately dense population of small to medium lymphocytes with oval to round nuclei, a central nucleolus, and angular cell shape; (c) Ki67 IHC highlights the follicles toward the periphery of the lymph node with higher mitotic activity than neoplastic lymphocytes in the paracortical region. Flow cytometrically, the lymphocytes were $CD3^-/CD5^{mod}/CD8^{hi}/CD18^{hi}/CD21^{low}/CD45^-/MHCII^{hi}$. Interpretation: T zone lymphoma.

important to note that such projections can also be seen in reactive lymphocytes or as an artifact of slide preparation. Histopathologically, there is an apparent paracortical expansion of small- to intermediate-sized T cells with moderately abundant cytoplasm, indented or cleaved nuclei, and indistinct nucleoli (Figure 72.5b). Instead of effacing the lymph-node architecture, these cells expand and displace nodal follicles until these are compressed against the capsule (Figure 72.5c). The capsule is minimally thinned, and neoplastic cells do not extend beyond the capsule. Mitotic activity is nearly absent among neoplastic cells and is lower than in remnant follicles.[158]

The TZL cells are consistently positive for cytoplasmic CD3, though cell surface CD3 may be low or absent.[36,44,158,159] Most TZL cells by flow cytometry are $CD8^+$, or $CD4^-/CD8^-$. Concurrent detection of CD4 and CD8 on neoplastic cells, or TZL that consists of subpopulations that are either $CD4^+$ or $CD8^+$ (likely two clones), has also been reported.[36,92,141] Cells of TZL are typically strongly positive for MHCII, which is a distinguishing feature from high-grade $CD4^+$ PTCL-NOS or LBTCL.[141]

Unique immunotypic features of TZL are absence of CD45 and faint to intermediate presence of CD21.[93,141] Absence of the CD45 antigen is due to reduced gene expression in neoplastic cells, and was also noted in presumed non-neoplastic TZL precursor cells.[68,91] Positivity for CD25 is an additional feature of TZL.[102,103] Whether absence of CD45 is a distinctive feature of TZL, or also occurs in other types of lymphoma, has been debated.[26,122] The vast majority of reports have attributed absence of CD45 on neoplastic lymphocytes to TZL.

Clinical disease is typically absent in dogs with this type of lymphoma. Forty-eight to 64% of dogs with TZL had lymphocytosis, and almost all had neoplastic cells in circulation.[44,92,104,141] Therefore, it is important to differentiate TZL from T-CLL, which may have similar cytomorphology. The presence of lymphocytosis does not appear to adversely affect survival.[44] TZL is associated with very long survival with and without chemotherapy. Median survival times ranged from 22.5 to 33.5 months.[44,92,102,159] In two studies, shorter median survival times of 55 and 180 days were observed in a few patients that were clinically ill at the time of diagnosis.[5,92] In a separate study, one of 16 dogs with TZL died due to progression of a concurrent diffuse large B-cell lymphoma (DLBCL).[102] In other studies, combination chemotherapy relative to no therapy provided no significant survival benefit for TZL and other indolent T-cell lymphomas.[36,157]

Cutaneous T-Cell Lymphoma

Cutaneous lymphomas are relatively uncommon extranodal neoplasms that, in both humans and dogs, have heterogeneous clinical presentations. Cutaneous lymphomas represent 1–2% of all canine skin tumors,[57,168] and are predominantly diseases of older animal with a mean age of 9–12 years.[35,108,111] Retroviral particles were noted in a cell line obtained from a dog with Sézary syndrome, but infectious agents have not convincingly been identified in canine lymphoma.[56] An association between

atopic dermatitis and cutaneous lymphoma has been noted in humans and dogs.[128,139]

Cutaneous lymphomas are subdivided into epitheliotropic and non-epitheliotropic forms based on the presence or absence of neoplastic lymphocytes in the epithelium of the epidermis and/or adnexa, respectively. Epitheliotropic lymphoma is more common than non-epitheliotropic lymphoma.[99] In both humans and dogs, T-cell origin is most common with denotation as CTCL.[35,111] Here, we have applied the WHO classification as a framework to organize the descriptive properties of CTCL in dogs, recognizing that not all of the current veterinary literature fits into this scheme. Entities in veterinary medicine with WHO classification counterparts are the epitheliotropic forms mycosis fungoides (MF), pagetoid reticulosis (PR) and Sézary syndrome, and the non-epitheliotropic form.

Cutaneous Epitheliotropic T-Cell Lymphoma Clinical features of MF and PR are variable and may consist of cutaneous nodules, generalized exudative dermatitis, and oral or mucocutaneous ulcers. The lesions were often associated with erythema, scaling, crusting, pruritus, ulceration, alopecia, hypopigmentation, plaque formation, and/or discrete masses.[45,108,168] Aspirates of discrete masses typically yield a uniform population of medium to large lymphocytes with roundish to irregular or lobulated nuclei (Figure 72.6), but do not allow distinction of subtypes of CTCL. Histopathologically, both MF and PR are neoplasms involving the epidermis, and MF also involves the underlying dermis.[16,35,45,108,133] Lymphocytes may form intradermal aggregates inaptly referred to as "Pautrier's microabscesses." Additional epidermal changes can include acanthosis, hyperkeratosis, spongiosis, ulceration, and secondary inflammation.[61] Prominent dermal involvement or invasion into the subcutis may be referred to as MF d'emblé or tumor-stage MF.[45,108]

Immunophenotypically, CTCL are $CD3^+$, and either $CD4^-/CD8^+$ (approximately 80%) or $CD4^-/CD8^-$ (approximately 20%). The TCR in MF cells is either αβ or γδ, but PR cells consistently express the γδ TCR.[108] Tumor cells are usually $MHCII^+$ and variably $CD5^+$.[36,111,108] Similar as in human CTCL, coexpression of CD3 and CD20 (a B-cell marker) has been reported in canine tumors.[14,41]

Most survival studies had low sample size and different therapies. Median survival was from 3 to 5 months without therapy or only prednisolone therapy, 6 months with lomustine therapy, 9 months with L-asparaginase therapy, and 11 months with synthetic retinoid therapy.[11,45,113,167] Surgical cure of solitary lesions has been reported.[45,168] Although lomustine therapy resulted in a 30% complete remission rate, differences in survival between dogs treated with lomustine, prednisolone, or no therapy were not significant.[45,85] A larger retrospective study (80 patients) showed median survival of 207 days with chemotherapy and 64 days without chemotherapy.[22] Patients with solitary lesions survived longer (median 231 days) than those with multiple lesions (median 104 days).[22] Of interest, patients with mucocutaneous/mucosal epitheliotropic lymphoma had a median survival of 490 days.[22]

Sézary Syndrome Sézary syndrome is a progressive form of MF defined by skin lesions similar to CTCL, generalized lymphadenopathy, and neoplastic T cells in lymph nodes and peripheral blood.[150] Sézary syndrome is rare in both humans and dogs.[48,56,136] Circulating neoplastic cells are usually recognized as abnormal on blood film review. Histologically, skin lesions are similar to those of MF.[48,56,136,150] The immunophenotype of canine

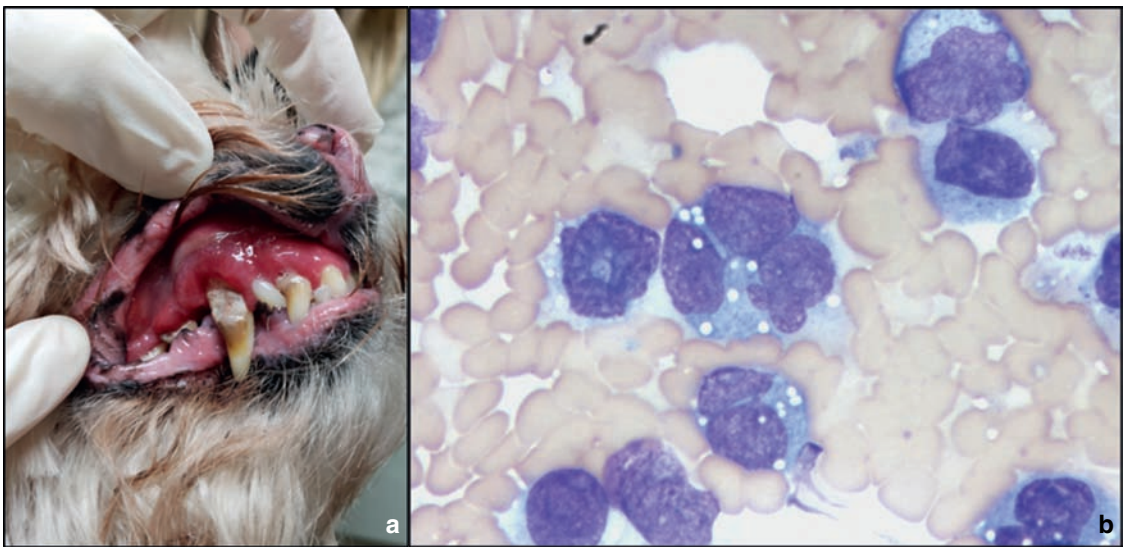


FIGURE 72.6 (a) Dog (13 years) with masses at the mucocutaneous junction, gingival disease, and planum depigmentation; (b) Aspirate of a mass consists of large lymphocytes with irregular, indented, or multilobed nuclei, pale blue cytoplasm, and cytoplasmic vacuoles. Histopathologic diagnosis: Epitheliotropic CTCL.

Sézary syndrome cells is not well described, but appears to be $CD3^+$ and $CD4^-/CD8^+$, similar to MF.[48,56,136]

Cutaneous Non-Epitheliotropic T-Cell Lymphoma Non-epitheliotropic lymphoma is the least common type of cutaneous lymphoma in dogs, and both B-cell and T-cell types have been reported.[2,77,109,129,155] Clinically, these can present similar to epitheliotropic canine CTCL, with frequently affected sites including the face, lower extremities, neck, and trunk.[109] Cytologically, non-epitheliotropic CTCL does not have distinctive features, but some forms have an extensive and pleomorphic inflammatory component. Histopathological findings are highly variable but characterized by dermal infiltration of large, atypical lymphocytes with minimal or absent epidermal involvement. Detection of CD3 can be variable or absent, while CD18 and CD45 are uniformly detected, rendering distinction between atypical lymphocytes and histiocytes challenging.[8,109] Limited information is available regarding additional immunophenotypic characteristics, though a few $CD4^-/CD8^+$ and $CD4^-/CD8^-$ tumors have been reported along with variable detection of CD5.[8,110]

Too few cases have been reported for meaningful survival analysis, but in one study six patients died from the disease after a median survival of 9 months, while one dog survived 3 years.[109] These findings suggest marked variation in the biology of non-epitheliotropic CTCL.

Enteropathy-Associated T-Cell Lymphoma

EATL refers to a primary lymphoma of the gastrointestinal tract. It is a relatively uncommon form of lymphoma in both humans and dogs, comprising 5–10% of human non-Hodgkin lymphomas and 5–7% of canine lymphoma.[31,37,150,163] The most commonly affected site in both dogs and humans is the small intestine, though EATL also occurs in stomach and colon.[31,33,51,52,150] In humans, EATL has been linked to celiac disease, though a similar correlation has not been identified in dogs.[150,164]

Grossly, GI lymphoma can be focal or multifocal, with thickening and ulceration of the intestines.[33] EATL is divided into types 1 and 2. EATL type 1 is more common in dogs and involves transmural infiltration by lymphocytes that are generally large and anaplastic. EATL type 2 involves discrete, patchy to complete effacement of the lamina propria by small, cytologically unremarkable lymphocytes that can be difficult to differentiate from reactive lymphocytes.[160] Epitheliotropism, including the presence of intraepithelial nests of five or more lymphocytes, occurs in 60–75% of EATL, and is helpful in differentiating EATL type 2 from inflammation.[33,82,160] In three cases, EATL type 1 cells were positive for CD3 and CD20 and had clonally rearranged the γ chain of the... TCR, implying that these were indeed EATL, and that CD20 should not be used to differentiate between T- and B-cell origin at this anatomic location.[118]

Very few studies have examined the immunophenotype of canine EATL. In one study, tumors were $CD4^+/CD8^-$ with γ TCR rearrangement.[36] This is different from human EATL, which is typically $CD4^-/CD8^+$ or $CD4^-/CD8^-$ with αβ TCR expression.[150] It has been suggested that EATL type 1 and 2 may differ in the frequency of cells positive for the transcription factor FOXP3.[90]

Most dogs with EATL type 1 have a grave prognosis, with reported median survival times ranging from 13–62 days, which may be skewed by a diagnosis late in the disease course.[36,51,143] On the other hand, dogs with EATL type 2 without treatment had a median survival of 279 days, and dogs treated with chemotherapy had median survival of 628 days.[32] In another study, a median survival of 424 days with therapy was noted.[82]

Subcutaneous Panniculitis-Like Lymphoma

In humans, subcutaneous panniculitis-like lymphoma is a rare tumor of cytotoxic lymphocytes that accounts for less than 1% of all non-Hodgkin lymphomas.[150] In dogs, it is similarly rare, with one report of five dogs.[117] In single or multiple subcutaneous masses, neoplastic lymphocytes invade the subcutaneous adipose tissue and mimic panniculitis. The neoplastic cells extend into the dermis and approach, but do not involve, the epidermis. The lymphocytes are small- to intermediate-sized with distinct nucleoli, a high mitotic index, and CD3 positivity.[117]

Anaplastic Large Cell Lymphoma

Anaplastic large cell lymphoma is a relatively common form of T-cell lymphoma in humans which is categorized by the presence or absence of a recurrent genetic translocation involving the anaplastic lymphoma kinase (ALK) gene.[150] The tumors consist of unique large anaplastic cells, often a mixed inflammatory infiltrate, and large Reed–Sternberg-like cells.[150] This is an uncommon lymphoma in dogs that, without IHC, is difficult to differentiate from anaplastic diffuse large B-cell lymphoma or histiocytic sarcoma.[1] In humans, these tumors are positive for CD30, and, remarkably, also for CD4 and cytotoxic proteins such as granzyme B and perforin.[150] The immunophenotype in dogs is not well studied.[150] Five cases reported as part of a large multicenter study had a favorable response to chemotherapy.[150,157]

Intravascular Large T-Cell Lymphoma

Although typically a B-cell neoplasm in humans, intravascular large cell lymphoma can also be of T- or NK-cell type.[21] This is a rare tumor in dogs, with both T- and B-cell types described.[83,95] Inimitably, neoplastic lymphocytes are noted within blood vessels rather than in hemolymphoid tissues. The cells then disseminate into organs, typically causing kidney disease and central nervous system signs due to infiltration of the brain or spinal cord.[95] Neoplastic cells can be found in circulation (Figure 72.7), though lymphocytosis has not been reported. The cells are large, pleomorphic, and can have granular morphology.[83,95]

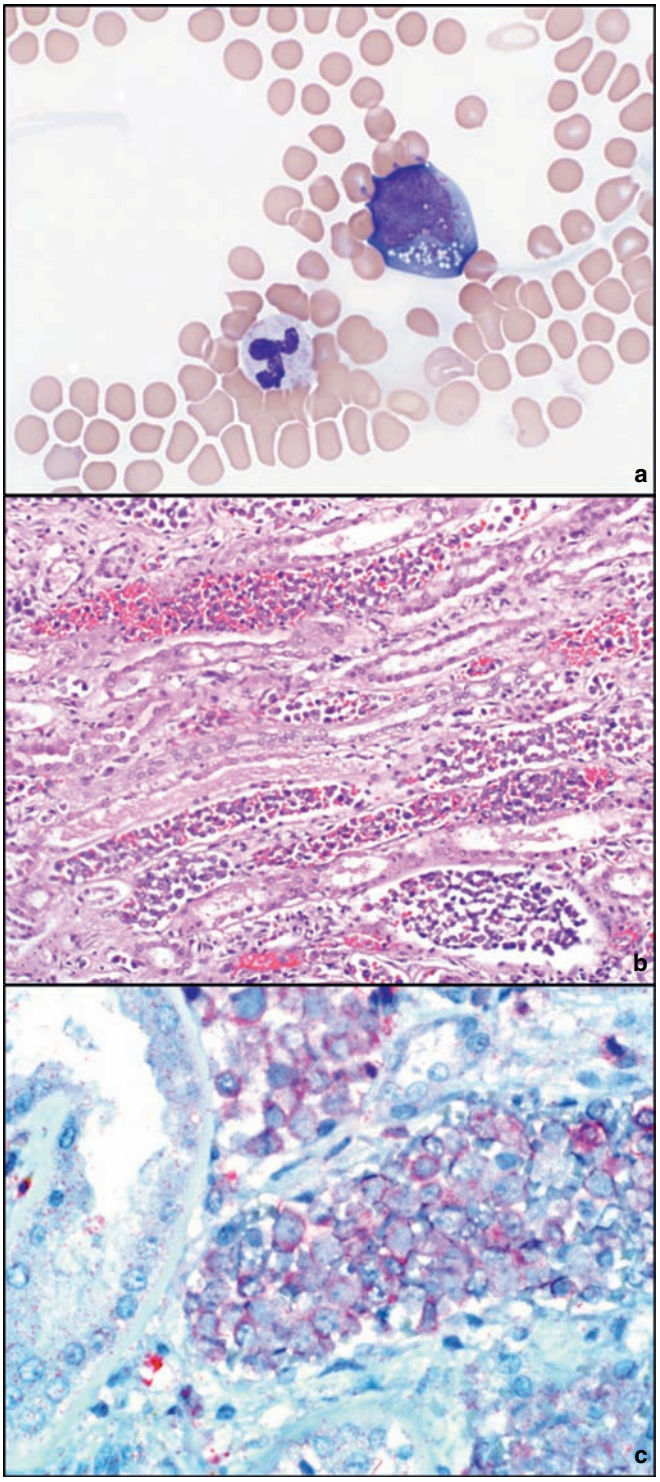


FIGURE 72.7 Samples from a 6-year-old dog with a 10-day history of hyporexia, vomiting, diarrhea, and weight loss. On presentation, the dog was tachypneic and had acute kidney injury. (a) There were rare, large, atypical lymphocytes with cytoplasmic granules and vacuoles on the blood film; (b) Renal biopsy showed venules filled with aggregates of large lymphocytes; (c) The cells were CD3-positive on IHC. Interpretation: Intravascular T-cell lymphoma. Postmortem, extensive intravascular neoplastic lymphocytes were identified throughout most tissues.

Extranodal T-Cell Lymphoma, Nasal Type

Extranodal nasal T-cell lymphoma is a rare tumor in humans with a higher incidence in East Asia and Latin America where the majority of cases are associated with Epstein–Barr virus (EBV) infection.[150] In dogs, nasal T-cell lymphoma is also very rare with only two reported cases.[69,155]

Hepatosplenic T-Cell Lymphoma

Analogous to the human disease, hepatosplenic T-cell lymphoma (HS-TCL) in animals is a rare T-cell neoplasm characterized by primary involvement of liver and spleen, absence of peripheral lymphadenomegaly, cytopenia, and an aggressive biological behavior. In animals, this entity has been primarily recognized in dogs.[24,54,71,147] The number of published cases is too low to identify a breed, sex, or age predisposition. Clinically, dogs present with fever, splenomegaly, progressive lethargy, anorexia, weight loss, and diarrhea or vomiting ranging from 1 week to 3 months prior to presentation. A variably regenerative anemia is a consistent finding, and thrombocytopenia and hypoproteinemia are seen in most cases. A lymphocytosis is generally not observed. Cytologically, neoplastic cells are intermediate to large and often contain cytoplasmic granules suggestive of a cytotoxic phenotype (Figure 72.8a).

Erythrophagocytosis or thrombophagocytosis by neoplastic cells and macrophages is frequently observed and is reflective of a secondary hemophagocytic syndrome (Figure 72.8b). Erythrophagocytosis secondary to HS-TCL must be differentiated from immune-mediated hemolytic anemia and hemophagocytic histiocytic sarcoma.[55,71] Histologically, neoplastic lymphocytes are centered on hepatic sinusoids and the splenic red pulp with occasional involvement of regional lymph nodes. The bone marrow is inconsistently involved, but peripheral lymph nodes are generally spared. The dominant immunophenotype of the neoplastic lymphocytes has been characterized as $CD3^+$, $CD4^-$, $CD8\alpha^+$, $CD8\beta^-$, TCR $\alpha\beta^-$, TCR $\gamma\delta^+$, $CD11d^+$, and granzyme B^+.[55,71] Based on the frequent expression of CD11d and TCR γδ, it has been proposed that HS-TCL in dogs arises from γδ T cells of the splenic red pulp.[55,71] The frequent expression of granzyme B additionally points toward a cytotoxic T-cell origin of the neoplastic cells.[55,71] All cases for which clonality testing has been performed had a clonally rearranged T-cell receptor gamma locus.[25,70,147] With one exception, the survival time in all published cases has been, regardless of therapy, short, ranging from a few days to less than a month.[25,55,71,147]

Together, these data support HS-TCL as a distinct WHO entity in dogs that closely resembles the human disease. Hepatosplenic T-cell lymphoma may exist in other species, but only isolated case reports exist in cats and horses.[19,131] The findings in a horse and a cat were consistent with the findings described for dogs with the exception that the cat responded to treatment and lived for 2.5 years.

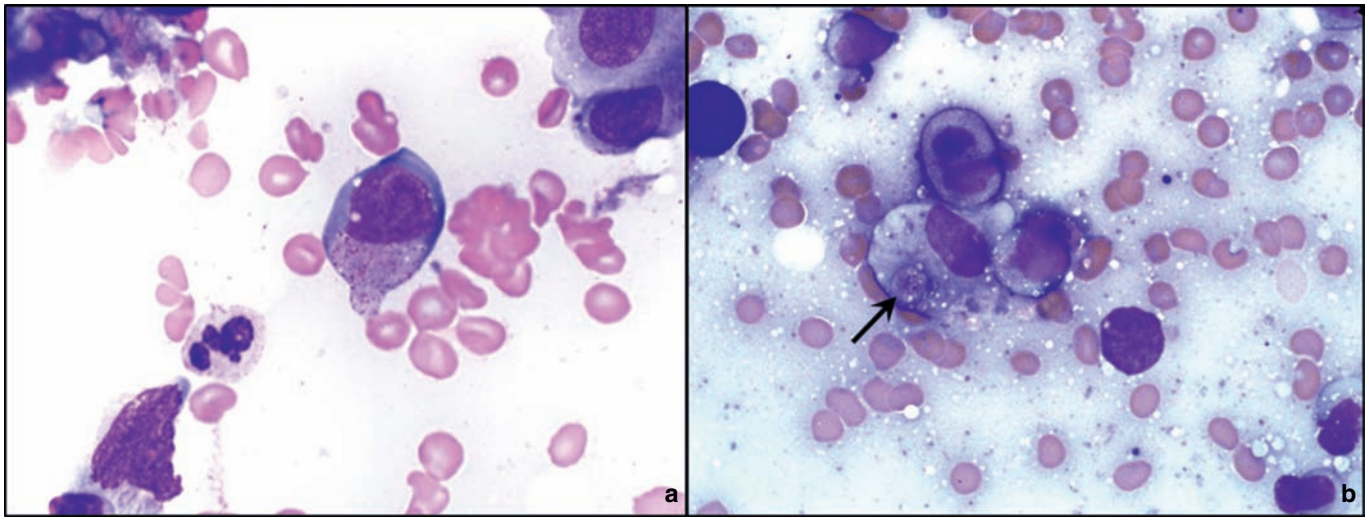


FIGURE 72.8 Splenic aspirates from an 11-year-old dog with marked anemia and thrombocytopenia. (a) Large lymphocytes with fine eosinophilic granules predominate; (b) Macrophages and lymphocytes containing platelets (arrow) or RBC were noted. Flow cytometrically, lymphocytes were $CD3^+$, $CD8^+$, $MHCII^{low}$. Interpretation: Hepatosplenic $CD8^+$ T-cell lymphoma.

Hepatocytotropic T-Cell Lymphoma

Hepatocytotropic T-cell lymphoma (HC-TCL) has been proposed as an entity in dogs that shares features with HS-TCL but is characterized by a more intimate association of neoplastic lymphocytes and hepatocytes (Figure 72.9), as well as differences in clinical presentation and immunophenotype.[71] Akin to HS-TCL, HC-TCL arises from γδ T cells and affects liver and spleen without causing peripheral lymph node enlargement. However, patients with HC-TCL present with severe jaundice and cholestasis. Anemia is not a primary feature and erythrophagocytosis and secondary hemophagocytic syndrome are not observed. Histologically, neoplastic lymphocytes invade hepatic cords and neoplastic cells are negative for the splenic red pulp marker CD11d. Hepatic lymphoma with emperipolesis has been described in cats, but additional data are needed to substantiate this group of elusive neoplasms as a distinct entity.[121,148]

FELINE T-CELL NEOPLASMS

Feline lymphoma is relatively common in cats, accounting for 10–50% of all feline tumors.[59] In prevalence studies prior to approximately 1990, many feline lymphomas were associated with feline leukemia virus (FeLV) infection, while data from the last few decades, since the advent of FeLV testing and vaccination, reflect a higher frequency of FeLV-negative lymphomas.[87] Recent data indicate that approximately 25% of feline lymphomas are of T-cell origin.[156] Both ALL and CLL in cats are predominantly of T-cell type.[13] As in dogs, there are several studies whereby the WHO classification has been adapted to characterize feline lymphoproliferative neoplasms.[162,174,175]

Precursor T-Cell Neoplasms

T-Acute Lymphoblastic Leukemia

Feline T-ALL is reported, but much less commonly than in humans and dogs. Limited information is available on this subtype, but few cases described were $CD3^+$ and/or $CD5^+$.[13]

Lymphoblastic T-Cell Lymphoma

Feline LBTCL is similar to the canine counterpart with a predilection for nodal and thymic/mediastinal sites. Prior to control of FeLV infection, LBLT accounted for up to 70% of feline lymphomas.[87] Lymphomagenesis due to FeLV infection results from insertion of a retrovirus promoter gene near the *c-myc* gene.[114] Spontaneous mediastinal LBTCL is also reported in cats, and is now more common than FeLV-induced LBTCL.[42] FeLV-associated LBTCL has a worse prognosis than non-FeLV-associated LBTCL with median survival times 25 and 373 days, respectively.[97,42]

Mature T-Cell Neoplasms

T-Cell Chronic Lymphocytic Leukemia

Over 90% of feline CLL are of T-cell origin, and virtually all are $CD4^+$.[18,166] Median age at the time of diagnosis is 12.5 years, and the most common clinical sign is weight loss; other signs include lethargy, vomiting, and diarrhea. Anemia or thrombocytopenia may be present, but are typically mild. Lymphocyte counts range from 10–325 × 10^9 cells/L, or rarely higher.[18] CLL cells are primarily of small size with mature condensed nuclear chromatin, slightly indented or cleaved nuclei, and indistinct nucleoli.[18,166] Infiltration of the

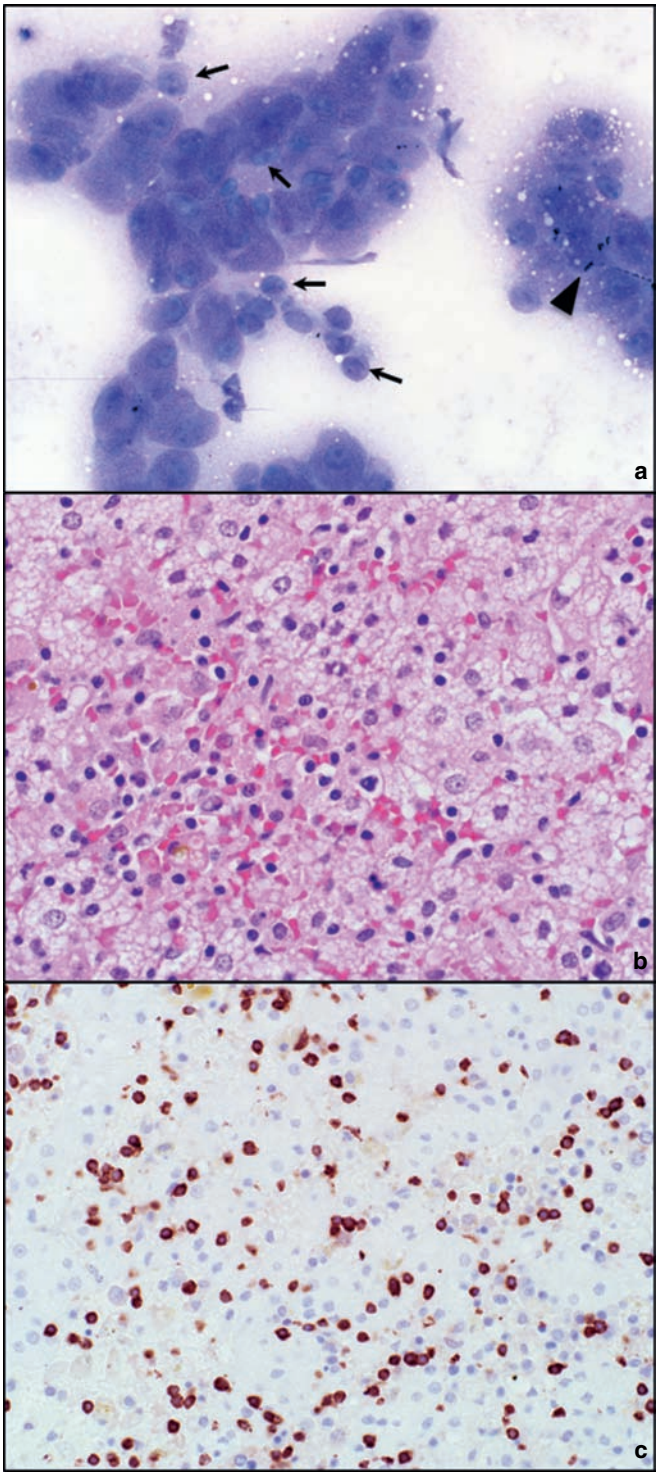


FIGURE 72.9 Samples from a 10-year-old dog with increased liver enzymes and jaundice. The liver and spleen were hyperechoic and irregular, respectively, on ultrasonic examination. (a) A liver aspirate consists of hepatocytes with occasional bile casts (arrowhead), and medium-size lymphocytes (arrows) in close relation to hepatocytes; (b) A liver biopsy shows numerous medium-size lymphocytes among and within hepatic cords. Similar cells were present throughout the splenic red pulp; (c) The cells were CD3-positive on IHC. Interpretation: Hepatocytotropic T-cell lymphoma.

bone marrow is variable suggesting that the neoplasm does not originate in the bone marrow.[18,166] Splenic involvement and concurrent EATL have been reported in cats with T-CLL.[18] Feline CLL generally has a favorable prognosis with median survival after diagnosis of 14.4 months.[18]

Peripheral T-Cell Lymphoma, Not Otherwise Specified

After EATL, PTCL-NOS is the second most common, accounting for 23% of feline lymphomas.[162] Clinical findings vary depending on the location of the tumor, and cytological and histopathological findings are similarly variable as in dogs.[1] Unlike in dogs, most cases of feline PTCL-NOS are extranodal, although rare nodal tumors are also observed. The primary extranodal location is the gastrointestinal tract, where PTCL-NOS needs to be distinguished histologically from EATL.[162] Non-epitheliotropic cutaneous, subcutaneous, laryngeal, upper respiratory, renal, and central nervous system PTCL-NOS have also been reported.[23,162,175] A minority of cutaneous vaccine-associated and tarsal lymphomas are of T-cell type, though B-cell lymphoma is more common in both locations.[17,130]

Since location does not reliably distinguish T-cell type from B-cell type, and immunophenotyping is not always performed in survival analysis, reliable median survival times are not available to separate these locations into distinct entities. One study showed overall median survival time of 6.1 months for cats with PTCL-NOS.[175]

Feline Large Granular Lymphocyte Lymphoma Feline LGL lymphoma is a unique type of PTCL-NOS that does not have a counterpart in the human WHO classification scheme. Age at the time of diagnosis is 10–11 years. Clinical signs are generally nonspecific but pronounced, and include inappetence, lethargy, weight loss, and vomiting. Frequently, a palpable abdominal mass is present. Complete blood cell counts often reveal a variable degree of anemia as well as neutrophilia, thrombocytopenia, and rarely eosinophilia.[43,78] An LGL lymphocytosis is common, and may be subtle or $>400 \times 10^9$/L.[132] Test results for feline immunodeficiency virus and FeLV are typically negative.[43,78] The feline gammaherpesvirus 1 has been detected in a single intestinal LGL lymphoma.[3]

Feline LGL lymphoma frequently affects more than one anatomic location, with a predilection for the small intestine, mesenteric lymph nodes, liver, and spleen. Cytologically, the neoplastic cells are typically intermediate size with characteristic prominent magenta/azurophilic cytoplasmic granules, round to indented/cleaved nuclei with fine granular to lacey chromatin and variably distinct nucleoli.[78] Immunophenotypically, LGL lymphoma is $CD3^+$, frequently $CD8\alpha\alpha^+$, and sometimes $CD4^+/CD8\alpha\alpha^+$ or $CD4^-/CD8^-$. Detection of the leukointegrin αE CD103 on the majority of LGL lymphocytes suggested small intestinal intraepithelial lymphocyte origin.[132]

Median survival for treated cats was <3 months from the time of diagnosis, and only 2 days for untreated cats, which may reflect diagnosis at an advanced disease stage.[127,132] In another study, it was suggested that lack of clinical abnormalities and lymphocytosis at the time of diagnosis were associated with longer survival.[43]

Extranodal Subcutaneous Lymphoma This subtype of PTCL-NOS in cats is relatively uncommon and does not have a WHO counterpart.[150] Approximately one third of subcutaneous lymphomas in cats are T-cell origin. Median age at time of diagnosis was 11 years, and lesions were present for several days to several months.[98] A small proportion of cats have lethargy, weight loss, and anorexia, and are retrovirus positive.[98]

The lesions consist of large, atypical lymphocytes infiltrating the deep dermis and subcutaneous fat. Frequent macrophages are present, creating a starry-sky appearance on histopathology. Extensive central coagulative necrosis is a consistent finding.[98]

Feline Enteropathy-Associated T-Cell Lymphoma

Unlike in humans and dogs, EATL is the most common form of lymphoma in cats, accounting for up to a third of all lymphomas and 50–84% of all gastrointestinal lymphomas.[87,107,124,162] The most commonly affected site is the small intestine, particularly the jejunum, while gastric and colonic involvement is much less common.[107] Although 60% of cats diagnosed with EATL have a prior history of inflammatory bowel disease, and some have lesions of both conditions in the intestines, whether and how frequently inflammation precedes neoplasia remains to be determined.[112]

Cats with EATL are typically older than 10 years of age and had a median age at diagnosis of 12.5 years.[107] Clinical signs mimic those of inflammatory bowel disease and include weight and muscle loss, inappetence, vomiting, and possibly diarrhea.[137] Lymphocytosis is present in 85% of cats with EATL of granular cell morphology.[132] Paraneoplastic eosinophilia and, less commonly, basophilia have also been reported in feline EATL.[9] Most cats have negative retrovirus test results.[107,124]

Feline EATL can be mucosal or transmural, with the former accounting for >80% of tumors. Mucosal tumors may present in a variety of distribution patterns, while the transmural lesions are most often focal or multifocal. Mucosal tumors consist primarily of small- to intermediate-sized lymphocytes suggestive of the EATL type 2 form.[107,174] These indolent lymphomas primarily affect the lamina propria without submucosal involvement and 60% have an increased number of intraepithelial lymphocytes that are more commonly in the villus than crypt epithelium. Most transmural lesions consist primarily of large lymphocytes that are granzyme B^+. In contrast to EATL type 2 lesions, EATL type 1 tumors have a heavy submucosal infiltration and, in advanced lesions, involve the muscularis propria and surrounding tissues. Thus, transmural lesions tend to be more consistent with EATL type 1, though they do not fully meet all histopathological criteria of this entity in other species.[107,174] Histopathologically, small cell lymphomas can be difficult to distinguish from inflammatory bowel disease, and features such as epitheliotropism, degree of infiltration, proportion of dilated crypts, severity of villus changes, proportion of plasma cells, and detection of clonally rearranged TCRs may be useful.[15] Aspirates of intestinal mass lesions or enlarged mesenteric lymph nodes may yield diagnostic specimens consisting predominantly of large lymphocytes that reflect EATL type 1, but they rarely allow distinction between inflammation and small cell lymphoma.

Feline EATL is defined by CD3 positivity, and IHC can assist with visualizing intraepithelial lymphocytes.[107] Detection of MHCII is variable, and further characterization is hampered by limited antibody availability.[15] Detection of a clonally rearranged T-cell receptor has a reported sensitivity and specificity of up to 90% and up to 100%, respectively, compared to histopathology and immunohistochemistry, but is dependent on factors such as primer coverage and amplicon resolution.[107,112] In one study, cross-lineage monoclonal rearrangement of the immunoglobulin heavy chain locus was noted in 9% of feline EATL; these cases also had polyclonal rearrangement of the TCR γδ locus.[4]

Treatment generally consists of prednisolone and chlorambucil, and, unsurprisingly, EATL type 2 is associated with longer survival than EATL type 1.[127,175,107] Survival was shorter with lomustine rather than glucocorticoid and chlorambucil rescue therapy (median survival 492 days).[127] Median survival for cats with EATL type 1 was 1.5 months, and for those cats with concurrent granular lymphocytosis it was only 19 days.[107,132]

Cutaneous T-Cell Lymphoma

Epitheliotropic Cutaneous T-Cell Lymphoma Feline CTCL is rare and compromises <1% of feline lymphomas and feline skin tumors.[50,162] The disease occurs in middle-aged to older cats, with the median age at diagnosis ranging from 10–13.5 years. Like dogs, disease progression is gradual. Clinical signs refer to isolated or generalized lesions, often affecting the face. The lesions include erythematous plaques or patches, scaly alopecic patches, and nonhealing ulcers or nodules.[46]

Mass lesions are amenable to cytological diagnosis (Figure 72.10), and characterized by large, atypical lymphocytes. Histopathological features include epitheliotropism, but intraepidermal lymphocyte aggregates appear to be less common than in dogs. The follicle epithelium is consistently but mildly affected. Spongiosis, parakeratosis, keratinocyte apoptosis, and inflammation may be present. Pagetoid reticulosis has not been identified in cats, but disease progression to Sézary syndrome has been reported.[46,176]

Neoplastic cells are consistently positive for CD3, and one case of CTCL was perforin-positive.[23,46,115]

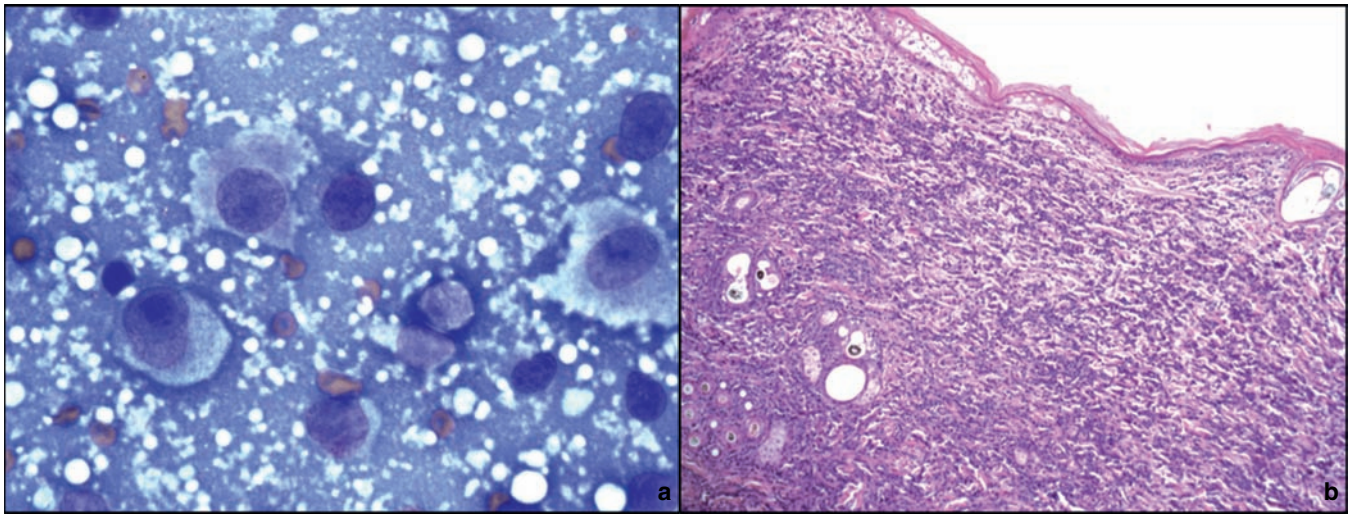


FIGURE 72.10 Samples from a cutaneous mass of a 9-year-old cat. (a) The aspirate consists of pleomorphic lymphocytes, including several very large lymphocytes with prominent nucleoli; (b) The mass consists of aggregates of large lymphocytes that infiltrate the dermis and superficial subcutis. Lymphocytes were $CD3^+$. Interpretation: Cutaneous T-cell lymphoma.

Tarsal Non-Epitheliotropic Cutaneous Lymphoma Cats are susceptible to a unique non-epitheliotropic cutaneous lymphoma of the tarsus. A majority of these are B-cell type, but some are of the PTCL-NOS subtype. Median age at time of diagnosis was 12 years, and lesions consisted either of individual or multiple masses proximal to each other. Systemic signs of illness were rare, and no association with retroviral infection was identified.[17] The tumors are characterized by sheets of intermediate to large lymphocytes effacing the affected tissue without epitheliotropism. Necrosis is common, tumors have a high mitotic rate, and are locally progressive.[17]

Cutaneous Anaplastic Large T-Cell Lymphoma Although non-epitheliotropic cutaneous lymphoma is more common than epitheliotropic lymphoma in cats, only a minority are of T-cell type.[17,130] A few of these tumors that fit into the WHO classification were consistent with the anaplastic large T-cell lymphoma subtype. Some were associated with vaccine injection sites.[130]

Angioimmunoblastic T-Cell Lymphoma

Feline angioimmunoblastic T-cell lymphoma is also very rare with only four cases reported. Of these, one was located in the eye and one was gastrointestinal; the remaining locations were not specified.[162,175] This type of lymphoma is characterized by proliferating high-endothelial venules with perivascular cuffing by immunoblastic lymphocytes.[1]

Extranodal T-Cell Lymphoma, Nasal Type

Nasal lymphoma in cats accounts for approximately 10–13% of all lymphomas, 20–40% of extranodal lymphomas, and 70% of all feline nasal tumors.[23,65,152,156] Unlike in humans, most nasal lymphomas in cats are B-cell type with T-cell lymphomas comprising <10%.[23,138,150,156] Nasal lymphoma is a disease of older cats, and lesions can be restricted to the nasal cavity or also affect the nasopharynx.[86,138] The most common clinical signs are nasal discharge and sneezing; additional signs may include unilateral or bilateral epistaxis, stridor or stertor, dyspnea, epiphora, ocular discharge, anorexia, and facial deformities.[138,152]

Aspirates of nasal masses can be readily diagnosed as lymphoma by cytology if an appropriate sample has been collected. Histopathologically, epitheliotropism may be noted in T-cell and B-cell nasal lymphoma, therefore immunohistochemistry is needed to differentiate these.[86] Cell size is variable.[86,138] In humans, these tumors are comprised of cytotoxic lymphocytes, and in some cat nasal lymphomas, cytoplasmic granules were also reported.[86,138] Mean survival with upper respiratory tract T-cell lymphoma was 53 days.[138]

Feline Intravascular Large T-Cell Lymphoma

A single case of intravascular large T-cell lymphoma was reported in a 7-year-old Siamese cat. The brain and kidneys were the most affected sites.[84]

EQUINE T-CELL NEOPLASMS

In horses, large surveys identified lymphoma as the third most common neoplasm and as the eighth most common cutaneous neoplasm.[75,140] Histomorphologic and immunohistochemical assessment of 203 tumor samples indicated that T-cell-rich B-cell lymphoma (TCRBCL) was the most common subtype (43%),

followed by PTCL (22%), DLBCL, and other less common subtypes.[39] Among 88 specimens of cutaneous lymphoma (which included 33 cases previously classified[39]), 84% were classified as TCRBCL and 13% as cutaneous T-cell lymphoma, with other infrequent subtypes.[101] In an earlier study of 37 cases of equine lymphoma, 26 (70%) were categorized as PTCL and five (14%) as TCRBCL.[100] Thus, there may be a discrepancy in the relative frequency of different subtypes of equine lymphoma, which may reflect sample bias, geographic prevalence of different subtypes, or differences in application of classifying criteria.

In veterinary pathology, TCRBCL is typically a histopathological diagnosis based on identification of fewer, large, often atypical, cells identified as B cells by CD20 or CD79a IHC, and numerous small lymphocytes that are $CD3^+$. In humans, entities with similar histomorphology may be nodular lymphocyte predominant Hodgkin lymphoma (NLPHL), which has indolent behavior and is restricted to a limited group of peripheral nodes, or T-cell/histiocyte-rich large B-cell lymphoma (THRLBCL), which is a rapidly progressive tumor.[60] Based on microdissected single-cell antigen receptor gene rearrangements and mutations, and application of a range of antibodies specific for different lymphocyte maturational stages, it is now recognized that NLPHL and THRLBCL in humans represent opposite ends of the spectrum of a particular type of B-cell lymphomas.[60] It is likely that equine TCRBCL, which has similarly variable histomorphology, often also including histiocytes, encompasses multiple overlapping entities. Immunohistochemical assessment of such complex neoplasms with just two antibodies is insufficient to confidently identify the neoplastic component, and until a greater range of antibodies and more comprehensive correlation with patient outcome are available, caution is indicated for assignment of the cellular origin of the neoplasm.

Frequently reported clinical features of equine lymphoma, regardless of T- or B-cell type, are weight loss, edema, and pyrexia.[39,73,100] Edema was unrelated to albumin concentration, and likely reflects hemodynamic effects of tumor burden. Systemic inflammation, as implied by hyperfibrinogenemia, hyperglobulinemia, and/or hypoalbuminemia, was present in the majority of horses with lymphoma; and cytopenia, most commonly anemia, was identified in 62%.[100] Therefore, many types of equine lymphoma are associated with systemic abnormalities.

Subtypes of T-cell lymphoma in horses are like those in dogs and cats. Diagnostic samples of intestinal T-cell lymphoma are challenging to obtain antemortem, but EATL types 1 and 2 have been identified in horses as in other species.[7] Mediastinal lymphoma is most often of T-cell type, and may be diagnosed by cytologic evaluation of pleural fluid samples (Figure 72.11). While TCRBCL was the most common subtype among cutaneous lymphomas, epitheliotropic T-cell lymphoma akin to MF in dogs and cats was second most common.[101]

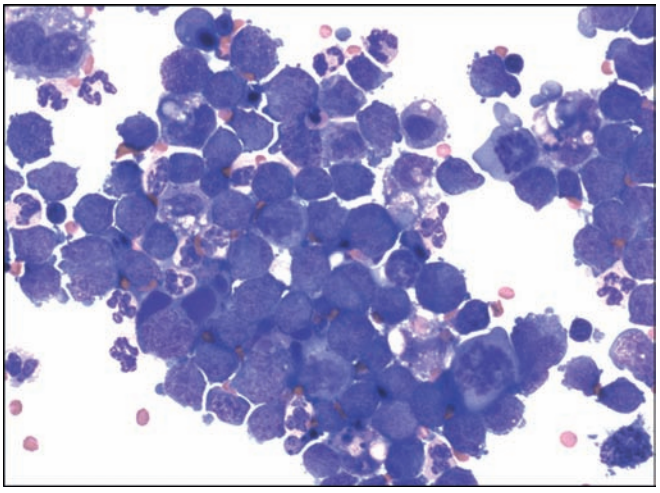


FIGURE 72.11 Cytocentrifuge preparation of pleural effusion fluid from a 6-year-old horse. The lymphocytes are very large in relation to RBC, and there are frequent mitoses and macrophages. At postmortem, the horse had a large mediastinal mass, and lymphocytes were $CD3^+$ by IHC. Interpretation: T-cell lymphoma.

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CHAPTER 73

Mast Cell Neoplasia

MELINDA S. CAMUS

Canine Mast Cell Tumor
- Signalment, Incidence, and Etiology
- History and Clinical Signs
- Diagnosis
- Staging
- Grading
  - Cytologic
  - Histologic
- Proliferation Markers
  - Mitotic Index
  - PCNAs
  - MCM7
  - Ki-67
  - AgNORs
- Other Prognostic Indicators
  - c-kit
  - Cell Morphometry

Feline Mast Cell Tumor
- Cutaneous
  - Signalment, Incidence, and Etiology
  - History and Clinical Signs
  - Diagnosis
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Mast Cell Tumors in Other Species

Acronyms and Abbreviations

AgNOR, argyrophilic nucleolar organizer region; CTMC, connective tissue mast cells; COX-2, cyclooxygenase-2; FGF, fibroblast growth factor; GI, gastrointestinal; H&E, hematoxylin & eosin; MCM7, minichromosome maintenance protein-7; MCT, mast cell tumor; MI, mitotic index; MNA, mean nuclear area; MMC, mucosal mast cells; MVD, microvascular density; PCNA, proliferating cell nuclear antigen; WHO, World Health Organization.

CANINE MAST CELL TUMOR

Signalment, Incidence, and Etiology

The mast cell tumor (MCT) accounts for approximately 20% of all canine skin tumors.[28,53] No age or sex predilection has been recognized, although increased breed-related incidence has been reported. Shar Peis reportedly have more biologically aggressive MCTs than Boxers and Pugs, who are predisposed to MCTs less prone to distant metastasis.[58,68] No definitive cause for MCT has been elucidated, although the possibility for a genetic cause is suspected, due to increased prevalence in certain breeds.[28]

History and Clinical Signs

MCTs demonstrate a wide variability of biologic behavior. They are most common in cutaneous tissues, with fewer subcutaneous and extracutaneous MCTs. Tumors in multiple locations typically represent distant metastasis rather than multiple primary tumors.[23] Owners often report that these masses wax and wane, as mast cell granules contain histamine, heparin, and vasoactive amines that can induce erythema, urticaria, and pruritus when released.[22] As release is triggered by physical manipulation, MCTs in locations that can be licked or scratched are most prone to intermittent redness and swelling.

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Occasionally, patients present with vomiting, abdominal pain, and evidence for gastrointestinal (GI) bleeding, as histamine release triggers upregulation of gastric acid production, via stimulation of H_2 receptors on gastric parietal cells. Chronic external blood loss due to ulceration can result in iron deficiency anemia.[23] Histamine levels can be quantified in plasma and are often elevated in patients with MCTs.[21]

Diagnosis

MCTs are readily diagnosed on cytologic examination. These neoplasms are composed of individualized mast cells with centralized round nuclei and abundant pale cytoplasm that contains numerous magenta granules, which often obscure the nucleus. When stained with aqueous solutions (e.g., Diff-Quik®), the granules may fail to uptake stain, potentially due to dissolution of granule contents. This artifactual change can be minimized by allowing slides to remain in the fixative component of the stain for at least 5 minutes, or by using a methanol-based Romanowsky stain. It is important to recognize that an absence of cytoplasmic granules may be due to cell degranulation, tumor microenvironment, or poor differentiation. Aspirates from MCTs which are degranulated often have abundant magenta granules in the background.

As canine mast cell granules contain interleukin (IL)-5, a cytokine which induces eosinophil migration, aspirates of most canine MCTs contain a mixture of both mast cells and eosinophils.[39] Additionally, tryptase-containing mast cells have been demonstrated to secrete fibroblast growth factors (FGF) that induce fibroblast proliferation and promote wound healing.[3,46] The presence of these plump mesenchymal cells can occasionally complicate the microscopic diagnosis, as the possibility for a neoplastic mesenchymal population (e.g., sarcoma) with a mast cell infiltrate must be excluded. Mast cells containing both tryptase and chymase demonstrate both collage proliferation and collagenolysis, which appear as brightly eosinophilic fibrous bands.[14,47]

Staging

The World Health Organization (WHO) staging system for MCTs consists of five tiers (0–IV) (see Table 73.1), with substages I and II which correlate with the absence or presence of clinical signs, respectively.[28] In the five-tiered system, dogs with stages 0 and I disease have prolonged survival when compared with dogs with higher-stage disease.[33] A consensus panel, which included members of the Veterinary Cancer Society and the European Society of Veterinary Oncology, disputes the organization of the WHO tiers, as dogs with stage II disease (single tumors) often exhibit worse outcomes than dogs with stage III disease (multiple tumors).[2,20,23] The recommended staging procedures vary among oncologists. However, it is generally accepted that easily resectable, well-differentiated MCTs confined to the skin warrant only aspiration of the draining lymph node (even if normal sized). However it is critical to ensure that the correct lymph node is aspirated through review of known lymphatic drainage or with the use of lymphoscintigraphy, as the node closest to the MCT may not be the draining node. In one study, only 60% of draining nodes would have been sampled if only the anatomically closest node had been sampled.[72]

Cytologic evaluation of lymph nodes for MCT metastasis can be challenging, as non-neoplastic mast cells are common lymph node residents and there is no definitive way to microscopically discriminate neoplastic from non-neoplastic mast cells. Relatively increased numbers of mast cells, while subjective, may suggest mast cell metastasis; however, manipulation of MCTs can cause recruitment of non-neoplastic mast cells to the draining node via the lymphatics.[23] For this reason, it is advised that lymph node aspiration occur prior to any physical MCT manipulation.[28] Although a system for evaluating the significance of mast cells in draining lymph nodes has been proposed, it remains largely unused due to its limited known prognostic utility.[25,34] Thus, cytopathologists often rely on overall number, mast cell pleomorphism, and arrangements in aggregates, to document microscopic metastasis. As architectural arrangement is more accurately assessed with histology, excisional biopsy of the draining lymph node is often performed. A four-tier histologic classification for nodal mast cell metastasis has shown promise in predicting disease progression[70] (see Table 73.2).

TABLE 73.1 WHO staging system for canine MCTs.[23]

Stage	Diagnostic criteria
0	One dermal tumor, incompletely removed, with no lymph node involvement
I	One dermal tumor, with no lymph node involvement
II	One dermal tumor, with lymph node involvement
III	Multiple dermal tumors or large, deep tumors with or without lymph node involvement
IV	Any tumor with distant (beyond local node) metastasis

TABLE 73.2 Histologic grading system for nodal metastasis in dogs with MCT.[70]

Stage	Diagnostic criteria
HN0	Zero to three isolated mast cells in sinuses and/or parenchyma per 400× field
HN1	More than three isolated mast cells in sinuses and/or parenchyma in a minimum for four 400× fields
HN2	Clusters of mast cells in sinuses and/or parenchymal or sinusoidal mast cell sheets
HN3	Disruption or effacement of nodal architecture by mast cells

Proposed interpretation: HN0 = non-metastatic; HN1 = pre-metastatic; HN2 = early metastasis; HN3 = overt metastasis.

With highly pleomorphic, known metastatic, or nonresectable MCTs, abdominal imaging is often utilized for detection of organomegaly. Although most ultrasonographically unremarkable abdomens lack mast cell metastasis, aspiration of normal-appearing liver and spleen may be warranted based on the presence of known or suspected nodal metastasis.[10,56,65] Although formerly recommended, buffy coat analysis is no longer advised in dogs, as mastocytemia is more likely the result of a non-neoplastic process than a neoplastic one.[29]

Grading

Cytologic

Grading of neoplasms involves evaluation of microscopic criteria to predict disease progression. While traditionally performed on histologic sections, cytologic grading schemes for canine cutaneous MCT (cMCT) have been developed. These grading systems vary from direct application of histologic criteria to the de novo development of a predictive cytologic grading algorithm. Although both types of cytologic grading systems correlate well with histology, they tend to overestimate the number of high-grade tumors.[12,19,56] This overestimation may result in unnecessarily aggressive staging, surgery, or, potentially, euthanasia. As such, cytologic grading is recommended as a screening test with a high likelihood that low cytologic grade correlates with relatively benign biologic behavior.[12]

The cytologic feature most predictive of biology is tumor cell granularity, as poorly granular tumors metastasize at a higher rate than highly granular tumors. The ability to recognize cells that are poorly granulated, rather than poorly stained or degranulated, is essential to cytologic grading. Other features associated with aggressive biologic behavior include anisokaryosis, binucleation, multinucleation, and bizarre nuclear morphology.[12,44] In highly granulated MCTs, where nuclear morphology is obscured, staining cytologic specimens with H&E, which minimizes granule staining, may be of value in assessing nuclear features.[44]

Histologic

Three histologic grading systems exist, two of which are commonly utilized: the three-tier (Patnaik) system and the two-tier (Kiupel) system.[24,37] The Patnaik system utilizes tumor cellularity, tumor cell morphology, mitotic index (MI), degree of tissue involvement, and stromal reaction, to assign a histologic grade of I–III.[37] Grade I tumors are associated with an excellent prognosis, requiring only complete surgical excision, whereas grade III tumors are more aggressive and require adjunctive chemotherapy. Using this scheme, most MCTs are assigned a histologic grade of II, which is a subtype associated with a 5–22% metastatic frequency.[9,54] This predominance of grade II tumors and a wide interpathologist variability limit the clinical utility of the Patnaik system.[35] The Kiupel grading system was developed, in part, to eliminate this "gray zone" of grade II tumors. The Kiupel system utilizes karyomegaly, mitotic index, multinucleation, and bizarre nuclear morphology as criteria for classifying MCTs as either high- or low-grade.[24] This system has been demonstrated to have high prognostic value with minimal interobserver variability.[54] Currently, as recommended by the Veterinary Cancer Society's Oncology–Pathology Working Group, most pathologists report both grades.[7] However, even using the strict criteria defined by both systems, grading inconsistencies remain, particularly with regard to margin evaluation and subcutaneous MCTs.[41]

Proliferation Markers

Indicators of cell proliferation rate have been utilized to prognosticate clinical outcome in MCT patients. These indicators include MI, the percentage of cells expressing proliferating cell nuclear antigen (PCNA), minichromosome maintenance protein-7 (MCM7), and/or Ki-67, and the presence of argyrophilic nucleolar organizer regions (AgNORs).[8,27] Each marker provides information about a different component of the cell cycle or proliferation rate. While consensus is lacking on their utility and practical application, all proliferation markers have been reported as useful prognostic indicators in dogs with MCTs.[22]

Mitotic Index

MI is assessed on H&E sections and represents the number of mitoses in 10 high-power (100× objective) fields. Although inter-region variability and differences in lens aperture make standardization difficult, the utility of MI as a predictor of outcome is well documented, with relatively low MI (e.g., <7) tumors associated with less-aggressive biologic behavior than tumors with a high MI.[15,49,66]

PCNAs

PCNAs are nonhistone nuclear proteins required for DNA synthesis and are most commonly detected during the S-phase of mitosis. They are detected using immunostaining, and semi-quantitative scoring methods are used to determine the fraction of proliferating cells.[59] Although higher PCNA estimates are reported in metastatic versus non-metastatic MCTs, PCNA is considered less useful than MI and AgNORs, perhaps because of lack of uniformity in quantification procedures and due to the presence of nonspecific staining.[28,59]

MCM7

Minichromosome maintenance proteins are a group of proteins essential for DNA replication. Proteins 2–7 are bound together and to chromatin in phase G_1 of the cell cycle and they are removed from the nucleus and remain in the cytoplasm during the S-phase and in subsequent phases. They are responsible for ensuring the appropriate order of mitosis and for restricting the cell to a single

mitosis per cycle. With the help of morphometric software, MCM7 can be quantified using immunostaining and an index generated from the number of positive versus negative cells. A higher MCM7 index has been associated with a shorter mean survival in canine cMCT.[8]

Ki-67

Ki-67 is a nuclear protein expressed in all cycling cells, but is absent in all resting cells (in G_0). As with PCNAs, assessment of the Ki-67 expression is done with immunohistochemistry.[69] The fraction of cells expressing Ki-67, termed the Ki-67 labeling index, has been positively correlated with disease progression in numerous neoplasms (including canine cMCT), but its stand-alone utility is limited.[23,57]

AgNORs

AgNORs are structures within nucleoli that are responsible for transcription of ribosomal RNA. Due to their affinity for silver, these areas stain black with a specialized silver stain. The number of AgNORs per nucleus has been associated with both cell proliferation rate and rate of tumor growth in canine cMCT.[69] However, no consensus cutoff score for AgNORs has been reached, thus diminishing its utility.[23] Use of a combined Ki-67 and AgNOR score has shown promise as a prognostic indicator, as it combines both the number of cycling cells (Ki-67) and the proliferation rate (AgNOR) into a single metric, which may prove useful in predicting biologic behavior.[62]

Other Prognostic Indicators

c-kit

KIT is a tyrosine kinase receptor normally located on the surface or within the membrane of numerous cells, including mast cells, hematopoietic stem cells, and melanocytes. When phosphorylated, KIT initiates an intracellular signaling cascade that induces cell proliferation, differentiation, and maturation.[50,51] KIT is detected in histologic sections using immunostaining and its pattern of localization (i.e., cytoplasmic versus membranous) is associated with receptor dysregulation.[23] Mutations in both the extracellular and the transmembrane domains of the c-kit gene have been identified in 25–30% of intermediate and high-grade MCTs. The cause of the high mutation of the c-kit gene in canine MCTs is unknown, but it is not demonstrated to be germline in nature, and is thus not heritable.[28] The detection of specific mutations is associated with differences in prognosis depending on mutation type. Thus, PCR for c-kit mutations, rather than immunologic detection of the KIT receptor, may become valuable as a prognostic tool in canine MCTs.[23]

Cell Morphometry

Cell morphometry is computer-assisted image analysis, which requires specialized software and advanced training. It can be applied to both cytologic and histologic specimens to determine mean nuclear area (MNA) and microvascular density (MVD), the latter with the concurrent immunostaining for factor-VIII-related antigen and cyclooxygenase-2 (COX-2). Using MNA analysis, larger nuclei have been correlated with shorter mean survival times, and higher MVD have been linked to higher histologic grade in canine MCT.[5,17,30,38,40,66]

FELINE MAST CELL TUMOR

Mast cell neoplasia in cats is best divided into three clinical entities: cutaneous, visceral, and intestinal. As these represent different clinical entities, each will be discussed separately.

Cutaneous

Signalment, Incidence, and Etiology

The MCT accounts for approximately 21% of all feline skin tumors.[71] There has been a dramatic increase in the number of feline cMCTs since 1950 and the global incidence may vary by geographic distribution.[28] Most cMCTs occur in cats over age four, although young Siamese cats may have an increased incidence of atypical MCTs.[23] No definitive age predilection has been identified. No definitive cause for feline MCT has been elucidated, although gain-of-function mutations in the c-kit gene are reported in up to 68% of cats with cutaneous MCT.[23] However, in one study of cats with multiple cMCTs, the majority of neoplasms contained different c-kit mutations within individual tumors, suggesting that additional factors play a role in the development of feline MCTs.[53]

History and Clinical Signs

Most feline cMCTs are solitary, although multiple clustered or diffuse lesions can exist.[28] Tumors most commonly occur on the head and neck, with fewer on the truck and extremities. Typical feline cMCTs are raised and alopecic, with variable degrees of erythema, although occasional plaque-like lesions resembling feline eosinophilic granulomas are described.[28]

Diagnosis

The majority of feline cMCTs are well-differentiated and easy to diagnose on cytology, although they are often less granular than their canine counterpart.[23] However, there is an atypical-appearing subtype of feline cMCT that contains few well-granulated mast cells admixed with scattered histiocytes, lymphocytes, and eosinophils.[28,71]

Staging

Although there is no standardized staging system for feline MCTs, a search for multiple cutaneous nodules is recommended. If more than one mass is noted,

aspiration of the spleen is advised, even if ultrasonographic evaluation is unremarkable, as splenic involvement is described in cats with multiple MCTs and splenectomy has been associated with prolonged survival time.[26]

Unlike in dogs, buffy coat evaluation may be valuable, as cats rarely have circulating mast cells in the absence of MCT.[16] Most cats with peripheral mastocytosis have splenic involvement, rather than only cutaneous MCTs.[61]

Grading

There is no standardized diagnostic approach, including cytologic or histologic grading scheme, for feline cMCT. Previous attempts to apply the canine Patnaik grading scheme were unhelpful.[11,32] While some recommend dividing these neoplasms into two types, mastocytic and histiocytic, others advocate for a system containing three types, namely, well-differentiated, pleomorphic, and atypical (histiocytic).[23,28] A European consensus group composed of both pathologists and oncologists suggests dividing these tumors into mastocytic and histiocytic types, with further division of the mastocytic type into well-differentiated and pleomorphic subtypes.[9]

Although the proposed terminology is confusing, the vast majority of feline cutaneous MCT are easily diagnosed. Occasionally, tumors of the atypical or histiocytic types lack characteristic granules and may require special stains (e.g., Giemsa or toluidine blue). These tumors occur rarely and may initially be confused with histiocytic inflammation or non-mast cell neoplasia.[23] Unlike their canine correlates, feline cMCTs typically contain low numbers of eosinophils and minimal collagenolysis.[23] This is likely due to the fact that the granules of feline mast cells lack eosinophil chemotactic factor and other collagenolytic substances.

While pleomorphism does not typically correlate with aggressive biologic behavior in feline cMCTs, a high mitotic index and high tumor burden (>5) have been associated with poor outcome, regardless of histologic type.[22,53] Immunohistochemical staining and most proliferation markers have proven minimally helpful in prognostication of feline cMCTs. While high Ki-67 expression has been associated with more biologically aggressive MCTs, no definitive cutoff score has been determined, limiting the utility of this index.[53]

Visceral

Signalment, Incidence, and Etiology

MCTs are one of the most common splenic lesions in cats, accounting up to 15% of all splenic pathology.[63] Older cats are typically affected and there is no known breed or sex predilection.[26,63] While the spleen is the primary site, concurrent involvement of the liver, intraabdominal lymph nodes, bone marrow, lung, and intestine has been reported.[28]

History and Clinical Signs

Cats with visceral MCT most commonly present with systemic signs, including vomiting, anorexia, and weight loss. Palpable splenomegaly and the presence of an eosinophil and/or mast-cell-rich effusion may be noted. GI ulceration with hemorrhage or hypotensive shock may be evident, due to the release of mast cell mediators.[28]

Diagnosis/Staging

Visceral MCTs are typically diagnosed with cytologic examination of effusions and splenic aspirates. In some cats with multicentric disease, there are no ultrasonographic lesions, while in other cases, discrete nodules are noted.[18] Buffy coat examination may be helpful both initially and to monitor response to therapy, as the number of circulating mast cells may positively correlate with response to therapy and disease progression.[28] Following splenectomy, median survival time for feline visceral MCTs is 12–19 months.[28]

Intestinal

Signalment, Incidence, and Etiology

Similar to cats with splenic MCTs, cats with intestinal MCT most commonly present with systemic signs, including vomiting, diarrhea, anorexia, and weight loss. One or more palpable intestinal masses may be present and the presence of an eosinophil and/or mast-cell-rich effusion may be noted.[28] eighty-five percent of feline intestinal MCTs are located within the small intestine, with roughly equal distribution between the duodenum, jejunum, and ileum. Colonic involvement is much less frequent.[28]

Diagnosis/Staging

Intestinal MCTs can be challenging to diagnose, as neoplastic cells often lack characteristic metachromatic granules on both cytologic and histologic preparations. This may be due to the fact that there are two different types of mast cells, connective tissue mast cells (CTMC) and mucosal mast cells (MMC), both of which reside in the intestinal tract and exhibit different MMCs (see Chapter 47), which comprise a large number of feline intestinal MCTs, and exhibit low granularity due to either poor differentiation or poor granule preservation. Use of non-formalin fixatives (e.g., Carnoy's) may better preserve mast cell granules in specimens stained with both H&E and special stains.[23,45]

As feline intestinal MCTs often spread to distant locations, both abdominal and thoracic imaging can be used to determine disease spread.[28] Buffy coat smears are less useful for monitoring disease progression and response to therapy, as peripheral mastocytosis is rarely seen in cats with intestinal MCT. Prognosis is typically poor in these cats, as most exhibit distant metastasis at the time of presentation.[28]

MAST CELL TUMORS IN OTHER SPECIES

MCTs have been reported in numerous veterinary species, including horses, cattle, pigs, ferrets, goats, snakes, and iguanas.[1,4,6,13,23,31,42,43,48,60,64]

In horses, MCTs are uncommon. They are overrepresented in males and Arabians and typically present as a single, variably appearing mass over the head, neck, or legs.[13,23] The vast majority are cured with surgical excision, although low numbers of equine MCTs reportedly spread to local lymph nodes.[43,48] Diagnosis is typically easily accomplished in both cytologic and histologic specimens, where both eosinophils and collagenolysis can be observed. Equine MCTs occasionally demonstrate dystrophic mineralization, which can complicate the diagnosis.[23] No definitive predictors of biologic behavior have been discovered for equine MCTs.[13,23]

MCTs in cattle are typically malignant and disseminate widely to both lymph nodes and viscera, although occasional solitary cMCTs have been reported.[1,23] Microscopic appearance of bovine MCTs is variable, ranging from well-granulated with abundant eosinophils to poorly granular with rare eosinophils. As such, a diagnosis of round cell neoplasia in cattle warrants special or immunohistochemical stains to distinguish MCT from histiocytic lesions.

Both solitary cutaneous and disseminated MCTs, including cases of mast cell leukemia, have been reported in pigs, although the overall incidence of MCTs is very low in this species.[6,31,60] Porcine MCTs may exhibit poor granularity, which may result from a predominance of mucosal mast cell-origin tumors and may require nontraditional fixatives for histologic specimens.[31,45] Due to the low prevalence of porcine MCTs, there is little information regarding prognosis or response to therapy.

MCTs are the most common cutaneous neoplasm of ferrets. MCTs in ferrets appear similar microscopically to those in cats and are relatively easy to diagnose.[4,35] Visceral and intestinal forms also exist. Despite their prevalence, the paucity of pet ferrets makes the overall number of these neoplasms low, limiting available prognostic and treatment information.

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CHAPTER 74

Histiocytic Proliferative Diseases of Dogs and Cats

PETER F. MOORE

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Acronyms and Abbreviations

APC, antigen-presenting cell; BG, Birbeck granules; CCH, canine cutaneous histiocytoma; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (lomustine); CH, cutaneous histiocytosis; CNS, central nervous system; DC, dendritic cell; EMH, extramedullary hemopoiesis; CTCL, cutaneous T cell lymphoma; FPH, feline progressive histiocytosis; HHS, hemophagocytic histiocytic sarcoma; HS, histiocytic sarcoma; IHC, immunohistochemistry; LC, Langerhans cell; cLCH, cutaneous Langerhans cell histiocytosis; MH, malignant histiocytosis; PAHS, periarticular/articular histiocytic sarcoma; pLCH, feline pulmonary Langerhans cell histiocytosis; SH, systemic histiocytosis; CDKN2A/B, cyclin dependent kinase inhibitor 2A/B; RB1, retinoblastoma protein 1; PTEN, phosphatase and tensin homolog; PTPN11, protein tyrosine phosphatase non-receptor type 11; MEK, mitogen-activated protein kinase kinase; MAPK, mitogen-activated protein kinase; PDGF, platelet derived growth factor.

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AN OVERVIEW OF CANINE AND FELINE HISTIOCYTIC DISEASES

There are several well-defined histiocytic proliferative diseases that have been recognized in dogs (Table 74.1).[55] These disorders represent a frustrating group of diseases because it may be difficult to differentiate them from granulomatous, reactive inflammatory disorders or from lymphoproliferative diseases in regular paraffin sections. The clinical presentation and behavior and responsiveness to therapy vary tremendously between the syndromes observed.

The reactive histiocytoses have only been identified in dogs; there is no known feline counterpart. The reactive histiocytoses, cutaneous histiocytosis (CH) and systemic histiocytosis (SH), are related disorders arising from activated interstitial dendritic cells (DCs). Reactive histiocytoses are histiocyte- and lymphocyte-rich inflammatory disorders associated with a degree of immune dysregulation, which promotes lesion persistence. Cutaneous histiocytosis presents with single or multiple lesions, which tend to wax and wane, and may even spontaneously regress. Some cases respond to prednisone, some respond to tetracycline and niacinamide, and the remainder persist and may require more

TABLE 74.1 Histiocytic diseases of dogs and cats.

Disease	Species	Cell of origin	Key morphological features	Immunophenotype
Cutaneous histiocytosis	Dog	iDC-activated	Vasocentric lesions are focused on mid-dermis to subcutis ("bottom-heavy"). Lesions are pleocellular but are dominated by histiocytes and lymphocytes. Lympho-histiocytic vasculitis is commonly observed. Histiocytes lack cytologic atypia, and multinucleated giant cells are rare. Skin-draining lymph nodes may be infiltrated.	CD1a, CD4, CD11c/CD18, CD90, Iba-1, CD204 (variable)
Systemic histiocytosis	Dog	iDC-activated	Lesions are identical to cutaneous histiocytosis in skin. Lesions extend to lymph nodes, ocular and nasal mucosae, and internal organs.	CD1a, CD4, CD11c/CD18, CD90, Iba-1, CD204 (variable)
Histiocytoma	Dog	LC	Lesions have an epidermal focus ("top-heavy"), and intra-epidermal foci are common. Histiocytes have diverse nuclear morphology (round, ovoid, indented, or complex nuclear contours). Multinucleated cells and cytologic atypia are rare.	CD1a, CD11c/CD18, E-cadherin, Iba-1, CD204 (negative)
Cutaneous Langerhans cell histiocytosis	Dog	LC	Multiple cutaneous lesions are observed. Metastasis to lymph nodes and internal sites is possible. Lesions are otherwise identical to histiocytoma, but may have a higher frequency of multinucleated cells and cytologic atypia.	Identical to histiocytoma
Histiocytic sarcoma	Dog, cat	iDC	Mass lesions are observed in spleen, lung, lymph node, periarticular, and other primary tissue sites. Histiocytes are pleomorphic, mononuclear, and multinucleated giant cells with marked cytological atypia.	CD1a, CD11c/CD18, Iba-1, CD204 (variable)
Histiocytic sarcoma—hemophagocytic	Dog, cat	Macrophage	Mass lesions are lacking. Diffuse splenomegaly and insidious infiltration of liver lung and bone marrow are consistently observed. Splenic red pulp is expanded by erythrophagocytic histiocytes. Mononuclear and multinucleated giant cells with cytologic atypia are common. Alternatively, histiocytes may have little cytological atypia.	CD1a (low), CD11d/CD18 (dog), Iba-1, CD204
Dendritic cell leukemia	Dog	iDC	Predominant blood and bone marrow involvement is observed. There is diffuse infiltration of spleen, lung, and liver. Histiocytes manifest moderate cytologic atypia in blood and tissues.	CD1a, CD11c/CD18, Iba-1*, CD204*
Feline progressive histiocytosis	Cat	iDC	Skin nodules and plaques are observed. Lesions occupy the dermis with an epidermal focus. Intra-epidermal foci (40%) occur. In early lesions, histiocytes have minimal cytologic atypia. In later lesions, histiocytes manifest cytological atypia as described for histiocytic sarcoma.	CD1a, CD11c*/CD18, CD5 (50%), Iba-1, CD204 (variable)
Pulmonary Langerhans cell histiocytosis	Cat	LC	There is multinodular to diffuse involvement of all lung lobes. Lesions consist of cohesive histiocytic infiltrates, which obliterate terminal airways and extend to pleural surfaces. Birbeck granules observed by TEM.	CD1a*, CD11c*/CD18, E-cadherin, Iba-1*, CD204*

TEM = transmission electron microscopy; LC = Langerhans cell; iDC = interstitial dendritic cell; CD1a* = expected, not assessed to date; CD11c* = expected but not yet assessable in cats; Iba-1* = not reported; CD204* = not reported.

aggressive immunosuppressive therapy. Systemic histiocytosis is a familial disease of Bernese Mountain Dogs that occurs sporadically in other breeds. SH presents with prominent skin manifestations identical to CH, but systemic involvement, including mucous membranes (ocular and nasal), lymphoid organs, lung, and bone marrow, may occur. Although the lesions may wax and wane, SH is a progressive disease that often requires continuous immunosuppressive therapy with cyclosporine or leflunomide.

Canine cutaneous histiocytoma (CCH), which exhibits evidence of Langerhans cell (LC) differentiation, usually occurs as a solitary lesion in young dogs and spontaneously regresses. Cutaneous Langerhans cell histiocytosis (cLCH) covers a spectrum of disease in which multiple cutaneous lesions are consistently observed. In some cases, lymph node and systemic metastasis are also observed. Individual skin lesions otherwise resemble those in CCH. Hence, these two diseases could be considered part of a larger spectrum of disease with diverse biological behavior. Feline counterparts of CCH and cLCH have not been reported.

The histiocytic sarcoma (HS) complex occurs in dogs and cats. Histiocytic sarcomas occur with a high incidence in Bernese Mountain Dogs, Rottweilers, Flat-coated Retrievers, Golden Retrievers, and sporadically in many other breeds. The incidence of HS in cats is much lower than in dogs. Histiocytic sarcomas occur as localized lesions in spleen, lymph nodes, lung, bone marrow, skin and subcutis, brain, and articular tissue of appendicular joints. HS may occur as multiple lesions in single organs (especially spleen and skin), and rapidly disseminate to involve multiple organs. This form of the disease was formerly called malignant histiocytosis (MH), but is now more appropriately termed disseminated HS. HSs mostly arise from interstitial DCs. However, the hemophagocytic form of HS is a distinctive clinicopathologic entity, which arises from splenic red pulp (± bone marrow) macrophages. Durable responses to chemotherapy are difficult to achieve.

There are two feline histiocytic diseases that do not have a canine equivalent: feline progressive histiocytosis (FPH) and pulmonary Langerhans cell histiocytosis (pLCH). FPH is the most common histiocytic disease in cats, begins as cutaneous nodules and plaques, and is essentially a low-grade HS with an initially indolent behavior. Later in the clinical course, the lesions are more aggressive. Pulmonary LCH is a disease of aged cats (10–15 years), which causes progressive respiratory failure due to extensive obliteration of the pulmonary parenchyma by an infiltrate dominated by LCs.

HISTIOCYTIC LINEAGES

The cellular spectrum covered by the term histiocyte has evolved over time. Histiocyte is now considered an overarching term to describe cells of DC or macrophage lineage.[16,26] Histiocytes differentiate from CD34+ stem cell precursors into macrophages and several DC lineages, which encompass LCs and interstitial DCs. Langerhans cells occur within epithelia of the skin and alimentary, respiratory, and reproductive tracts.[52] Interstitial DCs occur in perivascular locations in many organs except the brain, although they do occur in the meninges and choroid plexus.[22] Among the most studied DCs are those that occur in skin; these include epidermal LCs and dermal DCs, which are interstitial DCs. Dendritic cells that occur in T cell domains in peripheral lymphoid organs (lymph node and spleen) are known as interdigitating DCs. Interdigitating DCs in lymph nodes are comprised of resident DCs and migratory DCs. The migratory DCs arrive in lymphatics from tissues and consist of LCs and interstitial DCs.[85] The majority of canine and feline histiocytic diseases involve proliferations of cells of LC or interstitial DC lineage. Hemophagocytic histiocytic sarcoma (HHS) is currently the only histiocytic disease of dogs and cats that has been shown to originate from macrophages.[55]

Cytokines and growth factors that influence DC development include fms-like tyrosine kinase (FLT3) ligand, granulocyte–macrophage colony-stimulating factor (GM-CSF), stem cell factor, tumor necrosis factor alpha (TNF-α), interleukin 4 (IL-4), and transforming growth factor beta 1 (TGF-β1).[44,85] Macrophage development from CD34+ precursors is influenced by GM-CSF and macrophage colony-stimulating factor (M-CSF). Blood monocytes can differentiate into either macrophages under influence of M-CSF or into DCs under the influence of GM-CSF and IL-4.[83,84] Langerhans cell differentiation is critically dependent on TGF-β1 stimulation.[44,52,73] Epidermal keratinocytes constitutively secrete TGF-β1, which promotes the differentiation of LCs from dermal CD14+ precursor cells. These precursor cells upregulate C–C chemokine receptor 6 (CCR6) and migrate into the epidermis to become immature epidermal-resident LCs in response to locally produced macrophage inflammatory protein 3 alpha (MIP-3α or CCL20). Langerhans cells localize within epithelia via E-cadherin homotypic adhesion.[44,52] Langerhans cells, in the quiescent conditions that prevail in normal skin, are subject to slow turnover and are maintained by self-renewal or renewal from a dedicated dermal precursor cell.[44,52] Dermal interstitial DCs are subject to more rapid turnover and are repopulated by a blood-borne precursor (perhaps the monocyte) and by local self-renewal.[52,100] Culture of LCs in the presence of GM-CSF and IL-4 results in further differentiation of LCs into functionally mature DCs with enhanced potential to present antigen to T cells with appropriate costimulation. These latter conditions are present in states of cutaneous inflammation.[44]

IMMUNOPHENOTYPIC MARKERS OF HISTIOCYTES

Dendritic cells are the most potent antigen-presenting cells (APC) for induction of immune responses in naïve T cells. The development of canine- and feline-specific

monoclonal antibodies has enabled the identification of DCs and macrophages in canine and feline tissues, especially skin.[53,72,77] Dendritic cells occur in two major locations: (1) within the epidermis (LCs), and (2) within the dermis, especially adjacent to postcapillary venules (dermal interstitial DCs).[100] Canine and feline DCs express CD1a molecules which together with major histocompatibility complex (MHC) class I and class II molecules are responsible for presentation of peptides, lipids, and glycolipids to T cells.[47,53,61,77,79,97] Hence, DCs are best defined by their abundant expression of molecules essential to their function as APC. In skin, the family of CD1 proteins is largely restricted in expression to DCs; while MHC class I and II are more broadly expressed. Antigen presentation is only effective in activating naïve T cells in the presence of costimulation, which is mediated by B7 family members (CD80, CD86) expressed on DCs. Termination of immune responses is accomplished via signaling through the checkpoint inhibitors CTLA-4 and PD-1, which are expressed on T cells (Figure 74.1).[9,12,46,78]

The β-2 integrins (CD11/CD18) are critically important adhesion molecules, which are differentially expressed by all leukocytes. β-2 integrins are heterodimeric molecules consisting of one of four α subunits (CD11a–d) and a single β subunit (CD18 or β-2).[23,24,94] Canine-specific monoclonal antibodies for the known human β-2 integrin α subunits CD11a (αL), CD11b (αM), and CD11c (αX) were produced (Leukocyte Antigen Biology Laboratory, UC Davis—LABL). Also, a monoclonal antibody specific for a novel canine β-2 integrin α subunit was discovered.[24] This antibody was then used to characterize the canine protein, which led directly to the discovery of the human analog designated αD and by inference CD11d.[95] Subsequently, CD11d expression was used to define macrophage origin in HHS, which differed markedly from HS of interstitial DC origin, which expressed CD11c.[5,58]

CD11/CD18 expression is highly regulated in normal canine macrophages and DCs. CD11c is expressed by LCs and interstitial DCs; while macrophages predominately express CD11b (or CD11d in hematopoietic environments such as the splenic red pulp and bone marrow). A subset of dermal interstitial DCs also expresses CD11b.[23,24,72] In diseased tissues, these β-2 integrin expression patterns may be broadened. β-2 integrins are detectable in feline tissues; however, monoclonal antibodies specific for feline CD11a and CD11c are not available. Feline CD11b and CD11d are detectable with cross-reactive anticanine monoclonal antibodies (LABL). Canine and feline CD18 and canine CD11d are detectable by species-specific monoclonal antibodies that were developed for use in formalin-fixed paraffin-embedded (FFPE) tissue (LABL). These monoclonal antibodies have greatly facilitated the investigation of histiocytic diseases in dogs and cats.[2–5,13,21,28,53,58,61]

Histiocytes (macrophages and DC) express ionized calcium-binding adaptor molecule-1 (Iba-1), which is detectable in many species in FFPE tissue.[35,69] Macrophages express an array of scavenger receptors including the class A scavenger receptors, CD163 and CD204. The latter is more broadly, but not uniformly, expressed by macrophages.[37] For example, microglia in the brain do not express CD204, although it is expressed by perivascular macrophages. Epidermal LCs and interdigitating DCs in normal tissues do not express CD163 and CD204.[37,92] CD163 and CD204 are markers of M2 macrophages, which, in contrast to M1 macrophages, inhibit inflammation.[41] Dendritic cells in other locations can express CD163 and CD204.[10,50,98] Detailed characterization of the expression pattern of class A scavenge receptors on the diverse DC populations in dogs and

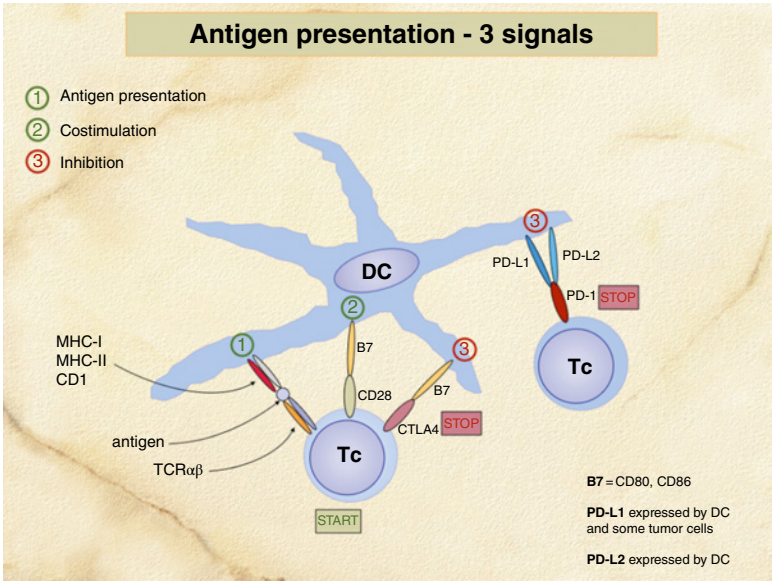


FIGURE 74.1 DCs express molecules involved in antigen presentation (CD1, MHC1, MHCII) and costimulation (B7 family: CD80 and CD86). DCs also signal through the checkpoint inhibitors expressed on T cells (CTLA-4 and PD-1), which leads to inhibition of immune responses.

cats is currently lacking. Langerhans cells and dermal interstitial DCs are also distinguishable by their differential expression of E-cadherin (LCs+) and Thy-1 (CD90) (dermal interstitial DCs+).

DENDRITIC CELL MIGRATION

The migration of DCs (as veiled cells) beyond the skin to the paracortex of lymph nodes, where they join forces with interdigitating DCs, occurs in the normal steady state and is enhanced in inflammatory states.[52,100] Successful interaction of DCs and T cells in response to antigenic challenge involves the orderly appearance of costimulatory molecules (B7 family—CD80 and CD86) on DCs, and their ligands—CD28 and cytotoxic T-lymphocyte antigen 4 (CTLA-4) on T cells (Figure 74.1).[9,78] In situ DCs have low expression of MHCII and costimulatory molecules and are more receptive to antigen uptake. Migratory DCs upregulate MHC class II and B7 family members and become more adept at antigen presentation to T cells.[9]

Aspects of the developmental and migratory program of DCs are recapitulated in canine and feline histiocytic diseases. Defective interaction of DCs and T cells may contribute to the development of canine CH and SH, which are related interstitial DC disorders that likely occur in the context of disordered immune regulation. The distant migratory potential of DCs is of immense clinical significance in the adverse prognosis of histiocytic sarcomas, which largely originate in interstitial DCs and rapidly disseminate via metastasis.

CANINE REACTIVE HISTIOCYTOSES

Reactive Histiocytoses—General Considerations

Reactive histiocytoses have been described in dogs only and are either confined to skin and draining lymph nodes (CH) or involve skin and extracutaneous sites (SH). Systemic histiocytosis was first described in Bernese Mountain Dogs and a familial association was apparent.[56] Histiocytic sarcomas occurred in the same families of Bernese Mountain Dogs as SH, but progression of SH to HS was not observed.[60] Systemic histiocytosis occurs in other large breeds of dogs. Overall, SH is a rare disease and CH is far more common. Cutaneous histiocytosis was first described in dogs as a waxing and waning dermatitis and panniculitis of unknown etiology, in which the lesions were dominated by histiocytes.[51]

The concept that CH and SH are related diseases emerged from a study in which an identical lesion topography and histiocyte immunophenotype were described.[3] Both are believed to be antigen-driven, but no etiologic agent/antigen has been discovered.[3] The lesions are dominated by activated dermal interstitial DCs and T cells, which frequently creates a lympho-histiocytic dermal vasculitis. The lymphoid component, which can vary throughout and between lesions, is enriched for CD8+ T cells.[3] The role played by these T cells is unknown, as spontaneous regression of SH or CH does not occur as it does in CCH. Instead, T cells, as an antigen-responsive element, may be involved in the proliferation, activation, and differentiation of DC via T cell-derived cytokines such as GM-CSF and TNF-α.[44,85] The hypothesis that an immunoregulatory deficit underlies the development and progression of CH and SH is bolstered by their clinical behavior and response to therapy. Both diseases are marked by spontaneous remissions and relapses (particularly early in the disease) and the only effective therapies are either immunosuppressive or immunomodulatory, including prednisolone, cyclosporine A, leflunomide, and tetracycline/niacinamide.[54,67] In light of their capacity to wax and wane and spontaneously resolve, assessing the effects of therapy in SH and CH is challenging.

CUTANEOUS HISTIOCYTOSIS VERSUS SYSTEMIC HISTIOCYTOSIS

Cutaneous histiocytosis is an inflammatory lympho-histiocytic proliferative disorder that primarily involves the skin and subcutis.[51] CH occurs in a number of breeds with no clear breed predisposition.[3,51,67] Lesions may involve the local lymph nodes, although the incidence of lymph node involvement is unknown (as lymph nodes are not commonly sampled when skin biopsies are taken unless there is marked lymphadenomegaly). The lesions most often occur as multiple cutaneous and subcutaneous nodules up to 4 cm diameter, and solitary lesions are uncommon. Overlying skin ulceration is common. Lesions may disappear spontaneously, or regress and appear at new sites simultaneously. Topographically, lesions may be found on the face, nose, neck, trunk, extremities (including foot pads), perineum, and scrotum.[3,51,67]

SH is a generalized histiocytic proliferative disease involving the skin, ocular and nasal mucosae, and peripheral lymph nodes. The disease predominately affects young to middle-aged dogs (2–8 years). Although SH was originally described in related Bernese Mountain Dogs, it has been observed, albeit less commonly, in the Rottweiler, Labrador Retriever, Basset Hound, Irish Wolfhound, and others.[3,56] Familial occurrence of SH is evident in Irish Wolfhounds in the Pacific Northwest (USA) (Moore—unpublished data). The clinical signs of SH vary with disease severity and extent, but may include anorexia, marked weight loss, stertorous respiration, and conjunctivitis with marked chemosis. Multiple cutaneous nodules may be distributed over the entire body, but, similar to CH, are especially prevalent in the scrotum, nasal bridge, nasal planum, and eyelids. Ulceration of the skin overlying the nodules is common and peripheral lymph nodes may be palpably enlarged by the histiocytic infiltrates.

Morphologic Features—Reactive Histiocytoses

In skin, the microscopies of SH and CH are essentially identical. In both, there is typically a "bottom-heavy" topography (Figure 74.2) characterized by extensive involvement of the deep dermis and subcutis with inconsistent involvement of the superficial dermis (with rare epidermotropism by histiocytes). Discrete perivascular lesions in the mid-dermis often coalesce in the deep dermis and subcutis. A lympho-histiocytic vasculitis (Figure 74.3) is common and may lead to vascular compromise, infarction of surrounding tissues, and ulceration of the overlying epidermis. The infiltrate is pleocellular, but histiocytes and lymphocytes outnumber neutrophils, plasma cells, and eosinophils. Histiocytes have large round to oval, indented, folded, or twisted nuclei and contain abundant pale eosinophilic cytoplasm with occasional vacuoles. Multinucleated histiocytes and histiocytes with bizarre, misshapen nuclei and mitotic figures, which are characteristic of HS, are lacking in CH and SH. In affected lymph nodes, histiocytes selectively invade the paracortex and sinuses. In severe lesions, the histiocytic infiltrate obliterates the cortex, trabeculi, and capsule with possible perinodal infiltration.

Inflamed non-epitheliotropic cutaneous T-cell lymphoma (CTCL) may be confused with CH.[57] The key discriminatory features are: (1) the identification of clusters of atypical T cells and (2) variable expression of CD3 by neoplastic T cells—so-called "CD3 antigen loss." Definitive diagnosis is reliant upon clonality testing, which will detect clonal T cell expansion within the reactive/inflammatory T cell and histiocyte infiltration.[38,57]

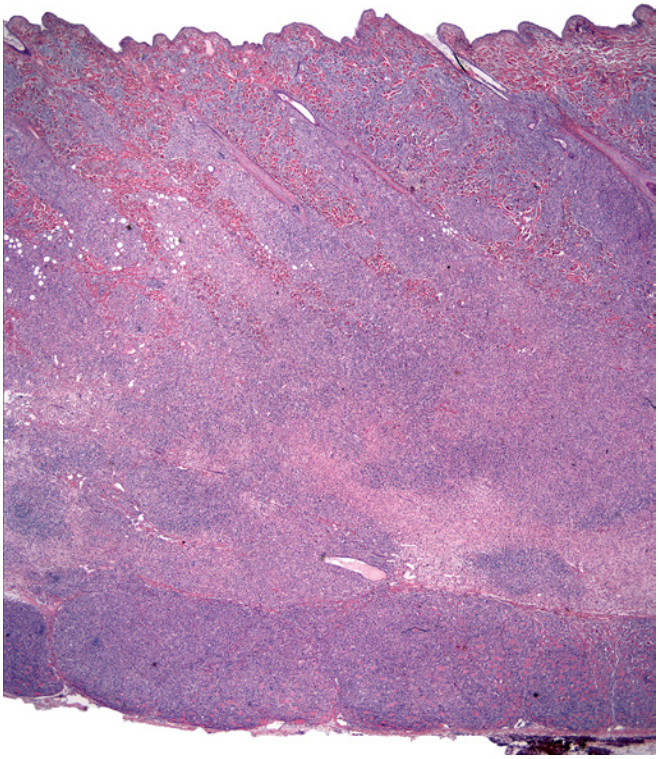


FIGURE 74.2 Cutaneous histiocytosis. Histiocytic infiltrates spare the superficial dermis and coalesce in the deep dermis and subcutis. H&E.

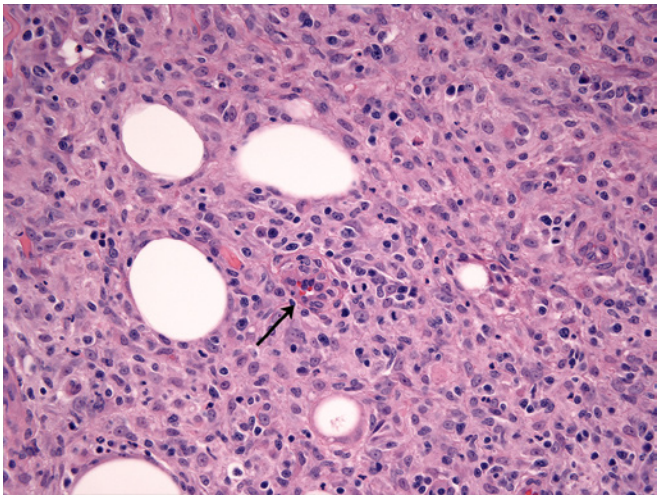


FIGURE 74.3 Cutaneous histiocytosis. Cohesive infiltrates of well-differentiated histiocytes and fewer lymphocytes in the subcutis. Lymphocytes and histiocytes infiltrate a vessel wall (arrow). H&E.

The complete distribution of SH lesions is often only fully appreciated at necropsy. Histiocytic lesions have been observed in lung, liver, bone marrow, spleen, peripheral and visceral lymph nodes, kidneys, testes, orbital tissues, nasal mucosa, and other sites. Like the skin, lesions in these sites are vasocentric and the infiltrates radiate to obliterate surrounding tissue. The lesions may only be microscopic, but mass formation has also been observed.

Immunophenotypic Features

The immunophenotypes of SH and CH, which is one of an activated interstitial DC, are virtually identical.[3] Histiocytes in both express markers expected of DCs, such as CD1a, C11c/CD18, and MHC class II. Histiocytes are $CD4^+$ (a marker of DC activation) and $CD90^+$ (Thy-1), which is expressed by normal dermal interstitial DCs, but lack expression of E-cadherin. Hence, the immunophenotype of histiocytes in CH and SH is the inverse of cutaneous histiocytoma and cLCH, which lack expression of CD4 and CD90, and distinct from HS, which lacks expression of CD4.[5,61] Of these antigens, only CD90, CD18, MHC class II, and E-cadherin are assessable in formalin-fixed tissue sections, whereas CD1a, CD4, and CD11c are not. More recently, the author has assessed expression of Iba-1 and CD204 in CH and SH lesions. Although Iba-1 was diffusely expressed by lesional histiocytes, CD204 expression was variable and most prominent in the dispersed histiocytes at the periphery of the lesions (Moore—unpublished data).

TREATMENT OPTIONS IN SH AND CH

A full evidence-based summary of the treatment of SH and CH is beyond the scope and intent of this chapter; however, the following are general concepts. Systemic histiocytosis is a difficult and frustrating condition to treat. The response to steroids is variable, although results are better for CH than for SH. Hence, steroids are worth trying given the expense of alternative treatments. Intractable cases, which may require maintenance therapy, are best treated with more aggressive immunosuppressive drugs, including cyclosporine A and leflunomide. However, published data are lacking. In some cases of CH and SH, spontaneous regression of lesions or episodic disease activation can occur. As such, aggressive immunosuppressive therapy may not be necessary until disease progression is evident or troublesome sites are involved. A retrospective evaluation of 32 cases of CH identified tetracycline and niacinamide as an effective treatment. Recurrence of lesions occurred in nine dogs; multiple recurrences occurred in seven of these dogs. At the conclusion of the study, 26 dogs were alive and lesion-free. Ten dogs were on maintenance therapy, most commonly with tetracycline/niacinamide (eight dogs). Recurrence of CH was more likely in dogs with lesions involving the nasal planum.[67]

CANINE CUTANEOUS HISTIOCYTOMA COMPLEX

Cutaneous Histiocytoma

Histiocytomas usually occur as solitary lesions, which often undergo spontaneous regression.[62,89] Multiple histiocytomas (i.e., cLCH) occur in <1% of cases and are more common in Shar Pei dogs.[2,89] Postexcisional recurrence is extremely rare and development of a histiocytoma at a new site occurs at extremely low incidence. Metastasis of solitary histiocytomas to local lymph nodes has been observed.[25,61] Regression of these lesions is the most common outcome, although systemic spread has also occurred.

Cutaneous histiocytomas originate from cells that manifest LC differentiation.[48,49,61] Demonstration of LC differentiation, in part, rests upon the identification of a cytoplasmic variant of the surface C-type lectin, langerin (CD207). Internalization of langerin mediates the formation of Birbeck granules (BGs), which are cytoplasmic structures that define LCs and distinguish them from interstitial DCs. Birbeck granules have been found in the LCs of species that express a functional langerin molecule, including mice, humans, pigs, cats, and many others.[52,65,77] However, BGs have not been observed in canine epidermal DCs, which are presumed to be LCs.[59] The lack of BGs in these presumed LCs may reflect unique features of canine langerin, including known structural abnormalities in its carbohydrate recognition domain, which is predicted to diminish mannose binding or its lack of exon 3, which encodes the neck region required for oligomerization and mannose binding.[65] In support of this, mice bearing langerin-lacking exon 3 do not form Birbeck granules.[74]

In accordance with their presumed LC origin, BGs have not been conclusively illustrated in CCH. Despite their lack of BGs, an LC origin to CCH is based on the aggregate of immunophenotypic and ultrastructural features.[7,31,39,49,61] Histiocytomas do exhibit ultrastructural features characteristic of DCs and also express CD1a and often E-cadherin, which are important functional markers expressed by LCs. Histiocytomas arise in the dermis and may invade the epidermis in a subset of lesions. Hence, histiocytomas do not arise directly from intra-epidermal LCs. It is quite possible that they arise from dermal precursors of LCs similar to the cells described by Larregina and others.[44] These CD14+ dermal cells downregulate CD14, and express CD1a, E-cadherin, and langerin following culture with TGF-β1. They also express CCR6 and are responsive to MIP-3α (CCL20), which is a chemokine produced by keratinocytes that facilitates migration of CCR6+ LCs into epidermis. CD14+ dermal cells did not possess BGs when first isolated, but did develop BGs after culture with TGF-β1. CD14+ LC precursors are found in perivascular locations in superficial and deep dermis. It is not known if an analogous CD14+ LC precursor exists in the dog, but the prospect that CCH might originate from a perivascular dermal LC precursor is an attractive hypothesis, which could also explain the stratified expression pattern of E-cadherin within some histiocytomas—stronger expression closer to the epidermis, which is a rich source of TGF-β1.[71]

Morphologic Features

Histiocytomas are often dome-shaped exophytic growths with complete or partial alopecia that are less than 2 cm in diameter. Topographically, the tumor infiltrate demonstrates a tropism for the superficial dermis and epidermis to create a "top-heavy" lesion. Tumor histiocytes may invade the epidermis as individual cells or nests of cells (Figure 74.4). In one study, invasion of the epidermis by individual histiocytes or nests of histiocytes occurred in >60% of lesions.[61] Although epidermal invasion is likely not as prevalent as that report suggests, it is a diagnostically useful feature and should be carefully evaluated. Importantly, such invasion may raise concern for epitheliotopic CTCL, and immunophenotyping using antibodies specific for CD3 and CD18 is necessary to resolve this dilemma. Tumor histiocytes may display a range of cytological features. Nuclei may be round to oval, indented, or convoluted with rare multinucleated forms, but anisokaryosis and hyperchromatic nuclei are unusual. The mitotic index in histiocytomas is variable, but is often high. The cytoplasm is usually abundant and eosinophilic.

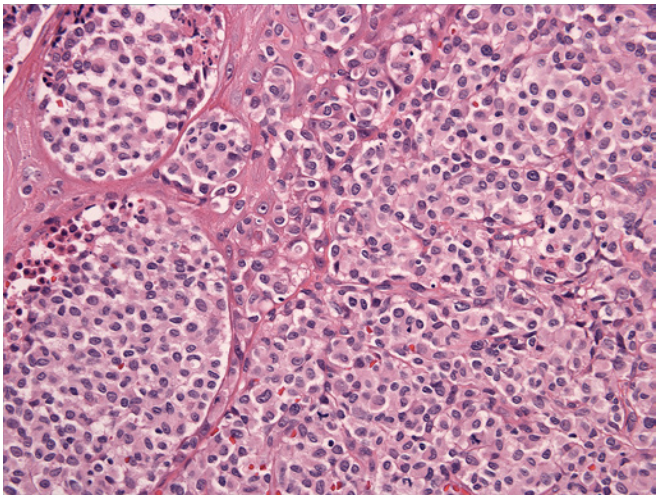


FIGURE 74.4 Canine cutaneous histiocytoma. Nest of histiocytes in the epidermis. H&E.

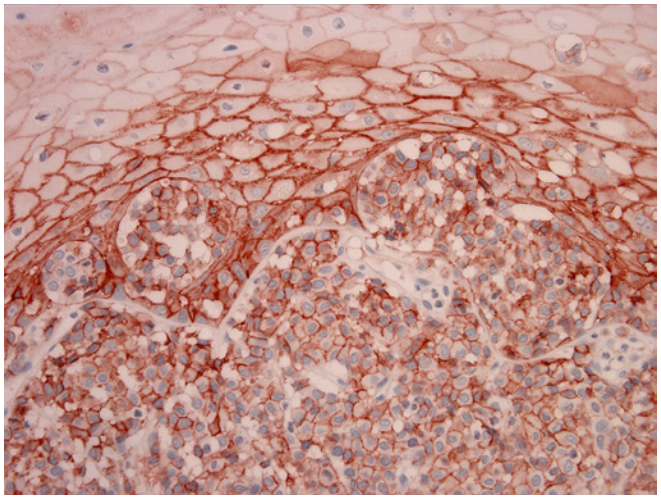


FIGURE 74.5 Canine cutaneous histiocytoma. E-cadherin expression by epitheliotropic histiocytes and by epidermal keratinocytes.

Immunophenotypic Features

The most comprehensive immunophenotype of canine histiocytomas was established using acetone-fixed sections from snap-frozen tumor tissue.[61] Based on this, and subsequent work, tumor histiocytes in canine histiocytoma have been found to diffusely express CD1a, CD11a/CD18, CD11c/CD18, CD44, CD45, and MHC class II, variably express CD11b/CD18 and CD54, and to lack expression of CD90 (Thy-1) and CD45RA.[79] Additionally, tumor histiocytes express E-cadherin, which is also expressed by normal LCs to localize in the epidermis via homotypic interaction with keratinocytes (Figure 74.5). The true incidence of E-cadherin expression in canine histiocytomas is not known, but it is very likely less than 100% observed in the published reports.[8,71] Histiocytomas lacking E-cadherin expression occur in our case series (Moore and Affolter—unpublished data). The staining pattern of E-cadherin may be limited to the histiocytic infiltrate immediately adjacent to the epidermis. Also, E-cadherin may be expressed by non-CCH, canine round cell tumors especially intracellularly, but also in the cell membrane.[71] Immunophenotyping with multiple markers can clearly resolve this issue, and E-cadherin expression remains a valuable indicator of LC differentiation in CCH. Furthermore, differential expression of CD90 and E-cadherin is useful in distinguishing proliferative diseases of interstitial DCs (CD90+) and LCs (E-cadherin+). Utilization of Iba-1 and CD204 adds further clarity in the diagnosis of CCH, as histiocytomas express Iba-1 but do not express CD204.[37]

CUTANEOUS LANGERHANS CELL HISTIOCYTOSIS

Histiocytomas are almost always solitary lesions, and the existence of multiple histiocytomas is uncommon. However, there are several reports of extensive skin involvement with multiple histiocytomas.[11,29,30,34,61,64] These cases may best fit under the umbrella of cLCH, as skin is the initial site of origin even when systemic disease eventuates. Cutaneous LCH is well documented in humans as a single-system disease and as a component of systemic disease.[63,80] Affected dogs may have literally hundreds of cutaneous lesions ranging from nodules to masses, which elevate the epidermis and may be accompanied by redness, alopecia, and ulceration. Lesions at mucocutaneous junctions and in tissues of the oral cavity may also occur. Although lesions are initially limited to skin, involvement of draining lymph nodes and, rarely, internal organs may occur.

Shar Pei dogs were overrepresented in a cohort of dogs afflicted by cLCH (about 20% of cases), but the disease occurred in many other breeds. Delayed regression of cLCH was common and lesions persisted for up to 10 months prior to regression. About 50% of the dogs with cLCH were euthanized due to complications in management of the extensive ulcerated lesions and failure of timely regression (Moore and Affolter—unpublished data). If cLCH involved lymph nodes, the prognosis was markedly worse. In all instances, these dogs were euthanized. The clinical course of dogs that experienced systemic spread of cLCH was even more rapid and all dogs were euthanized or died. Necropsies were performed on some dogs. Peripheral lymph nodes and lungs were consistently affected, but metastatic disease can be widespread and involve many other organs (Moore and Affolter—unpublished data). This spectrum of disease manifestation and diverse clinical behavior most resembles rapidly progressive multisystem LCH of humans.

Morphologic Features

Individual lesions of cLCH often do not differ markedly from CCH. The lesions are often large masses, which span the dermis and extend into the subcutis and muscle.

In aggressive cases, the lesions manifest a higher degree of cytologic atypia, but maintain tropism for the epidermis. Histiocytes may have more anisokaryosis and multinucleated cells than is typical for CCH, but these are not consistent features. Lymphatic invasion by neoplastic histiocytes portends lymph node involvement, which is an adverse prognostic indicator. Systemic lesions are consistently observed in lung where the infiltrates have a peribronchial distribution.

Immunophenotypic Features

The histiocytes in cLCH share the immunophenotype of histiocytes in CCH.

TREATMENT OF HISTIOCYTOMA COMPLEX

Solitary CCHs are either surgically removed or undergo spontaneous regression. Cutaneous LCH and cLCH with systemic involvement are refractory to therapeutic intervention, as immunosuppressives (e.g., corticosteroids and cyclosporine A) have not shown efficacy. Spontaneous remission is possible in cLCH and this occurs in about half of the cases, unless lymph node involvement occurs. In one published report, response of cLCH to immunomodulatory therapy with griseofulvin was reported.[64] Others have tried immunomodulation with levamisole without success in cLCH. Treatment with 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU or lomustine) was effective in causing lesion regression, but the responses were not durable.[55] A 6-year-old German Shorthaired Pointer with cLCH and internal disease was treated with toceranib and then masitinib after initial lomustine/vinblastine/l-asparaginase therapy failed (2 months). This dog enjoyed an extended survival (11 months after initiation of toceranib therapy) before succumbing to progressive disease. Although c-kit expression was not documented in this dog, unpublished data from the author suggest that another tyrosine kinase was likely active in his lesions. Further evaluation of toceranib and masitinib as a therapy for cLCH has not been conducted but is warranted.

HISTIOCYTIC SARCOMA COMPLEX

Histiocytic Sarcoma—General Considerations

HS are most often derived from cells with the immunophenotype of interstitial DCs, although the exact sublineages of DCs are not determined in most cases.[5] Specific candidates include interdigitating DCs in lymphoid tissues and perivascular interstitial DCs in other tissues. Given the ubiquitous distribution of these cells, HS can arise in almost any tissue. HS may be localized (i.e., originating at a single-tissue site) or disseminated (i.e., spread beyond the local draining lymph node to involve distant sites). Disseminated HS is the currently accepted terminology for what was originally reported as malignant histiocytosis. HS is principally a disease of dogs, although clinically identical, localized and disseminated HS has been infrequently reported in cats.[19,27,28,43,70]

The HS complex of diseases was first recognized in Bernese Mountain Dogs, in which a familial association was apparent.[60] Subsequent pedigree analyses support a polygenic mode of inheritance in this breed.[1,60,66] Although other breeds are predisposed to HS complex diseases, including Rottweilers, Labrador Retrievers, Golden Retrievers, and Flat-coated Retrievers, HS can occur sporadically in any breed.[5,18] Notably, the Pembroke Welsh Corgi has been associated with localized HS in the brain and in the lung.[35,36,91] Recently, studies of the genomic loci involved in HS in Bernese Mountain Dogs and Flat-coated Retrievers have identified abnormalities in tumor suppressor gene loci cyclin dependent kinase inhibitor 2A/B, (CDKN2A/B), retinoblastoma protein 1 (RB1), and phosphatase and tensin homolog (PTEN) that are broadly similar to those described in human HS.[33,82] Recently, a gain of function mutation in protein tyrosine phosphatase non-receptor type 11 (PTPN11) was identified in HS in Bernese Mountain Dogs; the same mutation had been identified in human HS. This mutation activates the MAPK pathway. Preliminary investigation of mitogen-activated protein kinase (MEK) inhibitor, trametinib, which targets the mitogen-activated protein kinase (MAPK) pathway, in canine HS cell lines and a mouse xenograft model shows promise.[88,90]

Clinical signs of HS are vague and include anorexia, weight loss, and lethargy. Other signs depend on the site of involvement, including cough and dyspnea with pulmonary involvement, seizures, incoordination and paralysis with central nervous system (CNS) involvement, and lameness with periarticular/articular HS (PAHS). Dogs with HS of interstitial DC origin often have mild, nonregenerative anemia.[1,58] More severe regenerative anemia, thrombocytopenia, hypoalbuminemia, and hypocholesterolemia have been consistently documented in HHS.[58] Hypercalcemia may also be encountered with HS, although the incidence is low (3%).[86]

Morphologic Features

Primary lesions of HS occur in spleen, lymph node, lung, bone marrow, CNS, skin and subcutis, and in periarticular and intra-articular tissues of the limbs. Secondary sites are widespread, but consistently include liver and lungs (with splenic primary), and hilar and mediastinal lymph nodes (with lung primary). In the original report of HS in Bernese Mountain Dogs, a high incidence of primary lung lesions was apparent.[60] Notably, pulmonary HS may have been mistaken for anaplastic large-cell carcinoma before the advent of lineage-specific cell markers. Similarly, approximately 50% of canine synovial tumors in one study were reclassified as HS by utilizing an anticanine CD18 monoclonal antibody (LABL).[5,21]

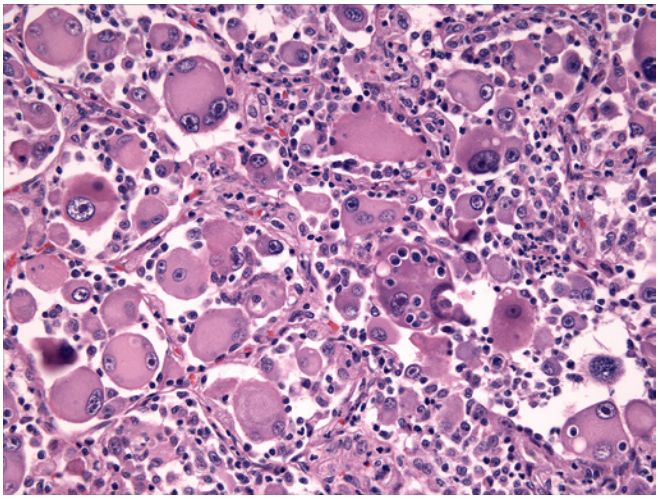


FIGURE 74.6 Histiocytic sarcoma. Cytologically atypical mononuclear and multinucleated giant cells in HS. Many cells are phagocytic. H&E.

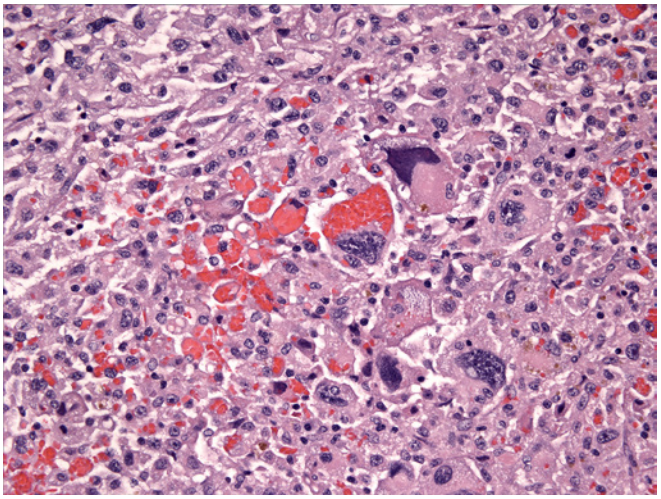


FIGURE 74.7 Hemophagocytic HS. Prominent erythrophagocytosis by cytologically atypical histiocytes in the splenic red pulp. H&E.

Lesions of HS that originate from interstitial DCs are typically destructive masses with a uniform, smooth-cut surface and are white/cream to tan in color. Lesions may be solitary or multiple within an organ (especially spleen). Periarticular/articular HS has a distinctive appearance: it occurs as multiple tan nodules located beneath the synovial lining (see in the following text). HHS, which originates from macrophages (see in the following text), does not initially form mass lesions in the primary sites (spleen and bone marrow). Typically, diffuse splenomegaly is observed, although ill-defined mass lesions may be seen. HHS may coincide with HS of interstitial DC origin and, in these instances, both diffuse splenomegaly and discrete mass formation are observed.[58]

HS lesions are composed of sheets of large, pleomorphic, mononuclear, and multinucleated giant cells, which have marked cytological atypia and numerous bizarre mitotic figures (Figure 74.6). Some lesions may consist of spindle cells, either alone or mixed with the mononuclear and multinucleated giant cells. Such lesions can resemble spindle cell sarcomas of diverse cell lineage (fibroblast, myofibroblast, and smooth muscle origin) and confirmation of histiocytic lineage requires immunohistochemistry (IHC).[5,35,37,69] Phagocytosis of red cells, leukocytes, and tumor cells occurs in HS, but less frequently than HHS (Figure 74.7).

Immunophenotypic Features

Immunostaining of most HS lesions reveals that tumor histiocytes express leukocyte surface molecules characteristic of interstitial DCs, including CD1a, MHC class II, and CD11c/CD18. In contrast to CH/SH, expression of CD4 by neoplastic histiocytes has not been observed in HS.[5] The β-2 subunit of the leuko-integrins (CD18) is readily demonstrable in formalin-fixed tissue sections of HS lesions (Figure 74.8). Importantly, as lymphomas may express abundant CD18, it is important to exclude a lymphoid origin (e.g., large-cell lymphoma) by staining

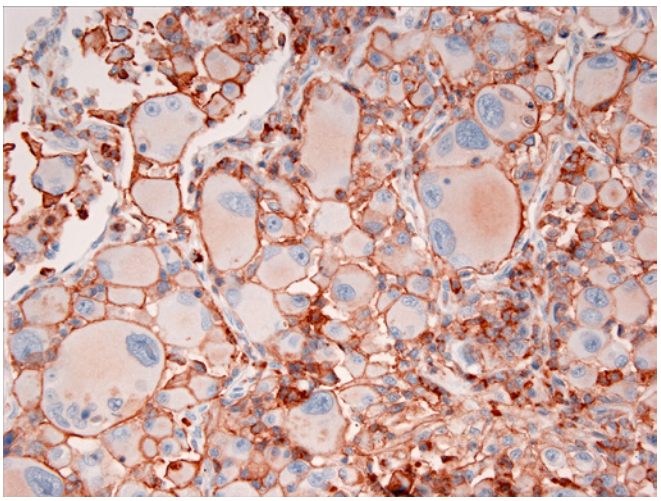


FIGURE 74.8 Histiocytic sarcoma. CD18 expression by atypical histiocytes. This most likely represents expression of CD11c/CD18.

for lymphocyte-antigen-receptor-associated molecules (CD3 and CD79a) and CD20.[5] Iba-1 is also expressed in HS lesions regardless of subtype and is not expressed by lymphocytes.[35,69] CD204 expression is inconsistent in HS, unless there is differentiation toward the macrophage lineage. In HHS, histiocytes express markers most consistent with macrophage differentiation including (CD11d/CD18) (Figure 74.9), Iba-1, and CD204 (Moore—unpublished data).[58]

HISTIOCYTIC SARCOMA—DISTINCTIVE SYNDROMES

There are several unique variants of HS that deserve separate description because of particular clinical or clinicopathological associations that are often crucial to successful diagnosis.

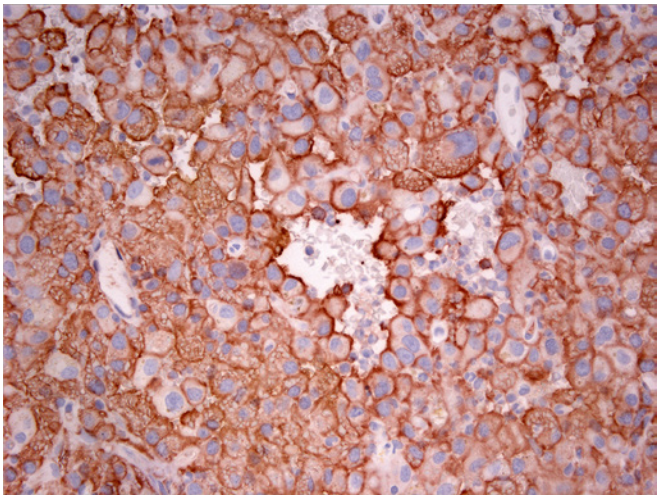


FIGURE 74.9 Hemophagocytic HS. CD11d expression by atypical histiocytes in the splenic red pulp—reflects expression of CD11d/CD18.

Hemophagocytic Histiocytic Sarcoma

Dogs with HHS present with a distinctive clinicopathologic syndrome characterized by a moderate to marked, regenerative, Coombs'-negative, hemolytic anemia and thrombocytopenia. They have mild hyperbilirubinemia without jaundice at initial presentation. Most dogs have hypoalbuminemia and often hypocholesterolemia. This clinical picture is often confused with immune-mediated hemolytic anemia and thrombocytopenia (Evans syndrome). Hemophagocytic HS carries the worst prognosis of all forms of HS with a median survival of only 4 weeks after diagnosis. Factors contributing to poor outcome include development of severe anemia and coagulopathy with disease progression.[58] In another study, in which the efficacy of CCNU was assessed as a treatment for HS, a cohort of dogs that presented with anemia, hypoalbuminemia, and thrombocytopenia had a median survival of 28 days or less (versus 106 days for all dogs). The form of HS was not further classified in this subset of dogs; laboratory data were consistent with HHS.[86]

Dogs with HHS have diffuse splenomegaly and lack grossly visible masses in sites of metastasis (e.g., liver and lung). This gross appearance is enforced by the growth pattern of the neoplastic histiocytes, which expand the splenic red pulp more or less diffusely. Neoplastic cells exhibit marked erythrophagocytosis and are accompanied by interspersed foci of extramedullary hematopoiesis. They invade red pulp sinuses and travel to the liver where they insidiously invade the sinusoids. Examination of sections of lung will reveal erythrophagocytic histiocytes within the pulmonary vasculature. The bone marrow is simultaneously infiltrated and erythrophagia is observed there as well. There are two reports of HS in cats, in which the lesions in three cats closely resembled the pattern and distribution of canine HHS.[28,43] Cytologically, the splenic neoplastic histiocytes often demonstrate more atypia than their bone marrow counterparts.[58] However, even the well-differentiated cells are aggressive, as evidenced by hepatic and pulmonary invasion.

Neoplastic histiocytes in HHS express a surface antigen profile similar to splenic red pulp and bone marrow macrophages. They express CD11d/CD18, Iba-1, CD204 and have low expression of CD11c and variable (negative to weak) CD1a expression. CD11d, CD18, Iba-1, and CD204 are assessable in FFPE sections in dogs, and this greatly facilitates the diagnosis of HHS (Figure 74.9) and the detection of often inconspicuous metastatic, intravascular lesions in liver and lung.[37,58,69] Feline HHS, based on lack of CD1 expression, is also thought to arise from macrophages.[28] Expression of CD11d and CD204 has not been thoroughly investigated due to the paucity of cases and the need for unfixed tissue.

PERIARTICULAR/ARTICULAR HISTIOCYTIC SARCOMA

PAHS is a localized form of HS, which occurs on the limbs. The lesions originate either within the joints (intra-articular) and/or adjacent to the joints (periarticular) of the appendicular skeleton. PAHS is the most common joint tumor of dogs and occurs in cats at much lower incidence. The stifle and elbow joints are most commonly affected, but lesions may occur in the coxofemoral joint, scapulohumeral joint, tarsus, and carpus. Rottweilers, Labrador Retrievers, Golden Retrievers, Flat-coated Retrievers, and Bernese Mountain Dogs have a high incidence of PAHS.[18,21,70,96] Invasion of bone can occur with advanced disease. Since skeletal lesions can be observed in disseminated HS, it is important to determine if the skeletal involvement is subsequent to a soft-tissue mass originating near the synovial tissues.[81] Early detection and treatment of PAHS are potentially curative, while disseminated HS has a worse prognosis.[5,40] However, PAHS is capable of widespread dissemination as reflected by the median survival of affected dogs with evidence of metastatic disease (about 5–8 months) versus those without (17 months).[21,40]

PAHS has a distinctive appearance: it occurs as multiple tan nodules located beneath the synovial lining that may encircle the affected joint. Hence, these lesions begin outside of the synovial space either within the joint and/or in a periarticular location. Microscopically, the lesions display a micronodular growth pattern that may coalesce into masses in advanced lesions. The lesions most often originate beneath the synovium, and the synovial lining is usually intact above the tumor. In advanced lesions, these topographical relationships may be obscured by effacing mass formation. Cytologically, the neoplastic cells in PAHS, through their atypia and pleomorphism, resemble HS in other sites. However, a marked inflammatory infiltrate is usually recognizable within the lesions. Lymphocytes (mostly T cells), neutrophils, and well-differentiated histiocytes are prevalent. Inflammation of the adjacent synovium with lymphocyte, plasma cells, and histiocytes is also observed.[21]

A history of cranial cruciate rupture or other traumatic joint injury is associated with the development of PAHS.[21,96] Synovitis, dominated by T cells and dendritic cells, has been reported in association with rupture of the cranial cruciate ligament.[45] While no causal relationship has been defined between synovial inflammation and the development of HS, there is ample evidence of confirming the progression of inflammation to neoplasia in other disease states.[20]

The immunophenotype of histiocytes in PAHS is most similar to that of perivascular interstitial DCs (CD1a+ CD11c/CD18+ Ib-1+ CD204–) rather than synovial type A cells (CD1a– CD11b/CD18+ Iba-1+ CD204+), which are intercalated between synovial type B cells (synovial fibroblasts) in the synovial lining. In FFPE tissue, PAHS lesions express CD18 and Iba-1 (Figure 74.8) with variable expression of CD204. It is important to note that this expression profile is not tumor-specific and is shared by inflammatory histiocytes. This distinction is key in the differentiating PAHS from synovial cell sarcoma. Synovial cell sarcoma is neoplasm that is likely derived from synovial type B cells (specialized synovial fibroblasts). Synovial fibroblasts are highly synthetic cells that can produce a diverse array of inflammatory mediators, including macrophage-recruiting chemokines.[75] Hence, it is not surprising that synovial cell sarcomas are often infiltrated by large numbers of CD18+ histiocytes. Importantly, in synovial cell sarcoma, a dominant CD18– neoplastic population is present among the many CD18+ histiocytes. This contrasts with the uninterrupted sheets of CD18+, cytologically atypical histiocytes in PAHS.

CENTRAL NERVOUS SYSTEM HISTIOCYTIC SARCOMA

The central nervous system may be infiltrated by HS as a primary location or as a consequence of metastatic spread in disseminated HS. Extracranial metastasis of primary CNS has not been reported. CNS HS most often originates in the leptomeninges, and the lesions are composed of large numbers of mixed inflammatory cells (lymphocytes, histiocytes, plasma cells, and neutrophils) and a variable number of cytologically atypical histiocytes. In two case series of CNS HS, Pembroke Welsh Corgis were overrepresented.[35,91]

CNS HS commonly presents as a solitary subdural mass and less commonly as diffuse meningeal infiltrates. The immunophenotype of CNS HS has yet to be fully clarified. In one report, the neoplastic histiocytes in the focal variant expressed MHC class II, Iba-1, and two macrophage scavenger receptors (CD163 and CD204). In this same report, the diffuse variants did not express either scavenger receptor.[35] These findings are slightly different than our published and unpublished data, which describe the expression of interstitial DC markers (CD1a, CD11c, and MHC class II) on neoplastic histiocytes.[5] Given that macrophage scavenger receptors are present on subsets of interstitial DCs, this could be one potential point of reconciliation of the divergent data.[50] Also, dendritic cells are located in the meninges and choroid plexi, but not in the brain, which would represent a resident population from which HS could develop.[22]

DENDRITIC CELL LEUKEMIA

Peripheral blood involvement in HS is rare and there have been only two reports of DC leukemia in dogs. In both dogs, large numbers (30,000–60,000 per microliter) of atypical histiocytes were observed in peripheral blood.[6,76] In both dogs, the DC lineage (CD1+ CD11c+ CD11d– MHCII+) was confirmed by immunophenotyping of cell smears or by flow cytometry. Diffuse infiltration of affected organs (bone marrow, spleen, lung, and liver) without mass formation was characteristic of a leukemic infiltrate. Although this pattern of infiltration mimics HHS, this condition does not involve peripheral blood and is associated with regenerative, extravascular hemolytic anemia and marked erythrophagocytosis by CD11d+ neoplastic histiocytes.

TREATMENT OF HS COMPLEX

Localized HS affecting skin and subcutis have been cured by early surgical excision, which in some instances has been successfully supplemented by local radiation therapy.[87] In the case of PAHS, amputation of the affected limb (preceded by appropriate staging) is enforced by the inoperable nature of the primary lesions. Disseminated HS is generally not a surgical disease as early metastasis is common. Chemotherapy with CCNU (lomustine) has been reported with outcome impacted by disease burden. In one report, although the median survival for all treated dogs was only 106 days, a small number of dogs with minimal residual disease had prolonged survival (>431 days). In this same study, the authors identified hypoalbuminemia and thrombocytopenia as negative prognostic variables, as affected dogs survived less than 1 month.[86] Notably, these dogs may have had HHS, which share these clinicopathologic features and poor survival.[58] Combination therapy using lomustine and doxorubicin has been used in an alternating treatment regime.[14]

The discovery of activating mutations in PTPN11 (SHP2) in Bernese Mountain Dogs has fueled interest in targeted therapies focused on MAPK pathway. MEK inhibitors including trametinib and cobimetanib are potential treatments for dogs with MAPK pathway activation. A clinical trial for dogs with HS and other cancer types is being conducted with trametinib at the University of Wisconsin. Several research groups are developing immunotherapies that could be used in the

future to treat HS and other cancers. These include chimeric antigen receptor (CAR) T cell therapy and antibody-directed immune checkpoint blockade. Targets for the former include B7-H3 (CD276), CD20, and CD19, and for the latter, PD-1, PD-L1, and CTLA-4 (Figure 74.1).[15,32,68]

FELINE PROGRESSIVE HISTIOCYTOSIS

FPH is a disease of middle-aged to older cats (7–17 years).[4] The initial presentation of FPH may be a solitary skin nodule, although usually multiple papules, nodules, or plaques develop, measuring up to 1.5 cm in diameter. The nodules are firm, nonpruritic, and nonpainful. The surface is often alopecic and may be ulcerated. The lesions are mostly located on the head, lower extremities, or trunk. Occasionally, the lesions are limited to one extremity. The lesions may wax and wane, but spontaneous regression does not occur. In FPH, a proportion of cats develop invasive, expansile masses in lymph nodes and internal organs including the lungs, kidneys, spleen, and liver.

FPH behaves as a low-grade HS and appears to originate from interstitial DCs.[4] The initial clinical course is indolent, but affected cats have a poor long-term prognosis. Surgical removal is not a good option, as lesions can reoccur at sites distant from the surgical excision. In a multicenter study of feline histiocytic disorders (HS and FPH), expression of platelet derived growth factor (PDGF)-beta was observed in the majority of cats including four of five cats with FPH. Extended survival following treatment with tyrosine kinase inhibitors masitinib and toceranib was reported in one cat; one cat responded to lomustine/cyclophosphamide, one cat responded briefly to lomustine/toceranib, and another responded to prednisolone. One cat achieved a spontaneous remission, which is highly unusual.[93]

Morphologic Features

Microscopic lesions consist of diffuse dermal histiocytic infiltrates, which may extend into the subcutis. Early in disease, the histiocytes are well-differentiated, and, as such, it is important to rule out infectious agents with special stains. However, later in the course, histiocytes are more atypical and consistent with HS (based on serial biopsies from the same cat). The overlying epidermis may be ulcerated and approximately 40% of lesions manifest epitheliotropism (Figure 74.10). Early in disease, histiocytes have irregular, vesicular nuclei and finely dispersed chromatin, but late-stage lesions are essentially morphologically identical to HS. Electron microscopic investigation of FPH in two cats did not demonstrate BGs (indicative of LC differentiation) in the neoplastic histiocytes. The mitotic activity varies and atypical mitoses are seen. A varied reactive infiltrate, composed of dispersed lymphocytes and fewer neutrophils, may be seen.

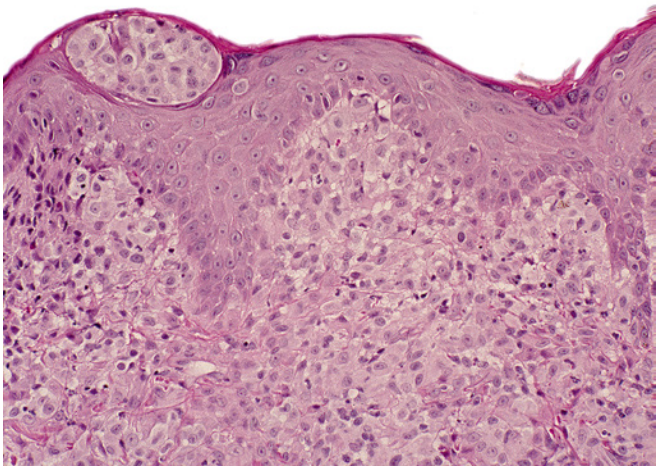


FIGURE 74.10 Feline progressive histiocytosis. Cohesive sheets of immature histiocytes infiltrate the superficial dermis and extend into the epidermis as a solitary focus. H&E.

Immunophenotypic Features

Neoplastic histiocytes in FPH express CD1a, CD18, MHC class II, and Iba-1 with rare E-cadherin expression (10% of cases), sporadic CD5 expression (approximately 50% of cases), and variable CD204 expression (Moore—unpublished data).[4] This immunophenotype is most consistent with an interstitial DC origin. Notably, CD5+ DCs have recently been identified in humans, they are potent inducers of cytotoxic T cells and Th22 cells. They have a role in the maintenance of barrier immunity in skin and mucosal sites.[42] The reactive lymphocytes that infiltrate the lesions mostly express CD3 and CD8.[4]

FELINE PULMONARY LANGERHANS CELL HISTIOCYTOSIS

Pulmonary LCH is an uncommon disease. The original report described three cats and, since then, four additional cases have been encountered by the chapter's author (Moore—unpublished data). All cases have been diagnosed at necropsy. It appears to be a disease of aged cats (10–15 years) characterized by progressive, terminal respiratory failure.[13] Affected cats present with severe respiratory distress characterized by tachypnea, increased respiratory effort, or open-mouthed breathing. Thoracic radiographs reveal a diffuse bronchointerstitial pattern with military-to-nodular opacities throughout all lung lobes. Given its rarity, the biology of feline pulmonary LCH is not fully clarified. A neoplastic origin is favored based on the cytological characteristics of the LC infiltrate and the consistent extrapulmonary lesions. However, molecular clonality studies were not conducted in support of that conclusion. Notably, pulmonary LCH occurs in humans, especially among smokers. In humans, it is believed to be a reactive disorder and often resolves after smoking cessation.

Although some lesions harbor clonal expansions of LCs, the majority of lesions demonstrate polyclonal LC proliferation.[99]

Morphologic Features

The lungs of affected cats are diffusely firm and entirely effaced by ill-defined, coalescing nodular masses (2–5 mm). When coalescing, the nodules efface larger areas of parenchyma and extended from peribronchiolar locations to the pleural surface. Extrapulmonary spread to pancreas and kidney is variably observed. The tracheobronchial lymph nodes and lymph nodes draining affected abdominal organs are also enlarged and diffusely effaced.

Pulmonary lesions are unique, and consist of cohesive histiocytic infiltrates within terminal and respiratory bronchioles (Figure 74.11). The infiltrates partially obliterate the airway walls, fill the lumens, and extend into adjacent alveolar ducts and alveoli. The cells are moderately pleomorphic in size and nuclear morphology, which is often complex (convoluted, twisted, or folded) (Figure 74.12). Transmission electron microscopy (TEM) revealed characteristic BGs in the cytoplasm of lesional histiocytes, which is consistent with an LC origin to these cells.

Immunophenotypic Features

The lesional histiocytes express CD18 and E-cadherin. CD1 expression has not been evaluated in pulmonary LCH, as only FFPE tissues have been available. Interestingly, E-cadherin expression is often markedly reduced in extrapulmonary lesions, which may indicate downregulation of E-cadherin expression associated with LC migration (as occurs in normal LCs when they migrate from epithelia to lymph nodes).

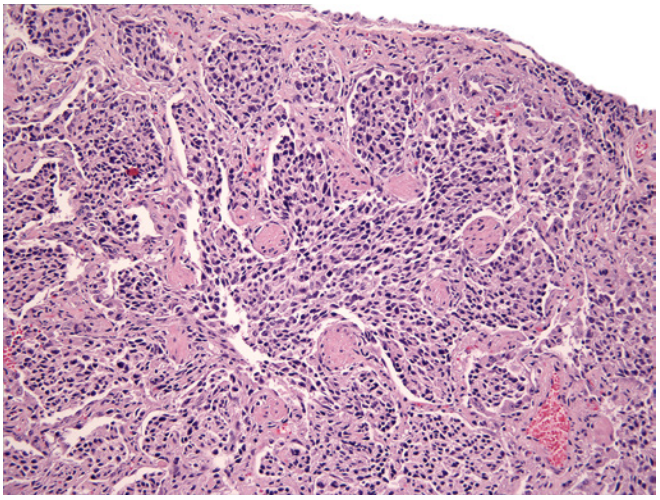


FIGURE 74.11 Feline pulmonary LCH. Cohesive sheets of histiocytes fill the alveolar duct and extend to the alveoli and pleura. H&E.

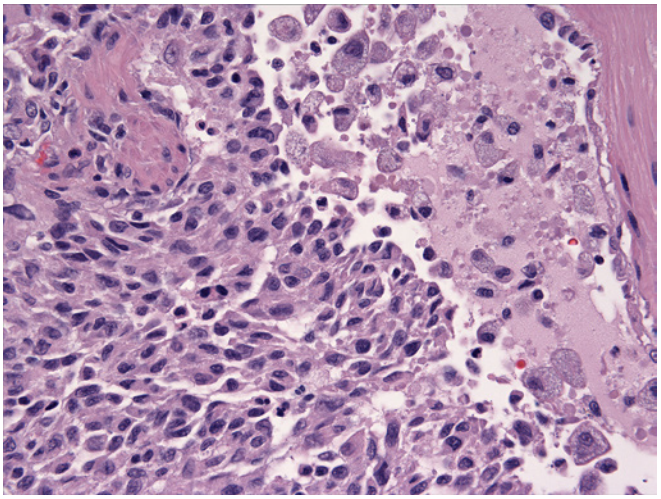


FIGURE 74.12 Feline pulmonary LCH. Moderately pleomorphic histiocytes with complex nuclear membranes invade the bronchial lumen, which is filled with foamy macrophages. H&E.

CONCLUSION

Histiocytic proliferative diseases occur as neoplastic processes and inflammatory processes with immune dysregulation in dogs. The latter do not occur in cats. Most histiocytic proliferative diseases originate from LCs or interstitial DCs in both dogs and cats. Macrophage differentiation has thus far only been demonstrated in HHS. The finer future characterization of histiocytic diseases will rely on the development of a greater array of markers of histiocytic lineage and the application of gene expression profiling on lesional histiocytes. Studies of this nature will hopefully lead to more effective targeted therapies for individual histiocytic diseases.

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SECTION VI
Platelets

CHAPTER 75

Thrombopoiesis

MARY K. BOUDREAUX and PETE W. CHRISTOPHERSON

Thrombopoiesis Overview
  Megakaryocyte–Erythroid Progenitor
  Bone Marrow Endothelial Cells
Transcription Factors
  GATA1 and GATA2
  Friend of GATA1, Friend Leukemia Integration 1, and GA-Binding Protein
  Core Binding Factors
  Nuclear Factor-Erythroid 2
Megakaryocyte Maturation and Platelet Formation
  Endomitosis
  Demarcation Membrane System and Proplatelet Formation
Thrombopoietin and Other Key Hormones
  Thrombopoietin
  Stromal-Cell-Derived Factor-1
  Other Cytokines, Growth Factors, and Proteins Involved with Thrombopoiesis
Megakaryocyte Ploidy and Platelet Mass among Species

Acronyms and Abbreviations

AMR, Ashwell–Morell receptor; Arp, actin-related protein; BFU-MK, burst-forming unit-megakaryocyte; BMEC, bone marrow endothelial cell; CalDAG-GEFI, calcium diacylglycerol guanine nucleotide exchange factor I; CBF, core binding factor; CD, cluster of differentiation; CFU-MK, colony-forming unit-megakaryocyte; CK2β, casein kinase 2 (beta subunit); CMP, common myeloid progenitor; CXCR, chemokine receptor; DMS, demarcation membrane system; E#, day of embryonic development, # indicates age of the embryo in days after conception; ETS, erythroblast transformation specific; FGF-4, fibroblast growth factor-4; FCR, receptor for the FC portion of immunoglobulin; FLI1, Friend leukemia integration 1; FOG1, Friend of GATA1; GA, guanine adenine nucleotides; GABP, GA-binding protein; GATA1 and 2, GATA-binding proteins 1 and 2; G-CSF, granulocyte colony-stimulating factor; GP, glycoprotein; GTP, guanosine triphosphate; IL, interleukin; LIF, leukemia inhibitory factor; Lin^-, lineage negative; JAK, Janus kinases; MAPK, mitogen-activated protein kinases; Meg-CFC, megakaryocyte-colony-forming cells; MEP, megakaryocyte–erythroid progenitor; Mpl, myeloproliferative leukemia; NF-E2, nuclear factor-erythroid 2; PECAM-1, platelet–endothelial cell adhesion molecule-1; PF4, platelet factor-4; PINCH1, particularly interesting new cysteine-histidine-rich protein 1; PIP4Kα, phosphatidylinositol-5-P-4-kinase α RUNX, runt-related transcription factor; SDF, stromal-cell-derived factor; STAT, signal transducers and activators of transcription; TPO, thrombopoietin; VCAM-1, vascular cell adhesion molecule-1; WASp, Wiskott–Aldrich syndrome protein; WAVE, WASp-family verprolin-homologous protein.

THROMBOPOIESIS OVERVIEW

Megakaryocyte–Erythroid Progenitor

Megakaryocytes are derived from a bipotent megakaryocyte–erythroid progenitor (MEP).[7] Although the MEP was once thought to arise from a committed common myeloid progenitor, the MEP is now believed to arise directly from an uncommitted short-term hematopoietic stem cell.[1] Primitive MEPs and megakaryocyte-colony-forming cells (Meg-CFCs) have been detected in mouse embryo yolk sacs and give rise to primitive glycoprotein (GP)Ibβ-positive platelets that are of large diameter, highly reticulated, and relatively hypogranular.[33] These early platelets can be detected in circulation in mouse embryos as early as embryo day (E) 10.5. By E11.5, definitive MEPs and Meg-CFCs within fetal liver become the predominant source of megakaryocyte

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progenitor expansion and produce platelets that are more similar in size and granularity to adult platelets. It is not known whether primitive platelets produced within the yolk sac represent a population of platelets distinct from those produced by the fetal liver and bone marrow.

Bipotent MEPs cannot be distinguished morphologically from small lymphocytes in bone marrow but have a unique cell surface protein expression pattern that includes interleukin (IL)-7Rα$^-$/lineage negative (Lin$^-$)/c-Kit+/Sca-1$^-$ clusters of differentiation (CD)34$^-$/FCRγ^{lo}.[18] Once commitment to megakaryocyte lineage occurs, cells begin to express CD41 and CD61 (integrin $\alpha_{IIb}\beta_3$ or GPIIb and GPIIIa), CD42 (glycoprotein Ib), and glycoprotein V. Two morphologically distinct colonies leading exclusively to megakaryocyte production have been identified in vitro: burst-forming unit-megakaryocyte (BFU-MK) and colony-forming unit-megakaryocyte (CFU-MK).[18]

Bone Marrow Endothelial Cells

The bone marrow can be divided into two anatomical and functional entities with respect to development of hematopoietic cells: the endosteal zone, where nondividing stem cells reside, and the more centrally located vascular niche that promotes stem cell differentiation and eventually mobilization of mature hematopoietic cells to the blood (see Section I). Originally, precursor cells were thought to migrate from the endosteal zone toward the vascular niche for further maturation and entrance into circulation.[11] Recent studies suggest there may be two separate pools of hematopoietic cells, including megakaryocytes.[30] Within the bone marrow, bone marrow endothelial cells (BMECs) support proliferation and differentiation of hematopoietic cells as a consequence of direct contact and release of cytokines. In turn, megakaryocytes nurture BMECs by secreting survival and growth factors. Platelet–endothelial cell adhesion molecule-1 (PECAM-1) may play a role in movement of megakaryocytes toward the vascular sinuses. Transendothelial migration and proplatelet formation and release are dependent on the interaction of megakaryocytes with BMECs via adhesion molecules, including vascular cell adhesion molecule-1 (VCAM-1) and integrin $\alpha_4\beta_1$.

TRANSCRIPTION FACTORS

Several transcription factors determined to be critical for thrombopoiesis in humans and mice likely play a similar regulatory role in other species.

GATA1 and GATA2

GATA-binding protein 1 (GATA1) is an X-chromosome-encoded transcription factor that plays a key role in megakaryopoiesis and erythropoiesis. GATA2 is a closely related transcription factor that has early effects on maintenance of hematopoietic stem cells and multipotential progenitors.[35] GATA1 possesses distinct structural elements necessary for binding promoters and cofactors involved in progenitor cell development and differentiation. Several erythroid and megakaryocyte-specific genes contain GATA motifs in critical *cis*-regulatory elements. For megakaryocytes, these genes include those encoding for GPIIb, GPIbα, GPIbβ, GPIX, platelet factor-4 (PF4), and the thrombopoietin receptor, myeloproliferative leukemia protein (cMpl, CD110). GATA1 activity is necessary for the divergence of megakaryocytic and erythroid progenitors from the MEP. Megakaryopoiesis is more sensitive to levels of GATA1 than erythropoiesis.[29] Several GATA1 mutations in humans are accompanied by a role reversal in which megakaryocytes are small and possess nuclei with reduced lobulation and erythroblasts are large and multinucleated.[5,9]

Friend of GATA1, Friend Leukemia Integration 1, and GA-Binding Protein

Friend of GATA1 (FOG1) is an essential cofactor of GATA1. Mutations in GATA1 that result in impairment of GATA1 binding with FOG1 result in X-linked macrothrombocytopenia in people. Friend leukemia integration 1 (FLI1) is a member of the erythroblast transformation specific (ETS)-family of transcription factors and interacts synergistically with GATA1 in the activation of megakaryocyte-specific genes. The heterodimeric guanine adenine nucleotide binding protein complex (GABP) is another ETS-binding factor that regulates gene expression in megakaryocytes. GABP, a complex composed of alpha and beta subunits, binds to promoters of early megakaryocyte-specific genes such as GPIIb and c-Mpl, whereas FLI1 primarily affects expression of late megakaryocyte-specific genes, including GPIbα, GPIX, and PF4. The alpha component of GABP mediates DNA binding of the complex, whereas the beta component mediates transcriptional activation and nuclear localization of GABPα.[26] GABP and FLI1 both can bind to promoters of early and late megakaryocyte-specific genes and regulate activity of the GATA1/FOG complex. This overlap in function allows partial compensation if either activity is lost.

Core Binding Factors

A family of transcription factors referred to as core binding factors (CBFs) is critical in megakaryopoiesis and megakaryocytic differentiation. The CBF family contains three DNA-binding proteins (runt-related transcription factors (RUNX1), RUNX2, and RUNX3) and a non-DNA-binding protein (CBFβ). RUNX1 is a heterodimer composed of core binding factor α-2 (CBF α-2) and CBFβ. RUNX1 interacts with GATA1 and has an essential role in early hematopoiesis and vasculogenesis.[32] RUNX1 may also play a role in lineage determination. RUNX1 mutations in humans are associated with thrombocytopenia and platelet function abnormalities associated with decreased dense granule formation.[21] In humans, CBF α-2 mutations have been

associated with decreased levels of protein kinase C-θ,[31] resulting in impaired activation of platelet integrin $\alpha_{IIb}\beta_3$.

Nuclear Factor-Erythroid 2

Nuclear factor-erythroid 2 (NF-E2) is a key transcription factor involved in enhancing early megakaryopoiesis and in promoting megakaryocyte maturation. NF-E2 is a heterodimer consisting of a hematopoietic-specific p45 subunit and two p18 subunits, MafG and MafK. Mice with deleted genes for NF-E2 are severely thrombocytopenic, and megakaryocytes have reduced granules, a disorganized demarcation membrane system, fail to form proplatelets, and are unable to generate signals necessary for fibrinogen binding.[28] Megakaryocyte targets for NF-E2 include genes encoding for thromboxane synthase, β1-tubulin, Rab27b, caspase-12, calcium diacylglycerol guanine nucleotide exchange factor I (CalDAG-GEFI), and particularly interesting new cysteine-histidine-rich protein 1 (PINCH1). PINCH1 is an adaptor protein that interacts with integrin-linked kinase, which in turn binds with cytoplasmic tails of integrins and thus may be important in mediating migration and interaction of megakaryocytes in the bone marrow microenvironment.[39]

MEGAKARYOCYTE MATURATION AND PLATELET FORMATION

In humans and domestic animals, megakaryocytes are located primarily in bone marrow. However, in adult mice, the spleen is considered a primary hematopoietic organ and generates all hematopoietic cell lineages, including megakaryocytes. Megakaryocytes undergo some degree of fragmentation in the pulmonary circulation in most mammalian species.

Endomitosis

During the early stages of megakaryocyte development, progenitor cells undergo typical cell division as they acquire lineage-specific markers. In later stages of development, megakaryocytes begin several rounds of endomitosis in which anaphase, telophase, and cytokinesis are skipped, resulting in DNA division without cell division.[37] Mature megakaryocytes are polyploid cells and DNA content or ploidy correlates with the number of platelets a megakaryocyte will ultimately produce. The progression from megakaryocytic cell division to endomitotic replication is due to the lack of formation of a normal functioning mitotic spindle.[14]

Stathmin expression may be necessary for induction of endomitosis in megakaryocytes. Stathmin is a phosphoprotein that promotes microtubule depolymerization and is phosphorylated and dephosphorylated in a cell-cycle-dependent fashion. Stathmin is phosphorylated/inactivated as cells enter mitosis to allow for assembly of the mitotic spindle and then dephosphorylated/activated at the end of mitosis to allow dissolution of the mitotic spindle. Studies with K562 erythroleukemia cells suggest that during early megakaryocyte development, stathmin expression levels are high, corresponding with normal cell division. During later stages, stathmin expression levels are reduced, resulting in abortive megakaryocytic mitosis and polyploidization.[14] Recently, ligand stimulation of CD226, an Ig superfamily protein found in the cell membrane of platelets and mononuclear leukocytes, has been demonstrated to mediate megakaryocyte activation, differentiation, and ploidy development.[40] Interestingly, silencing of CD226 downregulates expression of transcription factors GATA1 and FLI1, but not nuclear NF-E2.

Demarcation Membrane System and Proplatelet Formation

Terminal megakaryocyte differentiation occurs after endomitosis and is associated with tubular invagination of the plasma membrane resulting in formation of the demarcation membrane system (DMS).[27] The current model of platelet formation suggests that the DMS functions to provide a membrane reservoir for the formation and extension of proplatelets.[27] Prior to proplatelet formation, there is an accumulation of the phospholipid PI-4,5-P2 as a result of megakaryocyte lipid kinase activity. Specifically, phosphatidylinositol-5-P-4-kinase α (PIP4Kα) is thought to generate PI-4,5-P2 by acting on the substrate PI-5-P. PI-4,5-P2 is thought to trigger actin polymerization by enhancing binding of Wiskott–Aldrich syndrome protein (WASp) to actin-related protein (Arp2/3). WASp–Arp2/3 complex formation probably triggers Rho-family guanosine triphosphate (GTP)ses with activation of WASp-family verprolin-homologous protein (WAVE) resulting in actin fiber assembly.[25] Actin polymerization is associated with dilation and movement of the DMS to the cell periphery in preparation for microtubule assembly.[25]

Synthesis of platelet granules occurs simultaneously with formation of the DMS and granules are packaged and mobilized, as microtubule assembly occurs in the extension of proplatelets. The microtubule motor kinesin is believed to transport organelles and granules along microtubules.[24] Movement is bidirectional, as microtubules slide past each other. Once organelles and granules enter the proplatelet tip, they are captured there for packaging within the developing platelet.[24] In addition to organelles, spliceosomes and pre-mRNAs are packaged into newly forming platelets, thus providing platelets with the capacity for protein synthesis.

Microtubule assembly is a dynamic process: time-lapse photography shows alternating extension and retraction as well as bending and branching of proplatelet shafts. Platelets form from the tips of proplatelet shafts; thus, branching provides a mechanism for formation of more platelets per proplatelet extension. Branching and bifurcations of proplatelet extensions are mediated by actin forces. Elongation of proplatelets,

however, is mediated by dynein, a molecular motor protein that mediates microtubule sliding necessary for proplatelet extension.

Microtubules are hollow polymers formed by α/β-tubulin dimers. β1-Tubulin is the most abundant platelet β-tubulin isoform. β1-Tubulin-null mice demonstrate increased expression of other β-tubulin isoforms, nevertheless they are thrombocytopenic with reduced numbers of platelet marginal microtubule bands. Two missense mutations in β1-tubulin causing macrothrombocytopenia in dogs have been described: one in Cavalier King Charles Spaniels (and >25 other breeds), and a second seen mainly in Cairn, Norfolk, and Jack Russell Terriers[6,10] (P. W. Christopherson, personal observations).

Proplatelets are formed in a spiraling manner starting at the periphery and moving centripetally toward the nucleus. Proplatelets consist of long beaded strands that fragment in circulation when subjected to shear. Shear stress within bone marrow sinusoids probably assists in fragmentation of proplatelets from megakaryocyte bodies after proplatelets are extended through the subendothelium. Activation of proplatelets is thought to be avoided by extension of large, cytoplasmic fragments into the lumen of sinusoidal vessels.[15] Proplatelet formation consumes the entire megakaryocyte cytoplasm leaving behind only the nucleus which enters an apoptotic pathway.

THROMBOPOIETIN AND OTHER KEY HORMONES

Thrombopoietin

Thrombopoietin (TPO) is a key humoral regulator of platelet production.[3,19] TPO initially was identified as the ligand for c-Mpl, the normal mammalian proto-oncogene version of a murine myeloproliferative leukemia virus.[38] The open reading frame for human TPO protein, including the signal peptide, encodes 353 amino acids. In cats, dogs, horses, cattle, pigs, and mice, the open reading frame encodes 349, 352, 350, 349, 353, and 356 amino acids, respectively.[3,20] The amino acid sequence of TPO is conserved across species, demonstrating 79%, 85%, 89%, 83%, 82%, and 81% homology between human versus cat, dog, horse, cow, pig, and mouse, respectively.[12] The amino-terminal end of TPO is highly homologous with erythropoietin in all species and is the portion of the protein that engages the TPO receptor. The carboxy-terminal domain is highly glycosylated, which is necessary for TPO survival in circulation and for secretion of TPO from cells. Truncated forms of TPO are found in circulation, particularly in thrombocytopenic animals.[17] TPO is cleaved by thrombin at two sites, generating polypeptides that vary in activity. Cleavage of TPO at Arg^{191} in the carboxy-terminal domain results in a polypeptide with enhanced activity; however, activity is destroyed by thrombin cleavage at Arg^{117} within the amino-terminal domain.

Studies have documented that although TPO is critical for normal megakaryopoiesis, TPO also is important for overall hematopoiesis and plays a role in enhancing expansion of hematopoietic stem cells.[2] TPO binding to cMpl results in activation of Janus kinases (JAKs) and signal transducers and activators of transcription (STAT) factors STAT3 and STAT5. JAK2 is bound constitutively to cMpl and upon activation phosphorylates tyrosine residues within cMpl and within STATs, phosphatidylinositol3-kinase (PI3K), and mitogen-activated protein kinases (MAPKs). STAT3 and STAT5 are required for regulation of normal megakaryopoiesis and have been found to be dysregulated in some myeloproliferative disorders and in megakaryoblastic leukemias in humans.

TPO is synthesized primarily by the liver and to a lesser extent in kidney and by bone marrow stromal cells. TPO receptors are located on platelets, megakaryocytes, and vascular endothelial cells. Although the numbers of TPO receptors on endothelial cells far exceed the numbers found on megakaryocytes and platelets, endothelial TPO receptors are not involved in regulation of TPO levels. Under steady-state conditions, TPO is produced constitutively and binds to its ligand c-Mpl on platelets and megakaryocytes. Bound TPO is internalized and degraded and not available for stimulating thrombopoiesis above steady-state levels. TPO production is also regulated by the uptake of senescent platelets depleted of sialic acid by the Ashwell–Morell receptor (AMR).[13] The AMR is found exclusively on hepatocytes in mice and when activated, triggers downstream cell signaling leading to TPO production.

During episodes of thrombocytopenia, TPO levels increase, resulting in enhanced thrombopoiesis. The responsiveness of megakaryocytes to TPO is regulated somewhat by the maturity of the megakaryocyte. Megakaryocytes that are GPIIb+ GPIb+ undergo fewer endomitotic cycles in response to TPO compared to younger megakaryocytes that are GPIIb+ GPIb−. The enhanced endomitotic capability of GPIIb+ GPIb− megakaryocytes is due to lack of binding of 14-3-3ζ adaptor protein to the cytoplasmic tail of GPIbα. Lack of sequestration of 14-3-3ζ results in enhanced phosphorylation and activation of Akt by PI3K. Activated Akt increases the post-translational stability of cyclins, which play a role in promoting endomitosis and increasing ploidy. Although TPO production is constitutive under steady-state conditions, severe thrombocytopenia can result in enhanced production of TPO by bone marrow stromal cells. Inflammatory conditions can result in enhanced hepatic TPO production, mediated by IL-6, which induces reactive thrombocytosis.[16] During certain pathologic conditions, the steady-state mechanism of TPO production is altered, such that TPO levels are discordant with levels expected based on platelet numbers. In some cases of severe idiopathic thrombocytopenia, particularly where megakaryocytes are markedly increased in number and size, TPO levels may not be as high as expected due to binding of TPO to cMpl receptors on megakaryocytes (Figure 75.1).

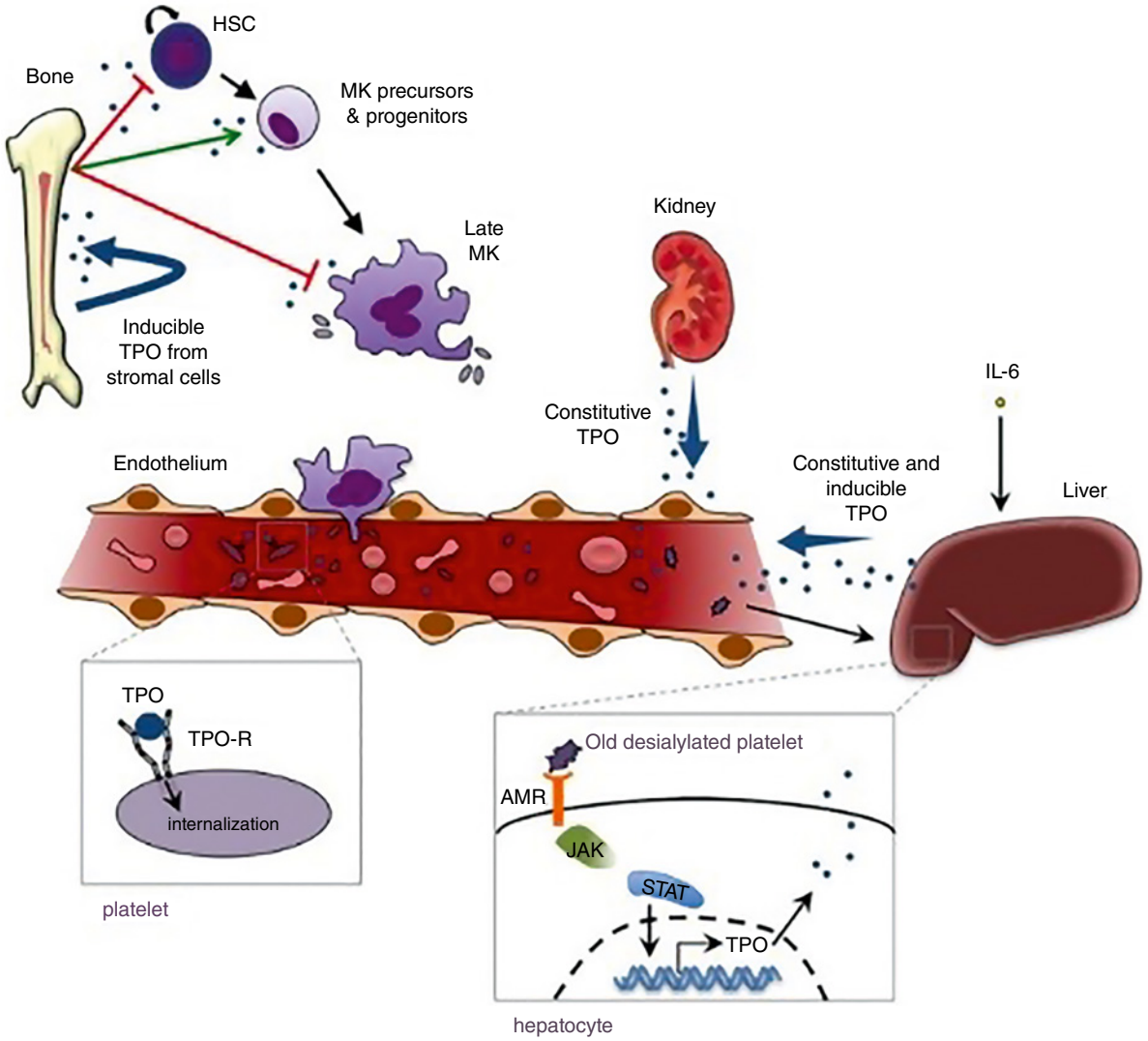


FIGURE 75.1 Thrombopoietin (TPO) production, function, and homeostasis. TPO is mainly produced by hepatic parenchymal cells and by endothelial cells of hepatic sinusoids. TPO production can also occur in proximal tubule cells of the kidney and in bone marrow stromal cells. The production is mainly constitutive (with no change in TPO mRNA levels), and regulation is mediated by negative feedback of the TPO receptor (TPO-R, c-mpl) expressing platelet pool that internalizes and destroys free circulating TPO. IL-6 is able to induce hepatic TPO mRNA production in reactive/inflammatory thrombocythemia. Additionally, binding of old desialylated platelets to the hepatic Ashwell–Morell receptor (AMR) promotes liver TPO mRNA production. TPO plays a dual role in hematopoiesis, including an inhibitory role (red lines with bars) acting to maintain stem cell quiescence and cell cycle arrest of late megakaryocytes (MKs), and a stimulatory role acting on immature precursors (common myeloid progenitor (CMP) and MEP) and promoting growth of very early progenitors of megakaryopoiesis (BFU-MK and CFU-MK). (Source: Varghese et al.[36] © 2017 Varghese, Defour, Pecquet and Constantinescu)

Stromal-Cell-Derived Factor-1

cMpl-null mice are markedly thrombocytopenic, but still capable of platelet production. Stromal-cell-derived factor-1 (SDF-1) can function alone or synergistically with TPO to enhance thrombopoiesis and may be responsible for maintaining low-level thrombopoiesis. SDF-1 interaction with chemokine receptor 4 (CXCR4) may be responsible for movement of megakaryocyte progenitors to the vascular niche necessary for initiation of thrombopoiesis. Movement of megakaryocytes to the vascular sinusoid compartment of bone marrow, mediated by SDF-1 and fibroblast growth factor-4 (FGF-4), induces megakaryocyte maturation and platelet formation.[5]

Other Cytokines, Growth Factors, and Proteins Involved with Thrombopoiesis

Although TPO is an important regulator of thrombopoiesis throughout all stages of megakaryocyte development, other cytokines act in synergy with TPO primarily in early stages of megakaryopoiesis. IL-3 synergizes with TPO to produce several hematopoietic lineages, but IL-3 effects are limited to the CFU-MK stage. Stem cell factor and granulocyte–macrophage

colony-stimulating factor act in synergy to enhance proliferation and expansion of bone marrow progenitors and megakaryocyte lines. Granulocyte colony-stimulating factor (G-CSF) combined with TPO can trigger hematopoietic stem cells to enter the cell cycle and support colony formation. In later stages of megakaryocyte development, G-CSF has been shown to suppress megakaryopoiesis. IL-11, IL-12, and erythropoietin also stimulate proliferation of megakaryocyte progenitors. PF4 has been shown to inhibit megakaryocytopoiesis in vitro. PF4 is stored in platelet alpha granules; aberrant platelet activation within bone marrow with subsequent PF4 release (e.g., radiation, chemotherapy) may contribute to thrombocytopenia. Earlier studies indicated that IL-1α and leukemia inhibitory factor (LIF) affect later stages of megakaryocyte maturation and platelet release. More recently, it has been shown that IL-1α can cause a more sudden platelet release reaction from megakaryocytes through the action of caspase-3, possibly during instances of sudden need.[23] Recent studies have shown that CK2β, a regulatory subunit of casein kinase 2, is vitally involved in thrombopoiesis regulation (and platelet activation) with roles in tubulin polymerization, megakaryocyte fragmentation, and proplatelet formation.[22] CK2β regulates calcium influx into platelet cytoplasm via inositol-triphosphate activity.

MEGAKARYOCYTE PLOIDY AND PLATELET MASS AMONG SPECIES

There are four to five times more megakaryocytes in mouse bone marrow than in human bone marrow, and mouse megakaryocytes are smaller. The difference in size is not related to ploidy distribution, since the modal ploidy value for both species is 16N. Megakaryocyte modal ploidy is 32N for dogs and rabbits. In cats, the modal value is evenly split between 32N and 64N.[8] In 1-week-old calves, the modal ploidy of cultured megakaryocytes was 64N, whereas in cattle aged 12–17 months the modal ploidy value was 32N.[34] Megakaryocyte ploidy is not consistently related to platelet mass and platelet mass is not constant across species. In dogs and cats, platelet size is inversely proportional to platelet number, whereas there is no correlation between platelet number and size in cows, goats, or ponies. Platelet number and size are directly proportional in horses. Mean platelet mass ranges from 1.8×10^6 to 2.5×10^6 fL/μL in cows, dogs, goats, and cats. In mice, platelet mass is 5.2×10^6 fL/μL, while in horses platelet mass is 0.72×10^6 fL/μL.[4]

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CHAPTER 76

Platelet Structure

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Membrane Structure
  Lipid Raft Microdomains
  Receptors
Cytoplasmic Structural and Membranous Elements
  Microtubules
  Open Canalicular System (OCS)
  Dense Tubular System (DTS)
  Mitochondria
Cytoskeleton
Granules
  Dense Granules
  Alpha Granules
  Lysosomal Granules

Acronyms and Abbvreviations

ADP, adenosine diphophosphate; Arp, actin-related protein; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; DTS, dense tubular system; GMP140, granule membrane protein 140; GP, glycoprotein; GTP, guanosine triphosphate; ICAM, intercellular adhesion molecule; LAMP, lysosomal-associated membrane protein; LIMP, lysosomal integral membrane protein; OCS, open canalicular system; PADGEM, platelet-activation-derived granule external membrane protein; PDGF, platelet derived growth factor; PECAM, platelet endothelial cell adhesion molecule; PGH2, prostaglandin H2; PMCA, plasma membrane calcium ATPase; PtdIns, phosphatidylinositol; SERCA, sarco/endoplasmic reticulum calcium ATPase; RGD, arginine glycine aspartic acid; STIM1, stromal interaction molecule 1; TSSC6, Tumor-suppressing subchromosomal transferable fragment cDNA 6; TXA, thromboxoane; VLA-2, very late activation antigen-2; VWF, von Willebrand factor; WASp, Wiskott–Aldrich syndrome protein.

MEMBRANE STRUCTURE

Lipid Raft Microdomains

Platelet membranes are similar to those of other cells and are characterized by phospholipids arranged in a bilayer forming a hydrophobic core. Interspersed within the fluid lipid matrix are densely compacted lipid raft microdomains primarily composed of sphingolipid and cholesterol molecules, and fewer numbers of protein molecules. These lipid rafts are capable of lateral mobility and enhance signaling in initial phases of platelet activation, and mediate cytoskeletal reorganization events critical for clot retraction.[9]

Receptors

Proteins and glycoproteins (GP), serving as specialized receptors important in platelet responses are present both within and outside membrane lipid rafts. GPIb-IX–V and GPVI, receptors involved in von Willebrand factor (VWF) and collagen binding, respectively, are examples of proteins primarily located within lipid rafts. Glycoprotein complex IIb–IIIa (GPIIb–IIIa), also known as integrin $\alpha_{IIb}\beta_3$,[3] is the most prevalent glycoprotein complex on the surface of platelets and is located outside of lipid rafts.[26] Although GPIIb–IIIa is not located within lipid raft microdomains, the complex does upregulate phosphatidylinositols, in particular

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phosphatidylinositol(4,5)bisphosphate (PtdIns(4,5)P2), as part of the outside-in signaling cascade. This process may be facilitated by interaction with tetraspanins located within microdomains distinct from lipid rafts.[34] PtdIns(4,5)P2 induces the recruitment of several actin-modulating proteins resulting in the interaction of the cytoskeleton with microdomains. The interaction of the cytoskeleton with lipid raft microdomains is necessary for sustaining the forces necessary for clot retraction.[9] GPIIb–IIIa receptors are the primary mediators of platelet aggregation by binding fibrinogen, although these complexes also bind VWF and assist with early stages of platelet adhesion to the subendothelium. The major receptors present on the surface of platelets are summarized in Table 76.1.

TABLE 76.1 Classification and function of major platelet membrane receptors.

Classification	Function	References
Integrins		
$\alpha_{IIb}\beta_3$ (GPIIb–IIIa; CD41/CD61)	Known as the fibrinogen receptor. 50,000–80,000 receptors per platelet. Mediator of platelet aggregation; platelet activation is required to induce a conformational change for ligand binding. Ligands include several arginine glycine aspartic acid (RGD)-containing proteins including fibrinogen, VWF, thrombospondin, fibronectin, and vitronectin	60
$\alpha_v\beta_3$ (CD51/CD61)	Known as the vitronectin receptor. 200–300 receptors per platelet. Platelet activation is required to induce a conformational change for ligand binding. Ligands include RGD-containing proteins such as vitronectin, fibrinogen, and osteopontin	4, 62
$\alpha_2\beta_1$ (GPIa–IIa; CD49b/CD29)	Known as very late activation antigen-2 (VLA2) on lymphocytes. 2000–3000 receptors per platelet. Major collagen adhesion receptor; platelet activation required for collagen binding via this receptor	48
$\alpha_5\beta_1$ (CD49e/CD29)	Known as the fibronectin receptor. Augments platelet adhesion; platelet activation is not required for fibronectin binding	56
$\alpha_6\beta_1$ (CD49f/CD29)	Known as the laminin receptor. Augments platelet adhesion; platelet activation is not required for laminin binding	30, 74
Leucine-rich repeat		
GPIbIX–V (CD42b/42c–CD42a–CD42d)	Known as the VWF receptor; also binds Mac-1 and P-selectin. Functions in platelet adhesion under high shear. 60,000 receptors per platelet	2
G-protein-coupled receptors		
Protease-activated receptors (PAR)	Function as thrombin receptors. Platelets have 3 PAR receptor types that vary with species. PAR1 and PAR4 are major human PARs; PAR3 and PAR4 major rodent PARs, other species less defined	10, 17
ADP receptors	P2Y1 and P2Y12 are expressed on platelets. P2Y1 is coupled to Gq and initiates calcium mobilization resulting in shape change and reversible platelet aggregation. P2Y12 is coupled to Gi resulting in inhibition of adenylate cyclase and lowering of cyclic adenosine monophosphate (cAMP). P2Y12 activation is associated with irreversible platelet aggregation	61
Thromboxane receptor (TXA_2)/prostaglandin H2 (PGH_2)	Two receptors are expressed, TPα and TPβ, which are coupled to G proteins; TPα is coupled to Gs, while TPβ is coupled to Gi. TXA_2 and PGH_2 interact with both receptor types. Both types of receptors are predicted to be present on platelets from cattle, horses, and cats, no evidence exists for TPβ receptors on dog platelets	80, M. K. Boudreaux, unpublished observations
Prostacyclin receptor (PGI_2)	PGI_2 receptors are coupled to Gs. Prostacyclin binding results in activation of adenylate cyclase, enhanced cAMP production, and inhibition of platelet activation	75
Platelet-activating factor (PAF) receptor	PAF mediates platelet shape change and aggregation. PAF receptors are coupled to Gi and Gq. PAF can also initiate signal-dependent translation pathways in human platelets	86
Adenosine receptor	Three receptor subtypes are expressed. Adenosine receptor activation results in cAMP elevation and inhibition of platelet aggregation	1, 32
Adrenergic receptor	Alpha-2A adrenergic receptors are present on platelets, which are coupled to Gi. Epinephrine binding results in reduction of cAMP and potentiation of platelet activation. May play a role in enhancing thrombus stability	66
Serotonin receptor	Coupled to G-proteins leading to calcium mobilization and enhancement of platelet activation. Two subtypes have been detected on human platelets	1

(*Continued*)

TABLE 76.1 (Continued)

Classification	Function	References
Immunoglobulin/ITAM/ITIM		
GPVI-FcRγ	Major collagen receptor. Composed of two immunoglobulin C2 loops, which bind collagen. Is complexed with FcRγ, which contains an ITAM that is tyrosine phosphorylated during platelet activation. Receptor contributes to platelet activation and aggregation and may contribute to maintaining blood vessel integrity during inflammation in an aggregation-independent fashion	11, 35
FcγRIIA	Immune receptor expressed on human but not mouse platelets. Binds IgG. Plays a role in heparin-induced thrombocytopenia	14
FcεRI	IgE immune receptor. Activation induces release of serotonin and RANTES and thus may play a role in allergic inflammatory responses	37
Intercellular adhesion molecule (ICAM)-2	Present on the surface of activated and nonactivated platelets. The only β2 integrin ligand on platelets; may play a role in leukocyte–platelet interactions	25
Platelet endothelial cell adhesion molecule (PECAM)-1 (CD31)	Composed of six C2 immunoglobulin domains, a transmembrane domain, and a short cytoplasmic tail containing two ITIM domains. Plays dual roles as an inhibitor and enhancer of platelet activation. Has an inhibitory effect on ITAM-based collagen responses and also promotes $\alpha_{IIb}\beta_3$-mediated outside-in signaling	47, 81
CD47	Thrombospondin receptor; also known as integrin-associated protein (IAP). TSP binding results in enhanced platelet activation via inhibition of nitric oxide/cyclic guanosine monophosphate (cGMP) signaling	41
Tetraspanins		
CD9	Present on resting platelet plasma membrane in association with $\alpha_{IIb}\beta_3$. May be involved in recruiting phosphatidylinositol kinase activity, specifically PI4K, to integrin-based signaling complexes	40
CD82	Studies in lymphocytes suggest a role in linking signaling through raft microdomains with the actin cytoskeleton.	22
CD151 (PETA-3)	Present on resting platelet plasma membrane in association with $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$. May play a role in integrin-mediated signaling	30
Tumor-suppressing subchromosomal transferable fragment cDNA 6 (TSSC6)	Associated with $\alpha_{IIb}\beta_3$ in resting platelets in cholesterol-rich microdomains that upon activation facilitate signal transduction events, including outside-in integrin signaling	34
Others		
P2X1	ATP receptor that enhances calcium entry. Plays a role in reinforcing and amplifying platelet activation	61
CD36 (GPIV, GPIIIb)	Ligands include thrombospondin, modified phospholipids, and long-chain fatty acids. Contributes to thrombus formation by binding to phosphatidylserine exposed on endothelial-cell-derived microparticles resulting in enhanced platelet activation	33
C1q	May play a role in classical complement pathway activation on platelet surfaces, particularly under high-shear conditions	63
Serotonin reuptake	Receptor is distinct from serotonin activation receptor. Responsible for serotonin transport across the platelet plasma membrane. Selective serotonin reuptake inhibitors inhibit platelet reactivity	72
Glycosyl phosphatidylinositol (GPI)-anchored proteins	At least five GPI-anchored glycoproteins are expressed. These include CD55, CD59, CD109, and prion protein. Platelets contain >96% of prion protein in human blood. Prion proteins are primarily present within alpha granule membranes and become expressed with platelet activation. Prion proteins are associated with platelet-derived microparticles and exosomes following thrombin-induced platelet activation	68
Thrombopoietin receptor (MPL)	A member of the tyrosine kinase receptor family. Important for regulating platelet production. Receptors are internalized when bound by TPO. TPO binding does not activate platelets, but does enhance platelet activation	53

ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; MPL, myeloproliferative leukemia protein; PETA-3, platelet and endothelial cell tetraspan antigen-3; RANTES, regulated on activation, normal T cell expressed, and presumably secreted; PI4K, phosphatidylinositol 4 kinase; TPO, thrombopoietin.

CYTOPLASMIC STRUCTURAL AND MEMBRANOUS ELEMENTS

Microtubules

A circumferential band of microtubules is located just beneath the platelet membrane.[36] Microtubules are hollow, cylindrical structures composed of protofilaments formed by alpha–beta tubulin dimers arranged in a helical head-to-tail fashion. Microtubules are responsible for the maintenance of the disc-shaped form of the circulating platelet.[44] Under physiologic conditions, β1-tubulin is solely expressed as a component of microtubules within megakaryocytes and is essential for the orderly fragmentation of platelets from the megakaryocyte cytoplasm.[70]

Open Canalicular System (OCS)

The surface-connected canalicular system or OCS connects to the surface of the platelet and extends deep within its cytoplasm in an extremely tortuous maze of open and interconnecting channels.[82] This system was thought to be a remnant of the demarcation membrane system of megakaryocytes, but the absence of the OCS in some mammals[87] and the presence of the OCS in thrombocytes of birds[77] and fish[19,52] have cast doubts to its origin. The OCS can act as a conduit for the uptake of particles and for the release of granule contents by activated platelets. Particles such as viruses or bacteria can be taken up by the OCS; this activity usually results in platelet activation. Alpha and lysosomal granules are documented to fuse and evacuate their contents into the OCS around engulfed bacteria or viruses, but communication with the outside is always maintained, unlike phagocytic vacuoles of leukocytes. Thus, platelets are not considered to be true phagocytes.[83] The OCS is everted to some extent during platelet activation, which increases the number of surface receptors available for ligand binding, since many of the same receptors found on the platelet surface also line the OCS.[28] Ruminant and equine platelets do not possess an OCS,[31] although their megakaryocytes do possess a demarcation membrane system.[78] Platelets from these species do not expand their surface area during activation, do not have a mechanism for uptake of particles, and release their granule contents by fusion of granules directly to the outer membrane. Figures 76.1 and 76.2 are platelet electron micrographs comparing overall appearance for several species.

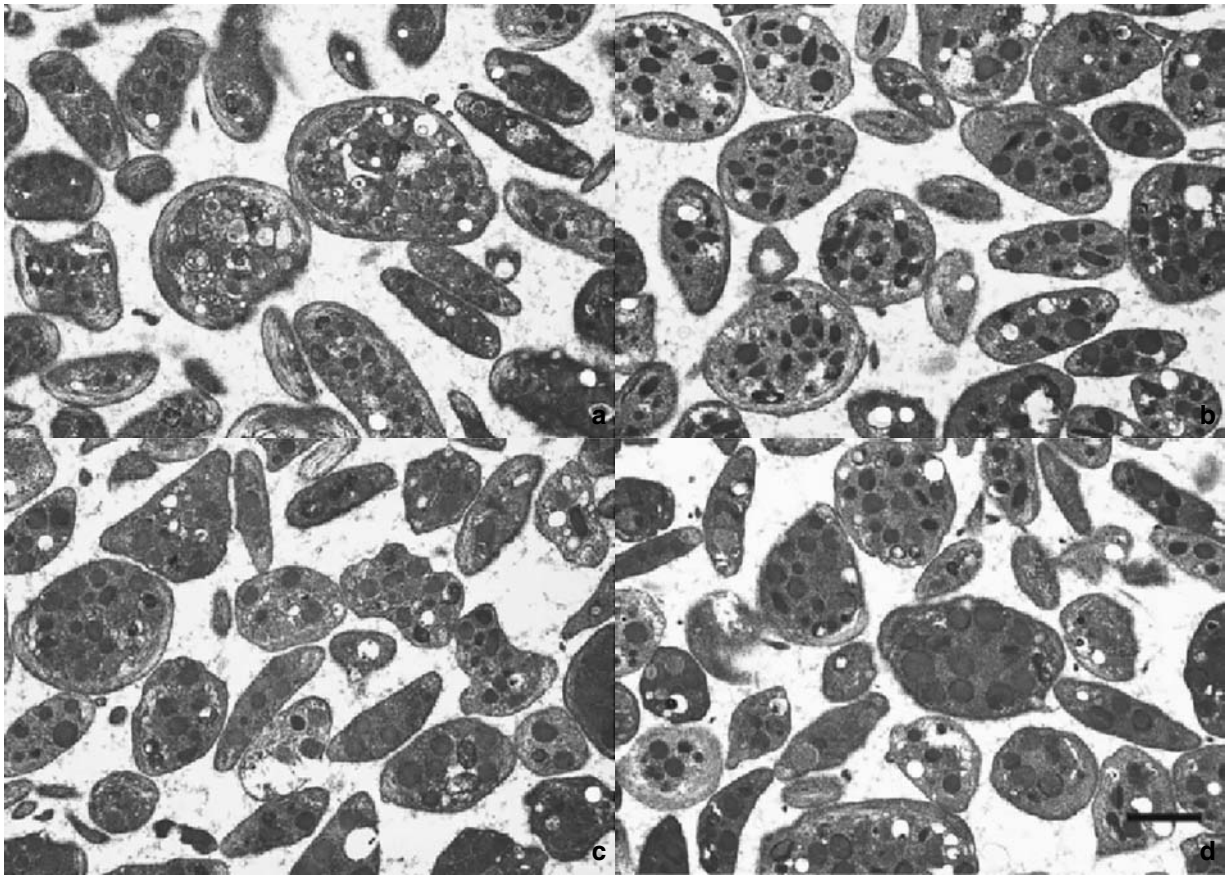


FIGURE 76.1 Electron micrographs of platelets obtained from (a) alpaca, (b) cow, (c) goat, and (d) sheep for overall comparison of platelet morphology. Alpha granules appear light to dark gray and are prevalent and variably sized and shaped in these species. The electron-dense content of most of the dense granules has been lost leaving vacuolar-like spaces. Microtubules can be observed in the periphery of some platelets. An open canalicular system is not present in these species. Bar = 2 μm. (**Source:** Courtesy of Dr. Maria Toivio-Kinnucan, Auburn University)

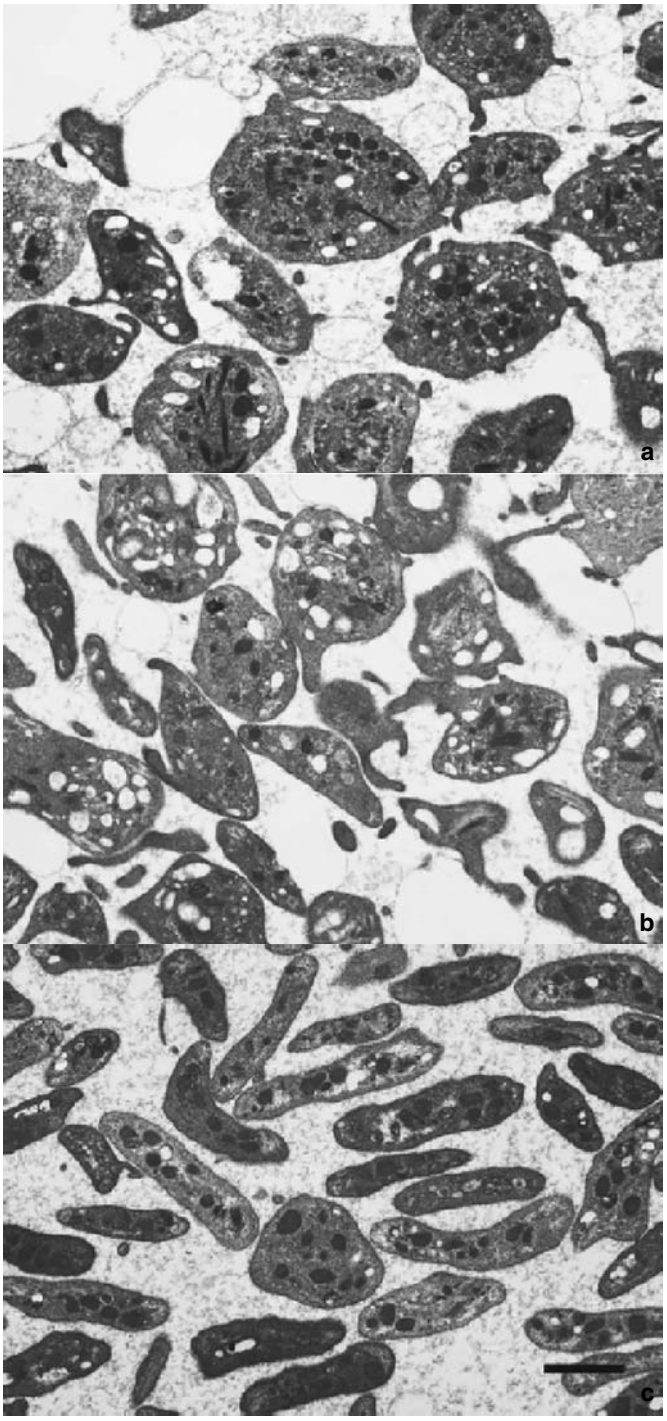


FIGURE 76.2 Electron micrographs of platelets obtained from (a) cat, (b) dog, and (c) horse. Alpha granules are numerous and variable in size and shape as seen with ruminant platelets. An open canalicular system is present in cat and dog platelets, but absent in horse platelets. Bar = 2 µm. (**Source:** Courtesy of Dr. Maria Toivio-Kinnucan, Auburn University)

Dense Tubular System (DTS)

The DTS exists as thin elongated membranes in the cytoplasm of resting platelets. The DTS is a remnant of the smooth endoplasmic reticulum of the megakaryocyte and is located near microtubules. Unlike the OCS, it does not communicate with plasma or granule membranes. DTS membranes contain cyclooxygenase and thromboxane synthase and thus participate in prostaglandin metabolism. The DTS is also a site for calcium sequestration by the platelet; calcium movement is facilitated by ATPase pumps termed sarco/endoplasmic reticulum calcium ATPases (SERCAs), which are tightly coupled to plasma membrane calcium ATPases (PMCAs). Plasma membrane calcium ATPases function to move calcium ions to the extracellular space, while SERCAs pump calcium ions into the DTS. Rapid calcium influx in response to release of intracellular stores, referred to as store-operated calcium entry, is mediated by a plasma membrane channel protein, Orai1, and a calcium sensor in the endoplasmic reticulum, stromal interaction molecule 1(STIM1).[79]

Mitochondria

Platelet mitochondria support the metabolic activities of resting platelets and play regulatory roles in platelet activation and apoptosis. Sustained high intraplatelet calcium leads to mitochondrial membrane potential collapse in a process linked to outer membrane phosphatidylserine exposure. Externalization of this negatively charged phospholipid supports assembly of active coagulation complexes and is a marker of platelet aging in circulation.[64]

CYTOSKELETON

Platelets possess a cytoskeleton on the cytoplasmic side of the plasma membrane composed of actin, spectrin (fodrin), adducin, filamin, and glycoprotein-Ib-IX–V (the VWF receptor). The membrane cytoskeleton interacts with an extensive cytoplasmic actin network. Adducin links the ends of cytoplasmic actin filaments to membrane-associated spectrin strands. Filamin subunits bind to actin and to the cytoplasmic tails of the GPIbα subunits resulting in strengthening of the interaction of the spectrin/actin network with the plasma membrane.[36] During platelet activation, adducin molecules dissociate from the ends of actin filaments allowing disassembly and reorganization of actin. Actin disassembly is promoted by gelsolin and cofilin. Gelsolin undergoes a conformational change in response to increased cytoplasmic calcium allowing the protein to interdigitate and disrupt actin filaments. Cofilin becomes activated as a result of dephosphorylation during platelet activation and functions to sever and promote actin disassembly along with gelsolin. Actin reassembly during the formation of filopodia and lamellipodia by activated platelets is mediated

by the actin-related protein (Arp) 2/3 complex. Proteins involved in activation of the Arp 2/3 complex include cortactin and Wiskott–Aldrich syndrome (WASp) family proteins. Actin reassembly and myosin II binding and activation are critical for platelet spreading, granule movement, granule release, and clot retraction.

GRANULES

Platelets have three main types of storage granules: dense, alpha, and lysosomal, and they are heterogeneous in content and morphology.[50] Alpha and dense granules are thought to be derived from a common multivesicular body intermediate, and thus their membranes share several common components[85] (Table 76.2).

Dense Granules

The dense granules or dense bodies, as their name implies, are electron-dense when viewed using an electron microscope, and serve as storage sites for adenine nucleotides, serotonin, calcium, and inorganic phosphates.[57,67] The adenine nucleotide (ADP/ATP) ratio is roughly 1.5 within dense granules. The pool is metabolically inert and not available as an energy source. Serotonin is actively transported from plasma into dense granule cores and the serotonin concentration within dense granules is 1000 times higher than plasma concentrations. Dense granules contain 70% of the total calcium within platelets. This calcium pool is not mobilized during platelet activation, unlike the calcium pool within the DTS. Inorganic phosphates, calcium, serotonin, and adenine nucleotides are held together tightly within dense granules as a result of intermolecular forces, thus contributing to their stability and density.[73] Human and pig platelet dense granules contain high levels of lysolecithin;[24] however, lysolecithin was not detectable in the dense granules of rabbit platelets.[20] Dense granules also contain ganglioside GM3 and phospholipids including phosphatidylethanolamine, phosphatidylinositol, phosphatidylcholine, and sphingosine.[15] Organelle proteomics has led to the discovery of 40 proteins within dense granules, many of which had not been known to be present within this organelle. Dense granule protein categories include cell signaling proteins, molecular chaperones, cytoskeletal proteins, proteins involved in glycolysis, and proteins involved in platelet function.[39] Dense granule membranes contain several receptors that become expressed on the plasma membrane as a result of dense granule fusion with the surface during the platelet release reaction. Some of these receptors are shared with other granule membranes and with the plasma membrane (Table 76.2).

TABLE 76.2 Granule membrane proteins.

Classification	References
Alpha granules	
Guanosine triphosphate (GTP)binding proteins	7, 49
Glycoproteins-Ib-IX–V	8
Glycoproteins-IIb–IIIa (CD41/CD61; $\alpha_{IIb}\beta_3$)	8
P-Selectin (CD62P)	42
Glucose transporter-3 (Glut-3)	38
PECAM-1	18
Vitronectin receptor	65
GPIV (CD36)	6
CD9	18
Osteonectin	12
Dense granules	
Hydrogen-ion-pumping ATPase	21
GTP-binding proteins	54
Glycoprotein-Ib	84
Glycoproteins-IIb–IIIa	84
P-Selectin	42
Lysosome-associated membrane proteins (LAMP-2 and LAMP-3/CD63)	42, 43
Vesicular monoamine transporter (VMAT)	13
Nucleotide transporter (MRP4)	45
Orphan sugar nucleotide transporter (Slc34d3)	16
Lysosomal granules	
LAMP-1 CD107a	59
LAMP-2 CD107b	59
LAMP-3 CD63, LIMP	59
LIMP II LGP85	59

Alpha Granules

Alpha granules, the largest and most numerous of the platelet granules, correspond to the azurophilic granules viewed by light microscopy. They are unique in terms of structure and packaging function. Structurally, alpha granules are composed of two major compartments, a dark centrally located nucleoid region and a peripherally located electron-lucent gray matrix region apparent at the electron microscopic level. The nucleoid region contains proteoglycans, which confer stability to the granules. The nucleoid region is where beta-thromboglobulin and platelet factor 4 as well as other proteins are localized. Von Willebrand factor, multimerin, and factor V colocalize within tubular structures located in the outer part of the gray matrix region. Fibrinogen, thrombospondin, and fibronectin are primarily located in the gray matrix region in an area between the nucleoid and outer gray matrix region. Compartmentalization of VWF and fibrinogen within alpha granules has been documented in mouse, human, and porcine platelets.[69] Compartmentalization of proteins may serve to allow platelets to differentially release proteins in response to activation.[71] Mouse alpha granules are more heterogeneous in terms of shape, size, and distribution compared to human alpha granules.[69] Alpha granules contain proteins that are synthesized by megakaryocytes in addition to plasma proteins that are endocytosed as platelets circulate in blood. Alpha granule proteins that are synthesized by megakaryocytes fall into two categories: megakaryocyte-specific (such as beta-thromboglobulin and platelet factor 4) and those

synthesized by other cells and concentrated within platelet alpha granules (such as factor V and platelet derived growth factor (PDGF).[76] The latter proteins are sometimes referred to as platelet-selective proteins. The most well-recognized protein endocytosed by platelets is fibrinogen whose uptake is mediated by the glycoprotein-IIb–IIIa (integrin $\alpha_{IIb}\beta_3$) receptor. Proteins present in low levels within alpha granules such as albumin and immunoglobulin are passively taken up by platelets and do not require specific receptors. A proteomic analysis of human platelet alpha granule proteins provided a list of 284 proteins, 44 of which have not been previously identified within platelet alpha granules.[55] Alpha granule membrane proteins, much like dense granule membrane proteins, become expressed on the platelet surface after platelet secretion as a result of fusion of granules with the OCS or outer platelet membrane. The most well-known alpha granule membrane protein expressed on the surface of activated platelets is P-selectin or CD62P (formerly known as granule membrane protein 140 (GMP140)[46] and as platelet-activation-derived granule external membrane protein (PADGEM)).[51] Since its discovery, P-selectin has also been detected on the membranes of dense granules.[42] Monoclonal antibodies have been developed to detect the presence of P-selectin on the surface of activated platelets in dogs.[27] Other proteins present within alpha granule membranes include GPIb-IX–V, GPIIb–IIIa, PECAM-1, vitronectin receptor, and GPIV (Table 76.2). Some of these membrane proteins have also been detected within the membranes of dense granules.[84]

Lysosomal Granules

Lysosomal granules contain acid-dependent hydrolases including glycosidases, proteases, and lipases.[23] Lysosomal granules can be identified in platelets and megakaryocytes using specific cytochemical stains.[5] Secondary lysosomes have been detected in bovine megakaryocytes and platelets.[58] Lysosomal membranes also contain proteins that become exteriorized during the platelet release reaction (Table 76.2). Membrane proteins, which include lysosomal integral membrane protein (LIMP or CD63) and lysosomal-associated membrane proteins 1 and 2 (LAMP-1 and LAMP-2),[29] are heavily glycosylated to protect them from hydrolytic enzymes stored within these granules.[59] While initially LAMPs were thought to be solely present within lysosomal granule membranes, LAMP-2 and LAMP-3 have been detected within dense granule membranes as well.[43] Additional lysosomal membrane proteins are present that mediate transport of ions and amino acids across the membrane to maintain an acidic luminal pH.

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CHAPTER 77

Platelet Signal Transduction and Activation Response

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Overview of Platelet Function
Platelet Receptors and Activation Pathways
  ADP/ATP Receptors
  Collagen Receptors
Thromboxane Receptors
  Thrombin Receptors
Platelet–Leukocyte Interactions and Thromboinflammation
Procoagulant Activity

Acronyms and Abbreviations

AA, arachidonic acid; CalDAG-GEFI, calcium diacylglycerol guanine nucleotide exchange factor I; CCL2, chemokine ligand-2; cPLA2, cytoplasmic phospholipase A2; CXCL8, chemokine IL-8; cyclic adenosine monophosphate (cAMP); DAG, 1,2-diacylglycerol; DTS, dense tubular system; GDP, guanosine diphosphate; GIRK, G-protein-gated inwardly rectifying potassium channel; GP, glycoprotein; GTP, guanosine triphosphate; ICAM-1, intercellular adhesion molecule-1; IP3, inositol triphosphate; ITAM, immunoreceptor tyrosine-based activation motif; LAT, linker for activation of T cells; LFA-1, lymphocyte function-associated antigen-1; Lin-11,Isl-1,Mec-3 (LIM); MLC, myosin light chain; NHE1, Na/H exchanger; PAK, p21-activated kinase; PAR, protease-activated receptor; PE, phosphatidylethanolamine; PI3K, phosphoinositide-3-kinase; PIP, phosphatidylinositol phosphate; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; PS, phosphatidylserine; PSGL-1, P-selectin glycoprotein ligand-1; ROCK, Rho-associated coiled-coil-containing kinase; SLP-76, Src homology domain-containing leukocyte phosphoprotein of 76 kDa; TF, tissue factor; TP, thromboxane receptor; TRAP, thrombin receptor-activating peptide; TXA, thromboxane A2; VCAM-1, vascular cell adhesion molecule-1.

OVERVIEW OF PLATELET FUNCTION

Platelets are the first line of defense against bleeding at sites of vascular injury and are major reactants in thrombotic, inflammatory, and neoplastic processes. Platelets have cell surface receptors that recognize signals from their environment and communicate those signals to a complex network of biomolecules that include ions, proteins, nucleotides, and phospholipids. While the details of many key networking pathways that elicit platelet responses are known, others remain to be elucidated. Platelet reactions orchestrated via these complex signaling networks include adhesion, aggregation, granule release, retraction, microvesiculation, and procoagulant expression.

When agonist molecules bind to platelet receptors, they induce inside-out signaling resulting in structural changes in glycoproteins on the platelet membrane surface that then allow binding of proteins that mediate platelet adhesion and aggregation (Figure 77.1; see also Chapter 76). In turn, binding of adhesive proteins to receptors results in outside-in signaling events that promote and enhance platelet granule release, platelet aggregate and fibrin formation, and clot retraction.

Cross-talk between platelet receptors and signaling molecules is a key component of platelet activation.

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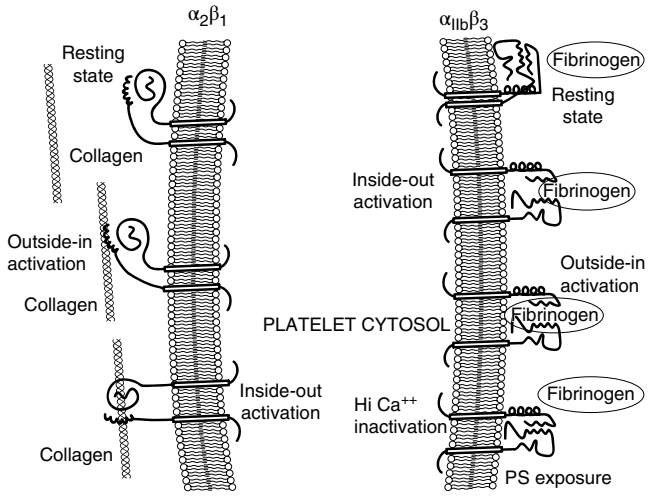


FIGURE 77.1 Platelet activation transforms the resting state of platelet membrane adhesion receptors for collagen ($\alpha_2\beta_1$) and fibrinogen ($\alpha_{IIb}\beta_3$) by altering their conformation states. Effector molecules generated by various signaling pathways in response to outside-in or inside-out signaling control the strength, reversibility, or irreversibility of collagen or fibrinogen binding. Formation of tight and irreversible complexes consolidates platelet adhesion and aggregation. When intraplatelet ionized Ca^{2+} is increased for prolonged periods, $\alpha_{IIb}\beta_3$ becomes secondarily inactivated and can no longer bind fibrinogen.

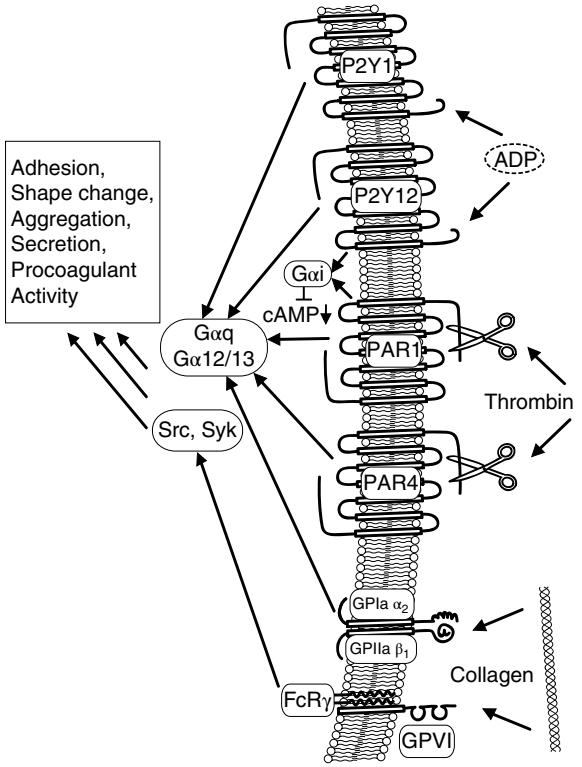


FIGURE 77.2 G-protein-coupled receptors (GPCRs) link platelet-signaling pathways. The extracellular domains of paired receptors for ADP ($P2Y_1$ and $P2Y_{12}$), thrombin (PAR1 and PAR4), and collagen ($\alpha_2\beta_1$ and GPVI) interact with their specific ligands to mediate outside-in signaling pathways. The thrombin receptor is activated when thrombin binds to and cleaves the extracellular *N*-terminal domain unmasking a new tethered *N* terminus that binds to the second extracellular loop of the receptor. In contrast to $\alpha_2\beta_1$, which is a GPCR, GPVI signals collagen-initiated platelet activation through sequential activation of Src and Syk family tyrosine kinases.

Adding to the complexity of platelet signaling events is the ability of platelets to synthesize proteins, referred to as signal-dependent translation.[72] This chapter reviews platelet-signaling events communicated through the major activation receptors present on platelets, as well as pathways and receptors involved in platelet–leukocyte interactions and expression of procoagulant activity.

PLATELET RECEPTORS AND ACTIVATION PATHWAYS

ADP/ATP Receptors

Platelets have three distinct P2 purinergic receptors, $P2Y_1$, $P2Y_{12}$, and $P2X_1$.[16] $P2Y_1$ and $P2Y_{12}$ are G-protein-coupled ADP receptors, mediating platelet shape change and aggregation responses. The $P2X_1$ receptor is a ligand-gated cation channel ATP receptor responsible for fast calcium entry, and may play a role in collagen and shear-induced platelet activation (Figure 77.2).[34,45,46]

$P2Y_1$ is coupled to a Gαq protein subunit; signaling via Gαq results in activation of phospholipase C β2 (PLCβ2), Rho A, Rac, and Src family kinases.[26] PLCβ2 mediates intracellular calcium mobilization and protein kinase C (PKC) activation via hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) within platelet membranes, to inositol triphosphate (IP3) and 1,2-diacylglycerol (DAG).[31,46] IP3 mediates calcium mobilization from intraplatelet stores sequestered within the dense tubular system (DTS). DAG and ionized calcium activate diacylglycerol guanine nucleotide exchange factor I (CalDAG-GEFI), which in turn facilitates the displacement of guanosine diphosphate (GDP) and loading of guanosine triphosphate (GTP) within Rap1b. GTP-bound Rap1b plays an important role in mediating the change in conformation of $\alpha_{IIb}\beta_3$ necessary for fibrinogen binding and platelet aggregation. The importance of CalDAG-GEFI downstream of PLCβ2 in mediating inside-out signaling events leading to activation of $\alpha_{IIb}\beta_3$ is illustrated in mice, dogs, and cattle with mutations in CalDAG-GEFI.[5,6,14] All of these animals have a bleeding diathesis and impaired platelet responses to ADP and collagen (see Chapter 84).

$P2Y_1$ activation results in platelet shape change as well as a reversible wave of platelet aggregation. Platelet shape change occurs as a result of both calcium-sensitive and calcium-insensitive pathways contributing to phosphorylation of myosin light chain (MLC).[48] The calcium-sensitive pathway is mediated by calcium-calmodulin. The calcium-insensitive pathway is mediated by Rho A/p160ROCK downstream of Gαq. Rho-associated coiled-coil-containing kinase (p160ROCK) is a serine/threonine protein kinase that is directly targeted by Rho

A. Activation of p160ROCK alters the balance between MLC phosphatases and MLC kinases in favor of MLC kinases, resulting in MLC-kinase-mediated phosphorylation of MLC necessary for platelet shape change.[15] The RhoA/p160ROCK pathway is important in mediating the disruption of the microtubule ring that transforms platelets from discs to spheres, and is also critical for maintaining the change in shape.[49] Rac, a Rho-family GTPase that is activated by $P2Y_1$, is thought to be important in mediating platelet spreading and anchoring via activation of a p21-activated kinase (PAK).[4] Activated PAK is thought to activate the serine/threonine kinase Lin-11, Isl-1, Mec-3 (LIM)which targets and phosphorylates cofilin, inhibiting cofilin's ability to depolymerize actin.[3]

Rac activation by $P2Y_1$ is potentiated by $P2Y_{12}$ signaling.[62] By contrast, $P2Y_1$ signals Src kinase activation and the inhibition of the phosphoinositide-3-kinase (PI3K)-dependent phase of $P2Y_{12}$ signaling. This inhibition is thought to be an important negative feedback mechanism regulating platelet activation mediated by ADP.[26]

$P2Y_{12}$ activation does not contribute to platelet shape change, but is associated with platelet aggregation and granule release, thromboxane generation, expression of procoagulant activity, and inhibition of adenylyl cyclase.[63] Engagement of $P2Y_{12}$ is also required for amplification of platelet activation induced by ADP and low concentrations of other platelet agonists including thromboxane A2 (TXA2), collagen, and thrombin. $P2Y_{12}$ is coupled to the heterotrimeric G protein complex $G\alpha i2\beta\gamma$.[70] ADP binding to $P2Y_{12}$ results in uncoupling of $G\alpha i2$ and $G\beta\gamma$, which act as separate signaling molecules. $G\alpha i2$ inhibits adenylate cyclase resulting in lowering of cyclic adenosine monophosphate (cAMP) levels. $G\beta\gamma$ dimers activate PI3K, Akt/protein kinase B, Rap1b, and G-protein-gated inwardly rectifying potassium channels (GIRKs).[34]

The PI3K isoforms that function downstream of $G\beta\gamma$ in platelets are PI3Kγ and PI3Kβ. The two isoforms seem to function in distinct but complementary roles leading to platelet aggregation and release. PI3Kγ plays a role in enhancing granule secretion, while PI3Kβ is necessary for sustaining platelet aggregation and formation of stable thrombi.[30] Akt/protein kinase B activation is thought to be mediated via PI3Kγ. Deficiency of Akt/protein kinase B in mice results in impaired platelet aggregation and release, and like PI3Kβ, it is required for formation of stable thrombi.[69] PI3Kγ and PI3Kβ, via generation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3), activate Rap1b, a critically important step in mediating the $\alpha_{IIb}\beta_3$ conformational change required for fibrinogen binding and platelet aggregation.[39]

GIRK channels containing GIRK1 and GIRK4 subunits are involved in the activation of Src kinases. Channel activation is coupled to $G\beta\gamma$ and ADP occupancy of the $P2Y_{12}$ receptor. Src kinases in turn phosphorylate and activate cytoplasmic phospholipase A2 (cPLA2).[60] $P2Y_1$ activation and signaling via Gq as well as signaling through $\alpha_{IIb}\beta_3$ are also necessary for cPLA2 activation.[32] When phosphorylated, cPLA2 is translocated from the cytoplasm to membranes where it functions to release membrane-bound arachidonic acid (AA) leading to the generation of thromboxane. Human patients with congenital $P2Y_{12}$ deficiency and $P2Y_{12}$ knockout mice have prolonged bleeding times and exhibit bleeding, illustrating the importance of this receptor in hemostasis. The absent aggregation response of $P2Y_{12}$-deficient platelets can be restored if platelets are activated concomitantly with ADP and epinephrine, presumably due to epinephrine being negatively coupled to adenylate cyclase in a manner similar to $P2Y_{12}$.[10] A mutation in the $P2Y_{12}$ gene found in Greater Swiss Mountain dogs has also been associated with an aggregation defect and signs of hemorrhage (see Chapter 84).

The $P2X_1$ receptor is an ATP receptor that is antagonized by ADP; early studies demonstrating $P2X_1$ receptor responses to ADP were later shown to be a result of ATP contamination of ADP preparations.[40] $P2X_1$ activation is associated with the change in conformation of platelets from discs to spheres with the rapidly reversible formation of multiple short pseudopodia.[54,55] ATP binding to $P2X_1$ is associated with a rapid, reversible calcium influx followed by rapid desensitization of the receptor.[44] Calcium influx is thought to activate MLC kinase which is responsible for mediating the shape change response. $P2X_1$ activation also results in centralization of granules without granule release and formation of platelet microaggregates.[21,65] Microaggregate formation is likely a result of calcium influx coupled with protein kinase C activation leading to Erk2 activation.[34] Like the $P2Y_{12}$ receptor, $P2X_1$ also participates in amplification of platelet responses to low concentrations of other agonists, including ADP, collagen, thrombin, and thromboxane.[21,23,44] Dense granule release of ATP induced by low concentrations of agonists triggers $P2X_1$-mediated calcium influx resulting in reinforcement and amplification of platelet activation. $P2X_1$ also seems to be essential in thrombus formation and growth at sites of vascular injury under high shear forces.[27]

Collagen Receptors

Platelets have two receptors primarily responsible for direct response to collagen; glycoprotein VI (GPVI) and integrin $\alpha_2\beta_1$[53] (Figure 77.2). The platelet glycoprotein-Ib-IX–V complex, which interacts with collagen indirectly, is critically important in initiating platelet contact with collagen under high shear conditions. The glycoprotein-Ib-IX–V complex mediates transient arrest of platelets from flowing blood and weak tethering of platelets on exposed subendothelial surfaces through the binding of von Willebrand factor (VWF) A1 domain to the GPIbα region of the receptor complex. As tethered platelets roll along exposed subendothelium, they encounter collagen fibrils, which bind to glycoprotein VI. This leads to inside-out signaling and activation of integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$, the release of ADP, and thromboxane formation, which in turn reinforce GPVI interactions.[43] Activated $\alpha_2\beta_1$, transformed to a high-affinity state, binds tightly to specific

sequences in collagen, allowing firm platelet adhesion and spreading.[35] Activated $\alpha_{IIb}\beta_3$, also transformed to a high-affinity state, reinforces firm platelet adhesion and supports platelet aggregation.[57]

Integrin $\alpha_2\beta_1$ and GPVI are thought to signal through a shared network of signal transduction proteins. The shared signaling pathway may explain why the absence of either GPVI or integrin $\alpha_2\beta_1$ does not result in a significant bleeding diathesis.

Glycoprotein VI is a member of the immunoglobulin superfamily type I transmembrane proteins; it is noncovalently associated with the Fc receptor gamma-chain (FcR γ-chain). The FcR γ-chain has an immunoreceptor tyrosine-based activation motif (ITAM). When stimulated with collagen, the FcRγ-chain is tyrosine phosphorylated by the Src kinases, Lyn and Fyn.[24] This leads to the recruitment and activation of Syk tyrosine kinases, which lead to the phosphorylation and activation of other signaling molecules.

Adaptor molecules such as linker for activation of T cells (LAT) and Src homology domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) coordinate assembly of a multiprotein signaling complex necessary for activation of phospholipase Cγ2 (PLCγ2) and PI3K. LAT is a membrane-associated adapter molecule that is anchored to lipid rafts in platelet membranes.[50] Activation of PLCγ2 leads to cleavage of phosphatidylinositol 4,5-bisphosphate into IP3 and DAG. IP3 mediates the release of calcium from stores within the dense tubular system. DAG and calcium bind to CalDAG-GEFI, resulting in activation of PKC and activation of Rap1b. Rap1b activation plays a role in inducing the change in conformation of $\alpha_{IIb}\beta_3$ to a high-affinity state necessary for binding of fibrinogen and platelet aggregation. Although GPIb-IX–V also stimulates PLCγ2 activation, the tyrosine phosphorylation pattern is distinct from that occurring via GPVI, resulting in weaker PLCγ2 activation and diminished phospholipase activity.[64]

Collagen-induced platelet aggregation is largely dependent on ADP signaling through $P2Y_{12}$. TXA generation and resulting platelet aggregation and release result in signaling through the $P2Y_{12}$ receptor by secreted ADP and a positive feedback on platelet secretion through the PI3K pathway.[46]

Thromboxane Receptors

TXA and prostaglandin H2 interact with the same receptors. Human platelets have two distinct thromboxane A2–prostaglandin H2 receptors, TPα and TPβ, that are products of alternative splicing. These receptors differ only in their C-terminal or tail domains.[29] TPα is a high-affinity binding site for thromboxane and mediates platelet shape change and intracellular calcium movement. TPα couples positively to adenylate cyclase via Gq and activates PLCβ which hydrolyzes PIP2, resulting in generation of IP3 and DAG. TPβ is a low-affinity binding site and mediates platelet aggregation and secretion. TPβ couples negatively to adenylate cyclase via Gi and inhibits cAMP formation. Most dogs demonstrate a weak AA response characterized by shape change and reversible platelet aggregation without granule release.[33] Addition of epinephrine prior to AA corrects the impaired aggregation and release response.[20] Canine platelets are predicted to lack TPβ receptors based on the absence within the dog genome of the coding region necessary for synthesizing the alternative C-terminal domain for TPβ. This may partially explain the generally weak response of canine platelets to AA stimulation in vitro. Epinephrine, by linking to Gi, may mimic the effects of TPβ, thus allowing for full platelet aggregation and release. A bovine TPα receptor has been cloned and characterized.[41]

Platelets generate TXA from AA. AA is liberated from dense tubular membrane phospholipids by the action of phospholipase A2 in response to agonist stimulation. Cytosolic cPLA2 is phosphorylated during thrombin, ADP, and collagen-induced platelet activation. Phosphorylation of cPLA2, in combination with a rise in intracellular calcium, results in cPLA2 translocation to the membrane where it releases arachidonic acid from the sn-2 position of membrane-bound phospholipids. Arachidonic acid is converted sequentially to prostaglandin G2 (PGG2), prostaglandin H2 (PGH2), and TXA via cyclooxygenase-1, prostaglandin hydroperoxidase, and thromboxane synthase. The resulting thromboxane generation results in platelet aggregation and dense granule release.

TXA synthase inhibitors do not inhibit platelet reactivity due to the enhanced production of prostaglandin H2 under these conditions and the ability of prostaglandin H2 to activate the receptor.[67]

Thrombin Receptors

Platelet thrombin receptors include the glycoprotein complex-Ib-IX–V and protease-activated receptors (PARs). The Ibα subunit of the Ib-IX–V complex has a thrombin-binding site that overlaps with the binding site for VWF.[52] The GPV subunit is cleaved and removed from the complex by thrombin during platelet activation. GPV is thought to negatively modulate platelet activation; cleavage of GPV is a necessary first step prior to binding of thrombin to Ibα. Thrombin binding to Ibα mediates platelet activation via induction of ADP release and signaling through $P2Y_{12}$. Ibα is also thought to act as a cofactor in the activation of PAR1 by thrombin.[17] Thrombin bound to Ibα is protected from inactivation by antithrombin.[18]

Protease-activated receptors are G-protein-coupled receptors that are characterized by the presence of a tethered ligand (Figure 77.2). Protease-activated receptors are activated when enzymes cleave at a specific site near the N-terminal exodomain resulting in formation of a tethered peptide sequence that can in turn bind to a G-protein-coupled receptor on the same molecule. Peptide sequences that match the tethered ligands generated by thrombin cleavage, referred to as thrombin receptor-activating peptides (TRAPs), have been used with varying success in vitro to activate animal platelets.[9,11] In most species, TRAPs are not as effective as the comparable tethered ligand in inducing platelet

aggregation or release. Three PAR types that vary with species have been identified in mammalian platelets. Human platelets have PAR1 and PAR4, and mouse platelets signal through PAR3 and PAR4, however PAR subtypes in other species are not well-defined. PAR1 couplings to Gq, G13, and Giz are thought to be of most importance in mediating thrombin effects on platelets.[12]

The PI3 kinase pathway is considered to be of importance in stabilizing thrombin-induced platelet aggregates, although thrombin can activate platelets independently of Gi activation. On human platelets, PAR1 binding results in a rapid spike in calcium influx, while PAR4 binding is associated with a slower and sustained calcium influx that is considered vital to eliciting secondary signals necessary for complete platelet activation.[13] Canine platelet PARs have not been fully evaluated; functional studies indicate that canine platelets possess PAR4 receptors and may possess PAR1.[5] Comparative genomics reveals sequence homology for all the PAR subtypes (PAR1 through PAR4) between dogs and people. Nevertheless, canine platelets do not respond to TRAPs designed from either human or canine sequence[19] (M. K. Boudreaux, personal observation). Mouse platelets lack PAR1 expression; however, PAR3 on mouse platelets acts as a cofactor in supporting cleavage and activation of PAR4. PAR3 alone is unable to mediate signaling leading to platelet activation, but rather acts as a cofactor for promoting PAR4 cleavage in the presence of low thrombin concentrations.[42] Recent studies have indicated that thrombin binding of both GPIb-IX and PAR receptors may be required for peak platelet activation.[22]

PLATELET–LEUKOCYTE INTERACTIONS AND THROMBOINFLAMMATION

The GPIbα subunit of platelet glycoprotein complex-Ib-IX–V is a ligand for endothelial P-selectin, as well as VWF, both of which translocate from Weibel–Palade bodies to endothelial surfaces in response to stimuli such as thrombin and histamine.[2,56,71] The binding site on GPIbα for P-selectin is very similar to the binding site of P-selectin glycoprotein ligand-1 (PSGL-1), which allows leukocytes to roll along the vascular endothelium. Both GPIbα and PSGL-1 are membrane mucins and possess a heavily *O*-glycosylated region that separates the ligand-binding region from the plasma membrane.[38] Both receptors also contain an acidic region immediately amino-terminal to the mucin-like region that is important for ligand binding.[36] Unlike PSGL-1, which requires extensive post-translational modification, including fucosylation of side chains in order to interact with P-selectin, GPIbα is capable of binding to endothelial P-selectin without modifications.

GPIbα can also bind to integrin αMβ2 (Mac-1) on neutrophils,[61] likely via the I domain within the αM subunit, which closely resembles the A1 domain of VWF. This interaction, which requires conversion of Mac-1 to the ligand-binding competent form, may contribute to neutrophil attachment to the endothelium or subendothelium at sites of endothelial activation or injury. Platelet P-selectin can bind to PSGL-1 on neutrophils, monocytes, and T-cells providing a mechanism for initial adhesion of leukocytes to platelets. Firm platelet–leukocyte adhesion is mediated by binding of Mac-1 to fibrinogen bound to the surface of activated platelets.

Firm leukocyte–platelet interactions can also be mediated by intracellular adhesion molecule-2 (ICAM-2) on platelets which can bind to αLβ2 (lymphocyte function-associated antigen-1 (LFA-1); CD11a/CD18) on leukocytes. Platelets can also release CD40L, which binds to CD40 on vascular endothelial cells resulting in chemokine secretion and upregulation of adhesion molecules.[28] Platelets also secrete IL-1β, which can induce endothelial cell secretion of IL-6, CXCL8, and CCL2 and upregulation of adhesion molecules such as E-selectin, vascular cell adhesion molecule-1 (VCAM-1), ICAM-1, and αvβ3.[25] In people and mice, pattern recognition receptors, particularly toll-like receptors, have been identified on platelets and provide a mechanism for platelet activation to participate in the innate immune response.[47] Together, all of these leukocyte interactions link platelets to the inflammatory process. The platelet transmembrane receptor C-type lectin receptor-2 (CLEC-2) and its primary ligand podoplanin have a prominent role in the prevention of blood–lymphatic mixing, but their interaction is also involved in thromboinflammation due to increased expression of podoplanin on macrophages and stromal cells.[51]

PROCOAGULANT ACTIVITY

Platelet activation triggers significant remodeling of the platelet cell surface. This is associated with the activation and clustering of receptors for adhesion molecules, receptor-mediated outside-in signaling, relocation and binding of proteins and other biomolecules from the cytosol to membrane surfaces, secretory organelle movement and fusion, and a dramatic redistribution of membrane phospholipids and formation and release of platelet-membrane-derived microparticles.

Mammalian cells and platelets exhibit an asymmetric distribution of aminophospholipids. In nonactivated platelets, negatively charged aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) are located primarily on the inner leaflet of the plasma membrane. An aminophospholipid translocase is responsible for transporting PS and PE to the inner membrane leaflet and maintaining asymmetry. When platelets are activated by stimuli that induce high intraplatelet calcium, such as collagen or thrombin alone or particularly when combined, PS is rapidly externalized to the outer membrane leaflet. Inhibition of translocase

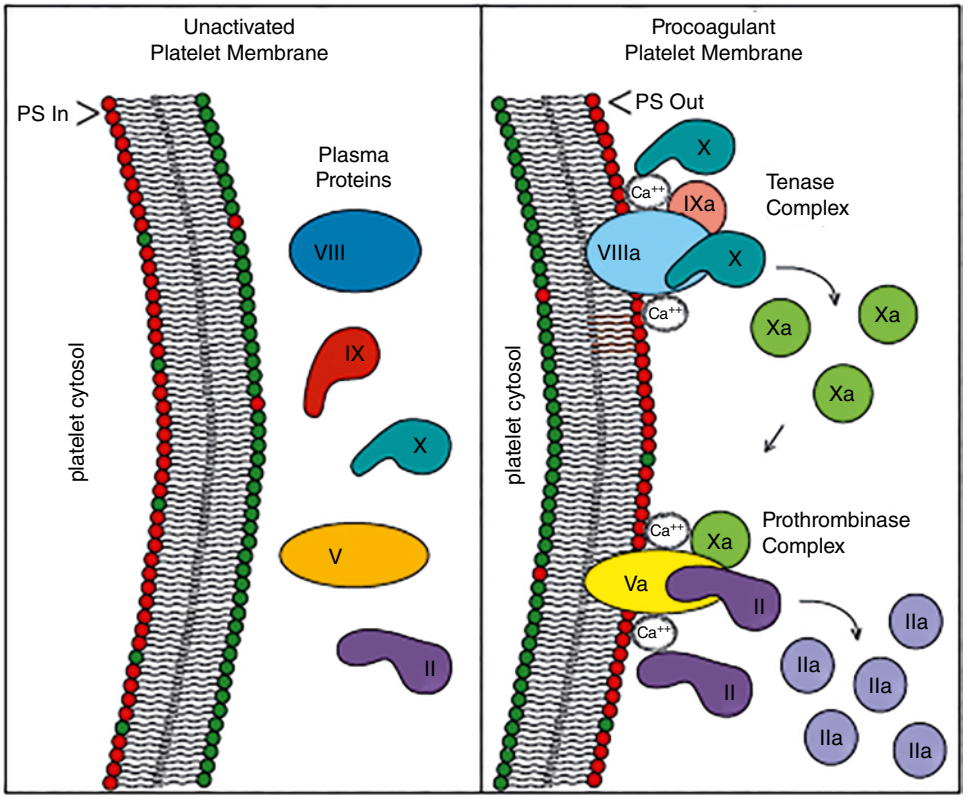


FIGURE 77.3 Signaling pathways activated by collagen and thrombin trigger externalization of phosphatidylserine (PS). When PS is externalized, specific coagulation proteins can bind to the platelet surface. The platelet then acts as a scaffold for assembly of the tenase and prothrombinase complexes, which accelerate thrombin (II_a) generation and clot formation at the site of vessel injury.

activity is believed to maintain PS externalization (Figure 77.3). Exposure of PS is also accompanied by the release of PS-enriched procoagulant microparticles.[68]

Platelets' ability to rapidly externalize PS by shuffling phospholipids between the two leaflets of the membrane bilayer is referred to as "scramblase" activity. The process is energy-independent and is now attributed to a calcium-dependent anoctamin family transmembrane protein, TMEM16F.[37] TMEM16F also plays a role in platelet PS externalization associated with cell death signals and apoptosis.[7]

The presence of PS on the surface of platelets is essential for platelet procoagulant activity. Rare bleeding disorders associated with a deficiency of platelet procoagulant activity have been reported in people and dogs.[7,66] Phosphatidylserine expression on platelets and platelet-derived microparticles facilitates the binding of coagulation proteins, which when anchored to PS and the activated platelet surface form procoagulant reaction complexes (see Chapter 87). These include the extrinsic tenase complex (factor VIIa + tissue factor (TF) + factor X), intrinsic tenase complex (factor X + factor IXa + factor VIIIa), and the prothrombinase complex (factor Va + factor Xa + factor II). Once assembled, the complexes allow for protein associations, which improve catalytic efficiency of the reactions. For example, the prothrombinase complex is about 300,000 times more efficient than Xa alone in generation of thrombin from prothrombin, and the intrinsic tenase complex is 100,000 times more active than factor IXa alone as an activator of factor X.

The assembly of enzyme and substrate complexes on the PS-enriched surface of activated platelets serves to localize and concentrate procoagulant factors at sites of injury. Since antithrombin preferentially targets uncomplexed reaction products for neutralization, the anchored reaction products are protected from neutralization and inhibition.

Studies have also shown that in human and porcine platelets, expression of procoagulant activity is dependent on a change in intracellular pH and Na influx, which occurs with activation of the Na/H exchanger (NHE1), and potentially other mechanisms.[58] NHE1 can be phosphorylated and activated by PKC. NHE1 also has two PIP2-binding domains, which are required for optimal activation, suggesting that PIP2 may play a role in mediating PS exposure in activated platelets.[1,8]

Activated platelets are also capable of synthesizing and expressing tissue factor on their surface, which contributes to the procoagulant activity of platelets. Platelets possess pre-mRNA transcripts, which are spliced and translated into protein upon platelet activation. Cdc2-like kinase-dependent splicing is thought to play a role in this mechanism.[59]

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CHAPTER 78

Platelet Kinetics and Laboratory Evaluation of Thrombocytopenia

ADI WASSERKRUG-NAOR

Platelet Kinetics
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Acronyms and Abbreviations

APTT, activated partial thromboplastin time; BFU-Meg, burst-forming unit-megakaryocyte; CFU-Meg, colony-forming unit-megakaryocyte; CKCS, Cavalier King Charles Spaniel; CMP, common myeloid progenitor cell; DIC, disseminated intravascular coagulation; DMS, demarcation membrane system; ER, endoplasmic reticulum; GM-CSF, granulocyte–monocyte colony-stimulating factor; HUS, hemolytic uremic syndrome; IL, interleukin; MPC, mean platelet component concentration; MPV, mean platelet volume; OCS, open canalicular system; OSPT, one-stage prothrombin time; PCDW, platelet component concentration distribution width; PCT, plateletcrit; PDW, platelet distribution width; PT, prothrombin time; SDF-1, stromal cell-derived factor-1; TTP, thrombotic thrombocytopenic purpura; VWF, von Willebrand factor.

PLATELET KINETICS

The last decade has seen significant progress in the understanding of megakaryocyte and platelet development, maturation, and functional life span. These mechanistic insights have deepened our knowledge of platelets' role in various pathological states and hold promise for discovery of novel therapeutic compounds.[92]

Megakaryocytopoiesis: Stem Cell Progenitors

The development of the megakaryocyte–erythroid progenitor cell is complex, and now believed to incorporate different hierarchical pathways (see Chapter 75). Unilineage progenitor cells (megakaryocyte–erythroid progenitor cells and others), and possibly megakaryocyte-committed progenitor cells, may develop directly from hematopoietic stem cells (HSC), without passing through myeloid progenitor cell lines. Phenotypic

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markers such as von Willebrand factor (VWF), CD41 (GpIIb), and CD61 (GPIIIa), previously thought to be expressed only after megakaryocyte differentiation, are now recognized at earlier developmental stages of HSC. These HSC may be programed to ultimately become megakaryocytes.[80,120]

The primitive megakaryocytic burst-forming unit (BFU-Meg) is the first defined megakaryocyte stage that has a high proliferative capacity, producing from 100 to 500 megakaryocytes per colony in a 1-week time period.[6,48] Following the BFU-Meg, the more mature megakaryocytic colony-forming unit (CFU-Meg) gives rise to approximately 3–50 megakaryocytes per colony. The BFU-Meg and CFU-Meg stages characteristically express CD34, CD33, and CD41, followed by later expression of CD61, CD42, and CD36. The first morphologically recognizable cell of the megakaryocyte lineage in marrow is the promegakaryoblast. Before this stage, the morphology of the earlier progenitors resembles a small lymphocyte. The promegakaryoblast has a high nuclear to cytoplasmic ratio, dark blue cytoplasm, and is approximately 10 μm in diameter.[48,117] The megakaryoblast has a kidney-bean-shaped nucleus, dark blue cytoplasm, and is approximately 15–50 μm in diameter. As the later stages of megakaryocyte precursors undergo endomitosis, the cells lose CD34 expression, and increase ploidy, cell size, and volume. The hematopoietic pluripotential stem cells and early megakaryocyte progenitor cells are positive for CD34 and have low expression of CD41.[48,53] Cells that become committed to the megakaryocyte lineage acquire the expression of CD61 and CD42, and expression of CD41 increases.[48,53] The promegakaryocyte loses its mitotic properties and goes through endomitosis to create a polyploid megakaryocyte. Genomic DNA duplication occurs in the endomitotic process, with nuclear envelope breakdown but lacking the final steps of mitotic condensation and separation of the chromosomes and cytoplasm.[89] Studies have shown that defective cytokinesis and karyokinesis prevent the megakaryocyte from completing mitosis.[6,68,92] The megakaryocyte can attain ploidy as high as 128 N, with a modal ploidy in most mammals of 16 N.[16] This process causes megakaryocytes to become the largest hematopoietic cells in the marrow, reaching diameters up to 150 μm. The higher ploidy likely corresponds to an increased cell volume, and ultimately larger numbers of platelets produced.[16]

Megakaryocytopoiesis: Platelet Release from the Bone Marrow

Each megakaryocyte is capable of generating several thousand platelets. In humans, daily platelet production is estimated to be approximately 35,000 ± 4300 platelets/μL or 1×10^{11} total platelets.[16,50,53] The sequence from megakaryocyte development to release of new platelets takes approximately 4–5 days.[50] In the marrow, mature megakaryocytes migrate to the sinusoids to be closely positioned near endothelial cells.[65] Long pseudopods extend from the megakaryocyte into marrow sinusoids and thereby facilitate megakaryocyte intravasation to the peripheral circulation.

The first cytoplasmic extension from the megakaryocyte into the blood stream was believed to be the proplatelet. However, recent studies by Brown et al. indicate that much larger extensions are seen in the blood. These protrusions contain internal membrane structure that fuses at multiple points with the plasma membrane, enabling mobility and forward movement into the bloodstream.[9] In this model, proplatelets represent a later stage of thrombopoiesis characterized by elongate processes formed via microtubule and membrane reorganization. Proplatelets subsequently become thinner and smaller as they form preplatelets, which later undergo fission events to generate discoid platelets that continue to mature and divide in the blood stream.

Several unique systems in megakaryocytes are needed for the development of multiple cytoplasmic fragments from one large cell. These systems rely on a well-developed cytoskeleton and the demarcation membrane system (DMS).[99] The cytoskeleton in megakaryocytes and platelets consists of key proteins including actin, myosin, and tubulin. Actin influences both the shape and the flexibility of platelets, as the actin filaments create an internal cytoskeleton connected to the plasma membrane and to each other.[81] The β1 tubulin is the major constituent protein in the microtubulin system and is a unique protein found only in megakaryocytes. Microtubules are mainly responsible for the extension of proplatelets from megakaryocytes, providing a site to which motor proteins bind. Actin proteins play a main role in generating a bifurcation from the proplatelet that will later separate to create a platelet.[41] The DMS develops in the course of megakaryocyte maturation, and represents a continuation of the plasma membrane that invaginates into the cell. The origin and formation of the DMS membrane has yet to be confirmed. A recent study provided evidence that the DMS is formed by a combination of invagination of the megakaryocyte plasma membrane and synthesis of additional membrane structures from intracellular stores, mainly the Golgi and endoplasmic reticulum (ER).[25] The excess membrane later surrounds the multiple small cytoplasmic platelets produced at later stages of megakaryocytopoiesis.[25,99]

The final process of platelet shedding occurs in the circulatory space,[16,108] with recent studies indicating that platelet release occurs within the bone marrow and also in the pulmonary vasculature.[60]

Thrombopoietin (TPO) is the major cytokine that regulates all stages of megakaryocyte and platelet production. In addition to thrombopoietin, megakaryocytes and platelet production are regulated by several growth factors and cytokines (see Chapters 75 and 82). Megakaryocyte progenitors are stimulated by interleukin (IL)-3, IL-6, IL-11, IL-12, granulocyte–monocyte colony-stimulating factor (GM-CSF), and erythropoietin.[37] Additional factors include IL-1, stem cell factor, and leukemia inhibitory factor.[16,37] Stromal cell-derived factor-1 (SDF-1) is thought to play a role in megakaryocyte migration.[39]

Platelet production is regulated by total platelet mass rather than platelet numbers. In situations of increased demand, platelet production can increase as much as 20-fold or higher.[53] Interactions between TPO and the TPO receptor, myeloproliferative leukemia protein (cMpl), play a major role in maintaining platelet mass.[77] Thrombopoietin is secreted primarily from the liver and upon release binds to its high-affinity receptor, cMpl, present on platelets and megakaryocytes. After binding, TPO is internalized and degraded and thus not available to stimulate further megakaryocytopoiesis above steady-state levels.[53,57] Hepatic uptake of senescent platelets, mediated by the Ashwell–Morell receptor (AMR), also stimulates TPO production (see Chapter 75).[62]

Platelet Life Span and Senescence

In health, a steady state exists between the number of platelets produced and platelets destroyed. Platelets circulate for 4–10 days in most mammalian species, with approximately 30% of the total platelet mass transiently compartmentalized in the spleen. As platelets age, they are removed from circulation by splenic and hepatic macrophages. Recent studies demonstrate that platelet life span is regulated by the Bcl-2 family of proteins in pro- and anti-apoptotic pathways similar to the regulation of nucleated cell aging.[61,85] As platelet proteasome peptidase activities decrease, the resultant increase in misfolded proteins causes increase in pro-apoptotic proteins, such as Bax.[76] The senescence signals of platelets include exposure of phosphatidylserine on the outer platelet membrane, which stimulates platelet phagocytosis by macrophages, and membrane glycoprotein desialylation facilitating clearance by hepatic AMR.[46,62,69] Similar to erythrocyte senescence, denatured or damaged membrane glycoproteins may display neoepitopes that are recognized by naturally occurring antibodies.[46] A smaller percentage of platelets are continually removed from circulation in their role of maintaining endothelial integrity.[35,46]

Platelet Granule Formation and Release

Platelets contain three major types of granules: α-granules, dense granules, and lysosomes. The formation of these granules starts in the megakaryocyte and continues through platelet maturation. Proteins are trafficked into granules via both synthesis in the ER and endocytosis, whereby plasma proteins are bound, absorbed, and loaded into the granules.[100]

The open canalicular system (OCS) is a continuous membrane structure similar to the DMS described in the megakaryocyte.[99] It is through these channels that plasma proteins and other substances are transported in and out of the platelet. Interestingly, some veterinary species including cow, horse, and camels lack the OCS. The significance of this finding is unclear, as platelet activation responses are generally similar for these species when compared to mouse, rat, rabbit, dog, and human that have the OCS. In species that lack the OCS, granule secretion occurs via fusion directly with the plasma membrane.[30] In species that have the OCS, secretion involves fusion of granules with the OCS, causing the release of granule contents through the OCS channels. The SNARE family proteins are key proteins that regulate fusion of the OCS and the granules.[99] Additionally, the OCS allows the platelets to expand and change shape once activated, though this property is retained also in species that lack the OCS.

Platelet Participation in Inflammation and Neoplasia

Beyond their hemostatic properties, platelets play important roles in inflammation, cellular growth and development, wound healing, angiogenesis, and cancer metastasis. Platelets participate in the inflammatory response through direct interactions with leukocytes and endothelial cells, release of cytokines and chemokines, and activation of the complement system (see Chapters 91 and 92).[110] Activated platelets express surface proteins, including P-selectin, that enhance adherence to or activation of inflammatory cells in addition to activation of complement system. Tumor cells also bind to platelets via P-selectin, thereby promoting microthrombus formation. Additionally, platelets assist in the transmigration of inflammatory cells through the endothelial cells, to reach extravascular sites of inflammation.

A correlation between increased platelet counts and poor prognosis has been described for many tumor types, supporting a role for platelets in promoting tumor metastasis. Platelet and tumor cell relationships are mechanistically complex.[3,11] Platelets protect and transport tumor cells in microthrombi through the blood stream, secrete mediators that enable tumor cell invasion through endothelium, and stimulate angiogenesis that assists in tumor survival. Platelets not only physically protect the tumor cells during transportation but also appear to downregulate the immune system.[11,70] In addition, platelets present the major histocompatibility complex class I, allowing tumor cells to be identified as self and escape natural killer cell recognition and destruction. The platelet–tumor relationship appears to be bidirectional, as tumor cells are capable of inducing thrombopoiesis.[70] To add to this complexity, platelet-derived microparticles can induce apoptosis of tumor cells by insertion of miRNA into the tumor and causing stimulation of suppressor genes.[71]

Nonmammalian Species

The cellular equivalent of platelets in birds, reptiles, amphibians, and fish is the thrombocyte. Thrombocytes are small, round, oval, or spindled nucleated cells. As in mammals, the primary function of thrombocytes is hemostasis, and thrombocytes are the second most common blood cell after erythrocytes. Avian thrombocytes may also be important in innate immunity, since these cells have phagocytic capability and can remove foreign material from blood (see Chapter 95).[38] In addition to

hemostasis, reptilian thrombocytes have been demonstrated to play a role in wound healing.[106]

Thrombopoiesis is similar in avian and reptilian species in that thrombocytes originate in the bone marrow from a distinct mononuclear cell. The stages in thrombopoiesis include thromboblasts, immature thrombocytes (early, mid, and late stages), and mature thrombocytes. In general, as thrombocytes develop, they decrease in size, the cytoplasm changes from basophilic to pale blue or colorless, and the cell and nuclear shape change from round to oval.

Thrombocyte numbers in most avian species range between 20,000 and 30,000/μL or approximately 10–15 thrombocytes per 1000 erythrocytes; two or more thrombocytes per monolayer area under oil immersion (100×) is considered normal or adequate in most birds.[14,28] In normal reptiles, thrombocyte numbers in blood range between 25 and 350 thrombocytes per 100 leukocytes.[106] Numbers of thrombocytes in fish blood range from 60,000 to 70,000/μL.[78] Manual counts for thrombocytes in fish can be performed using a hemacytometer, but given the tendency to clump, accurate counts are difficult to obtain. Thrombocytes of certain fish species do not appear to have phagocytic ability.[90]

MECHANISMS OF THROMBOCYTOPENIA

Thrombocytopenia, defined as a decrease in circulating platelet number, is the most common acquired hemostatic disorder in veterinary and human medicine. Thrombocytopenia results most often from decreased or defective platelet production or from increased platelet consumption or destruction. Less frequently, heredity thrombocytopenia or abnormal distribution of the platelets can be the mechanism of thrombocytopenia. Figure 78.1 presents an algorithm for clinical diagnosis of thrombocytopenia and mechanistic categories are described in the following sections.[123] The pathogenesis of thrombocytopenia is covered in more detail in Chapters 80 and 81.

Hereditary Thrombocytopenia

Hereditary thrombocytopenias have been described in several breeds of dog (see Chapter 84), with Cavalier King Charles Spaniels (CKCS) having the highest breed prevalence.[8] Additional affected breeds include Akita dogs, Norfolk and Cairn Terriers, and a relatively mild thrombocytopenia in Greyhounds.[32,42] Distinct mutations

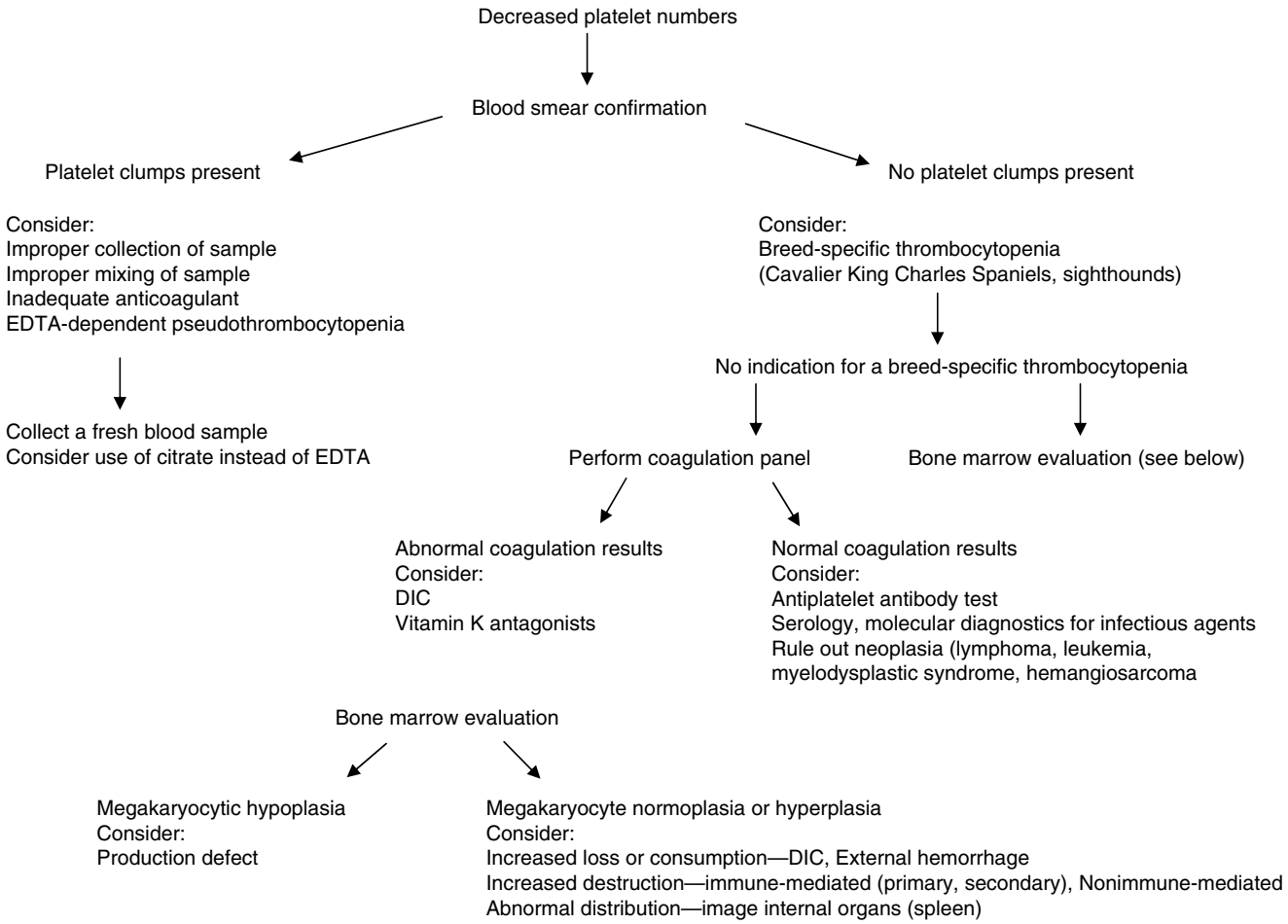


FIGURE 78.1 Diagnostic approach to thrombocytopenia.

in the gene encoding β1 tubulin have been identified as causing macrothrombocytopenia in CKCS and the related Norfolk and Cairn Terrier breeds.[21] Interestingly, members of the Felidae family display unique sequence variants in conserved regions of the β1 tubulin gene compared with other mammals.[7] This difference may be the underlying mechanism contributing to the high platelet volume variability seen in domestic and big cat species.

Decreased or Defective Platelet Production

Causes of decreased platelet production that result in thrombocytopenia can specifically target the megakaryocyte, but more frequently, other hematopoietic cells are also affected.

Acquired megakaryocyte hypoplasia or aplasia has been reported as a rare cause of isolated thrombocytopenia in dogs and cats, believed to have an immune-mediated component.[31,58,75] General etiologies of bone marrow hypoplastic disease of one or more hematopoietic cell lines include drugs, chemicals, or toxins that have cytotoxic effects or produce idiosyncratic reactions; irradiation resulting in cell death and marrow suppression; infection with certain viruses (e.g., canine parvovirus, feline leukemia virus, feline immunodeficiency virus, equine infectious anemia virus, bovine viral diarrhea virus, African swine fever virus), rickettsial agents, myelophthisis from primary or metastatic neoplasia or myelofibrosis, or myelonecrosis.

Increased Platelet Loss or Consumption

Thrombocytopenia secondary to rapid loss of platelets may occur with massive trauma, acute external hemorrhage, or exchange blood transfusion. In situations of trauma or external hemorrhage, thrombocytopenia is generally mild to moderate, transient, and usually reversible as hemorrhage is controlled.[101]

Accelerated consumption of platelets can develop in concert with widespread activation of the coagulation system, and syndromes of endothelial injury or abnormal VWF processing. Disseminated intravascular coagulation (DIC), thrombotic thrombocytopenic purpura (TTP), and hemolytic uremic syndrome (HUS) are complex syndromes characterized by widespread consumption of platelets, resulting in moderate to severe thrombocytopenia. Disseminated intravascular coagulation is a common complication seen in both veterinary and human medicine (see Chapter 92). Thrombotic thrombocytopenic purpura and HUS occur in humans, but are rare in veterinary species (see Chapter 93). A syndrome resembling HUS has been reported in dogs, cats, horses, and a heifer.[1,17,23,47,66,73,91] Animal models of TTP and HUS have been described.[88,94,116]

Disseminated Intravascular Coagulation

Disseminated intravascular coagulation occurs secondary to a wide variety of diseases, and is characterized clinically by microthrombosis and variable hemorrhage. Common disorders associated with DIC include systemic infectious or inflammatory disease, neoplasia, and severe tissue trauma. The pathophysiology of DIC is discussed in detail in Chapter 92.

Thrombotic Thrombocytopenic Purpura and Hemolytic Uremic Syndrome

In humans, TTP and HUS are the most common thrombotic microangiopathies. Thrombotic microangiopathies are characterized by endothelial damage, hemolytic anemia with schistocytosis, and thrombocytopenia associated with platelet-rich thrombus formation. Thrombotic thrombocytopenic purpura in people develops secondary to congenital or acquired (often immune-mediated) deficiencies of a disintegrin and metalloproteinase with thrombospondin type 1 motif, member 13 (ADAMTS13), the VWF-cleaving protease. In these patients, increased circulating ultrahigh-molecular-weight VWF multimers promote microvascular platelet thrombi formation.[107] Hemolytic uremic syndrome may occur either as a primary condition or as a secondary complication. Most cases of HUS occur in childhood after bloody diarrhea caused by *Shigella dysenteriae* serotype I or various *Escherichia coli* serotypes. Severe renal dysfunction is also a prominent feature of HUS. Plasminogen activator inhibitor type I and urine endothelin concentrations are increased in HUS patients.[4,101] Concentrations return to normal when patients enter remissions. Hemolytic uremic syndrome and TTP are discussed in more detail in Chapters 83 and 93.

Platelet Destruction: Immune-Mediated, Nonimmune-Mediated, and Complex Mechanisms

Immune-mediated destruction of platelets may be primary (idiopathic) or may occur in association with infectious agents, neoplasia, drugs, autoimmune, isoimmune, or neonatal-immune diseases. A diagnosis of idiopathic or primary immune-mediated thrombocytopenia is made after all other potential causes have been eliminated.

Immune destruction of platelet typically occurs when circulating platelets coated with antibody, antigen–antibody complexes, or complement are phagocytized by macrophages in the spleen, liver, and bone marrow, resulting in decreased platelet life span (see Chapter 80).

Platelet aggregation, phagocytosis, or lysis resulting in platelet destruction and thrombocytopenia can occur independently of immune-mediated events. Nonimmune-mediated platelet destruction occurs in some acute bacterial and viral infections and in cardiovascular disease (see Chapter 81).[34]

Platelet destruction in bacterial infections can occur as a result of platelet adherence or aggregation to activated monocytes or neutrophils. In Gram-negative infections, monocytes stimulated by endotoxin express tissue factor on their surface and generate thrombin.[95] Thrombin, a potent platelet agonist, causes platelet activation and aggregation. Platelets adhere to monocytes and are phagocytized. Exotoxins released from

Gram-positive bacteria may directly damage platelets and contribute to thrombocytopenia.[36,52,87,111]

The pathogenesis of thrombocytopenia associated with acute viral infections is often multifactorial, even though one mechanism may predominate. Direct platelet damage or lysis is one proposed mechanism of virus-associated thrombocytopenia. Myxovirus infections, including those due to Newcastle disease virus and influenza, decrease platelet survival by removal of platelet membrane sialic acid residues by viral neuraminidase.[109,112] Other viruses in which platelet damage is suspected include bovine viral diarrhea virus and hog cholera virus.[19,118]

In humans, platelet injury and clearance have been described secondary to arterial disease and vasculopathies, stenotic or prosthetic heart valves, or cardiac bypass surgery.[5,20,34] Turbulent circulation causes membrane damage and microparticle formation.[34] Interactions of platelets with damaged endothelial surfaces cause extensive platelet activation, clumping, and removal of platelets by the mononuclear phagocyte system.[5]

Many of the rickettsial diseases are associated with thrombocytopenia. Several mechanisms contribute to thrombocytopenia, including immune-mediated platelet destruction, direct damage, vasculitis, and platelet production deficits.

Abnormal Platelet Distribution

In health approximately 30–40% of the total circulating platelet pool may be stored in the spleen, referred to as physiologic platelet sequestration. The liver and bone marrow are additional sites of platelet sequestration.[2] Hypersplenism is a pathologic condition in which as much as 90% of circulating platelets become sequestered in the spleen. Hypersplenism is rare in animals, but a few suspected cases have been described.[49,56] In hypersplenism, thrombocytopenia is evident on a CBC, but the total numbers of platelets and platelet mass are actually normal. Platelet survival is usually normal, as is platelet production.[2,43] Hypersplenism is characterized by the presence of one or more cytopenias with corresponding bone marrow hyperplasia and significant splenomegaly.[35] Splenic size is an important factor determining the degree of thrombocytopenia. Although splenomegaly does not always result in thrombocytopenia, the spleen must be markedly enlarged to cause a severe decrease in circulating platelets.[82] All other causes of splenomegaly should be ruled out before a diagnosis of primary hypersplenism is made. Recommended treatment in these cases is splenectomy.

Severe hypothermia adversely affects platelet morphology and can cause a mild transient thrombocytopenia owing to pooling of platelets in the spleen.[49] Transient thrombocytopenia can also occur with endotoxemia and hypotension.

Pseudothrombocytopenia

Pseudothrombocytopenia (artifactual or spurious thrombocytopenia) is an in vitro phenomenon resulting from platelet clumping secondary to poor or difficult venipuncture and subsequent platelet activation or exposure of the blood to certain anticoagulants. Feline platelets are especially prone to activation during blood collection, with additional potentiating factors including a larger platelet size, a higher concentration of serotonin, irreversible aggregation and granule release when exposed to serotonin, and irreversible aggregation in response to low concentrations of ADP.[13,67,84] Because of their small size and the fact that many cats resist handling and restraint in unfamiliar settings, venipuncture is often challenging, which may increase the likelihood of platelet activation during collection.[78]

Pseudothrombocytopenia can also occur with overfilling of a blood collection vacutainer tube and subsequent improper or inadequate sample mixing.[83] EDTA is the anticoagulant most often implicated as causing pseudothrombocytopenia in people, as also described in horses, miniature pigs, and dogs.[33,44,86,119] Although there may be several factors, the leading mechanism for EDTA-dependent pseudothrombocytopenia involves autoantibody-mediated platelet agglutination.[26] Hidden epitopes become exposed within the platelet IIb–IIIa complexes as a result of complex destabilization occurring secondary to calcium removal from calcium-binding domains.[12,15,26] Platelet activation and clumping in samples collected in citrate anticoagulant has also been reported as a cause of spuriously low platelet counts in dogs.[108]

LABORATORY EVALUATION OF THROMBOCYTOPENIA

Complete Blood Count

A CBC should be the first test performed in any animal that is suspected of having a bleeding disorder. Generally, EDTA-anticoagulated blood yields stable platelet counts for 5 hours after collection when held at 20 °C and 24 hours when held at 4 °C; however, certain platelet parameters are affected by anticoagulant, time, or refrigeration.[74] Specific platelet parameters available on most automated CBCs include platelet count, mean platelet volume (MPV), and platelet distribution width (PDW). In addition to these automated parameters, microscopic examination of the blood smear for platelet morphology and size, and evidence of platelet clumping or adherence to leukocytes should be performed to confirm thrombocytopenia. Although manual methods are less accurate and precise compared to automated methods for platelet enumeration, they become necessary when platelet counts fall below the limit of sensitivity or linearity (usually 5000–20,000 platelets/μL) of a hematology analyzer. Manual platelet counts are best performed by experienced operators. If the platelet count is normal in an animal that has abnormal bleeding, petechiation, or ecchymosis, an abnormality in platelet function or a coagulopathy should be considered (see Chapters 84, 89, and 90).

Platelet Indices

Plateletcrit (PCT) may be a more reliable predictor of the clinical outcome of patients with thrombocytopenia than the number of platelets alone. In addition, examination of platelet indices may further enhance the diagnostic and the prognostic process in patients with thrombocytopenia.[115,122] The following section discusses the indices commonly used in human and veterinary medicine.

MPV estimates platelet size and is believed to be inversely proportional to platelet number in some species. Increases in MPV may be associated with regenerative response to thrombocytopenia, with some myeloproliferative diseases, and with hyperthyroidism.[27,102] Decreases in MPV have been reported in dogs with early manifestations of immune-mediated thrombocytopenia,[79] but are more often associated with bone marrow failure. Artifactual increases in MPV may arise when platelets are exposed to EDTA for prolonged periods of time, cooled to room temperature (25 °C), or refrigerated (4 °C), or if there is delayed exposure to an anticoagulant. While citrate anticoagulant yielded relatively stable MPV in blood stored at 37 °C in some studies,[40] canine citrate blood demonstrated increased MPV in a more recent study.[105] Evaluation of MPV in human medicine has proven useful in differentiating immune-mediated thrombocytopenia from other thrombocytopenic disorders and assisting in predicting the likelihood of clinical bleeding, with estimates of sensitivity of 93% and a specificity of 94.6%.[59] Clinical utility of MPV in veterinary medicine is not well-defined; however, a canine study reported that dogs with immune-mediated thrombocytopenia had higher MPV compared to a healthy population and that plateletcrit was an earlier marker for response to treatment than the platelet count alone.[96]

The use of platelet indices in nonthrombocytopenic disease has also been described.[10] Llewellyn et al. demonstrated that in dogs with surgical septic peritonitis, increased MPV is considered a negative prognostic factor.[64]

PDW represents an index of variation in platelet size. In humans, PDW was increased in patients with immune-mediated thrombocytopenia compared to patients with thrombocytopenia due to aplastic anemia; therefore, PDW may be useful in differentiating between hyperdestructive thrombocytopenia and hypoproductive thrombocytopenia due to aplastic anemia.[51] Additionally, the PDW can assist in differentiating reactive thrombocytosis from a myelodysplastic disease in humans, as the latter had a higher PDW in patients with thrombocytosis.[59]

The PCT (or thrombocrit) assesses total circulating platelet mass and is the percentage of the blood volume that is composed of platelets. It is determined by certain analyzers or calculated from platelet count and MPV. Expected values are usually less than 1%.

In dogs with experimentally induced endotoxemia and thrombocytopenia, the platelet count and the PCT were significantly decreased, and the MPV and PDW were increased.[121] PCT was found to be a better parameter than platelet count for assessing hemostatic status in CKCS, due to high breed prevalence of hereditary macrothrombocytopenia.[54,113]

Mean platelet component concentration (MPC) estimates the average platelet complexity by two-angle light scatter and is used to assess the presence of platelet granules, as indicators of platelet activation status and/or platelet maturation.[18] In veterinary studies, MPC was related to the activation state of feline platelets[122] and MPC was found to be decreased in septic foals, foals with DIC, and adult horses with inflammation, gastrointestinal obstruction, enteritis, ischemia, or thrombocytopenia.[97] Preanalytic variables that influence MPC include anticoagulant, sample age, storage temperature, and time.[29,105,114] Smith et al. reported that dogs with immune-mediated thrombocytopenia had decreased MPC; however, their data failed to replicate a previous report that found dogs with different inflammatory conditions had decreased MPC.[103]

Platelet component concentration distribution width (PCDW) is a measure of the variability of platelet density. Values of PCDW decrease due to platelet shape change from discoid to spherical as platelets undergo activation. PCDW also decreases with increasing storage time and may be useful for documenting the platelet storage lesion.[63] Although studies in thrombocytopenic animals are lacking, PCDW may help elucidate potential causes of thrombocytopenia secondary to platelet activation.

Immature platelet fraction (IPF) and reticulated platelets indicate the immature platelet fraction present in the blood, analogous to enumeration of reticulocytes in the red cell line. These parameters identify platelets that contain RNA remnants as a percentage of total platelet number and represent markers of megakaryocyte maturation and thrombopoiesis.[24,45] While immature platelets generally have increased MPV, reticulated platelets and IPF can more reliably identify immature platelets.[24,45] In cases of peripheral platelet destruction, the percentage of reticulated platelets or IPF is increased, whereas hypoproliferative thrombocytopenic patients have decreased percentages of reticulated platelets.[55] In veterinary studies, reticulated platelets were found to be increased in two horses that had DIC and several ponies experimentally infected with equine infectious anemia virus.[93] In dogs, preliminary studies have described methodologies for measuring reticulated platelets in clinical patients; however, diagnostic utility is not yet well-defined.[72,98,104]

Microscopic Evaluation of the Blood Smear

Microscopic examination of a blood film is necessary to evaluate platelet morphology and to ensure accuracy of automated platelet counts. Furthermore, decreased platelet counts should always be confirmed by microscopic examination of a blood smear, and, if necessary, the platelet count should be repeated on a freshly drawn blood sample. Delayed time of exposure to anticoagulation, improper mixing, or improper collection can all

contribute to falsely low platelet counts. EDTA-dependent pseudothrombocytopenia can be ruled out by restoration of normal platelet numbers and elimination of platelet clumps observed on blood smears when samples are drawn into citrate anticoagulant.

Evaluation of the blood smear should begin on low magnification to look for the presence of large platelet clumps. Often clumps are found on the feathered edge of the smear. If large clumps are found, this likely means that the number of platelets indicated by the hematology analyzer is falsely low. A general guideline for estimating platelet numbers on a blood smear is to determine the average number of platelets in 10 oil immersion fields using a 100× objective and multiply the average by 15–20 × 10^3 to obtain the estimated number of platelets per microliter.

Additional Diagnostic Tests

Once an animal is found to be thrombocytopenic, additional diagnostic testing is often necessary to elucidate the cause (see Chapters 80 and 81). Additional tests that are often performed include a coagulation panel, bone marrow analysis, serology or DNA analysis for infectious agents, and bacterial cultures.

Coagulation Panel

Determination of activated partial thromboplastin time (APTT), one-stage prothrombin time (OSPT; also referred to as prothrombin time or PT), and fibrinogen is ideal for characterizing patients with suspect hemostatic defects (see Chapter 88). Animals may be thrombocytopenic without additional abnormalities on a coagulation panel or they may have combined abnormalities suggestive of the underlying disease process. Among the common causes of thrombocytopenia, patients with primary immune thrombocytopenia typically have normal coagulation panel results, whereas patients with consumptive coagulopathy and DIC often demonstrate combined thrombocytopenia with abnormal coagulation results (see Chapter 92).

Bone Marrow Evaluation

Bone marrow evaluation of megakaryocytes may be necessary to determine if thrombocytopenia is secondary to a platelet production defect or is caused by peripheral platelet destruction or consumption. The typical finding in cases of defective platelet production is the absence, or decrease, in the number of megakaryocytes. Abnormal megakaryocyte morphology and size may also be observed. In most cases of peripheral platelet destruction, megakaryocyte numbers are normal to increased, often with concomitant increase in megakaryocyte size and ploidy. Aspiration and core biopsies of bone marrow offer distinct advantages. Cellular morphology is superior with aspiration biopsies, whereas core biopsies are more useful for evaluation of overall megakaryocyte numbers, myelofibrosis, or necrosis. For proper and complete bone marrow interpretation, a blood sample should be taken for a CBC at the time of bone marrow sampling. Sites for marrow collection include the iliac crest, greater tubercle of the humerus, and sternum, with species and size of the patient and operator preference determining the optimal choice.[22]

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CHAPTER 79

Evaluation of Platelet Function

PETE W. CHRISTOPHERSON and MARJORY B. BROOKS

Buccal Mucosal Bleeding Time
Platelet Aggregation
  Platelet Agonists
    ADP
    Collagen
    Arachidonic Acid
    Epinephrine
    Thrombin
  Light-Transmission/Optical Platelet Aggregation
  Platelet Secretion Assays
  Impedance/Whole Blood Platelet Aggregation
  Multiplate Analyzer
  Platelet Aggregation in Clinical Disorders
Platelet Function Analyzer—PFA100®, PFA200®
  Test Principle
  Factors Affecting Closure Time
Detection of Platelet Function Defects
    Hypofunction
    Hyperfunction
Flow Cytometry
  Cytometric Markers of Platelet Activation
  Leukocyte–Platelet Aggregates
  Automated Measurements
Additional Assays

Acronyms and Abbreviations

ADAMTS13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13; ADP, adenosine diphosphate; ATP, adenosine triphosphate; BMBT, buccal mucosal bleeding time; CADP, cartridges coated with collagen and ADP; CEPI, cartridges coated with collagen and epinephrine; CT, closure time; DDAVP, desmopressin acetate (1-(3-mercaptopropionic acid)-8-d-arginine vasopressin monoacetate (salt) trihydrate); FITC, fluorescein isothiocyanate; GP, glycoprotein; GPRP, synthetic peptide Gly-Pro-Arg-Pro; IMHA, immune mediated hemolytic anemia; LTA, light-transmission aggregation; MP, Multiplate Analyzer, MPC, mean platelet component concentration; NSAID, nonsteroidal anti-inflammatory drug; PAF, platelet-activating factor; PFA, platelet function analyzer; POC, point of care; PPP, platelet-poor plasma; PRP, platelet-rich plasma; SPD, storage pool disease; ST, S wave to T wave; VWF, von Willebrand factor; VWD, von Willebrand disease.

Platelet function tests are primarily used in clinical settings to identify platelet hypofunction and thereby diagnose intrinsic platelet defects, predict bleeding risk, and monitor antiplatelet drug therapy. As platelet activation and hyper-reactivity are increasingly recognized as contributing to many disease processes, assays to detect platelet hyperfunction have gained interest.

This chapter discusses platelet function tests with clinical veterinary applications, rather than tests primarily used in research settings. Table 79.1 summarizes the platelet function tests described in this chapter.

BUCCAL MUCOSAL BLEEDING TIME

The buccal mucosal bleeding time (BMBT) test or oral mucosal bleeding time has been the classic in vivo test to evaluate primary hemostasis in small animals (Table 79.2), although other types of bleeding time tests have been described.[58] The BMBT is performed by measuring the time for cessation of blood flow from a standardized incision in the oral mucosa made with a disposable, single-use, spring-loaded lancet.[30] The equine template bleeding time, performed by triggering the lancet on a forelimb, has been reported to have poor

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TABLE 79.1 Platelet function tests.

Name of test	Principle	Clinical applications	Reference
Bleeding time	In vivo cessation of blood flow	Screening point of care (POC)test	8, 30, 58, 63, 65
Flow cytometry	Measurement of platelet receptors and activation status by fluorescent markers	Diagnosis of platelet glycoprotein defects, detection of platelet activation in vivo or in response to agonists	7, 34, 38, 39, 49, 55, 59, 67, 69, 78
Full blood count	Automated impedance or flow-cytometry-based analysis of cells	Abnormalities in platelet number, size, and refractive index as measure of platelet activation status	56
Light transmission aggregometry	Low shear platelet-to-platelet aggregation in response to agonists	Diagnosis of acquired and inherited platelet aggregation defects, antiplatelet drug monitoring	14, 54, 62
PFA-100®	High shear platelet adhesion and aggregation during formation of a platelet plug	Detection of some inherited and acquired defects in primary hemostasis including moderate to severe VWD	11, 15, 52, 53, 66
Whole blood aggregation	Change in impedance in response to platelet agonists	Diagnosis of acquired and inherited platelet dysfunction	32, 68

TABLE 79.2 Normal bleeding times reported in animals.

		Normal bleeding time (seconds)			
Species	No.	Mean ± SD	Range	Method	Reference
Dogs	34	157 ± 29		Buccal mucosal	29
Dogs (Greyhounds)	61	129.5 ± 44.2		Buccal mucosal	62
Cats	14	114 ± 30		Oral mucosal	60
Horses	20	342 ± 114	136–894	Template	64

reproducibility, with coefficients of variation ranging from 26.8% to 45.5%.[65]

The BMBT has been used to screen patients preoperatively,[40] assess platelet function in an actively bleeding patient, characterize hereditary primary hemostatic disorders, and to evaluate drug effects.[5,8,30,50] In dogs affected with von Willebrand disease (VWD), the BMBT is reported to be prolonged by 85–100%, however, most reports consist of small case numbers.[8,12,30] Administration of desmopressin and cryoprecipitate has been reported to significantly shorten the BMBT, and the BMBT has been used to assess treatment response for dogs with VWD, and to monitor desmopressin acetate (1-(3-mercaptopropionic acid)-8-d-arginine vasopressin monoacetate (salt) trihydrate) (DDAVP) treatment for aspirin-induced impairment of platelet function.[12,13,18] The BMBT is reportedly prolonged in dogs with thrombocytopenia and severe azotemia.[30] However, in the author's experience, BMBT is not prolonged in dogs with congenital macrothrombocytopenia, presumably due to adequate total platelet mass. In a series of dogs with hereditary bleeding disorders, the BMBT was prolonged in dogs with primary hemostatic defects, i.e., thrombopathia and VWD, but normal in dogs with coagulation factor deficiencies.[8] Considerable variability in Greyhound BMBT has been described; with values that differed by ±2 minutes.[64] The BMBT correlated weakly with surgical blood loss in dogs;[40] and its value as a routine preoperative screening test in both human and veterinary medicine is debatable.[30,41] It has also been demonstrated that the type and size of lancet device used to perform a BMBT influence the results.[1] While the diagnostic utility of BMBT to predict bleeding risk is not established, the lack of availability of more sensitive or specific tests ensures its continued use in clinical veterinary medicine for point-of-care screening of primary hemostasis.

PLATELET AGGREGATION

Optical or turbidimetric platelet aggregation is the traditional "gold standard" method for testing platelet function in isolated platelet-rich plasma (PRP). The original assay was described in 1962 by Born and consisted of an absorptiometer with experiments performed at room temperature.[4] Whole blood aggregometry, based on changes in electrical impedance, was later developed as an alternate method with less-complex preanalytic processing. Regardless of method, aggregation is evaluated in response to addition of exogenous platelet agonists.

Platelet Agonists

ADP

ADP was the first known low-molecular-weight platelet aggregating agent. In human platelets, it induces shape change and reversible aggregation.

Secondary aggregation, sometimes observed after stimulation of PRP with ADP, is caused by secretion of dense granule constituents triggered by synthesis of thromboxane A_2. ADP plays a key role in platelet function by amplifying activation responses induced by other platelet agonists.

While considerable interspecies' differences in aggregation responses to various agonists exist, platelets from most species respond to stimulation by ADP at concentrations between 5 and 25 μmol/L.[54,62,68] Canine whole blood is an exception to this generality, since ADP does not induce consistent aggregation responses, possibly due to degradation of ADP by red blood cells and white blood cells.[32,57]

Collagen

Collagen, either of bovine or equine tendon origin, is typically the strongest of the agonists used in clinical laboratories. Collagen-induced platelet aggregation usually has a lag phase, during which the platelets adhere to the collagen fibrils and undergo shape change and release granule contents. Canine platelets respond in a dose-dependent manner to 1–20 μg/mL collagen.[32,54] Platelets from horses, cows, pigs, buffalo, and sheep all responded to collagen in one study, with equine platelets being the most sensitive.[62]

Arachidonic Acid

Arachidonic acid is converted to thromboxane A_2, a potent platelet agonist, through the actions of cyclooxygenase. The aggregation response induced by arachidonic acid is completely inhibited by aspirin in humans (see Chapter 94). Species differences exist in response to arachidonic acid stimulation, thereby limiting its value to evaluate the antiplatelet effects of aspirin and other nonsteroidal anti-inflammatory drugs (NSAID) on animal platelets.[14,32,62] Pelagalli[62] showed that arachidonic acid was less effective than ADP, collagen, or platelet-activating factor (PAF) in inducing aggregation of PRP from horses, buffaloes, pigs, and sheep, although a maximal aggregation response was achieved at 7.5 mmol/L. The use of arachidonic acid as an agonist in canine platelet aggregation experiments has been controversial. Jüttner[32] found no response to arachidonic acid at 0.5 and 1 mmol/L in any dogs tested, whereas others have found that canine platelets aggregate in response to arachidonic acid.[20,68] One study found that the variable platelet responses to arachidonic acid were breed-dependent,[14] and it has been suggested that the differences in sensitivity to arachidonic acid in dogs may reflect genetic differences in intracellular signaling pathways.[31]

Epinephrine

Epinephrine is the most unreliable of the agonists for platelet aggregation in animals. Epinephrine is usually regarded as ineffective by itself as an aggregation initiator, but has been used to enhance aggregation responses of other platelet agonists.

Thrombin

Thrombin is a potent platelet agonist; however, its ability to cleave fibrinogen to form a fibrin clot makes it a difficult agonist to use for platelet aggregation testing in plasma or whole blood milieux. The synthetic peptide, Gly-Pro-Arg-Pro (GPRP), which inhibits thrombin-induced fibrin polymerization but not platelet aggregation, can be added to reaction mixtures to facilitate evaluation of thrombin-induced activation response.[54]

Light-Transmission/Optical Platelet Aggregation

Light-transmission aggregation (LTA) is a specialized assay using warmed (37 °C) and stirred PRP or washed platelets, performed in photometers attached to a chart recorder or computer software that monitors sample turbidity over time. The instrument is standardized for each subject using PRP set as 0% aggregation and autologous platelet-poor plasma (PPP) set as 100% aggregation. As platelets aggregate in response to the addition of an exogenous platelet agonist, the sample becomes more clear and an increase in light transmission through the test sample is recorded (Figure 79.1).

Platelet aggregation response parameters include the percent maximal aggregation compared to PPP, the rate of aggregation (measured as the slope of the aggregation curve), and lag phase representing time from agonist addition to first aggregate formation.

Light-transmission aggregation is a very useful technique for characterization of platelet function defects. By using panels of agonists at various concentrations, it is possible to obtain a large amount of information on platelet receptor function, signaling pathways, and downstream response.[80] One limitation of LTA is its use of isolated platelets separated from other blood components and aggregated under low shear; conditions that do not mimic platelet adhesion, activation, and aggregation in damaged vessel walls.

Light-transmission aggregation can be affected by a variety of analytical and preanalytical variables. Meticulous venipuncture technique is required and evacuated collection tubes can be problematic due to ex vivo artifactual activation of platelets by the shear force of the vacuum. However, some laboratories use these tubes without causing significant ex vivo platelet activation.[47] Sodium citrate (0.102 mol/L, 0.129 mol/L, buffered or nonbuffered) at a ratio of nine parts blood to one part anticoagulant is the typical anticoagulant for platelet aggregation testing. Very low hematocrit can affect aggregation, as the final concentration of the citrate in plasma will be low compared to samples with normal hematocrit, thus yielding a higher concentration of free calcium in the plasma.[22]

The use of standardized platelet counts of PRP for platelet aggregation assays is somewhat controversial.[43,77] Aggregation response may vary in relation to platelet count;[29] however, diluting PRP with PPP can also influence results due to addition of activating substances in PPP that are released from platelets or other blood cells during processing.[42]

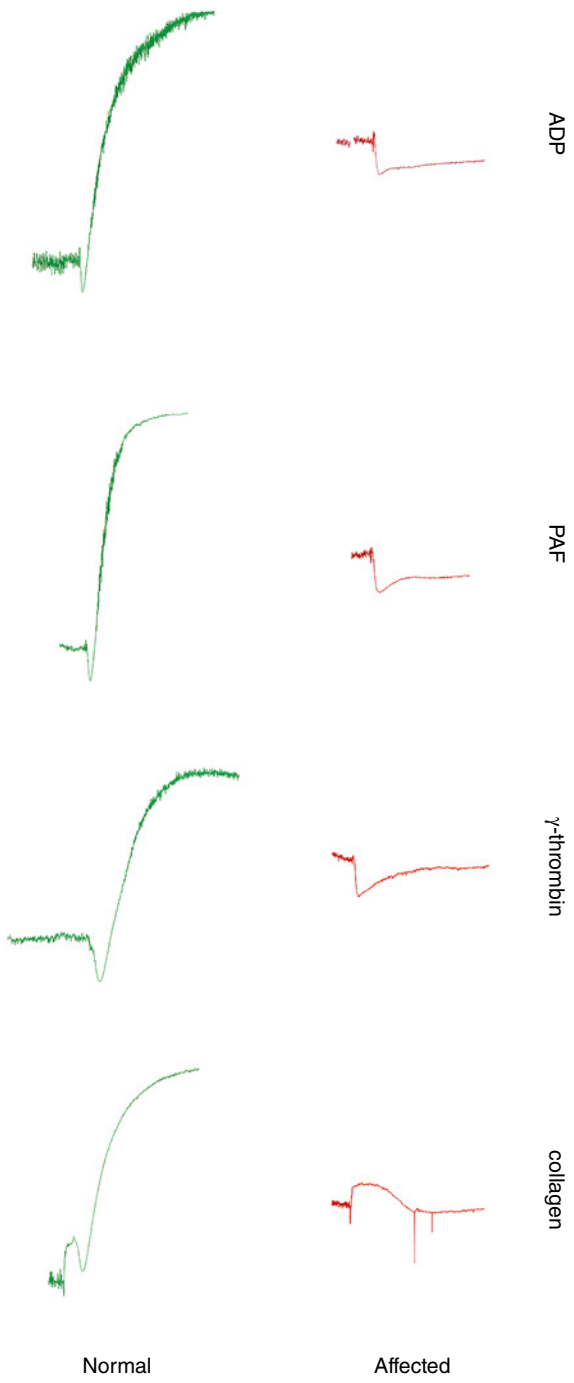


FIGURE 79.1 Normal platelet aggregation response to different agonists of a control dog (green; left-side tracings) versus a mixed breed dog with Glanzmann thrombasthenia (red; right-side tracings). Notice the universal lack of aggregation response to all agonists for the thrombasthenic dog. (Source: Haysom et al.[24])

Platelet Secretion Assays

Platelet dense granule release can be evaluated simultaneously with the aggregation response in a specialized aggregometer, called a lumiaggregometer. Lumiaggregation is based on the conversion of ADP, released from platelet dense granules, to ATP. This ATP then reacts with the firefly lantern extracts (luciferin and luciferase), generating adenyl-luciferin. Light is emitted when oxidation of adenyl-luciferin occurs. An ATP standard is used to calibrate the signal. Studies of platelet secretion allow assessment of signaling pathways culminating in both aggregation and degranulation. Secretion assays define the phenotype of storage pool disease and release defects, and allow diagnosis of platelet dysfunction in a subset of patients having clinical bleeding but normal aggregation tracings.[77]

Impedance/Whole Blood Platelet Aggregation

The impedance method can be used with either PRP or whole blood, although whole blood samples are used most widely. This method is based on the application of an alternating current across two electrodes and monitoring the change of resistance that is proportional to the size of the platelet thrombus that accumulates as platelets aggregate.[28] Compared with LTA, impedance methods use smaller blood volumes and require minimal preanalytic manipulation, thus preventing any loss of platelet subpopulations, or ex vivo platelet activation during centrifugation. Disadvantages include a relative insensitivity and inability to detect qualitative features of platelet activation, particularly shape change and the potential for inconsistent agonist response (P. W. Christopherson, personal observation).[32]

MULTIPLATE ANALYZER

The Multiplate system (Diapharma Group, Inc., West Chester, Ohio) is a newer generation impedance aggregometer that uses small volume (<500 µL) whole blood samples in single-use test cuvettes. The Multiplate measures platelet aggregation in hirudin- or heparin-anticoagulated blood exposed to various agonists.[33,48] The use of heparin is the preferred anticoagulant due to platelet autoaggregation induced by hirudin or citrate, and in one study, citrated samples had weak aggregation responses.[16,48]

Platelet Aggregation in Clinical Disorders

Clinical studies describe the use of aggregation in the evaluation of both inherited and acquired diseases. Glanzmann thrombasthenia, a quantitative defect in the glycoprotein complex IIb–IIIa (integrin $\alpha_{IIb}\beta_3$, fibrinogen receptor), has been identified in dogs and horses (see Chapter 84). The integrin $\alpha_{IIb}\beta_3$ is required for platelet aggregation, and the diagnostic features of Glanzmann thrombasthenia include absent aggregation to all physiologic agonists (Figure 79.1), and abnormal clot retraction.[6] Storage pool disease (SPD) is characterized by defective secretion of adenine nucleotides. Animals with dense-granule SPD may have impaired platelet aggregation response to ADP and collagen or normal platelet aggregation.[10] The SPD associated with Chediak–Higashi disease causes impaired platelet aggregation response to collagen and a virtually absent release of adenine nucleotides, measured by lumiaggregation.[27]

Platelet aggregation studies have been performed in animals with acquired platelet function defects secondary to uremia, immune-mediated thrombocytopenia, laminitis, infectious diseases, malignancies, heart disease, and drug-related disorders (see Chapter 85).[35,54,57,60,71] Multiplate has shown promise in the monitoring of aspirin and clopidogrel therapy in dogs[63] and has been used in the evaluation of platelet function in dogs with acute renal injury.[50]

PLATELET FUNCTION ANALYZER—PFA-100®, PFA-200®

Point-of-care instruments have been developed to replace bleeding time determinations as screening tests of primary hemostasis. The most widely used in veterinary medicine is the platelet function analyzer (PFA)-100® (Figure 79.2) and newer PFA-200® (Siemens, Munich, Germany). The test is designed to assess platelet adhesion and aggregation under conditions of high shear.

Test Principle

The PFA test is performed by pipetting citrated whole blood into a disposable cartridge. The blood sample is then drawn, under a constant vacuum, through a capillary tube and an aperture 150 µm in diameter. The membrane is coated with either collagen and epinephrine (CEPI) or collagen and ADP (CADP). The PFA-200 also offers a P2Y cartridge for assessment of P2Y12 receptor antagonism with certain drug therapies such as clopidogrel (Sysmex, Wymbush, Milton Keynes, UK). Activation of platelets and adhesion to the membrane are caused by the high shear rates (4000–5000 s^{-1}) generated in the PFA system and are mediated via a platelet surface glycoprotein-Ib-IX–V binding via von Willebrand factor (VWF) to collagen. Subsequent release of platelet granule contents and the presence of collagen and epinephrine or ADP on the membrane result in platelet aggregation. When the aperture is occluded by aggregated platelets, blood flow through the membrane stops. This is referred to as the closure time (CT).[36] Normal reference intervals for CT have been reported to be 53–98 seconds for CADP-CT and 92 to >300 seconds for CEPI-CT in dogs,[52] and 61–116 seconds for CADP-CT and 159 to >300 seconds for CEPI-CT in horses.[66] It is generally recommended that each laboratory establish its own normal range for CT, using samples drawn in a consistent concentration of sodium citrate for species controls and patients.



FIGURE 79.2 The PFA-100® instrument. (**Source:** Dade Behring)

Factors Affecting Closure Time

PFA CTs are sensitive to both platelet number and function. In dogs, there is an approximate linear relationship between CT and platelet count below 150 × 10^9/L.[53] However, no association has been found between platelet count and CT in Cavalier King Charles Spaniels with idiopathic inherited macrothrombocytopenia, likely due to normal total circulating platelet mass in these dogs.[72]

Closure time also increases with decreasing hematocrit in humans and dogs.[37,53] The exact threshold at which the CT is affected may vary between species. In dogs, a prolongation of the CT is seen at hematocrit values below 30–40%.[11,53] Plasma levels of VWF are an important determinant of CT because platelet aggregation at high shear rates depends on VWF concentration and function. In humans, it has been shown that plasma VWF levels modulate PFA CT to a greater extent than intrinsic platelet function.[23] An association between PFA-100 CT and VWF has been shown in healthy dogs,[70] and in dogs with inherited,[11,53] and acquired[71] von Willebrand disease.

The concentration of anticoagulant is an important determinant of CT in humans and dogs. Test times are longer in 0.129 mol/L (3.8%) citrate than in 0.109 mol/L (3.2%), probably due to greater calcium chelation. In dogs, the PFA-100 CT appears to be stable up to 8 hours after the blood sample is collected,[52] although testing within 2 hours is generally recommended. The manufacturer of the PFA-100 currently recommends testing within 4 hours for humans.

Detection of Platelet Function Defects

The PFA system is designed to assess human platelet function, thus species' differences in platelet response may complicate its use in animals. Very long CEPI-CTs and nonclosure (>300 seconds) are common for healthy, unmedicated dogs and horses.[11,53,66,72] It has been suggested that subjects with CEPI-CT > 300 seconds may

have an aspirin-like defect; however, normal plasma thromboxane B_2 levels were demonstrated in dogs with CEPI-CT > 300 seconds.[57] Unlike human platelet response, epinephrine does not consistently cause platelet aggregation in other species,[14] thus limiting clinical utility of CEPI determinations.

Hypofunction

The PFA is a sensitive test of von Willebrand disease in humans.[19] In two small case studies of dogs with von Willebrand's disease, all affected dogs had prolonged CADP-CT, suggesting a similar high sensitivity.[11,53] Callan and Giger[11] have reported a sensitivity of 95.7% and specificity of 100% for detecting primary hemostatic disorders (thrombocytopenia, thrombopathia, and type 1 von Willebrand disease) using the CADP cartridge; sensitivity was 95.7% and specificity 82.8% using the CEPI cartridge. The PFA has utility in monitoring responses to DDAVP in dogs with type 1 von Willebrand disease, where partial correction of CT typically is obtained. DDAVP administration has been found to significantly shorten the CADP-CT but not the CEPI-CT.[12]

Very few studies have documented the utility of the PFA system in detecting intrinsic platelet function disorders in animals. Callan and Giger[11] found that five out of six dogs with unspecified thrombopathia had prolonged CADP-CT, and a case report has described prolonged CADP-CT in a horse with Glanzmann thrombasthenia.[45] In people, the PFA CT is prolonged in cases of Glanzmann thrombasthenia (GPIIb–IIIa deficiency), Bernard–Soulier syndrome (GPIb-IX–V deficiency), and patients with δ-storage pool deficiency and primary secretion defects.[21,25]

Both the CADP-CT and CEPI-CT appear to be prolonged by acetylsalicylic acid in dogs and horses.[53,66] This finding contrasts with people. Aspirin's antiplatelet action in people is characterized by prolonged CEPI-CT, but normal (or near-normal) CADP-CT.[46] Dogs experimentally loaded with endotoxin had shortened CADP and CEPI-CT at 0.5 hours and prolonged CT 48 hours after inoculation. The initial shortened CT may reflect hemoconcentration more than actual platelet hyperfunction.[79] Dogs with heart diseases causing high-velocity blood flow (mitral valve regurgitation and subaortic stenosis) and an acquired type 2A von Willebrand disease have prolonged CADP and CEPI-CT (Figure 79.3). The mechanism has been suggested to be enhanced cleavage of VWF high-molecular-weight multimers by a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13), a plasma metalloprotease acting on VWF preferentially under conditions of high fluid shear stress.[70,71] A similar acquired defect with prolonged CT is described in humans with aortic stenosis.[73]

Hyperfunction

It is unclear whether shortening of the PFA CT truly reflects platelet hyperfunction/increased reactivity because there is lack of data that links shortened CT to clinical evidence of thrombosis or thrombosis-related outcome. Shorter CTs have been associated with S wave to T wave (ST) segment elevation myocardial infarcts in human patients, but data are lacking in veterinary medicine. Greyhounds have been found to have shorter CT compared to other dogs; however, this could be due to higher hematocrit found in this breed.[15] The PFA, in comparison to the Multiplate Analyzer and Plateletworks system, has shown to be useful in monitoring aspirin and clopidogrel therapy in dogs.[63]

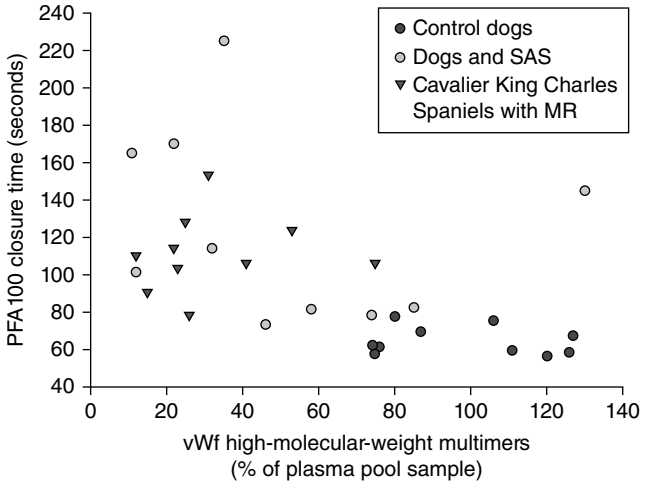


FIGURE 79.3 Platelet function analyzer closure times (Y axis) shown as a function of percentage high-molecular-weight von Willebrand factor multimers (X axis; sample relative to pool ratio). SAS, subaortic stenosis, MR, mitral regurgitation. (Source: Tarnow et al.[71])

FLOW CYTOMETRY

Flow cytometry is a versatile tool for study of platelet function. Flow cytometry may be used to: (1) measure the activation state of circulating platelets and their reactivity (by activation-dependent changes in the platelet surface, leukocyte–platelet aggregation, and procoagulant changes on the platelet surface), (2) diagnose platelet function defects (see Chapters 84 and 85), (3) monitor thrombopoiesis by quantifying reticulated platelets, (4) measure platelet count, (5) assess antiplatelet drug effects, and (6) detect platelet-bound antibodies. This section discusses the use of flow cytometry to characterize platelet activation status.

Flow cytometry measures specific characteristics of cells and cell fragments as they flow through a flow chamber and past the focused beam of a laser. The cells are usually labeled with a fluorescent compound or fluorescent-conjugated antibody, or can be analyzed unlabeled. When labeled cells pass through the laser, the laser light activates the fluorophore at the excitation wavelength, and the emitted fluorescence and the light scattering properties of each cell are detected. The intensity of the emitted light is directly proportional to the amount of bound fluorescent probe (see Chapter 143).

Flow cytometric assays evaluating platelet function can utilize washed platelets, PRP, or whole blood. Whole blood samples are typically labeled with a minimum of two fluorescent probes: a platelet-specific marker, to differentiate platelets from other cells in the blood, together with a "test" marker recognizing the activation parameter to be measured. Physiologic or nonphysiologic agonists can be used to assess in vitro platelet reactivity. Samples can be stabilized by fixation before or after addition of antibodies. Relevant isotype-matched antibodies or unstimulated cells may be used as labeling or experimental negative controls.[51]

The application of flow cytometry for evaluating platelet function in veterinary medicine is limited compared with human or murine studies by the relative lack of commercially available species-specific or cross-reactive monoclonal antibodies. Some platelet membrane proteins (e.g., integrin $\alpha_{IIb}\beta_3$) appear to be widely conserved across species, and many antihuman antibodies directed against this receptor are cross-reactive.[45,67,69]

Cytometric Markers of Platelet Activation

Flow cytometric assays gauge activation status of circulating platelets and platelet reactivity after in vitro agonist stimulation by detecting changes in surface expression of a receptor or other membrane component, or presence of a bound ligand. Detection of leukocyte–platelet aggregates and procoagulant platelet-derived microparticles has also been investigated as markers of platelet activation in veterinary medicine (Table 79.3).[51]

Markers of platelet activation include activation-dependent conformational changes in integrin $\alpha_{IIb}\beta_3$, exposure of granule membrane proteins, such as P-selectin, platelet surface binding of secreted platelet or plasma proteins (e.g., fibrinogen), and development of a procoagulant surface (assessed by Annexin V or lactadherin binding to phosphatidylserine).

Platelet P-selectin (CD62P) expression, denoting degranulation, is among the most common markers of platelet activation used in human medicine, and methods to detect this marker have been described for dogs, horses, and pigs.[38,50,55,67,69,78] Increased platelet surface P-selectin expression has been demonstrated in dogs with inflammatory disease,[56] and with immune-mediated hemolytic anemia.[75]

Assessment of fibrinogen binding has been described in dogs using a monoclonal antibody that recognizes a receptor-induced binding site (RIBS) on canine fibrinogen.[7,69] Moreover, exogenous fluorescein isothiocyanate (FITC)-labeled fibrinogen binding as a marker of platelet activation has been described in dogs and horses.[69,74]

Binding of an antifibrinogen antibody to platelet membrane surface fibrinogen as a marker of platelet activation has been described in horses and pigs.[34,39,59]

Detection of fluorescent-tagged Annexin V bound to outer platelet membrane phosphatidylserine has been described as a marker of procoagulant platelets in dogs and horses.[34,78] Annexin V detection is independent of species because it is a placental protein and not an antibody directed against a specific epitope. "Coated" platelet refers to a subpopulation of highly procoagulant cells generated by dual agonist stimulation with collagen and thrombin. These platelets are defined by the cytometric characteristics of phosphatidylserine exposure, bound α-granule proteins, membrane permeability to calcein, and shedding of membrane-derived microparticles. Coated platelet formation has been described in healthy dogs, and lack of this subpopulation is a feature of a hereditary canine platelet defect (Scott syndrome).[9]

Leukocyte–Platelet Aggregates

Leukocyte–platelet aggregates are formed via interaction between P-selectin exposed on the surface of activated platelets and the P-selectin glycoprotein ligand 1 counter receptor on the leukocyte surface.[51] Detection of leukocyte–platelet aggregates has been described in dogs (Figure 79.4) and horses,[67,69,78] and increased neutrophil–platelet aggregates has been associated with laminitis in ponies.[76]

Automated Measurements

Mean platelet component concentration (MPC) has been investigated as an automated measurement of platelet activity. The MPC estimates the refractive index of platelets. When activated platelets degranulate, the density

TABLE 79.3 Flow cytometric parameters of platelet activation.

Activation-dependent platelet changes	Method	Reference
Surface P-selectin exposure	Binding of monoclonal antibody AC1.2, 1E3, MD6 (dogs)	55, 69, 78
	Psel.KO.2.5, SwPsel.1.9 (pigs) CLBThromb/6, Psel.KO.2.7 (horse)	49, 67, 74
Endogenous fibrinogen binding	Binding of monoclonal antibody recognizing receptor-induced binding site on bound fibrinogen	7, 69
	Binding of an antifibrinogen antibody (polyclonal)	34, 39, 59
Exogenous fibrinogen binding	Binding of fluorophore-labeled species-specific fibrinogen	69
Development of a procoagulant surface (phosphatidylserine exposure)	Binding of Annexin V	34, 78
Exposure of lysosomal integral protein	Binding of monoclonal antibody CLBGran/12	78
Formation of leukocyte–platelet aggregates	Leukocyte–platelet aggregates	78
	Neutrophil–platelet aggregates	69, 76
	Monocyte–platelet aggregates	69

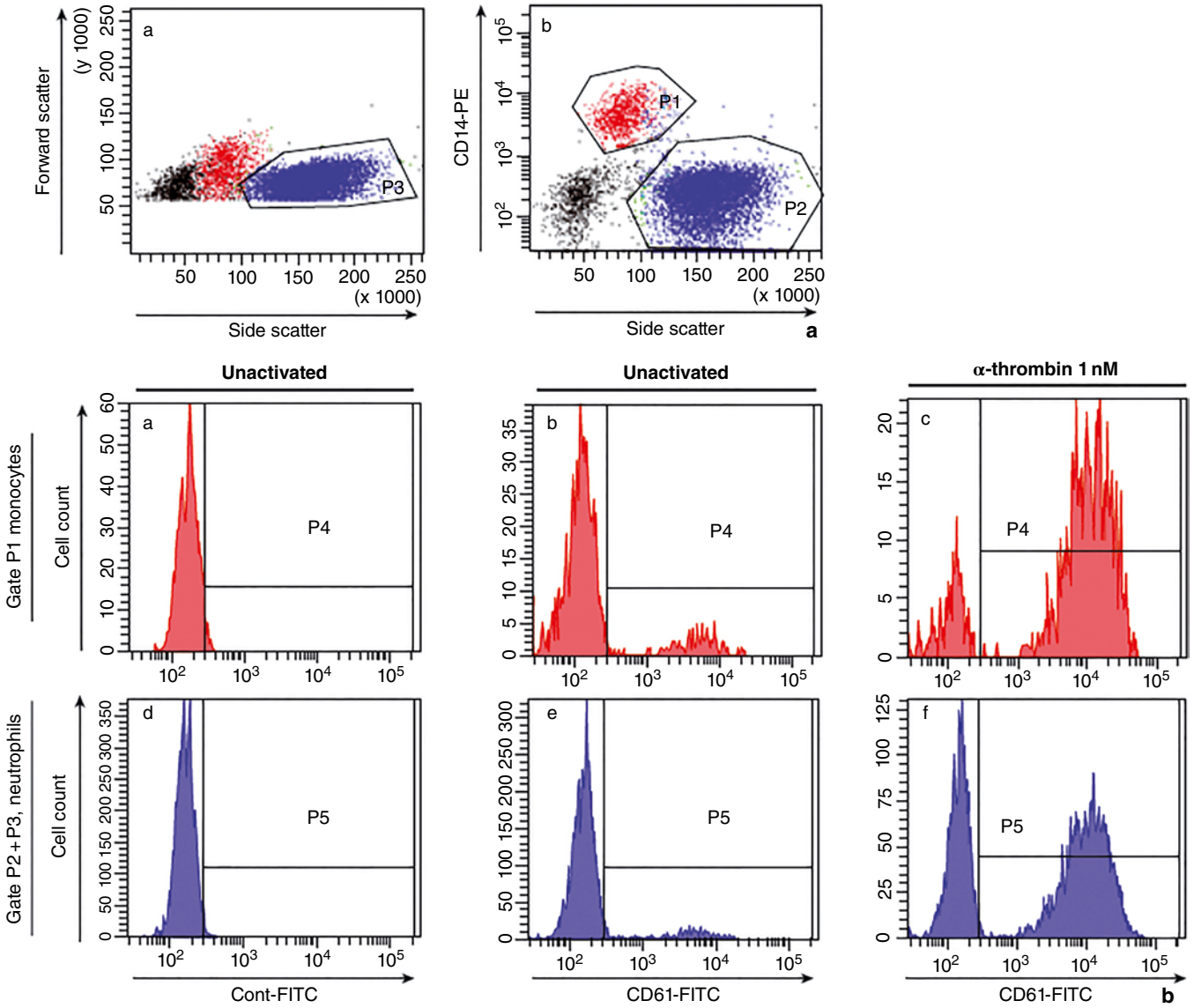


FIGURE 79.4 Flow cytometry gating logic for the detection of leukocyte–platelet aggregates in canine whole blood. (a) Monocytes and neutrophils were gated by a combination of light scattering and CD14-PE fluorescence. Bright CD14 and 90° (side) light scatter define monocytes (P1); dim CD14, forward, and 90° light scatter define neutrophils (P2 and P3); (b) Histograms are used to quantify leukocyte–platelet aggregates by percent CD61-FITC positive cells and CD61-FITC mean fluorescent intensity from P1 cells, monocytes (b-a, b-b, b-c) and P2 and P3 cells, neutrophils (b-d, b-e, b-f). Gates P4 and P5 are adjusted such that they include 1% of the isotype-control-stained (Cont-FITC) population (b-a, b-d). Unactivated samples had 9.6% monocyte–platelet aggregates (b-b), and 7.5% neutrophil–platelet aggregates (b-e). Activation with 1 nM α-thrombin gave 84.2% (b-c) and 59.7% neutrophil–platelet aggregates (b-f). (**Source:** Tarnow et al. [69])

decreases and the MPC decreases.[44] Decreased MPC has been associated with inflammatory disease in dogs, with a significant inverse correlation between platelet surface P-selectin expression and MPC.[56] Recent studies have suggested that MPC may be useful in prognosis prediction in dogs with immune mediated hemolytic anemia (IMHA).[81]

ADDITIONAL ASSAYS

Thrombelastography (TEG) is a viscoelastic point-of-care method that monitors the kinetics of thrombus formation and lysis in whole blood samples. Platelet mapping (TEG-PM, Haemonetics Corporation) is a modified assay designed to more specifically assess platelet function, particularly in patients receiving platelet inhibitory drugs. The assay is configured with different coagulation or platelet-activating agents designed to individually evaluate the contribution of coagulation factors, fibrinogen, and platelets to overall thrombus strength. Preliminary evaluations of the platelet mapping assay in dogs, cats, and horses have revealed some species' differences in agonist response versus human response, which, combined with the assay's high cost, may influence its clinical utility in animals.[2,3,74]

PlateletWorks (Helena Laboratories, Beaumont, TX) is a simple assay designed to evaluate platelet

activation response based on differential platelet counts in blood collection tubes containing platelet agonists versus an EDTA, baseline control tube. In pilot studies evaluating PlateletWorks in cats treated with clopidogrel, the assay successfully detected the drug's antiplatelet effects based on impaired collagen and ADP response post-treatment.[17,26]

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CHAPTER 80

Immune Thrombocytopenia

DANA N. LeVINE and MARJORY B. BROOKS

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Alloimmune Thrombocytopenias
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Treatment of Primary ITP
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Primary ITP Prognosis
Summary

Acronyms and Abbreviations

APC, antigen-presenting cell; ASH, American Society of Hematology; BUN, blood urea nitrogen; CV, coefficient of variation; FNAIT, fetal/neonatal alloimmune thrombocytopenia; GP, glycoprotein; HPA-1a, human platelet antigen-1a; IPF, immature platelet fraction; ITP, immune thrombocytopenia; IVIg, intravenous immunoglobulin; IWG, International Working Group; Mpl, myeloproliferative leukemia protein; MPS, mononuclear phagocytic system; MPV, mean platelet volume; PAIg, platelet-associated immunoglobulins; Tc, cytotoxic T cell; Th, T helper cell; Treg, regulatory T cell; TPO, thrombopoietin.

INTRODUCTION

Immune thrombocytopenia (ITP) is a common bleeding disorder in which autoimmune destruction of platelets and megakaryocytes results in severe thrombocytopenia. Although most commonly described in humans and dogs, ITP also affects cats and horses. Understanding of ITP pathogenesis is rapidly evolving. Traditionally considered to be an antibody-mediated disease, we now recognize ITP as a complex and heterogeneous syndrome that results from a combination of humoral and cell-mediated attack of platelets peripherally and of megakaryocytes in the bone marrow. This chapter focuses on ITP in dogs, the veterinary species in which it is most prevalent, and notes relevant differences in other species. Information from human ITP is referenced for knowledge gaps in veterinary medicine.

ITP NOMENCLATURE

In the medical literature, ITP historically stood for **i**diopathic **t**hrombocytopenic **p**urpura. However, this terminology was deemed inappropriate by the International Working Group (IWG) of human ITP experts, since bleeding signs are absent or minimal in most people with ITP.[93] The disease was retermed "immune thrombocytopenia" to emphasize its immune-mediated mechanism, but the same acronym was maintained (**i**mmune **t**hrombocyto**p**enia).[93] The veterinary literature includes

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the terminology "**i**mmune-**m**ediated **t**hrombocytopenia" and the abbreviation IMT. However, the IWG recommendations are applicable for the animal disease counterpart, thus use of the acronym ITP in veterinary medicine provides consistency for comparative studies.

PRIMARY ITP PATHOGENESIS

ITP is an autoimmune disease characterized by both platelet destruction and impaired megakaryocyte and platelet production.[20] The pathogenesis of the immune dysregulation resulting in ITP is incompletely understood, and likely complex. Very little is known about ITP pathogenesis in companion animals, thus the following summarizes human disease pathogenesis. Since Harrington's seminal 1951 experiment in which transfusion of ITP plasma to healthy people induced thrombocytopenia, ITP has been known as a humoral disease.[41,63] Autoantibodies targeting platelet surface glycoproteins (GPs) lead to platelet clearance by the mononuclear phagocytic system (MPS), primarily of the spleen and, to a lesser extent, the liver.[20] However, it is now recognized that T cells also play a central role in platelet destruction in ITP.[20,81,100,117] A proinflammatory T helper cell (Th)1, Th17, and Th22 cytokine milieu predominates in many ITP patients that together promote macrophage activation, autoreactive B cell development, and T cell cytotoxicity.[20,63] Furthermore, T and B regulatory cells that normally serve to maintain self-tolerance are dysfunctional in ITP, allowing the autoimmune response to persist.[20,49,65] Several studies have found a reduction in regulatory T cell (Treg) number and function in people with ITP, and one pilot study found a decrease in Treg number in ITP dogs compared to healthy controls.[7,103,110] Interestingly, Treg numbers recover when human and canine ITP patients are in remission even if patients were treated only with thrombopoietin (TPO) mimetics. This finding suggests an immunomodulatory role for platelets, perhaps by providing a transforming growth factor-β reservoir.[2,7,63,99]

Although antibody-mediated platelet destruction is often central in ITP pathogenesis, some human patients lack detectable platelet autoantibodies. These patients have cytotoxic T lymphocytes that induce platelet destruction via apoptosis and perforin/granzyme-mediated cytotoxicity.[20,79,117,118] In some patients, antibodies and T cells attack megakaryocytes, resulting in decreased platelet production further compounding platelet destruction (Figure 80.1).[48,118] Megakaryocytes of dogs with ITP display signs of injury including foaminess, vacuolation, and reduction of cytoplasmic granularity.[51] Complement may also contribute to the immune-mediated destruction of both platelets and megakaryocytes.[84]

In addition to immune targeting of megakaryocytes and platelets, TPO levels are often inappropriately normal in human patients with ITP. TPO is the major regulator of platelet production that is necessary for survival, proliferation, and differentiation of megakaryocytes to platelets.[1,17,40] Until recently, TPO was thought to be produced constitutively by the liver and cleared through binding to its receptor, the myeloproliferative leukemia protein (Mpl) receptor, on platelets and megakaryocytes.[40] Thus, TPO concentration should be inversely proportional to the platelet or "Mpl" mass.[40] Although the Mpl mass plays a role in TPO regulation, a more elegant system may explain the normal levels of circulating TPO documented in human ITP patients. As platelets age, they are desialylated and subsequently recognized and cleared by the hepatic Ashwell–Morell receptor.[40] This removal, in turn, drives hepatic TPO expression providing a feedback mechanism: as more platelets are cleared, more TPO is produced (see Chapter 75, Figure 75.1). However, in a murine model of ITP, platelets opsonized with antiplatelet antibody are cleared by the splenic and hepatic MPS, thus shunting platelets away from the Ashwell–Morell receptor and failing to trigger hepatic TPO production.[40] Circulating TPO levels in people with ITP are inadequate or unable to effectively stimulate platelet production.[1] TPO levels in dogs with ITP have not been established, as there are no available assays for circulating canine TPO.

A genetic susceptibility to autoimmunity is recognized in human medicine; however, familial ITP is very rare, complicating discovery of susceptibility loci in human ITP.[8] A breed predisposition to develop ITP is consistently reported in the veterinary literature, with an overrepresentation of Cocker Spaniels and Old English Sheepdogs.[60,77,90,113] Given this breed predisposition, it is very likely that genetic factors contribute to ITP pathogenesis. Presumably, ITP results from the combination of a genetic predisposition with an environmental or infectious trigger, which often remains unidentified.[20] Indeed, the trigger for initial loss of self-tolerance in ITP is not understood.

SECONDARY ITP

Antiplatelet antibody formation in patients with secondary ITP is associated with an identifiable antigenic stimulus, often in the form of a drug, infectious agent, or neoplastic disorder (Table 80.1). Although the presence of increased platelet-associated antibodies or immunoglobulins (PAIg) in patients with thrombocytopenia and an underlying disease or drug exposure is compatible with secondary ITP, it does not prove that the secondary disease definitively triggered ITP.

Any medication can cause secondary ITP, but penicillins, sulfonamides, and cephalosporin antibiotics are the most frequent culprits. Drug-induced thrombocytopenia usually involves the association of a drug-dependent antibody with a platelet membrane protein and the drug.[4] Drug-induced thrombocytopenia tends to occur 5–10 days following first-time drug exposure, but can occur more rapidly, especially with repeat drug exposure.[4]

Many infectious diseases cause thrombocytopenia. Infection-associated thrombocytopenia is often multifactorial in origin including vasculitis, bone marrow

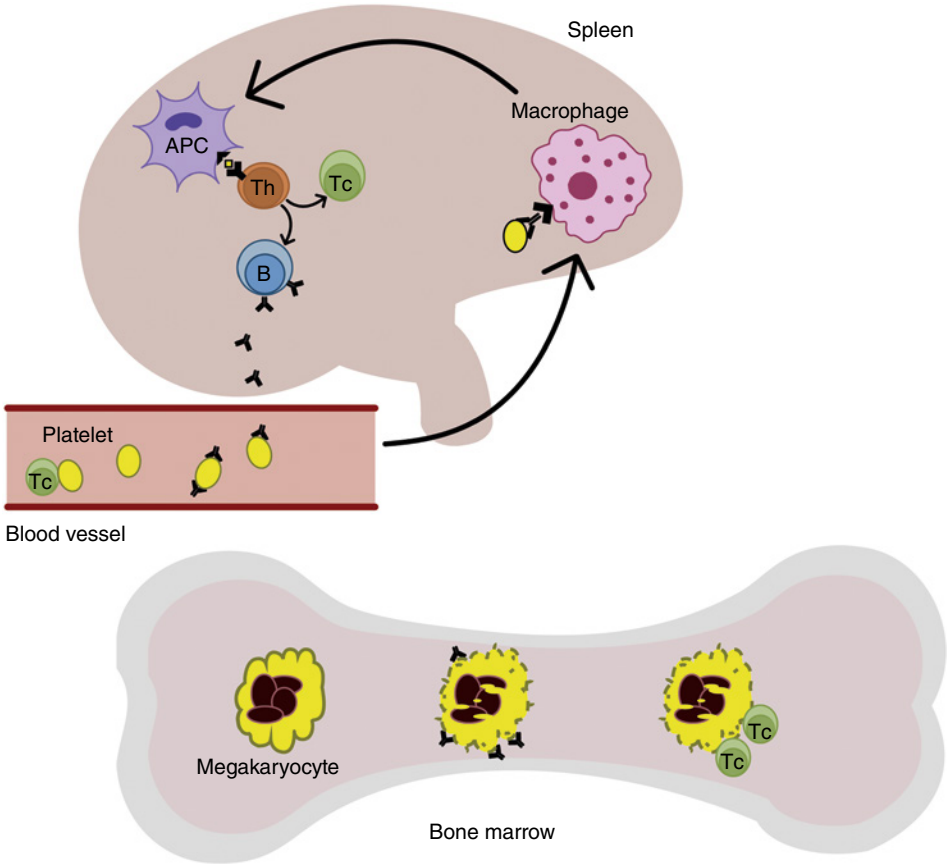


FIGURE 80.1 Mechanisms of platelet and megakaryocyte destruction in immune thrombocytopenia (ITP). ITP is a heterogeneous disease with a complex pathogenesis sustained by a positive-feedback loop. After loss of self-tolerance, T helper cells (Th) promote generation of autoantibody-producing B cells and/or autoreactive cytotoxic T cells (Tc). Autoantibodies can opsonize platelets, leading to their splenic and hepatic clearance by macrophages of the mononuclear phagocyte system. Macrophages can then serve as one type of antigen-presenting cell (APC), propagating the cycle. Dendritic cells can also serve as APCs. Tc can destroy platelets in the blood or spleen. Autoantibodies and autoreactive Tc may target megakaryocytes in the bone marrow, inhibiting platelet production. Thus, ITP is a disorder of both platelet destruction and decreased platelet production. (**Source:** Courtesy of Andrew Kingsbury, Center for Food Security and Public Health, Iowa State University)

suppression, and splenic sequestration. However, antiplatelet antibodies and subsequent platelet destruction likely play an important role, and PAIg are often reported in thrombocytopenic dogs and cats with many infectious agents, but especially vector-borne diseases.

Neoplasia-associated thrombocytopenia occurs through several mechanisms, including consumption from bleeding tumors, decreased production due to myelophthisis, and secondary ITP.[14] Though most studies report small numbers of patients, PAIg have been documented in patients with neoplasia. PAIg have also been documented in a few thrombocytopenic patients with non-neoplastic inflammatory diseases; however, the pathogenic role of the antibodies in these diseases is unknown.

In children, the measles, mumps, and rubella vaccine is linked to development of ITP and there is anecdotal evidence that vaccination may trigger ITP in dogs.[75] However, a retrospective, case-control study was unable to document an association between recent vaccination in dogs and development of ITP.[45]

ALLOIMMUNE THROMBOCYTOPENIAS

Alloimmune thrombocytopenia, a unique subtype of secondary ITP, results from exposure to foreign platelet antigens (alloantigens) and production of cognate antibodies (alloantibodies). Alloantibodies then mediate platelet opsonization and subsequent platelet destruction. Pregnancy and blood transfusion are the most common triggers of alloantibody production in people, resulting in fetal/neonatal alloimmune thrombocytopenia (FNAIT) or post-transfusion purpura.

FNAIT is a rare but potentially severe bleeding disorder that is caused by maternal IgG antibodies directed against a fetal/neonatal platelet antigen inherited from the father.[5] Passage of maternal antibodies occurs transplacentally or via colostrum. FNAIT is associated with a high risk of intracranial hemorrhage that can occur both antenatally or postnatally.[5] In veterinary medicine, NAIT has been described in pigs and horses, and experimentally in mules.[13,28,91]

TABLE 80.1 Reported causes of immune thrombocytopenia associated with antiplatelet antibodies.

Primary immune	Dogs, cats, and horses[3,55,70]
Secondary immune	
Infections	**Dogs**
	Ehrlichiosis,[29,62] granulocytic anaplasmosis,[18] Rocky Mountain spotted fever,[39] leishmaniasis,[107] *Babesia gibsoni*,[112] dirofilariasis,[62] anaplasmosis,[29] leptospirosis,[29,90] borreliosis,[90] prostatitis,[90] pyometra/endometritis[90]
	Cats
	Feline infectious peritonitis,[55] feline leukemia virus,[55] feline immunodeficiency virus,[55] pyelonephritis[55]
	Horses
	Equine infectious anemia[19,23]
Neoplasia	**Dogs**
	Lymphoma,[29,90] leukemia,[90] histiocytic sarcoma,[29] hemangiosarcoma,[90] mammary gland tumor[90]
	Cats
	Lymphoma,[55] leukemia[55]
	Horses
	Lymphoma[69]
Drugs	**Dogs**
	Sulfonamides,[56,62] cefazedone,[11] carprofen[72]
	Cats
	Propylthiouracil[86] (anti-RBC and antinuclear antibodies)
	Horses
	Penicillin,[70] trimethoprim–sulfadoxine[70]
Inflammatory	**Dogs**
	Hepatitis,[29] pancreatitis,[29] systemic inflammatory response syndrome[29]
	Cats
	Hepatitis,[55] fat necrosis[55]
Alloimmune	Neonatal alloimmune thrombocytopenia (horses, pigs),[13,28] post-transfusion purpura[62]

The antigens capable of triggering NAIT reside on platelet membrane glycoproteins and are well-characterized in humans. Fetal-maternal incompatibility of human platelet antigen-1a (HPA-1a) is the most common cause of NAIT in Caucasians.[85] The antigens responsible for FNAIT have not been characterized in veterinary medicine. Ideally, diagnosis of FNAIT is confirmed by identifying neonatal, but not maternal PAIg, and confirming maternal antibody reactivity with paternal and neonatal, but not maternal platelets. Therapy of FNAIT is recommended for severe thrombocytopenia, defined as <30,000 platelets/μL due to high risk of intracranial hemorrhage. Treatment options in human medicine include transient glucocorticoid therapy, intravenous immunoglobulin (IVIg) infusion (benefit unclear in a human systematic review), and platelet transfusions, ideally with maternal washed or type-compatible platelets.[5,85] As FNAIT is more severe with subsequent offspring, treatment algorithms based on risk stratification are used to guide human therapy, and avoidance of repeat breeding of incompatible dams and sires is recommended for veterinary patients.[85]

Post-transfusion purpura is a rare reaction defined by severe thrombocytopenia that develops 5–12 days following a platelet or red blood cell transfusion.[27] The patient must have been previously sensitized to the platelet alloantigen by pregnancy or transfusion, and re-exposure to the platelet antigen causes an anamnestic immune response.[27] In humans, post-transfusion purpura usually affects HPA-1-negative individuals.[27] The patient's own alloantigen-negative platelets, via an unknown mechanism, are also targeted leading to severe thrombocytopenia.[27] Recommended treatments in human medicine include steroids, IVIg, and plasma exchange. Prevention of recurrence includes use of washed red blood cell units or transfusion of platelet and red blood cell units from platelet antigen compatible donors.[27]

CLINICAL FEATURES OF PRIMARY ITP

Signalment

Primary ITP is most commonly described in dogs. Although two large retrospective studies of thrombocytopenic dogs found ITP in only 5% of the cases,[12,38] ITP is the most common cause of *severe*

thrombocytopenia in dogs. Dogs of any age, gender, or breed can develop primary ITP; however, affected dogs are more commonly young to middle age with reported median age of presentation ranging from 4 to 8 years.[12,60,77,90] In many studies, females outnumber male dogs.[12,60,90] Predisposed breeds include Cocker Spaniels, Toy Poodles, Miniature Poodles, Standard Poodles, Old English Sheepdogs, and in some reports, German Shepherds.[38,60,62,90,113] Primary ITP is rarely reported in cats.[9,32,50,55,106,114] Indeed, in a large retrospective study, thrombocytopenia was only reported in 1.2% of cats, and only one cat was diagnosed with ITP.[50] ITP in cats is often secondary to an underlying disease (Table 80.1). Similarly, primary ITP is rare in horses, and equine ITP is usually secondary to infections, drugs, or neoplasia, or is mediated by alloantibodies.

Clinical Presentation

Signs of ITP vary widely, including presentation for surface bleeding (i.e., hemorrhage involving skin and mucosal surfaces), or clinically inapparent with thrombocytopenia identified incidentally on blood count results. Overt hemorrhage is usually spontaneous, most often from cutaneous sites or oral mucosal petechiation, followed by gastrointestinal bleeding (Figure 80.2).[68,113] Abnormal bleeding can also occur at estrus, surgery, or venipuncture sites. Although spontaneous bleeding typically occurs at platelet counts below 30,000 platelets/μL, many ITP patients have no clinical signs of thrombocytopenia and platelet count is not a reliable predictor of bleeding risk for individual patients.[68] The cause of variable bleeding severity among patients with similar platelet counts is poorly understood and may represent combined and interacting influence of platelet activation by autoantibodies,[83] interference of antibodies with platelet function,[25,78] alterations in endothelial integrity secondary to thrombocytopenia,[36,58] and presence of circulating procoagulant microparticles.[52,105]

Cats may tolerate thrombocytopenia and demonstrate less-spontaneous bleeding than dogs.[50] This may be because feline platelets can be more readily activated and contain more serotonin, a platelet agonist, in their dense granules than dogs or humans.[55,73] Similarly, horses often lack overt hemorrhage despite severe thrombocytopenia.

Human primary ITP patients frequently complain of fatigue that is not correlated with bleeding or anemia,[42] whereas histories of veterinary patients are usually restricted to abnormal hemorrhage or weakness caused by blood loss anemia.

Physical examinations of ITP patients may be normal, but typical findings include petechiae and ecchymoses, oral bleeding, epistaxis, gastrointestinal hemorrhage (melena, hematochezia, and hematemesis), and/or hematuria.[68,77] Petechiae and ecchymoses are particularly common on the oral mucosa, abdomen, and pinnae. A thorough dilated fundus exam should be performed in all ITP patients, since ocular bleeding often occurs; ranging from mild scleral hemorrhage and pinpoint retinal bleeds to hyphema and severe retinal hemorrhage.[102] Findings related to anemia such as pallor, tachycardia, and systolic murmur may be present. Severe hemorrhage leading to anemia is most commonly associated with gastrointestinal bleeding, though it can result from epistaxis and hematuria.[77,113] There are some reports of fever in dogs with primary ITP, but fever may be due to undiagnosed secondary etiologies.[77,90] Splenomegaly may be present due to extramedullary hematopoiesis.[90] Although rare, intracranial and spinal cord hemorrhage can occur with resultant signs of altered mentation, or localized neurologic deficits referable to the site of hemorrhage.

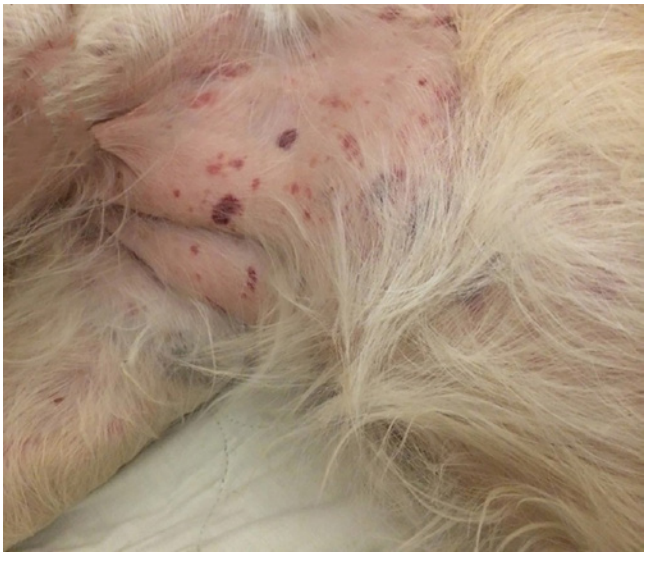


FIGURE 80.2 Abdominal petechiae and ecchymoses in a 9-year-old female spayed Labradoodle with primary ITP. (**Source:** Photograph courtesy of Dr. Kelly Makielski)

DIAGNOSIS OF PRIMARY ITP

In human medicine, primary ITP is defined as a platelet count of under 100,000/μL in the absence of other causes or disorders that may be associated with thrombocytopenia.[75] Similarly, in veterinary medicine, primary ITP is a diagnosis of exclusion. Other causes of thrombocytopenia including nonimmune platelet consumption, decreased production of platelets, and diseases associated with secondary ITP must be investigated (Chapter 81, Table 80.1).

The first step in an ITP workup is to confirm thrombocytopenia by performing a manual platelet count based on examination of a stained blood smear and ruling out platelet clumping. This is especially important in cats that often have pseudothrombocytopenia due to feline platelet reactivity and difficulty for some hematology analyzers to differentiate their similarly sized red blood cells and platelets.[116] EDTA and heparin can both result in platelet clumping and subsequent pseudothrombocytopenia in horses.[43,53] If

suspected, pseudothrombocytopenia should be ruled out by repeating a platelet count on a new citrate sample or, in cats, collecting blood directly into heparinized capillary tubes for fresh blood smear preparation accompanied by a manual platelet count.

Congenital macrothrombocytopenia should be suspected in dogs with chronic thrombocytopenia in the absence of bleeding signs (see Chapter 84). Hereditary macrothrombocytopenia due to β1 tubulin gene mutations has been identified in the Cavalier King Charles Spaniel, Norfolk and Cairn Terriers, and many other breeds.[33] Similarly, many healthy Greyhounds have platelet counts below typical canine reference intervals.[15]

In a recent study of 61 thrombocytopenic dogs, 57% were determined to have primary ITP, 28% secondary ITP due to lymphoid/myeloid neoplasia (9.8%), infectious disease (9.8%), liver disease (5%), or drug exposure (3%), and 15% had nonimmune thrombocytopenia attributed to bone marrow aplasia or consumptive coagulopathy.[59,68] Consumptive thrombocytopenia is typically ruled out with hemostasis tests to identify disseminated intravascular coagulation, or other thrombotic disorders. Investigations for secondary causes of ITP include review of medication and travel history, blood chemistry panel and urinalysis, imaging, and screening for relevant pathogens. Serologic testing and direct pathogen detection, including PCR tests, may be required for diagnosis of vector-borne disease, as one study documented that combining these methodologies increased sensitivity by up to 58%.[66]

While there is no gold-standard diagnostic test for ITP, platelet parameters derived from automatic blood cell counts and ancillary tests of platelet production or destruction have been used to support a diagnosis of ITP in veterinary and human medicine.

Platelet Count and Size

In several studies, platelet count was significantly lower in dogs with primary ITP than dogs with other causes of thrombocytopenia.[29,38,68] Even when comparing primary versus secondary ITP, thrombocytopenia was more severe in dogs with primary disease.[29,68] Although platelet counts overlap, severe (<20,000 platelets/μL) thrombocytopenia is suggestive of primary ITP.

High mean platelet volume (MPV) is generally a feature of young and more active platelets; however, appropriate validation studies have not yet demonstrated diagnostic utility of platelet size indices to differentiate causes of thrombocytopenia in people.[57] Dogs with ITP were found to have significantly higher MPV compared with healthy controls in one clinical study;[96] however, no differences were found in a second study using a different hematology analyzer.[82] Yet another study found MPV to be lower in dogs with primary ITP compared with dogs with nonimmune thrombocytopenia.[29] Low MPV may reflect relative increases in microparticles included in automated platelet counts, or impaired thrombopoiesis due to megakaryocyte-targeted destruction. Together, these studies indicate that increased MPV may be a feature of ITP in some cases, but the finding of normal to low MPV does not exclude the diagnosis of ITP.

Determination of MPV, however, may help differentiate ITP from hereditary macrothrombocytopenias. One human study found that an MPV of >12.4 fL differentiated hereditary macrothrombocytopenia from ITP with reasonable sensitivity (83%) and specificity (89%).[76] While discriminatory value of MPV has not been explored in dogs, determination of plateletcrit as a measure of total platelet mass has been described as a useful test to differentiate clinically relevant thrombocytopenia from hereditary macrothrombocytopenia in Cavalier King Charles Spaniels.[109]

Reticulated Platelets

Platelets newly released from megakaryocytes contain small amounts of RNA, and are referred to as reticulated platelets (see Chapters 75 and 78). Analogous to reticulocytes denoting erythropoiesis, circulating reticulated platelets are markers of megakaryopoiesis. Reticulated platelets were first characterized using flow cytometric techniques to detect thiazole orange staining of intraplatelet RNA. Although methodologically difficult to standardize, in humans, reticulated platelet assays have shown diagnostic utility for differentiating hypoproliferative thrombocytopenia from platelet consumptive and destructive disorders.[44] More recently, advanced, fully automated hematology analyzers (Sysmex XE and XN series, Sysmex Corporation; Abbott CELL-DYN Sapphire, Abbott Diagnostics) measure reticulated platelets in parameters referred to as immature platelet fraction (IPF) or percent reticulated platelets, respectively. These automated assays can be standardized and are better suited for routine and high-throughput testing. Some human studies of thrombocytopenic patients have shown increased IPF fractions in ITP patients; however, these studies have also revealed differences between analyzers that can limit usefulness of the assay.[24] A recent, well-designed prospective study assessing immature platelet indices on both the Sysmex XE and Abbott CD Sapphire found that while the Sysmex (not the Sapphire) demonstrated a significant increase in immature platelets in human ITP patients compared to those with bone marrow failure, both machines demonstrated broad overlap between the patient groups.[24] The authors speculated that this overlap is due to a shift toward immature platelets that occurs in any thrombocytopenic state regardless of etiology, analogous to the anemia "reticulocyte shift," and to decreased platelet production that occurs in many ITP patients.[24,71]

A canine study compared a flow cytometric, reticulated platelet assay with the Sysmex automated IPF assay in healthy and thrombocytopenic dogs.[82] The two methodologies demonstrated fair correlation and expected increases in immature platelet fractions in dogs with hyperproliferative thrombocytopenia; however, short (<18 hour) sample stability was noted for both methods along with high coefficients of

variation (CV). Another prospective study of dogs with thrombocytopenia determined that reticulated platelet percentages >8% (as determined by flow cytometry) combined with positive direct or indirect PAIg distinguished primary ITP from other causes of thrombocytopenia with high sensitivity but low specificity.[3] Overall, the utility of immature platelet fractions/reticulated platelets as ITP diagnostic tools appear low in both people and dogs.

Bone Marrow Evaluation

Bone marrow examination is not recommended in the initial evaluation of uncomplicated adult or childhood ITP; however, bone marrow examination is considered potentially useful for patients with refractory disease, atypical presentation, or age greater than 60 years.[89] Combined morphologic and flow cytometric assessments are performed in these patients and may reveal causes of secondary thrombocytopenia, lymphoproliferative/myeloproliferative disease, and other bone marrow disorders.[21] Bone marrow examinations are not typically included in the diagnostic workup of canine ITP in clinical reports.[77,90,98] A retrospective study of dogs with thrombocytopenia concluded that bone marrow cytology did not provide diagnostic or prognostic information in dogs with severe thrombocytopenia.[74] Similarly, a large retrospective study of dogs with ITP found that megakaryocyte hypoplasia did not impact survival or time to platelet count recovery.[77] One recent retrospective study of thrombocytopenic dogs contradicted these studies, showing that the finding of megakaryocytic hypoplasia on bone marrow exam was associated with bleeding and nonsurvival.[22] However, this study was small and statistical comparisons were not performed between groups. Possible indications for bone marrow examination in evaluation of ITP patients include suspect underlying marrow disease due to multiple cytopenias, poor response to therapy, or search for possible lymphoproliferative disease. When marrow evaluation is warranted, a sternal marrow aspirate can be considered as a less invasive alternative to the humerus or ilium.[26] Feline bone marrow examinations may be of higher yield in thrombocytopenic cats due to underlying viral infections (e.g., feline leukemia virus) and neoplasia.[50]

Platelet Autoantibodies

Autoantibodies directed against platelet antigens play a key role in platelet destruction; however, the presence of antiplatelet antibodies is not included in current consensus criteria for routine diagnosis of human ITP given that assays lack sensitivity and specificity.[89] Different assay configurations detect circulating antiplatelet antibodies (indirect methods) and membrane-bound PAIg (direct methods). Direct assay methods are generally more sensitive than indirect assays; however, platelets constitutively express immunoglobulin receptors that cause nonspecific antibody binding and complicate interpretation of a "positive" test.[87,108] Assay sensitivity is impeded by the fact that ITP is not always an antibody-mediated process. Up to 40% of human ITP patients lack detectable antiplatelet antibodies, presumably due to cytotoxic T-cell-mediated platelet destruction.[21] In human medicine, assay specificity is greatly enhanced by measuring platelet glycoprotein-specific autoantibodies. The platelet antigens primarily targeted by autoantibodies include GPIIb/IIIa (CD41/CD61, fibrinogen receptor), GPIb/IX (CD42c/CD42a, von Willebrand factor receptor), and GPV (CD42d).[87] The monoclonal antibody-specific immobilized platelet antigen assay (MAIPA) and bead-immobilized monoclonal antibody assays are configured to define each ITP patient's autoantibody specificity. The assays are based on detection of trimolecular complexes formed by a monoclonal antibody to a defined platelet glycoprotein, the patient's autoantibody, and the platelet membrane that carries the respective epitopes of each of these antibodies.[87,108] MAIPA specificity for ITP diagnosis is reported to be as high as 98%, but still lacks sensitivity (81% sensitive), potentially due to T cell as opposed to antibody-mediated autoimmunity.[87] Surveys of ITP patients reveal that autoantibodies targeting GPIIb/IIIa are the most common; found in approximately 70–80% of patients, with anti-GpIb complex antibodies found in 20–40% of patients.[63] The only case series describing the target antigen in dogs with ITP identified anti-GPIIb and GPIIIa antibodies in the serum of 4/17 (24%) of affected dogs.[61] Another study documented that plasma from a canine ITP patient immunoprecipitated glycoprotein Ib/IX.[97]

While PAIg assays are not used for diagnosis, recent studies demonstrate their potential value in optimizing treatment and monitoring response. Whereas antibodies to GPIIb/IIIa lead to macrophage clearance of platelets by the Fc receptor, anti-GPIb antibodies trigger platelet desialylation via platelet neuraminidase-1, which results in clearance via the hepatic Ashwell–Morell receptor (Figure 80.3).[40,63] Patients with anti-GPIb antibodies appear to be refractory to IVIg therapy due to non-Fc-receptor-mediated platelet clearance.[64] As demonstrated in recent case reports, these patients may instead respond to sialidase inhibitors like oseltamivir.[63,101] Although glycoprotein-specific PAIg and platelet desialylation testing is performed in research laboratories, these assays are not routinely performed in human medicine and have not been described in veterinary studies.

Flow cytometric assays to detect PAIg without defining target platelet antigen have been described in case series of canine thrombocytopenia.[3,29,112] As in human medicine, the diagnostic utility of canine PAIg assays for ITP has not been clearly demonstrated, mostly due to lack of sensitivity and specificity. A recent prospective study of dogs with thrombocytopenia determined that the sensitivity and specificity of direct and indirect platelet-associated IgG and IgM assays combined were 76.5% and 65.5%, respectively, for distinguishing primary ITP from other causes of

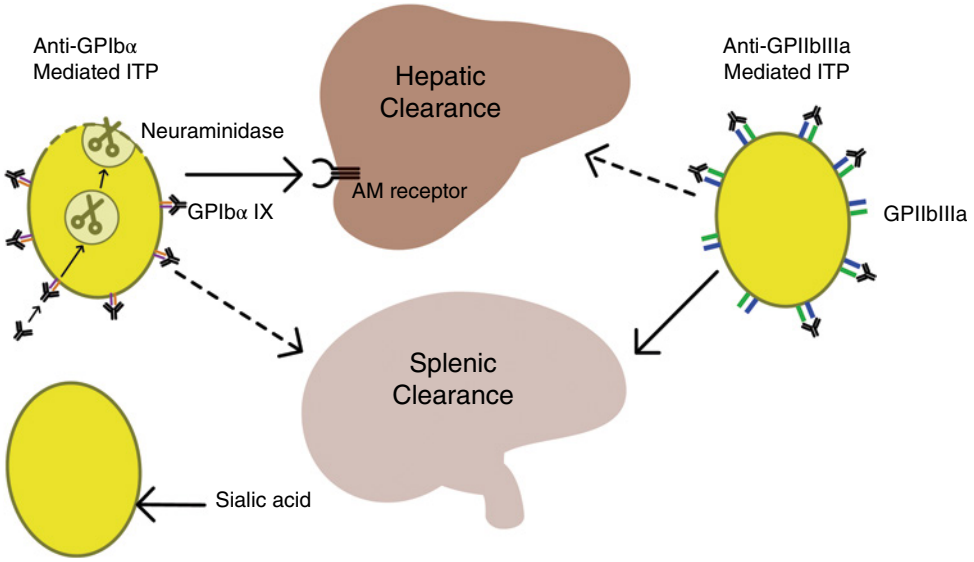


FIGURE 80.3 Autoantibody specificity determines pathway of platelet clearance in immune thrombocytopenia (ITP). Anti-GPIIb–IIIa antibodies lead to Fc-receptor-mediated mononuclear phagocyte clearance in the spleen and, less so, in the liver. Anti-GPIbα antibodies result in platelet activation and neuraminidase-1 surface translocation. Neuraminidase-1 in turn cleaves sialic acid residues from GPIbα and leads to Fc-independent hepatic clearance of the desialylated platelets via the Ashwell–Morell receptor (AM). (**Source:** Courtesy of Andrew Kingsbury, Center for Food Security and Public Health, Iowa State University)

thrombocytopenia.[3] Limitations of PAIg assays include a lack of standardization and defined controls, and non-specific antibody binding during sample storage. Furthermore, PAIg are well documented in many cases of canine secondary ITP, thus limiting the ability of PAIg testing to differentiate primary from secondary ITP (Chapter 81, Table 81.1).[29,112] Assays to define the targeted platelet glycoprotein could prove useful in dogs as in people to increase diagnostic specificity and potentially to guide treatment choice. To date, development of these assays is hampered by limited availability of commercial antibodies that recognize the main canine platelet membrane glycoproteins.[95]

Although PAIg testing is not specific for primary ITP, the finding of bound antibody is supportive of antibody-mediated platelet destruction, and persistent high PAIg may help guide treatment in cases where the patient has not responded to first-line drug therapy as expected.[21] The decision to begin tapering immunosuppressive therapy could also be guided by measurement of PAIg.[87] As in dogs, PAIg assays for cats and horses are not widely available, lack standardization, and play a limited role in ITP diagnosis at this time.

Circulating Thrombopoietin Levels

The recognition that many human ITP patients have inappropriately normal TPO levels and will respond to TPO mimetic drugs has revolutionized human ITP treatment. The diagnostic utility of TPO testing, however, has not been established[89] and TPO assays are primarily performed in research laboratories. Surveys of TPO levels in thrombocytopenic patients reveal overlap between values for consumptive thrombocytopenias and controls, but increases up to several thousand folds in patients with hypoproliferative thrombocytopenias.[1] While the utility of TPO levels for ITP diagnosis is uncertain, a potential prognostic relevance has been identified. Patients with relatively high TPO, defined as >95 pg/mL, were found to have only transient or partial response to TPO mimetic therapy.[67] Assays to measure canine TPO are not currently available.

TREATMENT OF PRIMARY ITP

Immunosuppression

Immunosuppression with glucocorticoids, often in combination with adjunctive immunosuppressive agents, is the mainstay of ITP treatment in dogs. Prednisone is generally started at 2 mg/kg/day, or 50–60 mg/m^2 for dogs over 25 kg. Once daily prednisone therapy compared to fractionated therapy may be associated with fewer side-effects.[104] Cats have inefficient prednisone absorption and/or conversion to prednisolone, the active drug form, thus prednisolone should be selected over prednisone for feline ITP treatment.[16] Interestingly, one feline ITP patient described in the literature responded to dexamethasone and not prednisolone for unknown reasons.[106]

There are no prospective, controlled studies to guide the selection of an adjunctive immunosuppressive agent in dogs with ITP. Empiric considerations for the use of adjunctive immunosuppressive agents in dogs include weight >25 kg with increased risk of severe steroid side-effects, severe hemorrhage requiring transfusion support, or failure of steroid therapy alone after 7 days. Commonly used immunosuppressive drugs for veterinary patients include cyclosporine, azathioprine, and mycophenolate

mofetil. Cyclosporine's immunosuppressive action can be monitored with a pharmacodynamic assay (Pharmacodynamic Laboratory, Mississippi State University College of Veterinary Medicine).[92] Azathioprine should not be used in cats, as they can develop severe, even fatal, drug-induced myelosuppression due to their low concentrations of thiopurine methyltransferase, limiting their ability to metabolize the drug.[30] Most horses with ITP respond to glucocorticoids, but azathioprine has been of benefit in refractory patients.[46,70] Use of single-agent mycophenolate was reported in five dogs with non-transfusion-dependent ITP in which corticosteroids were considered contraindicated.[115] There is currently no evidence of benefit to combined, triple immunosuppressive therapy, and triple therapy was associated with poor outcome in a retrospective study, thus combined therapy should be avoided.[77]

Therapy with Vincristine and Intravenous Immunoglobulin

Vincristine and IVIg have been shown to rapidly increase platelet counts in dogs with ITP, and are indicated for those with severe hemorrhage, especially gastrointestinal bleeding or life-threatening respiratory or central nervous system bleeding. Vincristine is speculated to increase platelet count by preventing microtubule polymerization and thereby accelerating megakaryocyte fragmentation and platelet release from bone marrow. In addition to accelerated thrombopoiesis, vincristine may impair phagocytosis of opsonized platelets.[6] In one prospective controlled study of severe ITP dogs, administration of vincristine (0.02 mg/kg) intravenously once in combination with prednisone was associated with more rapid increase in platelet count and shortened hospitalization compared to treatment with prednisone alone.[94]

Human IVIg is a sterile plasma derivative consisting primarily of IgG from healthy human donors. IVIg's mechanism of action in ITP is likely multifactorial and includes blocking antibody-mediated platelet clearance by saturating macrophage Fc receptors.[118] Other theoretical mechanisms include modulation of T-cell subsets to increase the proportion of regulatory T cells and inhibition of autoantibody production.[118] A prospective clinical trial of canine ITP demonstrated that treatment with IVIg combined with prednisone shortened time to achieve a target platelet count and hospitalization duration compared to prednisone treatment alone.[10] A subsequent study demonstrated that vincristine and IVIg are equally as effective in shortening platelet count recovery time and hospital duration in dogs with ITP.[6] Due to expense of IVIg, vincristine is usually the first emergent ITP therapy selected. In very severe cases, these therapies can be used in combination.

Novel Therapies

In people with ITP, frontline treatments include short courses of corticosteroids and IVIg, while second-line therapies include an anti-CD20 antibody (rituximab), splenectomy, and TPO receptor agonists.[75]

Treatment with TPO receptor agonists was adopted in human ITP based on recognition of inappropriately normal circulating TPO in this patient population. The TPO receptor agonists romiplostim (Nplate®) and eltrombopag (PROMACTA®) have revolutionized ITP treatment in people. Whether TPO is low in dogs or other species with ITP is unknown. Eltrombopag, a small molecule that binds to a transmembrane region of the TPO receptor, is not predicted to bind to the corresponding canine receptor.[54] By contrast, romiplostim, a peptide fusion protein, interacts at a conserved region of the canine TPO receptor with homology to the human protein. Romiplostim was used successfully in a pilot study of five dogs with ITP.[54] However, this study was not controlled, thus further research into canine TPO levels and romiplostim therapy in canine ITP patients is warranted.

Splenectomy is also a second-line ITP therapy in human medicine with reported response rates of 60% lifelong remission.[21] There is limited data on splenectomy efficacy in canine ITP; however, a recent retrospective study reported significant increases in platelet counts post-splenectomy, an 80% (12/15 dogs) 20-day survival rate, and 72% of surviving dogs requiring no long-term medical therapy.[34]

CD20 antibodies are believed to target autoantibody production via a B-cell-depleting effect. Rituximab, a chimeric anti-human CD20 antibody, has a response rate of 50–60% in people, but is associated with fewer sustained remissions than splenectomy. Despite its efficacy, rituximab can be associated with significant immunosuppression and other side-effects such as progressive multifocal leucoencephalopathy.[21] Rituximab does not bind canine B cells and there are currently no available anti-CD20 antibodies for veterinary patients.[47]

Transfusion Therapy

Blood transfusions may be required for ITP patients with signs referable to severe bleeding at critical anatomic sites or blood loss anemia. Although transfused platelets may have short circulatory half-life and not significantly alter platelet count, they may provide essential hemostasis at critical sites of bleeding. Hemorrhage from the gastrointestinal tract may be severe enough to warrant packed red cell or fresh whole blood transfusion. Human ITP guidelines recommend that platelet transfusions be reserved for those patients experiencing hemorrhagic bleeding or requiring invasive surgery.[75] A recent study determined that platelet transfusions, in the absence of these conditions, were not associated with improved clinical outcomes in human ITP patients.[35]

Platelet transfusion products include fresh whole blood, fresh platelet-rich plasma, fresh platelet concentrate (the standard product utilized in human blood banks), cryopreserved platelets, and a novel lyophilized canine platelet product, StablePlateRx™ (see Chapters 86 and 101). Fresh whole blood is the most readily available platelet source in many clinical settings; however, development of a commercial shelf-stable

product, StablePlateRx™, may provide wider access to platelet replacement therapy and enable more proactive transfusion of patients. Analyses of a prospective randomized controlled multicenter study comparing this product to cryopreserved platelets in bleeding thrombocytopenic dogs are currently underway.

ITP Treatment Goals

Treatment goals of ITP are not straightforward due to its clinical heterogeneity with highly variable signs of bleeding. Much of the overall disease burden of ITP is caused by uniform administration of high-intensity and long-term immunosuppressive therapy and potential for severe side-effects including secondary infections and bone marrow suppression. Indeed, in severe human ITP, mortality results equally from refractory hemorrhage as it does from secondary infections in immunosuppressed patients.[88]

While veterinarians routinely aim to normalize platelet counts, American Society of Hematology (ASH) guidelines do not recommend treating adult ITP patients unless platelet counts are less than 30,000 platelets/μL or there is active bleeding.[75] Additionally, ASH guidelines recommend, in the absence of bleeding signs, a treatment goal of over 30,000 platelets/μL.[75] Reconsidering ITP treatment goals in veterinary medicine to better align them with human recommendations may be warranted and may result in less treatment-associated morbidity.

Markers of ITP Disease Severity

Risk of spontaneous bleeding is generally associated with platelet counts less than 30,000/μL.[21] Although this threshold gives some indication of bleeding risk, not all patients with ITP bleed at low platelet counts, while others bleed excessively. Clinical bleeding assessment tools including the recently developed canine bleeding assessment tool, "DOGiBAT," may better reflect disease severity than do platelet counts.[68,80] Validated biomarkers of disease severity to guide treatment intensity are lacking; however, in human medicine, flow cytometric platelet function assays, thromboelastometry, and absolute IPF have shown promise in predicting bleeding in ITP patients.[21,31,37] A few disease severity markers have been recognized in dogs. In a retrospective study of 73 dogs, elevated blood urea nitrogen (BUN) on admission was associated with decreased survival (57% survival to discharge vs. 85% survival for dogs with BUN in the reference range).[77] Similarly, dogs with melena on admission were much less likely to survive to discharge (60%) compared to dogs without melena (90%) and were more likely to require a transfusion.[77]

PRIMARY ITP PROGNOSIS

The overall prognosis for dogs with ITP is good. Most dogs achieve hemostatic platelet counts within 5–7 days of therapy, though some patients have prolonged recovery times.[10,77,94] Reported mortality rates range from 10 to 30%.[60,90] While most dogs can eventually be weaned off immunosuppressive therapy over a range of months to years, others relapse following recovery (9–58% recurrence rate) requiring recurrent or chronic immunosuppressive therapy.[77,90,113] However, many studies define relapse as any decrease in platelet count, not necessarily a clinically significant decrease. Recalibrating to a goal of a safe platelet count would likely be associated with lower relapse rates.

Although uncommon in cats, reported rate of survival to hospital discharge for feline primary ITP is 85%.[9,32,50,55,106,114] While most cats experienced long-term survival ranging from 15 months to over 5 years, frequent relapses and development of diabetes mellitus secondary to chronic steroid therapy were described in one cases series.[9,106,114] Another cat with ITP developed a *Nocardia* infection presumably secondary to chronic immunosuppressive therapy.[32]

Reports of equine ITP describe a favorable prognosis and many cases with short disease duration. Recoveries often occur within 14–21 days, suggesting the presence of an unidentified secondary etiology.[111] More chronic ITP courses requiring prolonged glucocorticoid therapy are also described in horses.[111]

SUMMARY

Despite advances in our understanding, much about ITP pathogenesis and the inciting cause of loss of self-tolerance remain unknown. Consequently, diagnostic tests for ITP are lacking and few biomarkers exist to predict disease severity or help individualize treatment. Future research is needed to continue unraveling ITP pathogenesis and ultimately fill these gaps for safer, more targeted treatment and improved patient outcomes.

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CHAPTER 81

Nonimmune-Mediated Thrombocytopenia

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Acronyms and Abbreviations

ADAMTS13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13; APTT, activated partial thromboplastin time; AT, antithrombin; BVD, bovine viral diarrhea; CBC, complete blood count; CDV, canine distemper virus; DIC, disseminated intravascular coagulation; EDTA, ethylenediaminetetraacetic acid; EIA, equine infectious anemia; ELISA, enzyme-linked immunosorbent assay; EVA, equine viral arteritis; FDP, fibrin/fibrinogen degradation product; FeLV, feline leukemia virus; FIV, feline immunodeficiency virus; FIP, feline infectious peritonitis virus; GI, gastrointestinal; GPIIb–IIIa, glycoprotein IIb–IIIa; HUS, hemolytic uremic syndrome; ITP, immune-mediated thrombocytopenia; LDL, low density lipoprotein; MPC, mean platelet component; MPS, mononuclear phagocytic system; MPV, mean platelet volume; PCR, polymerase chain reaction; PSAIg, platelet-surface-associated immunoglobulin; PT, prothrombin time; SFTSV, severe fever with thrombocytopenia syndrome virus; TPO, thrombopoietin; TTP, thrombotic thrombocytopenic purpura; VEE, Venezuelan equine encephalitis; VLDL, very low density lipoprotein; VWF, von Willebrand factor.

INTRODUCTION

Thrombocytopenia is defined as a platelet count below the lower limit of the reference interval. Thrombocytopenia is common in many species and is generally an acquired process accompanying an underlying disease or disorder.[12,30,51,104] Uncomplicated, mild to moderate thrombocytopenia is usually subclinical and clinical signs (e.g., fever, lethargy, anorexia) are related to the underlying condition. Thrombocytopenia is typically identified from review of a blood smear or a CBC. Spontaneous bleeding due to thrombocytopenia is rare when platelet concentrations are greater than 30,000–50,000/μL; however, some studies have suggested that platelet count alone is unreliable in ascertaining bleeding risk.[73]

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Animals with severe thrombocytopenia or thrombocytopenia complicated by coagulopathy, vasculopathy, or platelet dysfunction often present with signs of primary hemostatic failure.[5] Findings may include petechiae or ecchymoses (often on the ventrum and inguinal region, or mucous membranes), and epistaxis, hematuria, gastrointestinal, gingival, vaginal, or ocular hemorrhage. Excess hemorrhage may develop following trauma, surgery, and venipuncture.

Platelet concentration in the blood reflects a balance between platelet production and platelet consumption, destruction, or redistribution to the vasculature of organs (see Chapter 78). In most healthy animals, platelet life spans are approximately 5–10 days. Platelets are continually consumed during repair of small vascular defects and senescent platelets are removed primarily by splenic macrophages. Platelet life spans are significantly longer in splenectomized dogs when compared to nonsplenectomized controls.[22]

Human studies have shown that 10^{11} platelets are produced every day and population turnover occurs every 8–9 days. In response to increased demand, platelet production can increase 20-fold or more.[54] Platelet production is affected by a number of growth factors (see Chapter 75); however, thrombopoietin (TPO) is the primary regulator of megakaryopoiesis and thrombopoiesis.[27,72] Thrombopoietin is primarily produced by hepatocytes, stromal cells in the bone marrow, and renal epithelial cells. In healthy animals, TPO is constitutively produced and the blood concentration is generally inversely related to platelet mass. Interleukin-6 has been shown to induce hepatic TPO production in inflammatory states.[27] Thrombopoietin binds to receptors on platelets and megakaryocytes. When circulating platelet mass is decreased, the concentration of unbound TPO available to stimulate megakaryopoiesis and thrombopoiesis is increased. Production of TPO is increased in some disease states (see Chapter 82).[15,108]

TABLE 81.1 Diagnostic tests for thrombocytopenia.

Assay	Comments[a]
Complete blood count	
Platelet concentration	Automated or manual Confirm and track thrombocytopenia
Mean platelet volume	Increase suggests enhanced thrombopoiesis
Mean platelet component	Decrease suggests platelet activation
Blood smear examination	Detect platelet clumps Estimate platelet concentration Detect organisms or neoplastic cells Detect large platelets—suggests increased production Evaluate for inflammatory leukogram
Hemostasis profile	Evaluate for consumptive coagulopathy (DIC)
Bone marrow examination	Assess megakaryocyte density and morphology Detect organisms, neoplastic cells, necrosis, inflammation, myelofibrosis
Serology	Detect exposure to infectious agents
PCR	Detect infectious agents
Flow cytometry	Detect PSAIg Measure reticulated platelets to assess platelet production Detect activation markers

[a]DIC, disseminated intravascular coagulation; PSAIg, platelet-surface-associated immunoglobulin.
Source: Based on Paltrinieri et al.[86]

DIAGNOSIS OF THROMBOCYTOPENIA

Common diagnostic tests are listed in Table 81.1 (and Chapter 78). When thrombocytopenia is detected by an automated hematology analyzer, it is important to examine a blood smear to check for platelet clumps, estimate platelet concentration to confirm the thrombocytopenia, and evaluate platelet morphology.[86]

Pseudothrombocytopenia

Pseudothrombocytopenia occurs when platelets are excluded from counting for any reason.[8] Small platelet clumps may not be detected by automated analyzers; thus, falsely lowering measured platelet concentrations. Most instruments will flag possible clumps and platelet histograms can be evaluated for abnormalities. A study in cats reported that platelet concentrations determined with an impedance hematology analyzer were often decreased; however, examination of a blood smear confirmed thrombocytopenia in only 3.1% of cats.[83] The disparity was attributed to platelet clumps and lack of size-separation of platelets from erythrocytes.[43,60] When clumps are detected in a sample, the measured platelet concentration is inaccurate, but may reflect the minimum number of platelets. Small clumps can be mistakenly counted as leukocytes by certain analyzers. Platelet concentration determined by analyzers that use a buffy-coat technique are less affected by platelet clumping than impedance hematology analyzers.[60]

Atraumatic venipuncture, with proper blood collection and handling techniques, is critical to minimize platelet activation and clumping. Various additives to inhibit platelet aggregation have also been investigated including prostaglandins, theophylline, adenosine, and dipyridamole, along with techniques such as vortex mixing samples.[43] Potent prostaglandin analogs, including iloprost, have demonstrated efficacy in normalizing platelet counts, but do not eliminate all clumping.[95,126]

Platelet clumps may form ex vivo in an EDTA-dependent manner.[68] In people, EDTA-related calcium chelation may result in a conformational change in glycoprotein IIb–IIIa (GPIIb–IIIa) that exposes cryptoantigens that bind to nonpathologic, but activating autoantibodies. EDTA-dependent pseudothrombocytopenia has rarely been reported in dogs, horses, and pigs.[31] Collection of blood in a citrate tube may clarify whether thrombocytopenia is due to EDTA;[68,84,117] however, aggregation can occur with citrate due to calcium chelation's role in the etiogenesis. Blood collection in fluoride oxalate tubes, or addition of amikacin or kanamycin, has been reported to resolve this pseudothrombocytopenia in humans.[68] Avoidance of delays in sample processing and low temperatures are practical measures to minimize clumping and hence pseudothrombocytopenia.[31]

Platelet satellitism can also contribute to falsely low platelet counts.[8,68] Platelets accumulate around neutrophils due to bridges formed by autoantibodies bound to platelet GPIIb–IIIa and neutrophilic FcγRIII receptors, usually in the presence of EDTA. This can rarely be seen with other leukocytes via unknown mechanisms.[8,68] Macrothrombocytosis may produce low platelet concentrations because large platelets are excluded by some automated hematology analyzers. Plateletcrit or manual platelet count may provide a more accurate platelet concentration.[54,125] Other causes of pseudothrombocytopenia include cold agglutinin disease and the therapeutic use of monoclonal antibodies in humans.[68] Pseudothrombocytopenia does not cause an increased bleeding tendency; however, the failure to recognize this artifact could lead to complications of unnecessary testing and treatment.

True Thrombocytopenia

Qualitative and quantitative data are available from a routine CBC to further characterize true thrombocytopenias. Increased numbers of macroplatelets on a blood smear suggest increased thrombopoiesis and may be associated with increased mean platelet volume (MPV). Increased MPV (exclusive of breed-related macrothrombocytopenia) suggests adequate to increased thrombopoiesis.[65] Decreased mean platelet component (MPC) suggests in vivo platelet activation. However, platelet volume indices have not been proven to help elucidate the pathophysiologic cause of thrombocytopenia.[11,102]

Test selection to define the cause of thrombocytopenia depends on clinical presentation and screening test results (see Chapter 78).[91] A complete hemostasis profile (prothrombin time [PT], activated partial thromboplastin time [APTT], fibrinogen concentration, fibrin degradation products [FDPs], D-dimer, and antithrombin activity) is used to characterize disseminated intravascular coagulation (DIC) (see Chapters 88 and 92). Examination of blood and cytology smears of bone marrow samples may reveal infectious organisms or neoplastic cells. Serology or PCR assays may identify infectious disease. Flow cytometry is used to detect immature reticulated platelets, platelet-surface-associated immunoglobulin (PSAIg), or activated platelets. Measurement of reticulated platelets may help differentiate thrombocytopenia due to shortened platelet survival from thrombocytopenia due to platelet production disorders.[29,129] Reticulated platelets were increased in dogs and horses with destructive or consumptive thrombocytopenia.[99,103] Identification of PSAIg may support a diagnosis of immune-mediated disease, but these assays do not differentiate primary from secondary immune-mediated thrombocytopenia (ITP).[129] Detection of activated platelets suggests prothrombotic states or DIC.[103]

Bone marrow examination aids in differentiating thrombocytopenia due to production disorders based on decreased or absent megakaryocytes.[94] Neoplastic cells or etiologic agents may be identified. Megakaryocytes are normal or increased if thrombocytopenia is due to platelet destruction or consumption. However, megakaryocytes can be unevenly distributed in bone marrow aspirates and core biopsies are often required for accurate assessment.[112] A study in thrombocytopenic dogs reported that bone marrow cytology rarely provided useful diagnostic or prognostic information, suggesting that bone marrow aspiration was most likely to be useful in refractory thrombocytopenia or patients with bicytopenia or pancytopenia.[77] Immunofluorescent antimegakaryocyte antibody assays can be performed on bone marrow cytology preparations; however, the sensitivity and specificity of these assays are low.[121]

BASIC MECHANISMS FOR THROMBOCYTOPENIA

Platelet Production Disorders

Decreased platelet production may result from disorders that specifically target megakaryocytes or from diseases that have direct effects on multiple hematopoietic cell lines, or by altering the microenvironment in the bone marrow (see Chapters 75 and 78). Bicytopenia or pancytopenia suggests bone marrow disease.[94]

Selective megakaryocytic hypoplasia is uncommon. Acquired immune-mediated amegakaryocytic thrombocytopenia has been reported in dogs and cats.[21] Megakaryocytic hypoplasia can be associated with drugs, infections, or neoplasia. Neoplasia may be metastatic to the bone marrow or hemic in origin, causing replacement of the normal hematopoietic cells. Other causes of hypoplasia include irradiation and myelonecrosis. Hereditary megakaryocytic hypoplasia is documented in people, but rarely reported in veterinary species.[82]

High-quality bone marrow samples are required to adequately assess bone marrow production. Due to variable megakaryocyte distribution within cytology samples, core biopsy samples are recommended for definitive diagnosis of megakaryocytic hypoplasia.[94]

Congenital or Breed-Associated Thrombocytopenia

Thrombocytopenia is an incidental finding in certain dog breeds (see Chapter 84). Greyhounds typically have a mild to moderate thrombocytopenia (platelet concentrations >100,000/μL) when compared to non-breed-specific reference intervals.[131] In one greyhound study, none of the samples tested had detectable PSAIg, indicating that the thrombocytopenia is not immune-mediated.[101] The stem cell competition theory is a possible mechanism, i.e., platelets and erythrocytes originate from a bipotential stem cell such that Greyhounds with high red cell concentrations have correspondingly lower platelet counts. Greyhounds also have relatively large spleens, suggesting that sequestration is a contributing factor.[54,131]

Canine hereditary macrothrombocytopenias due to mutations in the gene encoding beta-1-tubulin have been described in several breeds (see Chapter 84). These defects result in unstable microtubules and altered proplatelet formation. A common mutation has been identified in approximately 30–50% of the Cavalier King Charles spaniels screened.[23] The same mutation has been identified sporadically in Labrador retrievers, labradoodles, shih tzu, Chihuahua, Maltese, Jack Russell terriers, Havanese, poodles, English toy spaniels, boxers, cocker spaniels, and bichon frise, and a distinct beta-1-tubulin mutation has also been documented in Norfolk and Cairn terriers.[39] PennGen provides a searchable database of veterinary laboratories that offer genetic tests for these hereditary macrothrombocytopenias, and other hereditary disorders in dogs and cats.

Macroplatelets result in overlap in size between platelets and erythrocytes in automated analyzers. Consequently, plateletcrit may provide a more reliable estimator of platelet mass, since plateletcrits are typically within reference limits in macrothrombocytopenic patients versus those with true thrombocytopenia and normal platelet size.[125,127]

May–Hegglin anomaly is a rare congenital macrothrombocytopenia due to a mutation in the gene encoding nonmuscle myosin heavy chain IIA (*MYH9*). The trait is also characterized by neutrophil inclusions, and has been reported once in veterinary medicine in a pug mix (see Chapter 84).[36] Other inherited thrombocytopenias are likely present in veterinary species, since numerous mutations in various transcription factors involved in thrombopoiesis and the thrombopoietin receptor gene, *MPL*, have been identified in human hereditary thrombocytopenias.[82]

Platelet Loss

External hemorrhage may result in mild to moderate thrombocytopenia. Platelet concentration decreased by up to 50% in dogs with experimental acute, severe blood loss. Thrombocytopenia due to blood loss is usually self-limiting and resolves when hemorrhage ceases.[62] When severe thrombocytopenia occurs in conjunction with hemorrhage, it is likely that hemorrhage is due to thrombocytopenia rather than thrombocytopenia resulting from hemorrhage.

Dogs and cats presenting with severe hemorrhage due to anticoagulant rodenticide toxicity may have variable and potentially severe thrombocytopenia.[57,58,66,106] Likely mechanisms for thrombocytopenia include loss secondary to hemorrhage combined with platelet consumption.[106]

Platelet Consumption

Accelerated platelet consumption occurs with disorders that cause widespread damage to endothelial cells or trigger massive activation of coagulation via other mechanisms. In people, well-characterized platelet consumptive disorders include DIC, vasculitis, and thrombotic microangiopathies, such as thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS) (see Chapter 93).[61]

Disseminated Intravascular Coagulation

DIC is common in veterinary species (see Chapter 92). Patients with DIC typically have laboratory abnormalities in coagulation and fibrinolytic pathways.[116] DIC is never a primary condition; it occurs secondary to a variety of disorders characterized by excessive and unregulated coagulation. DIC varies from overt or uncompensated to nonovert or compensated. In nonovert DIC, excess thrombin generation is balanced by inhibitory pathways. In overt DIC, widespread microthrombi formation may lead to consumption of coagulation factors and platelets, and result in bleeding. and bleeding. Clinical signs vary from subclinical in nonovert DIC to signs of severe, life-threatening hemorrhage, shock, or multiorgan failure in overt DIC. Clinical signs referable to the underlying condition are often present. The consequences of hemorrhage are easily recognized; however, clinical signs and death are often attributable to tissue hypoxia and organ dysfunction resulting from microthrombosis.[116]

Specific disorders associated with DIC include infections (e.g., septicemia, endotoxemia, leptospirosis), malignant neoplasia, severe tissue injury (e.g., trauma, surgery, burns), immune-mediated disorders (e.g., immune-mediated hemolytic anemia), conditions that cause blood stasis (e.g., shock, severe dehydration), heatstroke, systemic hypersensitivity reactions, pancreatitis, and hepatic failure.[6,16,41,116] Snake venoms may directly activate coagulation factors or induce DIC from tissue injury.[46]

Thrombocytopenia accompanying DIC varies from mild to severe. No single test is diagnostic for DIC (see Chapters 88 and 92).[42,116] Laboratory criteria include thrombocytopenia, coagulopathy (prolonged APTT or PT, hypofibrinogenemia), enhanced fibrinolysis (increased FDPs or D-dimer), loss of natural anticoagulants (e.g., decreased AT activity), and the presence of schistocytes or keratocytes on a blood smear.[42,71,116] Various veterinary scoring systems exist and a recent canine study suggested that applying

in-house coagulation assay reference intervals improved diagnosis and predictions of mortality from overt DIC.[42]

Thrombotic Microangiopathies

Thrombotic microangiopathies are uncommon, life-threatening conditions characterized by disseminated microvascular thromboses, secondary ischemic organ damage, microangiopathic hemolytic anemia with schistocytes, and thrombocytopenia (see Chapter 93). Unlike DIC, microangiopathies are rarely accompanied by abnormalities in coagulation and fibrinolysis assays. The underlying pathogenesis is heterogeneous, occurring secondary to hereditary and acquired disorders.[60]

HUS is often associated with renal insufficiency. The most common form in human beings occurs in children after infectious diarrhea, particularly involving Shiga-toxin-producing *Escherichia coli* (STEC).[60] Atypical forms of HUS in humans are associated with inherited or acquired defects in complement regulation, or following organ transplantation and cyclosporine therapy. Although rare, HUS has been described in dogs, cats, cattle, and horses.[3,25,28,49,96] Possible HUS was seen in three cats following renal transplantation and cyclosporine administration.[3] Ruminants are often the source of *E. coli* infections, but other domestic species, including dogs and cats, carry STEC and have been implicated in possible zoonotic transmission.[60] Cases of human HUS have also been documented secondary to infection with *Capnocytophaga canimorsus* from dog- or cat-bite wounds.[113,114]

TTP is characterized by frequent neurologic dysfunction, moderate severity renal insufficiency, and microthrombi rich in platelets and von Willebrand factor (VWF). Many human TTP cases are now believed to result from inherited or acquired deficiency of a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13), a metalloprotease that cleaves VWF and prevents circulation of the most thrombogenic, high-molecular-weight multimers of VWF (see Chapters 83 and 93).[113] TTP has not been well documented in veterinary species; however, there are murine and primate models of experimentally induced TTP. A human ELISA, recently validated for measuring canine ADAMTS13 activity, may facilitate recognition of TTP in dogs.[74]

Platelet Destruction

Immune-Mediated Platelet Destruction

ITP is common in dogs and sporadically recognized in other species (see Chapter 80).[29] ITP occurs secondary to antibody-mediated complement-induced platelet lysis or removal by the mononuclear phagocytic system (MPS). Direct T-cell-mediated cytotoxicity, impaired T regulatory cell activity, and impaired megakaryopoiesis and thrombopoiesis are also implicated in ITP pathogenesis.[109] In people with primary ITP, IgG or IgM autoantibodies targeting epitopes of glycoprotein complexes GPIIb/IIIa, GPV, and/or GPIb/IX are most common.[109] Autoantigens are rarely identified in animals with primary ITP; however, antibodies targeting GPIIb/IIIa and GPIb were detected in a canine study.[129] In secondary ITP, the immune response is associated with an underlying disorder such as systemic immune-mediated diseases (e.g., immune-mediated hemolytic anemia), neoplasia (e.g., lymphoma, hemic neoplasia, various solid tumors), drug administration, or infections.[29]

Nonimmune-Mediated Platelet Destruction

Nonimmune platelet destruction occurs in disorders that cause platelet aggregation, phagocytosis, or lysis independent of antibody or complement. Nonimmune platelet destruction is often a feature of thrombocytopenia due to infections, drugs, or neoplasia. Mechanisms include direct platelet damage causing lysis or premature removal, release platelet agonists, cytokine-mediated activation of the MPS leading to enhanced platelet phagocytosis, hemophagocytic syndrome, or phagocytosis by neoplastic cells.

Hemophagocytic Syndrome

Hemophagocytic syndrome, or lymphohistiocytosis, is characterized by a non-neoplastic proliferation of activated macrophages and lymphocytes and hypersecretion of cytokines.[2,47,50,85] It can be a primary disorder, e.g., due to a genetic defect in lymphocyte cytotoxicity, or an acquired disorder secondary to infectious, immune-mediated, neoplastic or metabolic disease.[92,105] Clinical signs include fever (interleukin-1-mediated), peripheral cytopenias (often anemia and thrombocytopenia, less commonly neutropenia), hepatosplenomegaly, hyperferritinemia, hypoalbuminemia, hypofibrinogenemia, and often increased liver enzymes.[85] The cytopenias result from combined cytokine-mediated bone marrow suppression and phagocytosis. Hypertriglyceridemia is seen in humans (particularly children), presumably mediated by increased acute phase proteins, such as serum amyloid A, that enhance low density lipoprotein (LDL) removal from the circulation. Hypocholesterolemia, attributed to increased uptake or use of cholesterol, is more common in veterinary patients. Additionally, inflammatory cytokines can reduce lipoprotein lipase activity, thereby decreasing conversion of very low density lipoprotein (VLDL) to LDL and lowering cholesterol through decreased lipoproteins and cholesterol content.[79] Signs of hemophagocytic syndrome overlap with diagnostic criteria for several other disease states including systemic inflammatory response syndrome, viral infections, and neoplasia. In humans, the diagnosis is based on the presence of at least five out of eight criteria established by the Histiocyte Society.[34,47] Hemophagocytosis may be apparent in bone marrow, spleen, and/or lymph node samples, and serial sampling may be required to identify this phenomenon. Documentation of hemophagocytosis is not required for diagnosis in humans if other criteria are fulfilled, and hemophagocytosis is not pathognomonic.

Hemophagocytic syndrome can be mild to fatal. Several distinct disease genes have been associated with primary hemophagocytic lymphohistiocytosis (HLH) in humans and in mouse models. All are involved in the production or release of cytotoxic granules from T lymphocytes, including a mutation in the gene encoding perforin.[92,105] Chronic inflammation is suspected to play a role in inciting the acquired syndrome, with associated immune disorders in humans including lupus and systemic juvenile idiopathic arthritis.[92,105] Lymphoma (primarily T cell, but also B cell) is a common neoplastic trigger, presumably mediated by persistent antigenic stimulation and cytokine release by the neoplastic cells. Infectious diseases associated with HLH include leishmaniasis, tuberculosis, and salmonellosis. An underlying condition is not always identified. In veterinary patients, the major diagnostic dilemma is differentiating this syndrome cytologically from histiocytic sarcoma or forms of T-cell lymphoma.[1,78] Immunohistochemistry on fresh frozen tissue can aid in definitive diagnosis of neoplasia, but its availability is limited. Flow cytometry may also help differentiate HLH from lymphoma; however, clinical differentiation is often based on response to therapy.

Platelet Distribution Disorders

Total platelet mass consists of platelets in circulation and platelets reversibly sequestered or marginated in the vasculature of tissues, particularly the spleen, liver, and bone marrow. In healthy animals, 30% or more of total platelet mass may be stored in the spleen.[128] Redistribution thrombocytopenia refers to reversible platelet sequestration in tissues. Total platelet mass is not affected; thus, thrombopoiesis is not influenced. Platelet pooling has been reported in the liver and spleen of dogs with hypothermia and in the lungs of endotoxemic dogs.[115,130] Mild to moderate thrombocytopenia may result from sequestration in any condition associated with hepatosplenomegaly and increased blood pooling. For example, resolution of thrombocytopenia has been reported in dogs with splenic torsion after surgical correction.[118]

Hypersplenism, resulting in cytopenias, may occur in patients with portosystemic shunting, even in the absence of splenomegaly.[14,70] The condition recurs following splenectomy, which can also increase the risk of portal vein thrombosis. In spite of cytopenias, hypersplenism is often clinically inapparent.[14] Hypersplenism was recently described in a miniature horse with pancytopenia, but the histopathologic description of the spleen was more consistent with a diagnosis of hemophagocytic syndrome. The differentiation of these syndromes is challenging, as increased macrophage activation has been described as a component of hypersplenism.[24,97]

Platelet sequestration is not expected to cause signs of hemorrhage, unless complicated by platelet dysfunction or coagulopathy. Diagnosis of redistribution thrombocytopenia relies on discovery of the cause of organomegaly and ruling out other causes of thrombocytopenia.

DISEASE CATEGORIES OF THROMBOCYTOPENIA

Thrombocytopenia in veterinary species is frequently associated with infection, neoplasia, drug therapy, and liver disease.[30,45,51,104] The pathogenesis of thrombocytopenia in many of these disorders is likely multifactorial.

Infectious Thrombocytopenia

Thrombocytopenia frequently accompanies infections (see Table 81.2).[26,30,45,51,104] Many organisms impair thrombopoiesis by direct infection of bone marrow megakaryocytes, stimulation of an immune response targeting megakaryocytes, or inciting bone marrow inflammation and production of myelosuppressive cytokines. Additional pathophysiologic mechanisms may include increased platelet consumption due to platelet activation and clearance, DIC, direct infection of platelets causing destruction or removal by the MPS, secondary ITP, or platelet sequestration in the spleen or other tissues.[26,37]

Endotoxemia and inflammatory cytokines elicited by bacterial infections may directly activate endothelial cells, neutrophils, monocytes, and platelets. These activated cells upregulate production and release of platelet agonists, including thrombin and platelet-activating factor. Activated platelets in turn promote tissue factor expression by monocytes, activation of the coagulation cascade, and, via expression of P selectin, induce platelet–leukocyte aggregates.[26,63] Damaged endothelial cells decrease production of inhibitory substances such as prostaglandin I_2. Inflammatory cytokines activate the MPS leading to platelet phagocytosis.[63]

Rickettsial bacteria are commonly associated with thrombocytopenia.[80] Acute infection with *Ehrlichia canis* is associated with ITP and nonimmune platelet destruction in dogs. Chronic infection is associated with bone marrow hypoplasia. Dogs experimentally infected with *E. canis* demonstrated thrombocytopenia, platelet activation, and antiplatelet antibodies, and were hypercoagulable and hypofibrinolytic. Platelet activation and hypercoagulability may explain lack of overt bleeding in some profoundly thrombocytopenic dogs.[110] Other rickettsial bacteria associated with thrombocytopenia include *Anaplasma platys* in dogs, *Rickettsia rickettsii* in dogs, *Neorickettsia risticii* in horses, and *Anaplasma phagocytophilum* in several species. *A. phagocytophilum*, the cause of granulocytic anaplasmosis (granulocytic ehrlichiosis), directly infects megakaryocytes without altering platelet production, but may evoke an antiplatelet immune response.[57 and 58] The pathogenesis of thrombocytopenia in *A. platys* infection is suspected to be direct injury to platelets by replicating organisms, and to formation of antiplatelet antibodies.[124] Vasculitis and platelet consumption may result in thrombocytopenia in *R. rickettsii* infection. The cause of thrombocytopenia in *N. risticii* infection in horses likely involves consumption due to DIC.[33] Other

TABLE 81.2 Causes of thrombocytopenia in dogs, cats, horses, and cattle.

Species	Category	Specific examples
Dog	Infectious	Viruses: distemper, herpes virus, infectious hepatitis, parvovirus, SFTSV Bacteria: septicemia, endotoxemia, *Anaplasma phagocytophilum*, *A. platys*, *Ehrlichia canis*, *Rickettsia rickettsii*, leptospirosis, salmonellosis, bartonellosis, borreliosis Fungi: histoplasmosis, candidiasis Protozoa: babesiosis, leishmaniasis Parasites: heartworm disease
	Neoplasia	Hemangiosarcoma, lymphoproliferative and myeloproliferative neoplasia, various solid tissue neoplasms
	Drugs/toxins	Antibiotics (e.g., sulfonamides, cephalosporin), chemotherapeutic drugs (e.g., cyclophosphamide, doxorubicin), anti-inflammatory drugs (e.g., phenylbutazone, carprofen), phenobarbital, thiacetarsamide, estrogen, snakebites
	Miscellaneous	ITP, DIC, distribution disorders
Cat	Infectious	Viruses: FeLV, FIV, FIP, parvovirus Bacteria: septicemia, endotoxemia, *A. phagocytophilum*, *Mycoplasma* sp., salmonellosis, bartonellosis Fungi: histoplasmosis Protozoa: cytauxzoonosis, toxoplasmosis Parasites: heartworm disease
	Neoplasia	Lymphoproliferative and myeloproliferative neoplasia, various solid tissue neoplasms
	Drugs/toxins	Chemotherapeutic drugs, griseofulvin, methimazole, propylthiouracil
	Miscellaneous	ITP, DIC, distribution disorders
Horse	Infectious	Viruses: EIA, EVA, VEE Bacteria: septicemia, endotoxemia, *A. phagocytophilum*, *Neorickettsia risticii*, salmonellosis
	Neoplasia	Lymphoproliferative and myeloproliferative neoplasia, various solid tissue neoplasms
	Drugs/toxins	Phenylbutazone, trimethoprim, penicillin, trichothecene mycotoxin, snakebites
	Miscellaneous	ITP, neonatal alloimmune thrombocytopenia, DIC, gastrointestinal disorders, distribution disorders
Cattle	Infectious	Viruses: BVD Bacteria: septic metritis/mastitis, septicemia, endotoxemia, salmonellosis Protozoa: babesiosis, theileriosis
	Drugs/toxins	Bracken fern toxicity, trichothecene mycotoxin
	Miscellaneous	ITP, DIC, distribution disorders, displaced abomasums

bacterial infections (e.g., leptospirosis, salmonellosis, borreliosis, bartonellosis) are sporadically associated with thrombocytopenia.[56,88]

Sepsis-associated thrombocytopenia is a marker of disease severity and a prognostic indicator; thus, platelet counts are included in human sepsis scoring systems.[26,63] The role of platelets in sepsis and the mechanisms that cause thrombocytopenia are not fully elucidated. Platelets participate in innate immunity and can internalize bacteria and viruses; however, whether this helps control infection or facilitates spread is unclear.[63] Thrombopoiesis appears to be upregulated during sepsis as evidenced by increased TPO and reticulated platelets.[63] Platelet activation mediated by endotoxin signaling through platelet and inflammatory cell toll-like receptors may lead to increased platelet consumption. Platelets also accumulate in the liver during sepsis and are subject to hemophagocytosis.

Viral infections are well-recognized causes of thrombocytopenia in animals. In dogs, thrombocytopenia occurs with canine distemper virus (CDV), herpes virus, infectious hepatitis, and parvovirus infection. Thrombocytopenia in CDV infection results from viral damage to platelets, secondary ITP, and decreased production due to infection of megakaryocytes.[4] Thrombocytopenia associated with herpes virus or infectious hepatitis virus infection results from endothelial damage and DIC.[119] In cats, thrombocytopenia is associated with feline infectious peritonitis (FIP), feline leukemia virus (FeLV), feline immunodeficiency virus (FIV), and parvovirus infection.[30] Thrombocytopenia in FIP is multifactorial and results from vasculitis, secondary ITP, direct viral damage to platelets, and DIC.[13] In horses, thrombocytopenia occurs with equine infectious anemia (EIA), equine viral arteritis (EVA), and Venezuelan equine encephalitis (VEE) virus infection.[104] Thrombocytopenia in EIA results from cytokine-mediated impairment of platelet production, secondary ITP, and nonimmune platelet destruction.[98] In cattle, thrombocytopenia is associated with bovine viral diarrhea (BVD) virus and primarily results from megakaryocyte damage and decreased thrombopoiesis, combined with peripheral

platelet consumption.[9,20] In pigs, thrombocytopenia follows infection with African swine fever or classical swine fever. In classical swine fever, thrombocytopenia likely results from increased vascular permeability, direct damage to platelets, platelet activation, impaired megakaryopoiesis, and platelet removal mediated by increased cytokine production due to MPS infection.[10,100] A new tick-borne virus, severe fever with thrombocytopenia syndrome virus (SFTSV), has been reported in Asia (particularly China). Clinical signs in humans include fever, gastrointestinal (GI) signs, dyspnea, hemorrhage, neurologic signs, lymphadenopathy, leukopenia, and thrombocytopenia.[120] The virus is carried by *Haemaphysalis longicornis* ticks and is a member of the *Bunyaviridae* family.[69] The SFTSV is zoonotic, infecting domestic and wild animals, and birds are suspected to spread the virus.[52]

Other agents associated with thrombocytopenia include protozoa (e.g., babesiosis, cytauxzoonosis, leishmaniasis, theileriosis, and toxoplasmosis), fungi (e.g., disseminated candidiasis, histoplasmosis), and metazoan parasites (e.g., heartworm infection in dogs and cats). Babesiosis often causes profound thrombocytopenia. Surprisingly, overt hemorrhage is uncommon, likely due to increased platelet activation, and thrombocytopenia is unrelated to survival.[40] Increased MPV has been observed in dogs infected with *Babesia spp*. Thrombocytopenia may involve immune-mediated platelet destruction, splenic sequestration, and DIC.[38]

Thrombocytopenia secondary to infection varies from mild to severe. Diagnostic tests include direct pathogen identification from microscopic examination of blood, bone marrow, or cytology smears, molecular techniques, via culture, or serology.

Drug-Induced Thrombocytopenia

Thrombocytopenia may follow exposure to drugs or toxins; however, confirmation of causation is difficult (see Chapters 15 and 80).[18] Drugs associated with thrombocytopenia in veterinary species are listed in Table 81.2. Decreased platelet production and increased platelet clearance, via immune-mediated and nonimmune-mediated processes, may cause drug-related thrombocytopenia. Chemotherapeutic drugs, including antimetabolites and alkylating agents, cause dose-dependent, predictable myelosuppression leading to pancytopenia. Chemotherapeutic-induced thrombocytopenia is usually transient and resolves after drug withdrawal or dosage adjustment; however, overdosage may result in irreversible bone marrow damage.[48] Other drugs (e.g., potentiated sulfonamides) are associated with idiosyncratic and unpredictable thrombocytopenia. Some drugs increase platelet clearance via nonimmune mechanisms such as macrophage or growth-factor-mediated endothelial activation.[18]

Drug-dependent antibodies may induce thrombocytopenia by targeting platelets and/or megakaryocytes, or by causing ADAMTS13 deficiency and resultant VWF-mediated platelet-rich thrombus formation.[18] Additionally, platelet apoptosis may play a role.[111] Pathologic antibodies may bind to a drug or metabolite adsorbed to the platelet membrane, a drug–platelet antigen complex, or a hidden antigen newly exposed on the platelet membrane. The MPS then clears platelets with bound antibody. Rarely, drug-independent ITP persists after drug withdrawal. PSAIg has been detected in dogs following administration of sulfonamides and in horses following administration of trimethoprim and penicillin.[64,76] Heparin-induced thrombocytopenia is characterized by large vessel thrombosis and thrombocytopenia. The pathogenesis involves antiplatelet factor 4/heparin antibodies; however, the syndrome has not been described in veterinary patients.[44]

Some drugs directly activate platelets causing increased consumption, or injure platelets causing removal by the MPS. Snake venoms may directly aggregate platelets, interact with platelet glycoproteins, induce thrombin-like substances that cause platelet aggregation, or result in platelet sequestration at sites of envenomation.[46]

Diagnosis of drug-associated thrombocytopenia generally relies on a history of drug exposure and normalization of platelet concentration (usually within 1–2 weeks) following cessation of drug therapy.

Neoplasia-Associated Thrombocytopenia

Thrombocytopenia is commonly associated with neoplasia.[17] A large study of thrombocytopenic dogs reported a 20.8% incidence of neoplasia,[12] with similar incidence in thrombocytopenic cats.[30] Common tumor types include lymphoma, carcinoma, hemangiosarcoma, and other hematopoietic neoplasia. Thrombocytopenia is frequently multifactorial; secondary to chemotherapy, decreased platelet production, or DIC.[67,122] Chemotherapeutic drugs cause thrombocytopenia via megakaryocyte toxicity, promoting immune-mediated destruction, splenic sequestration, and via thrombotic microangiopathy. Inadvertent bone marrow irradiation may also cause thrombocytopenia.[90]

In addition to chemotherapy, decreased platelet production may result from myelophthisis, secretion of estrogen by neoplastic cells, and bone marrow infiltration by hematologic neoplasia. Solid tumor infiltrates are less common, but may be seen with prostatic, mammary, pulmonary, and gastric tumors. The presence of splenic extramedullary hematopoiesis may give clues of underlying bone marrow disease due to rubricytosis and other morphologic changes in the peripheral blood, e.g., ovalocytes.[90] Neoplastic processes that cause significant hepatosplenomegaly, e.g., lymphoma, mast cell neoplasia, hemangiosarcoma, can result in platelet sequestration.[17] Thrombocytopenia secondary to DIC frequently complicates neoplasia. In one study, over 10% of dogs with malignant neoplasia had DIC, particularly hemangiosarcoma, mammary carcinoma, and pulmonary adenocarcinoma.[75] Neoplasia is the most commonly recognized cause of DIC in cats.[32]

Secondary ITP may cause thrombocytopenia in hemic and nonhemic neoplastic disorders. In humans, ITP is most commonly associated with lymphoproliferative diseases such as Hodgkin's lymphoma, large granular

leukemia, and chronic lymphocytic leukemia.[90] Other proposed mechanisms for neoplasia-induced thrombocytopenia include distribution disorders with platelet pooling (particularly with large vascular tumors such as hemangiosarcoma), platelet loss with tumor-associated hemorrhage, or platelet destruction by neoplastic cells. Thrombocytopenia was identified in 88% of dogs with hemophagocytic histiocytic sarcoma, presumably mediated by platelet phagocytosis by neoplastic cells.[79]

Clinical signs of neoplasia-induced thrombocytopenia are related to the underlying neoplasm, with variable hemorrhage. Diagnosis is based upon cytologic or histopathologic confirmation of neoplasia. Severe thrombocytopenia may influence treatment; however, a recent study demonstrated no increase in hemorrhagic complications in thrombocytopenic veterinary patients administered standard chemotherapy doses.[35]

Liver-Disease-Associated Thrombocytopenia

Thrombocytopenia of multifactorial causes is common in humans and animals with chronic and acute liver disease.[87] Severity of thrombocytopenia may have prognostic significance.[107] Proposed mechanisms include portal hypertension and splenomegaly with platelet sequestration, decreased TPO synthesis, and secondary ITP, particularly with viral hepatopathies. Thrombocytopenia can complicate diagnosis and treatment of liver disease. One study in dogs and cats showed that a platelet count less than 80,000/L was a significant risk factor for complications of percutaneous ultrasound guided biopsy,[7] and another study found that platelet count less than 150,000/L was associated with transfusion and open laparotomy in dogs undergoing laparoscopic liver biopsy.[89] In addition to platelet transfusion (see Chapter 86), treatment options in people include recombinant cytokines and TPO receptor agonists.[87]

Dilutional Thrombocytopenia

Dilutional thrombocytopenia has been reported in humans after major surgery or following massive transfusion, and in dogs following massive transfusion. In a study of 15 dogs that received either half of their estimated blood volume within 3 hours, or more than their total blood volume in 24 hours, all developed moderate thrombocytopenia (many had mild thrombocytopenia prior to transfusion) and electrolyte, acid base, and coagulation changes.[62] Thrombocytopenia in these cases likely results from transfusion of stored blood products devoid of platelets, large volume crystalloid administration, and platelet consumption due to the underlying disease process.[62]

Gestational Thrombocytopenia

Gestational thrombocytopenia occurs in 11.6% of human pregnancies and is usually mild, with minimal consequences for mother or child. However, platelet counts <100,000/L are associated with a higher incidence of neonatal thrombocytopenia and risk of recurrence in future pregnancies. The causes of thrombocytopenia may include hemodilution and increased platelet clearance.[19] Gestational thrombocytopenia is rare in animals, with reports limited to mice and cows,[123] and transient thrombocytopenia in a rabbit following amniotic fluid embolization in the absence of DIC.[93]

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CHAPTER 82

Thrombocytosis and Essential Thrombocythemia

JULIE ALLEN and TRACY STOKOL

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Acronyms and Abbreviations

CALR, calreticulin; CML, chronic myeloid leukemia; CMPD, chronic myeloproliferative disease; ET, essential thrombocythemia; GM-CSF, granulocyte–monocyte colony-stimulating factor; IDA, iron deficiency anemia; IL, interleukin; JAK2, janus kinase 2; MDS, myelodysplastic syndrome; MPL, myeloproliferative leukemia protein; MPN, myeloproliferative neoplasm; MPV, mean platelet volume; PCT, plateletcrit; PDW, platelet distribution width; PMF, primary myelofibrosis; PV, polycythemia vera; PVSG, Polycythemia Vera Study Group; SD, standard deviation; TPO, thrombopoietin; WHO, World Health Organization.

INTRODUCTION

Thrombocytosis, or thrombocythemia, is specifically defined as a high platelet count above the reference interval established for a given species. The methods available for measuring platelet count include estimates from stained peripheral blood smears, manual hemocytometer counts, quantitative buffy coat analysis, and automated methods based on impedance or laser-induced light scatter. Methods vary in precision and accuracy and the same sample may yield different values using different methods.[18,51,73] Therefore, most veterinary laboratories establish reference intervals specific for their own equipment for routinely analyzed species. Interpretation of platelet count is more difficult for species lacking laboratory-defined intervals. Knowledge of the normal physiology of the animal, combined with published reference intervals, can be used as a guide to result interpretation. Platelet counts in healthy animals vary markedly between species. Small rodents have relatively high counts, whereas horses have relatively low counts such that a normal platelet count in a mouse would be regarded as extreme thrombocytosis in a horse. The criteria for classifying thrombocytosis vary among clinical reports, making assessment of the frequency of the condition challenging. One review in dogs defined thrombocytosis as a platelet count >600 × $10^3/\mu L$,[47] whereas other studies categorized >500 × $10^3/\mu L$[4,91] as a thrombocytosis, with reported incidence ranging from 4.6–7.2%.[4,47,91] However, counts can be misleading as platelet mass (size × volume) may be a more clinically relevant indicator of hemostatic function, and platelet counts and platelet size often vary inversely. For example, Cavalier King Charles spaniels with breed-related macrothrombocytopenia have low platelet counts but normal platelet mass due to the large size of their platelets. As a result, assessment of plateletcrit (PCT) may be a more accurate method to assess platelet mass.[84]

Pseudohyperkalemia is an in vitro artifact of thrombocytosis that may develop in serum samples due to release of intracellular potassium during clotting.[19,40,57]

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Potassium concentrations can be 0.6 ± 0.2 mEq/L (mean ± SD) higher in serum than plasma in dogs with platelet counts between 150 and 600 × 10^3/μL. This difference increases (1.6 ± 0.76 mEq/L) in dogs with higher counts (>600 × 10^3/μL) and can be quite marked (up to 5 mEq/L) in dogs with extreme thrombocytosis (>1000 × 10^3 platelets/μL).[19,40,58] To avoid this artifact, heparinized plasma is the preferred sample for potassium measurement.

Thrombocytosis may represent an artifact (pseudothrombocytosis), or physiologic thrombocytosis due to altered trafficking of platelets (i.e., release from storage pools) or increased platelet production (enhanced thrombopoiesis). Enhanced thrombopoiesis can be a secondary or reactive process, due to an increase in thrombopoietic factors from an underlying disease, or a primary disorder (Table 82.1).

TABLE 82.1 Causes and associated mechanisms of thrombocytosis.

Cause	Mechanism
Pseudothrombocytosis	
Red blood cell ghosts or fragments, fragile leukocytes, microorganisms, or lipemia	Nonplatelet cell fragments or particles counted as platelets
Physiologic	
Epinephrine (trauma, exercise, excitement)	Splenic contraction
Postsplenectomy	Lack of sequestration
Reactive	
Inflammation, infection, hematopoietic and nonhematopoietic neoplasia, trauma, rebound from thrombocytopenia, postsplenectomy	Cytokine-mediated—directly or indirectly through TPO production. Enhanced megakaryopoiesis
Iron-deficiency-related	Unknown
Drug-induced, e.g., vincristine, epinephrine	Drug-dependent: vincristine increases megakaryopoiesis and megakaryocyte fragmentation, whereas epinephrine causes splenic contraction
Primary	
Chronic myeloproliferative disease: Essential thrombocythemia, chronic myeloid leukemia, chronic basophilic leukemia, polycythemia vera, primary myelofibrosis	Acquired or inherited genetic mutations causing TPO-independent proliferation, e.g., JAK2 signaling mutations
Acute megakaryocytic leukemia	Acquired or inherited mutations
Hereditary/familial[a]	Common disease genes: *JAK2*, *CALR*, *MPL*, *THPO*, *TET2*

[a] Not reported in animals.

To understand the pathophysiology of thrombocytosis, the factors governing thrombopoiesis are briefly reviewed in the following text.

THROMBOPOIESIS: OVERVIEW

Platelets are produced from megakaryocytes in the bone marrow in adult animals by a unique process that involves fragmentation of long cytoplasmic extensions (proplatelets) that the megakaryocyte inserts between bone marrow sinusoidal endothelial cells[29,30] (see Chapters 75 and 78). In adult mice, the spleen is the primary platelet-producing organ and there is evidence that the lung may play a role in platelet synthesis, particularly in states of thrombocytopenia or relative marrow stem cell deficiency in murine models.[36] Megakaryocytes are formed from progenitor cells derived from hematopoietic stem cells. Megakaryocytes undergo endomitosis to become polyploid before maturing and packaging their contents into proplatelets.[27] Recent studies suggest that megakaryocytes at all stages of development are distributed throughout the bone marrow and most reside adjacent to vessels.[71] Platelets can also be released via rupture of mature megakaryocyte membranes if there is an acute demand or inflammation. The latter appears to be mediated by interleukin (IL)-1α.[48] It has been estimated that approximately 2000–3000 platelets are produced from each megakaryocyte, with the residual megakaryocyte nucleus degraded by phagocytes within the bone marrow.[27]

Thrombopoietin (TPO) is the main thrombopoietic hormone, produced primarily in the liver and kidney. Genetically engineered mice deficient in TPO or its receptor, MPL (product of myeloproliferative leukemia virus oncogene, *c-Mpl*), have platelet counts 10–15% of normal, supporting the presence of additional thrombopoietic factors.[29–31] Thrombopoietin binds to MPL to mediate downstream responses through the nonreceptor tyrosine kinase, janus kinase 2 (JAK2), which in turn activates the transcription factors, signal transducer and activator of transcription (STAT)3 and STAT5.[28] Signal transduction also occurs through Ras and Akt pathways.[29] The thrombopoietin receptor, MPL, is highly expressed on megakaryocytes and platelets, but is also expressed on other cell types, including hematopoietic stem cells. Other transcription factors, including GATA-1, RUNX1 and PU.1, are also involved in megakaryopoiesis.[20,41,81] Thrombopoietin mainly acts as a differentiation factor, stimulating stem cells to differentiate along the megakaryocytic lineage. It acts in concert (additively or synergistically) with other hematopoietic cytokines, including stem cell factor, IL-3, and erythropoietin, to promote megakaryocyte proliferation and polyploidy. Serum TPO levels are thought to be regulated by binding to MPL on platelets, with subsequent internalization and degradation. In a process referred to as the "sponge theory,"[39,70] circulating TPO levels are low in patients with thrombocytosis due to its binding

and internalization by high platelet mass. Conversely, thrombocytopenia results in relatively little TPO removal because of the few platelets in the blood.[33,70] Interleukin-6 can increase hepatic TPO production as does binding of senescent platelets to the Ashwell–Morell receptor on hepatocytes.[29] Insulin-like growth factor-1 can facilitate thrombopoiesis by promoting hematopoietic stem cell expansion, megakaryocyte differentiation, proplatelet formation, and platelet release mainly through activation of the Akt signaling pathway. The latter seems to be independent of TPO signaling, and requires the help of steroid receptor coactivator-3.[16] Growth hormone also seems to play a role in regulating late-stage thrombopoiesis. Additionally, microRNAs can affect megakaryocyte differentiation, particularly miR-28 and miR-180.[83]

The precise mechanisms controlling platelet release from proplatelets remain under investigation; however, this process involves adhesion of megakaryocytes to subendothelial matrix components (particularly fibronectin and thrombospondin), cytoskeletal elements (actin, microtubules), matrix metalloproteinases, and shear forces (which facilitate proplatelet fragmentation) (see Chapter 78).[39] By contrast, type I collagen seems to suppress proplatelet production.[39] The role of the bone marrow microenvironment is therefore extremely important. Stromal cell-derived factor-1, also known as chemokine 12, is considered vital for attracting megakaryocytes and other hematopoietic cells and stimulating their growth.[38] Other important thrombopoietic cytokines include the IL-6 superfamily members (IL-6, IL-11, leukemia inhibitory factor), granulocyte–monocyte colony-stimulating factor (GM-CSF), IL-1α, IL-3, and IL-12.[28] von Willebrand factor bound to GPIb/V/IX receptor seems to influence proplatelet formation.[81] Apoptosis has been speculated to play a role in platelet formation and activation;[13] however, recent studies have shown that the intrinsic apoptotic pathway and caspase-9 do not seem to be important.[90] Casein kinase 2 has been detected in platelets and megakaryocytes and is required for megakaryopoiesis and calcium-triggered platelet activation.[41,88] The final stages of platelet production appear to be TPO-independent and in vitro withdrawal of TPO is reported to stimulate proplatelet formation.

Alterations in any of the numerous factors that affect thrombopoiesis can therefore lead to thrombocytosis, including increased concentrations of thrombopoietic cytokines, ligand-independent MPL activation, and abnormal activation of the JAK2/STAT and PI3K–Akt signaling pathways.

CAUSES OF THROMBOCYTOSIS

Pseudothrombocytosis

Pseudothrombocytosis occurs if small, fragmented, or hemolyzed erythrocytes, leukocyte fragments (from normal or leukemic cells), or particulate cellular debris including bacteria, protozoa, fungi, cryoglobulin, or lipids are erroneously counted as platelets (Figure 82.1).[21,43,92] In humans, Howell–Jolly bodies and erythrocyte Pappenheimer bodies (iron inclusions) have been misidentified as platelets with older hematology analyzers.[43,92] Hemolysis can produce ghost cells, which are erroneously enumerated as platelets, as reported for equine blood stored at 4 °C.[17] Although this artifact can be analyzer-dependent, it can potentially occur with any automated method. Thus, it is important to always verify platelet counts by estimating numbers from stained blood smears. Although estimates are of questionable accuracy,[51] smear examination can quickly confirm an increased platelet count, identify morphologic defects in platelets, and potentially reveal abnormalities that may cause pseudothrombocytosis.

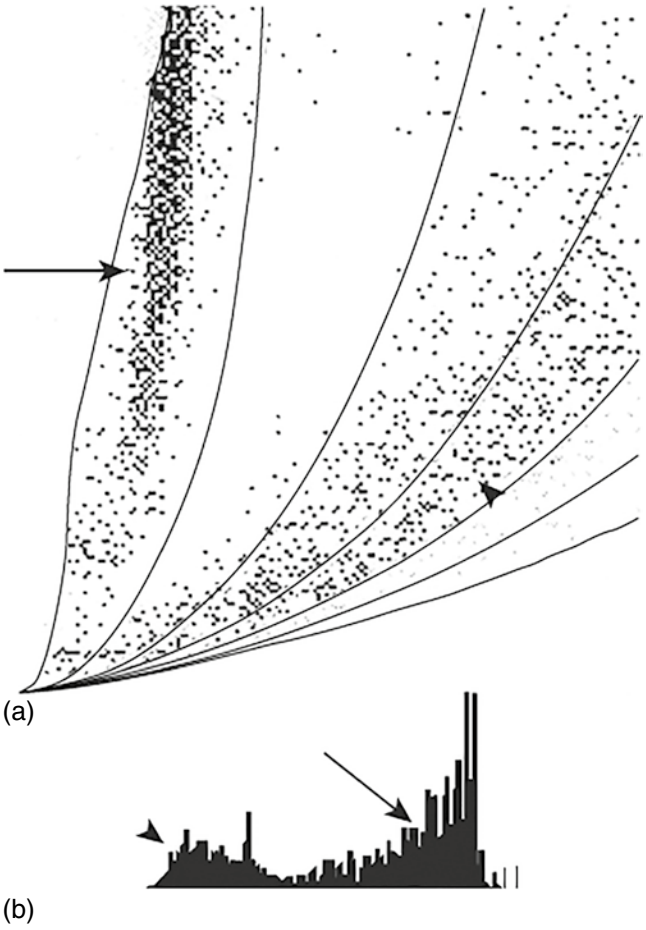


FIGURE 82.1 Pseudothrombocytosis in a hemolyzed sample from a dog with immune-mediated hemolytic anemia. (a) Hemolyzed red blood cells (arrow) are the events entering the upper left portion of the platelet cytogram (forward scatter versus side scatter dotplot) and are counted as platelets on ADVIA hematology analyzers (laser-light-scatter-based system); (b) The hemolyzed erythrocytes can also be visualized as a second population of macrocytic cells (arrow) in the platelet histogram (cell number versus cell volume) and will falsely increase the mean platelet volume (MPV) and platelet distribution width (PDW). The "true" platelet clusters are identified by the arrowhead in both cytograms. (Sources: Frotscher et al. [21]; Van Der Meer et al. [43]; Zandecki et al.[92])

Physiologic Thrombocytosis

Up to one-third of platelets are normally sequestered in the splenic red pulp.[87] These stores can be released upon splenic contraction mediated by epinephrine, although the effects in dogs are variable.[50] A transient or persistent thrombocytosis may occur postsplenectomy in humans,[35] and this has been described as an inconsistent finding in dogs for several months following splenectomy.[59] Some authors have also suggested the presence of an adrenaline-responsive, marginated platelet pool in the lungs, although this pool is likely small.[83]

Reactive Thrombocytosis

Reactive thrombocytosis (or secondary thrombocytosis) is due to cytokine stimulation of thrombopoiesis. It is the most common cause of thrombocytosis in humans and animals and occurs secondary to various disorders (Table 82.1).[4,47,61,68,69,91] Thrombocytosis in humans is typically asymptomatic. In a study of 890 adult subjects, 94% had reactive thrombocytosis of which over 50% were attributed to an infectious cause.[60] Several retrospective studies in dogs have shown that inflammation and neoplasia are the most common diseases associated with thrombocytosis, although different platelet count thresholds defined thrombocytosis in these studies.[4,47,91] Carcinoma is the most commonly associated tumor, closely followed by lymphoma. For patients with lymphoma, however, the waters are often muddied by the use of therapeutic agents such as corticosteroids and vincristine that may also contribute to thrombocytosis (see later section on drugs).[91] The platelet count can also rebound following treatment of any tumor with chemotherapeutic drugs. Drug effects may also be seen with immune-mediated disorders (the most common inflammatory disease associated with thrombocytosis in a number of studies) where corticosteroids are often used for therapy. A study of thrombocytosis in horses revealed infectious or inflammatory diseases, such as primary bacterial infection (peritonitis, pleuropneumonia, septic arthritis) and colitis, predominating as the underlying cause.[69] A retrospective study of thrombocytosis in 50 cats (defined as platelet counts greater than >700 × 10^9/L) again revealed that infection or inflammation were the most common diseases associated with reactive thrombocytosis, with the gastrointestinal tract and endocrine system being the most common organ systems affected.[61]

Paraneoplastic thrombocytosis has been seen with a variety of tumors, particularly solid tumors, such as human ovarian carcinoma.[37,56,89] Thrombocytosis can serve as a biomarker for cancer, sometimes appearing before the tumor is evident.[67] Tumors can increase TPO production, often via cytokines such as IL-6, leading to increased platelet production. Tumors may "hijack" platelet functions to promote progression and metastasis. Platelets can help neoplastic cells evade the immune system by coating circulating tumor cells; some tumor cells even mimic platelets by displaying similar markers.[82] Platelet-derived factors aid extravasation and transmigration of tumor cells and facilitate angiogenesis.[56] Studies in humans have shown that cancer patients with higher platelet counts had higher IL-6 and TPO levels and shorter progression-free survival times.[10,37,56] Therefore, current research is focusing on whether reducing platelet counts or inhibiting their function could block platelet-associated carcinogenesis. Patients taking aspirin for cardiovascular disease have a decreased risk of cancer metastasis;[64] however, more studies are needed into the role of platelets in neoplastic diseases.

Depending on the underlying cause, reactive thrombocytosis can be transient or persistent, so documenting a thrombocytosis more than once (particularly if done less than 1–2 months apart) does not exclude a reactive condition. In addition, the increase in platelet count can be quite marked in reactive conditions (>1000 × 10^3/μL) (Figure 82.2).

A plethora of cytokines are potentially responsible for reactive thrombocytosis, including TPO, IL-6, GM-CSF, IL-3, IL-11, and erythropoietin.[1] High cytokine levels may result from constitutive secretion by tumors or upregulation triggered by inflammation, infection, or neoplasia. The inflammatory cytokine IL-6 is considered one of the main mediators of reactive thrombocytosis. Concentrations of IL-6 levels are increased in patients with thrombocytosis due to inflammation and neoplasia and persistently high levels are supportive of reactive thrombocytosis.[77] IL-6 is thought to stimulate platelet

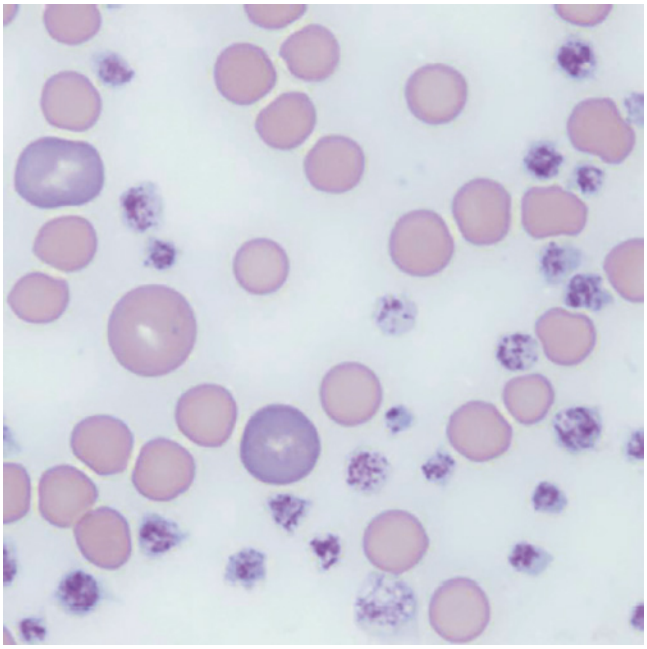


FIGURE 82.2 Peripheral blood smear from a 12-week-old kitten with iron deficiency anemia secondary to flea infestation. Many platelets, which vary markedly in size, are seen in the smear. The kitten had an extreme thrombocytosis (platelet count = 1900 × 10^3/μL), which was presumably reactive. Modified Wright's stain; 1000× magnification. (Source: Courtesy of Dr. Maria Toivio-Kinnucan, Auburn University)

production indirectly through induction of hepatocyte TPO production.[14] Measurement of cytokines, such as IL-6, could therefore be performed to confirm or refute the presence of reactive thrombocytosis.[1,10] However, cytokine measurement is often not practical and high values often normalize by the time thrombocytosis is identified. Surrogate markers of inflammation are often used, such as the measurement of acute phase proteins such as C-reactive protein.[15] Measurement of TPO is often not helpful in differentiating reactive from clonal thrombocytosis due to overlap of levels between the two groups;[11] however, clinical findings may aid in differentiation between these two disorders.

Iron Deficiency Anemia

Thrombocytosis, via an unknown mechanism, is common in patients with iron deficiency anemia (IDA), with platelet counts returning to within reference intervals after iron supplementation. Cytokine levels are often not increased in patients with iron deficiency compared to those with reactive thrombocytosis, leading to a preliminary hypothesis that iron deficiency somehow encourages megakaryocyte proliferation independent of thrombopoietin, IL-6, and IL-11.[26,32] A recent study in human blood donors found minimal correlation between serum ferritin and platelet counts,[26] a finding replicated in other studies.[32] Some authors have noted that platelet counts correlated instead with serum iron and transferrin saturation, and higher platelet counts, PCT, and MPV were observed in IDA patients having more severe and increasingly hypochromic anemias.[53] The expected increase in erythropoietin levels in IDA patients has been proposed to contribute to thrombocytosis through its homology with TPO and possible off-target action. In support of this theory, administration of erythropoietin results in thrombocytosis in healthy controls and renal failure patients. It is not clear, however, whether erythropoietin-induced red cell production results in iron deficiency or thrombocytopoiesis represents a more direct effect. A combined effect is possible because a mild increase in platelet count is still seen with concurrent iron supplementation.[72] On the other hand, a consistent relationship between erythropoietin levels and thrombocytosis has not been identified in IDA, and some severe IDA patients actually develop thrombocytopenia.[32] Although erythropoietin and TPO receptors display some homology, erythropoietin has not been shown to bind MPL in experimental studies.[12,45] The two hormones may act synergistically to increase platelet counts in IDA. Further mechanistic studies are needed to understand the effects of erythropoietin on thrombopoiesis and the likely multifactorial basis of thrombocytosis associated with IDA.

Drug-Induced Thrombocytosis

Numerous drugs have been associated with thrombocytosis in humans, including the vinca alkaloids (especially vincristine), gemcitabine, iron sulfate, ciprofloxacin, piperacillin/tazobactam, all-trans retinoic acid, clozapine, low-molecular-weight heparin, epinephrine, and erythropoietin.[86] It is possible that the thrombocytosis documented in patients on drug therapy is secondary to their underlying disease or a rebound from thrombocytopenia, rather than a drug response per se. Among the drugs utilized in veterinary medicine, vincristine is often administered for its effects on thrombopoiesis in the treatment of immune-mediated thrombocytopenia.[66] Erythropoietin is often used in the management of chronic renal failure and has also been associated with thrombocytosis as mentioned in the earlier text. Low-molecular-weight heparin (LMWH) has more recently come into use for the prevention and treatment of thromboembolism in cats with aortic (systemic) thromboembolism and dogs with immune-mediated hemolytic anemia. Thrombocytosis, of unknown etiology, has been described in humans treated with LMWH, but not in domestic species.[86] Epinephrine given to dogs in an experimental study induced a rapid (within a few minutes), transient (less than 60 minutes) thrombocytosis through splenic contraction, although there was large interanimal variability in the response.[50] The reported effects of glucocorticoids on platelet count are conflicting. Although hyperadrenocorticism has been associated with thrombocytosis in dogs,[52] several studies have not shown a consistent increase in platelet counts in healthy dogs given daily doses of up to 2 mg/kg prednisolone.[46,63]

The main differential diagnosis for reactive thrombocytosis in animals is essential thrombocythemia (ET). However, there are few reported cases of ET in dogs and cats,[7,23] indicating that thrombocytosis in most animals (like humans) is likely reactive.

ESSENTIAL THROMBOCYTHEMIA IN HUMANS

Essential thrombocythemia is a clonal disorder of hematopoiesis that primarily affects megakaryocytes. It is classified as a BCR-ABL-negative myeloproliferative neoplasm (MPN) or chronic myeloproliferative disease (CMPD), along with polycythemia vera (PV) and primary myelofibrosis (PMF) by the World Health Organization (WHO).[5,6,74,78] All of these disorders are characterized by mutations in *JAK2*, calreticulin (*CALR*), or *MPL*. Essential thrombocythemia is the most frequently diagnosed MPN and usually affects older patients (>60 years), with a higher prevalence in females (although males have an increased risk of venous thrombosis).[31,74] Up to two-thirds of ET patients are asymptomatic at diagnosis and their median survival time of 20 years is longer than PV and PMF.[74,78] Affected individuals most often suffer from microvascular thrombosis, but may develop macrovascular thrombosis (predominantly occurring in arteries supplying the brain, heart, and extremities) or, paradoxically, hemorrhage. Weight loss, pruritus, fatigue, and rarely splenomegaly are also noted.[79]

Essential thrombocythemia is characterized by a marked thrombocytosis in peripheral blood and bone

marrow megakaryocytic hyperplasia.[5] Leukocyte and erythrocyte counts are generally within normal limits. A left shift in neutrophils, marked dysplasia in any lineage, or dacryocytes suggests an alternate MPN diagnosis. Platelets can vary markedly in size with giant forms, but they are usually not dysplastic. A histologic hallmark of ET is the presence of large to giant megakaryocytes with deeply or hyperlobulated, occasionally staghorn-like nuclei, found singly or in small loose clusters diffusely throughout a normocellular to slightly hypercellular marrow. Other evidence of dysplasia is largely lacking and there is no or <5% reticulin fibrosis/grade 1 fibrosis (based on established criteria at the onset of disease).[5,74,80] The main differential diagnoses for ET are reactive thrombocytosis (which is far more common) and other MPN described in the following text.

The discovery of various mutations in *JAK2*, *CALR*, and *MPL* has advanced our understanding of the pathogenesis of ET and related MPN. Janus-associated kinase-2 has a critical role in megakaryocyte and other hematopoietic cell signaling, upregulates anti-apoptotic proteins, participates in *MPL* trafficking, and induces hematopoietic cytokine (e.g., IL-3, TPO) production.[2] The main *JAK2* mutation results in a phenylalanine for valine substitution at residue 617 (V617F) of the autoinhibitory domain and results in constitutive *JAK2* activation. The V617F mutation is not specific for ET and also occurs in PV (up to 90% incidence) and PMF, perhaps explaining the phenotypic and biologic similarities among these neoplasms.[2,74] Testing for the V617F mutation has been added to a proposed diagnostic scheme for ET (Table 82.2). However, ET is genetically heterogeneous with *JAK2* V167F found in at most 55% of ET patients. Mutations in the gene encoding *CALR* occur in 15–24% of ET patients (and 25–35% of PMF patients).[6,74] This multifunctional protein acts as a major calcium-binding (storage) protein in the lumen of the endoplasmic reticulum and it is found in the nucleus with a putative role in transcription regulation. Additionally, 4% of ET patients, 8% of PMF patients, and rarely PV patients have *MPL* mutations.[6,74] Mutations in *JAK2* also occur in other tumors, such as chronic myelomonocytic leukemia, acute myeloid leukemia, myelodysplastic syndrome (MDS), and chronic myeloid leukemia (CML), whereas *MPL* mutations can be seen in congenital amegakaryocytic thrombocytopenia. There is also emerging evidence of mutations in other genes in ET patients that affect cytokine signaling, thereby disrupting transcriptional and epigenetic regulation and RNA splicing. The absence of a mutation in a known disease gene in a suspect ET patient does not rule out ET because up to 20% of human patients are negative for all defined mutations.[5,6,74] Bone marrow sampling is required in these patients to evaluate for the typical morphologic features of ET and differentiate it from other MPNs (see Table 82.2).

Although patients with ET have reduced life spans compared to age- and sex-matched healthy individuals, survival times are approximately 33 years in patients under 60 years of age at diagnosis. Thus, ET is considered an indolent disease, compared to other MPN.[74,76]

TABLE 82.2 WHO criteria for essential thrombocythemia in humans.

Major criteria	Minor criterion
1. Platelet count >450 × 10^3/μL 2. Bone marrow biopsy showing proliferation of megakaryocytes with increased numbers of enlarged, mature megakaryocytes with hyperlobulated nuclei. No significant hyperplasia of other lineages, a left shift in neutrophil granulopoiesis or erythropoiesis, and usually no reticulin fibrosis (minor or grade-1 reticulin fibrosis is rare) 3. Not meeting WHO criteria for BCR-ABL+ CML, PV, PMF, MDS, or other myeloid neoplasms 4. Presence of *JAK2*, *CALR*, or *MPL* mutation	Presence of a clonal marker or absence of evidence for reactive thrombocytosis

Diagnosis of ET requires meeting all four major criteria or the first three major criteria and the minor criterion.

Unlike CML, ET evolves into acute leukemia in only 5% of cases, but can transform into PMF. Thrombohemorrhagic symptoms contribute to morbidity and mortality in ET patients; consequently, cytoreductive therapy is recommended for patients likely to develop these complications. Such therapy includes platelet apheresis, hydroxyurea, anagrelide (a quinazoline derivative that inhibits megakaryocyte proliferation and differentiation), busulfan, pipobroman, and interferon-α.[79] Aspirin is frequently administered to patients at risk for thrombosis. Newer drugs targeting generic or JAK2 kinases may have promise in treating ET.[22,85]

Differentiating Essential Thrombocythemia from Reactive Thrombocytosis

In humans, laboratory and clinical criteria are used to distinguish reactive thrombocytosis from ET (Table 82.3). Although platelet counts trend higher in ET, the degree of thrombocytosis cannot be relied upon for differentiation. There have been numerous studies evaluating MPV in MPN versus reactive thrombocytosis, but results have been conflicting.[34] However, a normal platelet distribution width (PDW) has a negative predictive value of 99.8% for MPN, i.e., more indicative of a reactive process.[34] Although a recent study found increased reticulated platelets in patients with ET versus healthy controls, the reticulated platelet percentage did not differentiate ET from other causes in patients with thrombocytosis.[54] A higher risk of thrombosis, associated with hyperaggregability, was seen in patients with increased reticulated platelets.[54,60] A diagnosis of ET is also favored by a history of thrombohemorrhagic events, mild to

TABLE 82.3 Criteria for differentiating reactive thrombocytosis from essential thrombocythemia in humans.

Criteria	Reactive thrombocytosis	Essential thrombocythemia
Clinical		
Underlying disease	Yes	No
Thrombosis or hemorrhage	No[a]	Yes
Splenomegaly	No	Mild to moderate (due to extramedullary hematopoiesis)
Laboratory		
Platelet morphology	Normal	Normal to giant platelets, may see hypogranular platelets
Bone marrow[b]	Normocellular to hypercellular (possible concurrent granulocytic hyperplasia) Megakaryocytic hyperplasia (normal size, morphology)	Normocellular megakaryocytic hyperplasia (large cells, hyperlobulated or "staghorn" nuclei seen in loose clusters)
Inflammatory markers (IL-6, fibrinogen, C-reactive protein)	High (may be normal)	Normal (may be high)
TPO levels	High or normal	High or normal[c]
Platelet aggregation	Normal	Abnormal (particularly to epinephrine) in 63–100%

[a] May occur if initiated by underlying disease.
[b] Although bone marrow changes are emphasized in the WHO classification scheme for ET, they may be quite subtle and are not present in all patients.
[c] High TPO levels in ET have been attributed to downregulation of MPL on platelets and megakaryocytes.

moderate nonprogressive splenomegaly (due to extramedullary hematopoiesis), no evidence of inflammation, abnormally large megakaryocytes in the bone marrow, and identification of *JAK2*, *CALR*, or *MPL* mutations.[2,11,31]

Differentiating Essential Thrombocythemia from other Chronic Myeloproliferative Disorders

Thrombocytosis is a common feature of all MPNs, although the incidence and degree of increase differ between them. There is also substantial overlap in clinical presentation, biology, and laboratory features between these diseases.[2,3,5,6] Differentiation first involves eliminating CML and MDS. The former is largely excluded by identifying typical changes in blood (moderate to severe nonregenerative anemia, marked leukocytosis with many blasts) and presence of the Philadelphia chromosome (BCR-ABL), although BCR-ABL-negative CMLs do occur.[3,78] Substantial dysplasia and genetic abnormalities, such as the del(5q), enable the diagnosis of most cases of MDS, including most of the MDS/MPN overlap syndromes.[3] Differentiating ET from MPNs such as PV and PMF can be challenging.[6] In 1986, the Polycythemia Vera Study Group (PVSG) developed diagnostic criteria for ET. The diagnosis was one of exclusion; i.e., ruling out reactive thrombocytosis (particularly due to iron deficiency), PV, and CML (mainly based on lack of the Philadelphia chromosome). In 2001, the WHO established a diagnostic scheme for ET. Like the PVSG criteria, ET was primarily a diagnosis of exclusion, with bone marrow histopathology added to exclude early or prefibrotic PMF. However, the WHO scheme excluded approximately 30% of ET patients with platelet counts below the designated cutoff (600,000/μL). The revised, 2008 WHO proposal used a platelet cutoff of 450×10^3/μL, incorporated testing for *JAK2*, *CALR*, and *MPL* mutations,[6,49] and emphasized the importance of differentiating ET from prefibrotic PMF as the latter has a much poorer prognosis.[3,6]

Although genetic testing is helpful, bone marrow evaluation is also key for differentiating ET from PV and PMF. In contrast to ET, PV is characterized by proliferation of all hematopoietic lineages with pleomorphic megakaryocytes.[74] "Masked" PV can appear similar to *JAK2*-mutated ET, as these patients are initially not polycythemic (due to concurrent iron deficiency), but the bone marrow changes can help differentiate between them.[74] In PMF, megakaryocytes are increased, abnormally distributed (adjacent to bony trabeculae and in sinuses), and display substantial atypia (dense clusters, nuclear:cytoplasmic asynchrony, irregularly folded, and hyperchromatic to hypochromatic nuclei), and there is usually obvious reticulin and/or collagen fibrosis.[6,74]

Prefibrotic PMF and MDS/MPN with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T) are even more challenging, as they can appear very similar to ET.[6,78] In prefibrotic PMF, the bone marrow has a normal to small amount of reticulin (grade 0 or 1) like ET, but megakaryocytes are atypical as in full-blown PMF.[6,78] Additionally, patients with prefibrotic PMF often have granulocytic hyperplasia and erythroid hypoplasia, features lacking in ET patients. Prefibrotic PMF patients also have higher white blood cell counts, lower hemoglobin levels, higher lactate dehydrogenase activities, few peripheral erythroblasts or myeloblasts, and an increased incidence of palpable splenomegaly.[5,49] In MDS/MPN with ring sideroblasts and thrombocytosis, megakaryocytes have morphologic changes that resemble those seen in ET or PMF and patients can have *JAK2*, or less frequently *CALR* or *MPL*, mutations. However, affected patients have refractory anemia and dyserythropoiesis (15% or more of the erythroid precursors are ring sideroblasts), and can have additional mutations in the spliceosome gene, *SF3B1*.[3,6]

ESSENTIAL THROMBOCYTHEMIA IN ANIMALS

Many of the reported individual case reports of ET in animals do not fit current definitions of ET in humans and are likely reactive thrombocytosis, acute megakaryoblastic leukemia, or CML. In two cases that may be true ET (in a dog and a cat), PVSG criteria were used for diagnosis.[7,23] Both animals had a marked persistent thrombocytosis (>600 × 10^3 platelets/μL for >1 month), megakaryocytic hyperplasia, normal TPO levels, and no evidence of dysplasia, iron deficiency, or underlying inflammation. It is possible that ET is a different disorder in animals than in humans and diagnostic criteria for humans (including genetic mutations) may not be valid in animals, although the V617F *JAK2* mutation has been documented in dogs with PV.[9]

The main differential diagnosis for ET in animals is a reactive or cytokine-driven thrombocytosis, which is far more common regardless of platelet count.[4,47,61,69,91] Essential thrombocythemia is an extremely rare diagnosis in animals and, as in humans, remains one of exclusion. It is very difficult to differentiate ET from other chronic myeloproliferative diseases (e.g., CML), which are also quite rare. A diagnosis of ET should only be entertained in animals with a sustained (more than 1–2 months) and unexplained thrombocytosis and no evidence of CML or MDS (low numbers of myeloblasts, and no dysplasia, leukocytosis, or severe anemia). It is possible that the increased availability of sensitive markers of inflammation, such as acute-phase reactant proteins,[15] may help distinguish between reactive thrombocytosis and ET in animals. Testing for *JAK2* mutations is also available at some institutions for research purposes,[9] and may become more widely available with the advent of commercial genetic test services.

Thrombocytosis has also been reported with acute megakaryoblastic leukemia and other CMPD, including basophilic leukemia in animals.[42,62]

FAMILIAL/INHERITED THROMBOCYTOSIS

In humans, this rare disorder has been attributed to germline mutations in genes involved in thrombopoiesis (*MPL*, *TPO*, *JAK2*), resulting in non-neoplastic polyclonal thrombopoiesis.[2,8,55] High platelet counts are observed at birth or an early age. Affected individuals may be asymptomatic or suffer from thrombohemorrhagic complications.

CONSEQUENCES OF THROMBOCYTOSIS

Thrombohemorrhagic events represent the most serious consequence of thrombocytosis.[11,75] Reactive thrombocytosis is often self-limiting without specific treatment, although intervention might be considered for paraneoplastic cases.[25,37] Reactive thrombocytosis occasionally results in venous thrombosis in people, usually in concert with diseases or conditions such as hypercholesterolemia. Human patients with clonal thrombocytosis are often treated to minimize the possibility of thrombotic complications.[11,24,31] The latter comprise microvascular thromboses, with signs of headache, syncope, and erythromelalgia, to macrovascular thromboses causing stroke or transient ischemic attacks.[11] Age (>60 years), prior history of thrombosis, concurrent leukocytosis, and presence of *JAK2* mutations are all associated with an increased risk in patients with ET.[11,24] Antiplatelet therapy and measures to decrease concurrent risk factors are therefore often part of the therapeutic plan in ET patients. Paradoxically, some patients with clonal thrombocytosis can experience bleeding, typically from mucocutaneous junctions.[11] This is most often seen in patients with extremely high platelet counts (>1500 × 10^3/μL). The cause of hemorrhage is multifactorial and due partly to platelet function defects and acquired von Willebrand disease associated with increased platelet adsorption of large von Willebrand factor multimers and increased proteolysis of von Willebrand factor.[44,65,79] There is less information regarding the risk of bleeding or thrombosis in veterinary patients, with rare reports of thrombosis with paraneoplastic thrombocytosis.[25] Low-dose aspirin or clopidogrel could be considered in at-risk patients.

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CHAPTER 83

von Willebrand Disease

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Disease Mechanism
Disease Classification
  VWD Subtypes
  Affected Species and Breeds
Inheritance and Expression Patterns
  Hereditary VWD
  Acquired VWD
von Willebrand Factor Assays
  Quantitative VWF Assays
  Functional and Structural VWF Assays
  Point-of-Care Analyses
Clinical Management of VWD
  Clinical Signs
  Diagnostic Evaluation
  Treatment of VWD
  Transfusion Therapy Guidelines
  Nontransfusion Therapy

Acronyms and Abbreviations

AVWS, acquired von Willebrand syndrome; ADAMTS13, a disintegrin and metalloprotease with thrombospondin repeats; DDAVP, deamino-8-D-arginine vasopressin; FVIII, coagulation factor VIII; GP, glycoprotein; MW, molecular weight; OMIM, online Mendelian inheritance in man; PFA, platelet function analyzer; ULVWF, ultralarge VWF; VWD, von Willebrand disease; VWF, von Willebrand factor; VWF:Ag, von Willebrand factor antigen; VWF:CB, von Willebrand factor collagen binding; VWF:RCo, von Willebrand factor ristocetin cofactor activity.

Von Willebrand disease (VWD) is the most common hereditary bleeding disorder in dogs[1,13] and people,[8,15,21] and the trait has been identified in many other species. The disease is heterogeneous, caused by a variety of defects in von Willebrand factor (VWF) protein that influence bleeding severity and complicate clinical diagnosis and management.[15,21,25]

DISEASE MECHANISM

The bleeding tendency of VWD is caused by quantitative and functional deficiencies of VWF, a large multimeric plasma glycoprotein (GP) required for platelet adhesion at sites of vessel injury (Figure 83.1).[8,15,17]

Endothelial cells are the major site of VWF synthesis and storage.[7,17] Platelets provide a secondary pool of VWF in some species; however, canine platelets contain only trace amounts of VWF.[5] Mature VWF circulates as long strands of subunits (multimers) assembled within the endoplasmic reticulum and Golgi apparatus. Initially, two VWF monomers join to form dimers, followed by the association of dimers into heterogeneous, variably sized multimers including ultralarge VWF (ULVWF). Multimers range in molecular weight (MW) size from 500 to 20,000 kDa. Endothelial cells secrete VWF via steady-state constitutive pathways and release the protein from storage organelles in response to stimuli such as thrombin and epinephrine.

Under normal blood flow conditions, VWF circulates as a globular protein that exhibits low affinity for platelets. Hydrodynamic forces generated at injury sites facilitate unfolding and elongation of VWF resulting in efficient binding of VWF's A2 domain to subendothelial collagen (see Figure 83.2). As the immobilized VWF is subject to

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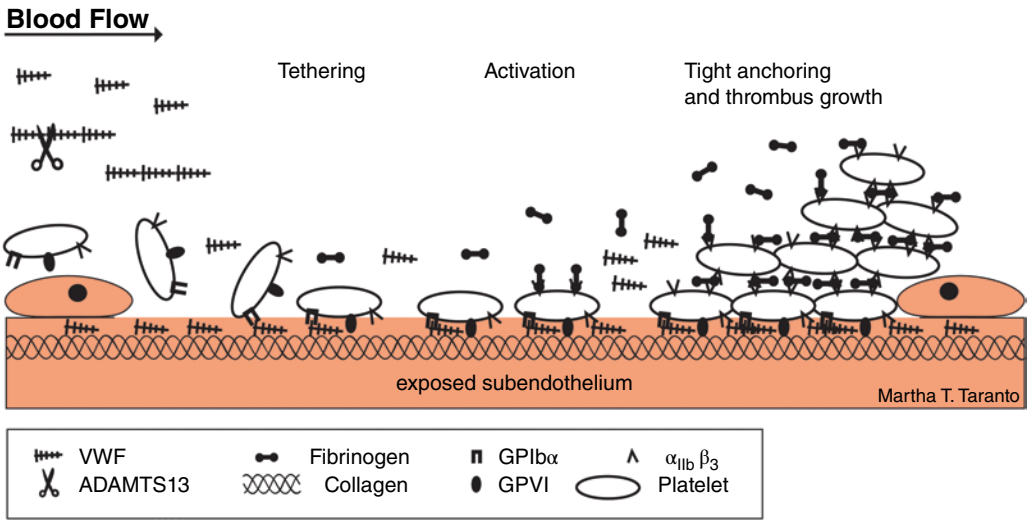


FIGURE 83.1 Schematic diagram illustrating the role of VWF in platelet adhesion, aggregation, and thrombus growth. At the site of blood vessel injury, platelets transiently adhere to subendothelial VWF via platelet GPIbα receptors. This slows platelet movement and triggers the engagement of platelet receptors, including the collagen receptor, GPVI. Receptor engagement results in platelet activation and stable attachment. Activated platelets secrete additional prostimulatory molecules and bind to plasma fibrinogen and VWF to form a tight anchoring matrix for platelet recruitment and ultimately thrombus formation. Upon release from endothelial cells, ultralarge VWF multimers are rapidly cleaved by ADAMTS13. (**Sources:** Favaloro[8]; Leebeek and Eikenboom[15]; Lenting et al.[17])

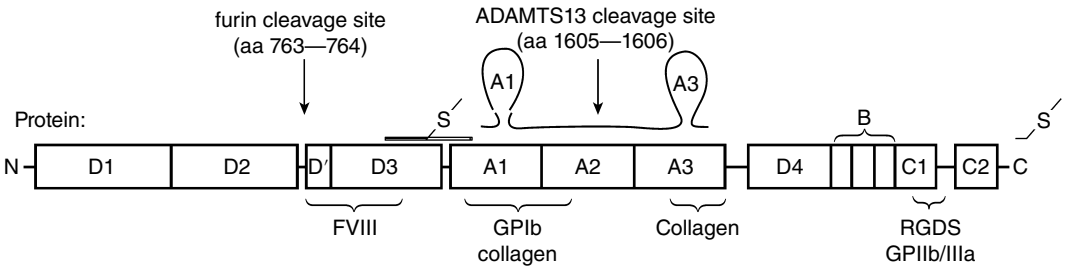


FIGURE 83.2 Domain structure of VWF protein. Functional regions representing binding sites to factor VIII (FVIII), platelet GPIb and GPIIb/IIIa (at R-G-D-S sites), and collagen are indicated below the protein sequence and cleavage sites for furin and ADAMTS13 are indicated above. The amino acid numbering represents human VWF protein.

increasing tensile forces, its A1 domain converts to a high-affinity binding site for platelet GPIbα. This platelet–VWF engagement leads to integrin-independent platelet adhesion and aggregation. VWF and fibrinogen mediate intraplatelet bridging upon activation of platelet integrin GPIIb–IIIa. High-MW VWF multimers are most effective in supporting platelet adhesion. Deficiency of VWF, or loss of high-MW forms, results in failure of platelet plug assembly, especially under high shear in the microvasculature. In addition to this role, VWF acts as a carrier for coagulation factor VIII (FVIII), circulating with FVIII in a noncovalent complex.

After secretion, ULVWF requires processing to prevent spontaneous interaction with platelets and potential formation of occlusive platelet thrombi. The protease, a disintegrin and metalloprotease with thrombospondin repeats (ADAMTS13), has a central role in this process (Figure 83.1).[7,17] The action of ADAMTS13 on globular VWF takes place slowly in plasma (12 hour VWF half-life); however, anchored VWF is rapidly cleaved after shear-induced unfolding and elongation.

DISEASE CLASSIFICATION

VWD Subtypes

Von Willebrand disease in people is classified into one of three categories based on plasma VWF concentration, function, and multimer structure (see Table 83.1).[8,15,24] This classification scheme can be applied to VWD in animals.[2,3,10,19,21,30] Type 1 VWD is a partial quantitative deficiency characterized by low VWF concentration (<50% of normal). In this form, the protein consists of a full complement of functional multimers and clinical severity generally correlates with reduction in VWF concentration.[8,15,21,25]

Type 2 VWD encompasses a group of VWF functional variants. Although four distinct subtypes are found in people, type 2A VWD is the only naturally occurring variant described in animals.[3,8,14,22,29,32] In this form, preferential loss of high-MW multimers accompanies low plasma VWF concentration. Consequently, residual VWF's ability to support platelet adhesion is greatly

TABLE 83.1 Classification of von Willebrand disease.

Type	VWF defect	Affected species (breeds)
1	Partial quantitative deficiency, residual VWF has normal structure and function	Dog (Airedale, Akita, Bernese Mountain Dog, Dachshund, Doberman Pinscher, German Shepherd, Golden Retriever, Greyhound, Irish Wolfhound, Kerry Blue Terrier, Manchester Terrier, Miniature Pinscher, Papillon, Poodles, Schnauzer, Welsh Corgi, other purebred and mixed breed dogs) Horse (Arabian) Mouse (RIIIS/J), genetically engineered (R1205H, Y1584C)
2A	Selective loss of large VWF multimers, decreased VWF–platelet and collagen interactions	Dog (German Shorthaired Pointer, German Wirehaired Pointer) Cow (Simmental) Horse (Quarter Horse, Thoroughbred) Mouse, genetically engineered (R1597W)
2B	Increased VWF affinity for platelet glycoprotein Ib	Mouse, genetically engineered (R1306W, V1316M, R1341Q)
2M	Impaired VWF binding to platelet glycoprotein Ib, normal multimer structure	Mouse, genetically engineered (VWFR1326H)
2N	Decreased VWF binding to FVIII resulting in rapid FVIII clearance	Mouse, genetically engineered (R763A, R854Q, R816W)
3	Complete VWF deficiency	Dog (Dutch Kooiker, Scottish Terrier, Shetland Sheepdog; sporadic cases in Australian Shepherd, Border Collie, Chesapeake Bay Retriever, Cocker Spaniel, Eskimo dog, Labrador Retriever, Maltese, Pitbull, and mixed breed) Cat (Himalayan, domestic cats) Pig (Poland, China) Primate (rhesus monkey) Mouse, genetically engineered (*VWF* –/–)

Sources: Favaloro[8]; Leebeek and Eikenboom[15]; Riehl et al.[24]

reduced. Genetically altered mouse strains have been developed that display all of the type 2 VWD phenotypes (2A, 2B, 2M, and 2N).[20] In addition to mutations in the VWF gene (*VWF*), defects in the platelet receptor GPIbα that enhance its VWF binding affinity produce a disease phenotype similar to type 2B VWD.[6]

Type 3 VWD refers to a complete absence of plasma VWF. In many species, lack of VWF is associated with concomitant reduction in FVIII.[10,19,20] However, dogs affected with type 3 VWD have relatively mild reduction in FVIII, presumably due to species differences in FVIII stability.[23,27]

Affected Species and Breeds

Clinical expression of VWD is best characterized in dogs;[1,3,4,5,13,18,23,24,28] however, family studies and case reports describe VWD in horses,[22] nonhuman primates,[19] cats[2,9] cows,[29] and laboratory strains of pigs[10] and mice,[16] as well as genetically engineered murine models[20] (Table 83.1). Type 1 VWD is the most common form in dogs and people, with cases identified in many purebred and mixed-breed dogs (Table 83.1). Type 2 VWD represents 10–20% of human cases[8,15]; however, this subtype has been found only in two related canine breeds (German Wirehaired and Shorthaired Pointers) in addition to horses and cattle. The most severe form, type 3 VWD, has been described as a familial trait in several breeds (Kooiker, Scottish Terrier, Shetland Sheepdog),[1,23,31] and sporadic cases occur in other breeds and species (Table 83.1). The true prevalence of VWD within affected breeds is difficult to determine. In the absence of random surveys, diagnosis bias favors screening affected individuals and their relatives. Selective breeding may eliminate the trait within a certain family, in spite of relatively high overall breed prevalence. The breed variants listed in Table 83.1 include published reports and recent case submissions to the authors' laboratory; however, new cases may develop in previously unaffected species and breeds.

INHERITANCE AND EXPRESSION PATTERNS

Molecular characterization of VWD in people reveals a variety of mutation types and numerous distinct mutations throughout the VWF gene (*VWF*).[6,12,15] In general, mutations causative for type 2 VWD are clustered in *VWF* regions required for multimer assembly and stability, or VWF–platelet interactions. Common mutations in type 3 patients include frameshift, nonsense, and large deletions that disrupt VWF synthesis. Mutations causative for type 1 VWD are found throughout *VWF*. Additionally, some type 1 variants, described in mice and people, are caused by mutations unlinked to the *VWF* locus that influence VWF protein stability or rate of synthesis.[12,16,20]

Hereditary VWD

Hereditary VWD is an autosomal trait; males and females transmit and express the trait with equal frequency. Most type 2 VWD kindreds and type 3 VWD

TABLE 83.2 Transmission and expression of recessive von Willebrand disease.

Parental status	Offspring status: predicted ratios[a]
Clear (VV) × clear (VV)	All clear (VV)
Clear (VV) × carrier (VV^m)	½ Clear (VV) & ½ carrier (VV^m)
Carrier (VV^m) × carrier (VV^m)	¼ Clear (VV) & ½ carrier (VV^m) & ¼ affected (V^mV^m)
Clear (VV) × affected (V^mV^m)	All carrier (VV^m)
Carrier (VV^m) × affected (V^mV^m)	½ Carrier (VV^m) & ½ affected (V^mV^m)
Affected (V^mV^m) × affected (V^mV^m)	All affected (V^mV^m)

[a] (VV) = VWD clear, male or female, no signs of bleeding; (VV^m) = VWD carrier, male or female, no signs of bleeding; (V^mV^m) = VWD-affected, male or female, clinical bleeding due to abnormal primary hemostasis.

families demonstrate simple recessive inheritance patterns.[15] Homozygotes inherit mutant *VWF* alleles from both parents and invariably express a bleeding tendency, whereas heterozygotes have one normal and one mutant *VWF* allele and are clinically normal (see Table 83.2).

Published reports describe two distinct mutations causative for canine breed variants of type 3 VWD.[23,31] A deletion mutation within exon 4 (c.255Cdel) and a splice-site mutation at the boundary of exon 16 (Int16G>A) have been identified in Scottish Terriers and Dutch Kooikers, respectively. A single base change (c.1657T>G) that generates a variant protein (p.Trp553Gly) has been associated with expression of type 2 VWD in German Shorthaired and Wirehaired Pointers.[32] Direct DNA tests that detect these mutations and other canine *VWF* mutations are commercially available for molecular diagnosis and carrier detection of VWD (links at PennGen). Due to the molecular heterogeneity of the VWD trait and many nonpathogenic *VWF* polymorphisms, selection of genetic tests requires knowledge of the relevant breed phenotype and confirmation that the sequence variant detected segregates with VWF deficiency and clinical expression of abnormal bleeding in that breed or line. Heterozygous carriers of types 2 and 3 VWD are expected to have low plasma VWF concentration, with values generally less than 50% of normal. Type-3-VWD-affected individuals, by definition, lack circulating VWF.[1,3,13,15]

The inheritance and expression of type 1 VWD is complex and typically dominant or dominant with incomplete penetrance.[4,6,12,15,24,25] The causative mutations underlying this phenotype in people are often classified as "dominant-negative," characterized by the presence of an abnormal gene product that affects VWF processing or clearance. Incomplete penetrance refers to a variability of disease expression among individuals carrying the same mutation. A range of clinical severity is also a common feature within canine breed variants of type 1 VWD.[1,4,13] A splice-site mutation at the end of exon 43 (c.7437G>A) has been associated with VWF deficiency in Doberman Pinschers,[11] and has been identified in many other breeds (see PennGen). While low plasma VWF concentration is a major risk factor for expression of canine type 1 VWD, the relationship of homozygosity or heterozygosity to expression of a bleeding tendency has not yet been fully characterized. Additional loci may influence disease penetrance, as in the human disease counterpart.[6,12,15]

Acquired VWD

Acquired von Willebrand syndrome (AVWS) refers to VWF quantitative and functional defects that develop as a consequence of other primary disease conditions.[8,15] The pathogenesis of AVWS in people includes antibody-mediated clearance secondary to immune disorders, shear-induced proteolysis due to cardiac disease, and increased VWF–platelet binding in thrombotic and neoplastic syndromes. In addition to these defined mechanisms, AVWS has been described in people (and animals) in association with hypothyroidism and treatment with plasma expanders (e.g., hydroxyethyl starch, dextran). An AVWS, characterized by loss of high-MW VWF multimers, has been reported in dogs with cardiac valvular lesions causing turbulent flow.[30] Screening to detect VWF defects is therefore warranted in animals with signs of mucosal hemorrhage and primary diseases associated with AVWS. Similarly, correction of acquired disorders that reduce VWF plasma half-life or impair its function may prevent exacerbation of hereditary VWD.

VON WILLEBRAND FACTOR ASSAYS

Definitive diagnosis of VWD is based on specific assay of plasma VWF.[8,15,21] Species differences in VWF antigenic structure and function require species-specific assays, or validation of human assays, for veterinary applications. Valid results also depend on collection techniques that prevent platelet activation and clot formation, conditions that deplete and degrade VWF in the sample specimen.[8]

Quantitative VWF Assays

VWF antigen (VWF:Ag) refers to the immunologic quantitation of VWF concentration. Determination of VWF:Ag is the initial step in characterizing VWD. The most sensitive and accurate assay methods in current use are ELISA and automated latex immunoassay (LIA).[8,21] Test results of VWF (and clotting factors) are conventionally reported as percentage of "normal." The VWF content of each test sample is compared with that of a reference standard having an assigned value of 100% or 100 units per deciliter (U/dL). Assay standards and reference intervals for animal VWF:Ag must be established by each testing laboratory. In

general, plasma VWF:Ag below 50% (<50 U/dL) indicates VWF deficiency.

Functional and Structural VWF Assays

Functional VWF assays measure various aspects of VWF interaction with platelets, collagen, or FVIII. The ristocetin cofactor assay (VWF:RCo) measures VWF-dependent platelet agglutination. This property of VWF depends on the presence of high-MW multimers and reflects VWF interaction with platelet GPIbα. VWF:RCo values are disproportionately decreased compared to VWF:Ag in people with types 2A, 2B, and 2M VWD.[8,15] VWF:RCo assays are difficult to standardize for clinical diagnosis in people and require modification for use in animals. Collagen-binding assays (VWF:CB) measure a functional property of VWF that also depends on the presence of high-MW VWF multimers. VWF:CB assays have begun to replace VWF:RCo for initial screening to detect type 2 VWD. These newer assays are more readily standardized among laboratories, and have been adapted for clinical assessment of VWD subtypes in dogs.[8,26]

VWF multimers are visualized using protein electrophoresis for size separation, followed by direct hybridization with anti-VWF antibodies, or transfer to a membrane (western blot) before antibody labeling. Multimer analyses are generally performed in research settings for detailed VWF characterization and VWD subtype classification of newly diagnosed breed or species variants.[3-5,8,10,15,22]

Point-of-Care Analyses

Bleeding time tests are in vivo measures of primary hemostasis performed by making a superficial incision in a capillary bed and monitoring the time to cessation of blood flow. Prolongation of bleeding time is compatible with severe forms of VWD (and platelet function defects); however, the bleeding time endpoint is also influenced by platelet count, hematocrit, and other conditions affecting blood viscosity.[8] In addition to nonspecificity, bleeding time tests have fallen out of use in human medicine because of high interoperator variability, patient discomfort, and poor predictive values.

The platelet function analyzer (PFA-100®, Dade Behring) is a table-top instrument that measures platelet plug formation in whole blood under high shear flow.[8,18] The test endpoint (closure time) refers to occlusion of an aperture coated with collagen and ADP or epinephrine. The instrument was developed as a noninvasive test of platelet and VWF function and has been evaluated in dogs and horses. The PFA-100® ADP/collagen closure time is sensitive to VWF deficiency and dysfunction in dogs, with reports of prolonged closure times in dogs with types 1, 2, and 3 VWD.[13,18] Many of the nonspecific conditions that influence bleeding time (e.g., thrombocytopenia, anemia) also affect the closure time endpoint, thereby limiting its use as a screening test in some clinical situations.

CLINICAL MANAGEMENT OF VWD

Clinical Signs

Clinical signs of VWD in all species include mucosal hemorrhage, cutaneous bruising, and prolonged bleeding after surgery or trauma. Specific sites of mucosal hemorrhage in VWD case series include epistaxis, hematuria, gastrointestinal hemorrhage, prolonged estral bleeding, and gingival bleeding at deciduous teeth.[1,3,4,15,25] These signs are typical of primary hemostatic defects, commonly seen in patients with thrombocytopenia, platelet dysfunction, and VWD. Small petechiae, however, are rarely seen in VWD patients. The bleeding tendency of type 1 VWD ranges from mild to severe. Mildly affected patients infrequently develop spontaneous hemorrhage, but may experience abnormal bleeding after surgery or trauma. Types 2 and 3 VWD are more severe, characterized by spontaneous and postinjury hemorrhage. Neonatal deaths caused by hemorrhage may occur, but many animals survive to adulthood, albeit with transfusion support. The anatomic site of tissue injury influences the consequences of hemorrhage. Minor hemorrhage into critical sites such as the central nervous system or respiratory tract may produce severe morbidity and mortality. Oronasal and urinary tract tissues are rich in fibrinolysins, and injuries to these tissues often cause more prolonged bleeding than superficial wounds.

Diagnostic Evaluation

Thrombocytopenia and coagulation factor deficiencies are common causes of hemorrhage; therefore, the initial evaluation of any patient suspected of having a bleeding diathesis should include platelet count and coagulation screening tests (Figure 83.3).

Bleeding scores derived from standardized questionnaires have been proposed to improve the diagnostic utility of VWD screening in people.[21] A brief hemostasis questionnaire is also useful as a screening tool to identify veterinary patients with hereditary or drug-induced bleeding disorders (see Table 83.3). If preliminary results reveal normal platelet count and coagulation panel results, then point-of-care tests of primary hemostasis

TABLE 83.3 Screening questionnaire to aid in the diagnosis of bleeding disorders.

1	Has this animal ever had spontaneous bleeding from nose, mouth, urinary tract, or noticeable bleeding at teething?
2	Has this animal ever had a surgical procedure, dentistry, or traumatic injury—did bleeding complications occur?
3	Has this animal ever required a transfusion because of anemia or hemorrhage?
4	Is this animal receiving medication or dietary supplements?
5	Are you aware of any bleeding problems in relatives or in this breed?

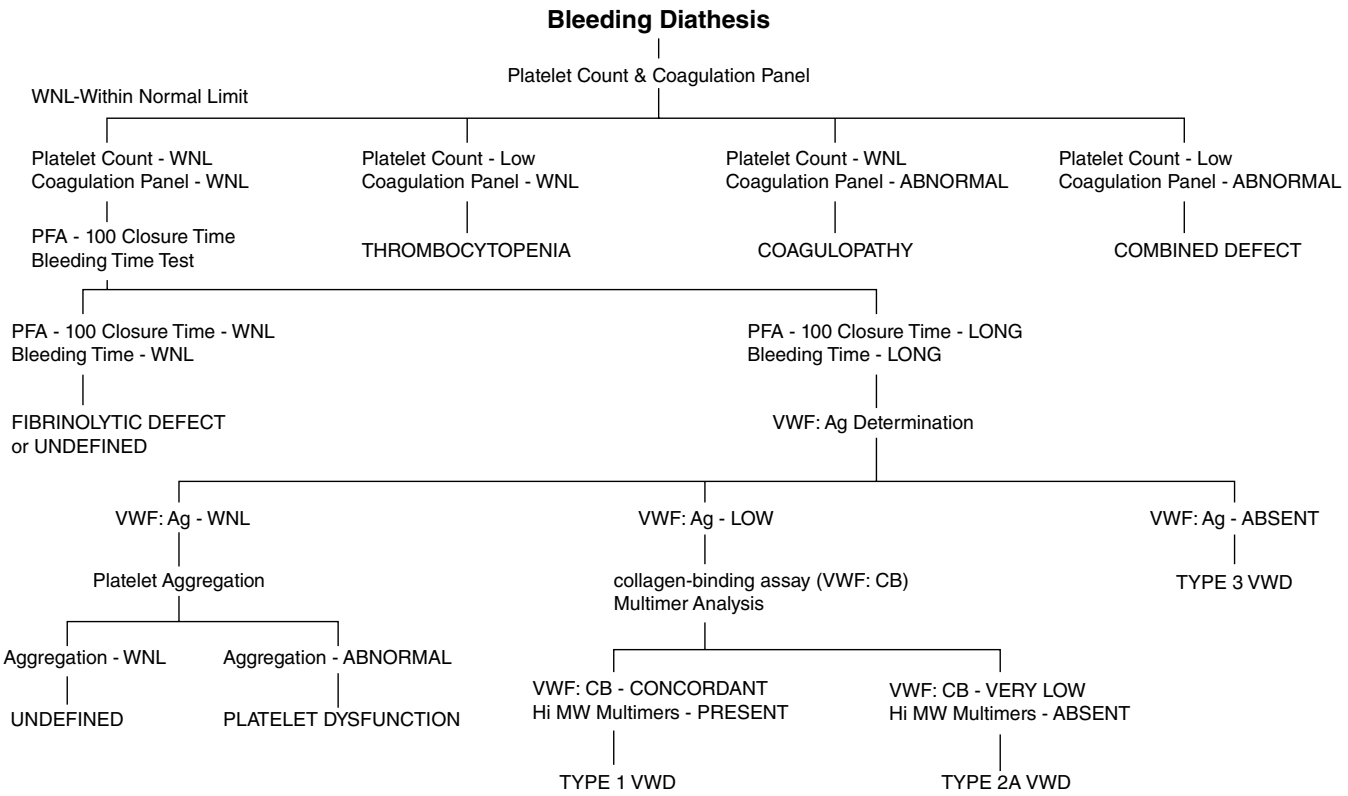


FIGURE 83.3 Diagnostic algorithm for assessment of VWD and other bleeding disorders.

and/or VWF:Ag should be performed to screen for VWD (see Figure 83.3). Prolongation of in vivo bleeding time (beyond 4–6 minutes) and PFA-100® collagen/ADP closure time (beyond 2 minutes) is compatible with VWD, and the finding of low plasma VWF (VWF:Ag < 50%) confirms VWF deficiency. A complete lack of plasma VWF (VWF:Ag < 0.1%) is definitive for severe, type 3 VWD. Diagnosis of type 2 VWD is based on quantitative deficiency, combined with additional structural and functional abnormalities (Table 83.1). In particular, a disproportionate defect of collagen binding (ratio of VWF:Ag/VWF:CB > 2) provides evidence of type 2A VWD. In patients with low, but functional VWF, the risk of abnormal hemorrhage ranges along the continuum of VWF:Ag values. In dogs and people, clinical expression of a bleeding tendency is typically associated with VWF:Ag < 25%, whereas VWF:Ag close to 50% is often clinically inapparent.[1,4,12,15] The current management strategy for type 1 VWD in people considers VWF deficiency a biomarker for hemostatic risk, with consideration of mutation type, and other parameters of intrinsic platelet function as potential modifying factors influencing clinical expression.[15,21]

Treatment of VWD

Management of VWD is directed at controlling active hemorrhage and preventing hemorrhagic complications in case of surgery. The clinical heterogeneity of VWD requires an individualized approach to account for disease classification and underlying conditions that might influence disease expression.

Transfusion Therapy Guidelines

Transfusion is the mainstay of emergency treatment and prophylaxis of VWD (see Chapters 86, 94, and 100). Plasma cryoprecipitate is the most specific component for rapid VWF replacement therapy, with fresh frozen plasma an acceptable alternative (see Chapter 100). The most successful strategy is early, high-dose transfusion to rapidly increase plasma VWF to levels capable of supporting platelet adhesion. Patients with types 2 and 3 VWD almost invariably require transfusion to undergo invasive procedures or stop an active bleed. Transfusion for type 1 VWD can be assessed based on clinical history, severity of VWF deficiency, any underlying disease, and the nature of the hemostatic stress. Patients with blood-loss anemia or requiring major surgical procedures should be transfused, whereas those with normal hematocrits or minor, cutaneous injuries may be successfully managed with limited or no transfusion. Severely affected patients may require repeated transfusion, with lower dosages at longer intervals to sustain hemostasis for 24–48 hours after initial control of bleeding. Stabilization of hematocrit and cessation of active hemorrhage are signs that hemostatic levels of VWF have been attained.

Nontransfusion Therapy

Hereditary VWD causes a lifelong bleeding tendency; therefore, invasive procedures are best avoided. Desmopressin acetate (deamino-8-d-arginine vasopressin [DDAVP]) is a synthetic vasopressin analog used as a procoagulant pharmacologic agent for type 1 VWD, mild forms of hemophilia A, and platelet defects in people (see Chapters 86 and 94). Desmopressin has been recommended as preoperative prophylaxis for type-1-VWD-affected dogs, although experimental studies reveal relatively small increases in plasma VWF concentration compared to the human response.[5] Close monitoring is therefore required, with transfusion available if desmopressin response is inadequate. In case of injury or nonelective surgery, local wound care may help support hemostasis and reduce transfusion requirements (see Chapter 94). Cautery, topical tissue adhesive, multilayer closure, and pressure wraps help to reduce blood loss at focal injury sites.

Signs of VWD can be exacerbated by concurrent thrombocytopenia and other disease conditions that affect hemostasis. Uremia, hyperproteinemia, anemia, hypothyroidism, and liver disease are among the more common disease conditions associated with primary hemostatic failure. Medical therapy to correct these disorders, and primary diseases associated with AVWS, is therefore important in the general management of VWD. Antiplatelet and anticoagulant drugs (e.g., sulfa drugs, nonsteroidal anti-inflammatory agents, heparin, dextran, and other plasma expanders) should be given cautiously, if at all, to patients with clinical signs of VWD.

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ON-LINE RESOURCES

1. **Comparative Coagulation Laboratory**. Animal von Willebrand factor assays. https://www.vet.cornell.edu/animal-health-diagnostic-center/laboratories/comparative-coagulation
2. **NIH**. Guidelines on diagnosis and management of VWD. http://www.nhlbi.nih.gov/guidelines/vwd/vwd.pdf
3. **OMIM**. Comprehensive summary and links to molecular and biochemical characterization of VWF and VWD. https://omim.org/entry/613160
4. **PennGen**. Searchable database of canine and feline genetic tests. https://www.vet.upenn.edu/research/academic-departments/clinical-sciences-advanced-medicine/research-labs-centers/penngen/tests-worldwide

CHAPTER 84

Inherited Platelet Disorders

MARY K. BOUDREAUX and PETE W. CHRISTOPHERSON

Overview—Intrinsic Versus Extrinsic Platelet Disorders
Inherited Macrothrombocytopenia
Membrane Receptor Disorders
  Glanzmann Thrombasthenia (GT)
  P2Y12 ADP Receptor Disorder
  Bernard Soulier Syndrome
Storage Pool Disorders
  Cyclic Hematopoiesis
  Chediak–Higashi Syndrome (CHS)
  Dense Granule Defect
Signal Transduction Disorders
  Calcium Diacylglycerol Guanine Nucleotide Exchange Factor I (CalDAG-GEFI) Thrombopathias
  Kindlin-3
Platelet Procoagulant Deficiency
  Scott Syndrome
Diagnosis and Management

Acronyms and Abbreviations

ADAMTS13, a disintegrin and metalloprotease with thrombospondin repeats; AP3β1, adaptor protein complex 3 beta 1; ADP, adenosine diphosphate; ATP, adenosine triphosphate; BSS, Bernard Soulier syndrome; CalDAG-GEFI, calcium diacylglycerol guanine nucleotide exchange factor I; CAP-1, canine activated platelet-1; CHS, Chediak–Higashi syndrome; CKCS, Cavalier King Charles Spaniel; DDAVP, 1-desamino-8-D-arginine vasopressin; GDP, guanosine diphosphate; GEF, guanine nucleotide exchange factor; GP1b, glycoprotein 1b; GT, Glanzmann thrombasthenia; GTP, guanosine triphosphate; MPV, mean platelet volume; PAF, platelet activating factor; PAR4, protease-activated receptor 4; PS, phosphatidylserine; PFA-100, platelet function analyzer 100; RGD, arginylglycylaspartic acid; RIBS, receptor-induced binding site; VWD, von Willebrand disease; VWF, von Willebrand factor

OVERVIEW—INTRINSIC VERSUS EXTRINSIC PLATELET DISORDERS

Intrinsic platelet disorders are characterized by defects involving membrane receptor glycoproteins, secretory organelles, signal transduction proteins, or structural proteins that result in either platelet dysfunction (thrombopathia) or thrombocytopenia. A variety of inherited intrinsic platelet disorders have been documented in veterinary medicine at the functional, biochemical, and molecular levels (Table 84.1). Extrinsic platelet disorders are characterized by abnormalities in plasma or subendothelial proteins that are essential for platelet adhesion or aggregation. Extrinsic platelet disorders include von Willebrand disease (VWD, see Chapter 83) and hypofibrinogenemia or dysfibrinogenemia. Intrinsic and extrinsic platelet function disorders may be clinically indistinguishable. In vivo bleeding times are typically prolonged in both types of disorders, while coagulation screening tests are normal. Clinical signs suggestive of platelet disorders include petechial and ecchymotic hemorrhages and mucosal bleeding such as gingival bleeding, epistaxis, and urinary and gastrointestinal tract hemorrhage. Excessive bleeding during permanent tooth eruption is common, and may be an early indication of a bleeding diathesis. Clinical severity can vary widely, including unprovoked hemorrhage or excessive bleeding after surgery or trauma. In milder forms, clinical signs may be limited to periodic bruising or petechial hemorrhage. Internal hemorrhage within the brain or spinal cord may result in seizures, paralysis, or acute death. Unilateral or bilateral epistaxis may be spontaneous or associated with drug therapy that inhibits platelet function. Blood products and management strategies

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TABLE 84.1 Inherited intrinsic platelet disorders in dogs, cats, cattle, and horses.

Disorder	Breeds	Gene	Mutation
Macrothrombocytopenia			
	Cavalier King Charles Spaniel, other canine breeds	*TUBB1*	r.745g>a; p.Asp249Asn
	Cairn, Norfolk, and Jack Russell Terriers	*TUBB1*	r.5g>a; p.Arg2His
	Akita	unknown	
Macrothrombocytopenia with neutrophilic inclusions (May–Hegglin anomaly)			
	Pug	*MYH9*	r.5521g>a; p.Glu1841Lys
Glanzmann thrombasthenia			
	Great Pyrenees	*ITGA2B*	r. 1,373_1,374insGGTGCCACAGACAT
	Otterhound	*ITGA2B*	r.1100g>c; p. Asp367His
	Mixed breed	*ITGA2B*	r.1264c>t; p.Arg422ter
	Golden Retriever	*ITGA2B*	r.1831delc p.(Gln611fs)
	DSH feline	*unknown*	unknown
	Thoroughbred horse	*ITGA2B*	r.122g>c; p.Arg41Pro
	Quarter Horse	*ITGA2B*	r.122g>c; p.Arg41Pro g.6478_6488delCAGGTGAGGA
	Peruvian Paso	*ITGA2B*	g.6478_6488delCAGGTGAGGA
	Oldenburg horse	*ITGA2B*	r.122g>c; p.Arg41Pro
ADP receptor (P2Y12) defect			
	Greater Swiss Mountain Dog	*P2RY12*	r.525_527delctc; p.176delSer
Bernard Soulier syndrome			
	Cocker Spaniel	*GP9*	g.3,025,814_3,028,273del2460
Cyclic hematopoiesis			
	Gray Collie	*AB3B1*	r.2543dupa; p.803Ter
Chediak–Higashi syndrome			
	Persian cats, Aleutian mink, blue fox, Brangus cattle	unknown	
	Japanese Black cattle	*LYST/CHS1*	r. 6065a>g; p.His2015Arg
Dense granule defect			
	American Cocker Spaniel	unknown	
CalDAG-GEFI platelet disorders			
	Basset Hound	*RASGRP2*	r. 509_511deltct; p.170delPhe
	Spitz	*RASGRP2*	r.452dupa; p.Arg150fs
	Landseer-ECT	*RASGRP2*	r. 982c>t; p.Arg328Ter
	Simmental cattle	*RASGRP2*	r.701t>c; p.Leu234Pro
	Arabian horse	*RASGRP2*	r.687c>g; p.His229Gln
Kindlin-3 disorder			
	German Shepherd	*FERMT3*	r.1654_1655insaagacggctgcc, p.Leu452_Ala453insArgArgLeuPro
Platelet procoagulant deficiency (Scott syndrome)			
	German Shepherd	*TMEM16F*	g.8912219G>A; p.Pro612Ter

Source: Sequence variant nomenclature http://varnomen.hgvs.org/ accessed 05/23/19

for intrinsic and extrinsic platelet disorders differ; therefore, correct classification is necessary for appropriate treatment of each patient (see Chapters 86 and 100).

INHERITED MACROTHROMBOCYTOPENIA

Cavalier King Charles Spaniels (CKCS) have high prevalence of an autosomally inherited macrothrombocytopenia that does not cause a bleeding diathesis.[24,28,53,58,60] Platelet counts typically range from 30,000 to 100,000/μL in affected (homozygous) dogs with slightly higher counts in carrier (heterozygous) dogs, and variable numbers of large platelets with high mean platelet volume (MPV) present in dogs with either genotype. Affected CKCS have been reported as having hyporesponsive and hyperresponsive platelets; discrepancies likely due to different assays, methodologies, and the CKCS populations evaluated. Prolonged platelet function analyzer 100 (PFA-100) closure times reported in some CKCS are likely secondary to acquired VWD caused by concomitant mitral valve regurgitation, rather than inherited macrothrombocytopenia.[63] The high shear conditions generated with mitral valve regurgitation and other types of heart valve disorders can result in enhanced cleavage of von Willebrand factor (VWF) by a disintegrin and metalloprotease with thrombospondin repeats (ADAMTS13), an enzyme that cleaves VWF under high shear conditions (see Chapter 83).[64] The finding of low plasma VWF concentration and disproportionate loss of VWF collagen-binding activity are characteristic of this form of acquired VWD.

The large platelets in affected CKCS can be equal in size, or larger than erythrocytes, and tend to be spherical to oval in shape with prominent alpha granule staining. A missense mutation at coding nucleotide 745 (r.745g>a) in the β1-tubulin gene (*TUBB1*) is causative for inherited macrothrombocytopenia in CKCS.[25,28] The mutation codes for the substitution of an asparagine for aspartic acid at amino acid position 249 (Table 84.1). Aspartic acid 249 is part of a microtubule intra-protofilament binding site and the change in charge at this position is thought to impair microtubule assembly necessary for normal proplatelet formation and platelet production by megakaryocytes. Prevalence of the trait in CKS in the United States is high; a survey found 90% of the CKCS were carrying one or two copies of the *TUBB1* mutation.[28] In the same study, 65% of CKCS in Ireland were also heterozygous or homozygous for the mutation.

Since the discovery of the TUBB1 mutation in CKCS, the same mutation has been detected in 28 other dog breeds (M. K. Boudreaux and P. W. Christopherson, personal observation). A second *TUBB1* mutation has also been discovered in Cairn and Norfolk Terriers.[33] The mutation is within exon 1 (r.5g>a) and changes the encoded amino acid from an arginine to a histidine. This mutation is thought to have an effect on guanosine triphosphate (GTP) binding and has also been identified in Jack Russell Terriers (M. K. Boudreaux and P. W. Christopherson, personal observations). A pug with macrothrombocytopenia and neutrophilic cytoplasmic inclusions, analogous to May–Hegglin anomaly, has been described.[30] The dog's neutrophil function was normal, and platelets demonstrated reduced response to ADP. Partial sequencing of the human May–Hegglin disease gene, myosin-9 (*MYH9*), revealed that the dog was heterozygous for a nonsynonymous single nucleotide variant in exon 38 (r.5521g>a), a mutation previously described in a human May–Hegglin patient.

Disease genes other than *TUBB1* and *MYH9* have been identified as the cause of hereditary macrothrombocytopenias in people. A similar molecular heterogeneity is likely in dogs. Of note, a congenital macrothrombocytopenia has recently been described in Akita dogs who did not have the previously identified canine *TUBB1* mutations[29,36] (M. K. Boudreaux and P. W. Christopherson, personal observations).

MEMBRANE RECEPTOR DISORDERS

Glanzmann Thrombasthenia (GT)

Glanzmann thrombasthenia (formerly known as thrombasthenic thrombopathia) is characterized by absence or marked reduction in platelet glycoprotein complex IIb–IIIa (integrin $\alpha_{IIb}\beta_3$). This complex functions as the platelet fibrinogen receptor.[51] In people, GT is categorized into three main types: type I, type II, and variant. In type I, less than 5% of the receptor is detectable on platelet surfaces and clot retraction is absent. In type II, 10–20% of the receptor is present on platelet surfaces and clot retraction is detectable but reduced. In variant GT, the receptor is present but dysfunctional; clot retraction may or may not be detectable. In veterinary medicine, type I GT has been described in horses and dogs.[5,27, 45,48] The $\alpha_{IIb}\beta_3$ complex primarily binds fibrinogen; however, it can also bind other arginylglycylaspartic acid (RGD)-containing adhesive proteins, including VWF. Glanzmann thrombasthenia is inherited as an autosomal recessive trait and is characterized by the inability of platelets to aggregate in response to all agonists, including thrombin. The alpha and beta subunits of the fibrinogen receptor are encoded by separate genes; however, both are necessary for intracellular transport and formation of a stable complex on the platelet surface. All cases of GT described in domestic animals have been due to mutations in *ITGA2B*, the gene encoding the α_{IIb} subunit; mutations in the gene encoding β_3 have not yet been identified[7,8,19,20,37,44] (P. W. Christopherson, personal observations). Most mutations are in areas of the gene encoding for calcium-binding domains within α_{IIb} (Table 84.1). α_{IIb} subunits are not detected in flow cytometric assays using antibodies specific to the α_{IIb} subunit. Flow cytometric studies using antibodies to the β_3 subunit or to the $\alpha_{IIb}\beta_3$ complex may detect β_3 subunits associated with vitronectin receptors ($\alpha_v\beta_3$), which share

the β_3 subunit and may be increased in these patients.[50] Platelets isolated from dogs with GT do not bind canine activated platelet-1 (CAP-1), a monoclonal antibody specific for a receptor-induced binding site (RIBS) epitope on canine fibrinogen, when activated by strong agonists.[6]

Glanzmann thrombasthenia has been identified in Otterhounds, Great Pyrenees, Golden Retrievers, and mixed-breed dogs, and in horses in England, Australia, Canada, Japan, and the United States. The prevalence of carriers for the mutation in Otterhounds is between 25% and 30% based on records of genetic testing at Auburn University. Prevalence estimates in other breeds are unknown; however, Great Pyrenees carriers have been detected in several states, including Mississippi, Alabama, Florida, Missouri, Minnesota, Indiana, Illinois, Oklahoma, and Washington, suggesting that the causative mutation may be widespread in the breed. Equine Glanzmann thrombasthenic patients identified in Australia and Japan were not characterized at the molecular level;[49,62] however, an affected Quarter Horse diagnosed at Auburn University was compound heterozygous for two distinct mutations in *ITGA2B*. One mutation was identical to that described in horses in England and Canada. The second mutation was a 10-base deletion spanning the exon 11–intron 11 splice site. A 17-year-old Peruvian Paso mare with GT was documented to be homozygous for the same 10-base deletion.[56] The prevalence of mutations causing GT in horses is unknown. In 2018, a case of GT in a domestic short-haired cat was discovered at the University of California, Davis.[67]

P2Y12 ADP Receptor Disorder

Epistaxis and abnormal surgical hemorrhage has been described in Greater Swiss Mountain dogs homozygous and heterozygous for a deletion mutation in *P2RY12*, the gene encoding the major platelet ADP receptor.[12,31] In these reports, clinically affected dogs demonstrated impaired in vitro platelet activation and/or aggregation response to ADP stimulation. Familial screening of the index case revealed dogs homozygous and heterozygous for the mutation with no histories of abnormal hemorrhage. Similarly, highly variable penetrance of *P2RY12* mutations has been described for human cases.[17]

Bernard Soulier Syndrome

A mild to severe bleeding disorder resembling Bernard Soulier syndrome (BSS) has been identified in Cocker Spaniels.[34] Clinical signs included epistaxis, gingival hemorrhage, delayed post-operative hemostasis, and hematoma formation at venipuncture sites. Clinically affected dogs are homozygous for a large deletion mutation in *GP9* that codes for the VWF receptor, glycoprotein Ib (GP1b). Phenotypically, the trait comprises characteristic clinicopathologic features of BSS including macrothrombocytopenia, defective platelet adhesion, and abnormal GP1b expression.[34]

STORAGE POOL DISORDERS

Cyclic Hematopoiesis

Cyclic hematopoiesis is an autosomal recessive disorder of Gray Collies characterized by cyclic fluctuations in the number of circulating neutrophils, reticulocytes, and platelets.[18,26,46] Melanocytes are also affected in this disorder resulting in the gray color of affected animals. A bone marrow stem cell defect results in neutropenic episodes occurring approximately every 12 days. Mortality is high; most puppies die prior to 6 months of age due to fulminating infection. Thrombocytopenia does not occur, and platelet numbers fluctuate between 300,000 and 700,000/μL. Platelet dense granules are absent. Platelet reactivity to several agonists is defective; clot retraction and platelet adhesiveness are impaired. A mutation in *AB3B1*, the gene encoding adaptor protein complex 3 beta 1 subunit (AP3β1), has been linked to this disorder.[2] AP3β1 directs trans-Golgi export of transmembrane cargo proteins to granules. Recombinant granulocyte colony-stimulating factor has been used successfully in an experimental treatment trial of affected dogs.[66]

Chediak–Higashi Syndrome (CHS)

Chediak–Higashi syndrome is an autosomal recessive genetic disorder characterized by abnormal leukocyte, melanocyte, and platelet granulation. Affected animals have partial oculocutaneous albinism, increased susceptibility to infection, and a bleeding diathesis as a result of impaired platelet function. Platelets of affected animals lack dense granules and are deficient in granule storage pools of adenine nucleotides, serotonin, and divalent cations. Platelet aggregation in response to collagen, which is largely dependent on thromboxane generation and granule release of ADP, is markedly reduced in affected animals.[38] The disease was identified in a line of Persian cats: all of the affected animals exhibited a "blue smoke" hair color and pale irises with the development of bilateral nuclear cataracts in several animals as early as 3 months of age.[42] Affected cats experienced prolonged bleeding at incision sites and hematoma at venipuncture sites. Chediak–Higashi syndrome has also been diagnosed in Aleutian mink,[41] three breeds of cattle,[1,52,66] blue foxes,[59] killer whales,[55] and mice.[47] CHS in Japanese Black cattle, mice, and people has been linked to mutations in the lysosomal trafficking regulator gene (*LYST*), which encodes a 425-kDa cytoplasmic protein that may be involved with incorporation of proteins into lysosomal membranes.[43] Molecular studies have not been reported in Brangus or Hereford cattle or other species affected with CHS.

Dense Granule Defect

A dense granule defect has been described in a family of American Cocker Spaniels.[14] Affected dogs had a moderate to severe bleeding diathesis, particularly after trauma or surgery. Three of five affected dogs had

impaired platelet aggregation responses to ADP and collagen. Platelet[14] C-serotonin uptake and retention were normal; ATP content was also normal. ADP content was low resulting in an increased ATP/ADP ratio. Platelet morphology at the electron microscopic level was normal suggesting a functional dense granule defect. Molecular studies were not conducted and the disorder has not been recognized in Cocker Spaniels since the initial report.

SIGNAL TRANSDUCTION DISORDERS

Calcium Diacylglycerol Guanine Nucleotide Exchange Factor I (CalDAG-GEFI) Thrombopathias

Basset Hounds, Eskimo Spitz, Landseers of European Continental Type (ECT), and Simmental cattle have been identified with inherited signal-transduction platelet disorders, referred to as thrombopathias, that are virtually identical in presentation at the clinical and functional level.[4,16,32,35,41,57,61] Platelet aggregation responses to ADP and collagen are minimal to absent in affected animals. Platelet aggregation in response to thrombin is rate-impaired and is characterized by a lag phase, lengthening the time to complete aggregation. This thrombin activation response is sufficient to support normal clot retraction. Flow cytometric assays using antibodies to αIIb or β3 show normal receptor density. CAP-1 binding is absent on ADP or platelet activating factor (PAF-)activated thrombopathic canine platelets.[9]

The molecular basis for thrombopathia in Basset Hounds, Spitz, Landseers of ECT, and Simmental cattle is due to distinct mutations in *RASGRP2* that encodes CalDAG-GEFI.[9,10] All of the mutations are within areas encoding structurally conserved regions (SCRs) of the catalytic domain (Table 84.1). CalDAG-GEFI is a signal transduction protein that functions as a guanine nucleotide exchange factor (GEF) and facilitates guanosine diphosphate (GDP)/GTP switching by Rap1b. Rap1b is a Ras-related low-molecular-mass guanine-nucleotide-binding protein (GTPase) that is activated by the binding of GTP. Activated Rap1b is critically involved in activation and change of conformation of integrin $\alpha_{IIb}\beta_3$ necessary for fibrinogen binding and ensuing platelet aggregation.[3] The ability of thrombin to activate mutant platelets is thought to be attributable to the ability of protease-activated receptor 4 (PAR4) signaling to bypass CalDAG-GEFI and activate Rap1b directly or act via a pathway independent of Rap1b.[9,23]

The *RASGRP2* mutation in Basset Hounds appears to be widespread; results of DNA testing at Auburn University indicate that between 25% and 30% of Basset Hounds are carriers for the mutation. The mutation is more prevalent in Basset Hound lines used for show rather than for hunting and has also been identified in a Basset–Shar-Pei (Ba-Shar) dog.[21] Landseer-ECT is a European canine breed with prevalence of *RASGRP2* mutations based on testing at Auburn University, of around 25%. Distinct mutations of unknown or lower prevalence have also been identified in Eskimo Spitz, Simmental cattle, and in an Arabian horse (Table 84.1). Although CalDAG-GEFI is also expressed in brain tissue, neurological signs in people or animals with CalDAG-GEFI disorders have not been reported. CalDAG-GEFI may play a role in signal transduction events in other hematopoietic cells, including neutrophils. Redundant pathways, however, are likely responsible for preventing significant impairment of neutrophil function in animals with CalDAG-GEFI mutations (Table 84.2).

Signal transduction defects are believed to represent the most common group of hereditary intrinsic platelet defects in people. Due to the complexity in platelet signaling pathways, functional characterization of these defects requires comprehensive analyses of platelet activation response to a variety of agonists to identify defective pathways and likely candidate disease genes.[15,54]

Kindlin-3

A mutation in *FERMT3*, the gene encoding Kindlin-3, has been identified in a German Shepherd dog and a German Shepherd–Rottweiler cross with histories of prolonged bleeding, persistently high leukocyte counts, and susceptibility to infections.[11,39] Kindlin-3 is a signal transduction protein shared by all hematopoietic cells, which plays an essential role in integrin activation.

PLATELET PROCOAGULANT DEFICIENCY

Scott Syndrome

A defect of platelet procoagulant activity, comparable to the human platelet defect Scott syndrome, has been identified in German Shepherd dogs.[13,40] Clinical signs include abnormal post-traumatic and postoperative bleeding, and spontaneous epistaxis, hematoma formation, and hyphema. Affected dogs demonstrate normal

TABLE 84.2 Amino acids within structurally conserved regions (SCR) of the catalytic domain of CalDAG-GEFI.

Species	SCR	Mutation[a]
Basset and Spitz	SCR1	F**D**HLEPLELAEHLTYLEYRS**F**CKI
Cow	SCR2	RAGVITHFVHVAEE**L**LHLQNFNTLMAVVGGLSHSSISRLKETH
Horse	SCR2	RAAVITHFV**H**VAEKLLELQNFNTLMAVVGGLSHSSISRLKETH
Landseer-ECT	SCR4	**R**LNGAK

[a] Underlined/bold amino acids are sites of mutations described in dogs, cattle, and horses.

buccal mucosal bleeding times and normal platelet aggregation, adhesion, secretion, and clot retraction; however, calcium-induced platelet membrane externalization of phosphatidylserine (PS) and microvesiculation are markedly reduced. Consequently, Scott syndrome platelets do not fully support procoagulant complex assembly. The molecular basis for this disorder is a splice-site mutation in *TMEM16F*, the gene encoding a transmembrane calcium-dependent lipid scramblase, referred to as anoctamin-6 or *TMEM16F*.[13]

DIAGNOSIS AND MANAGEMENT

Hereditary platelet defects are generally uncommon disorders that require an index of suspicion for timely diagnosis. These traits are typically found in purebred animals due to propagation of breed-specific mutations from breed founders or popular sires. However, de novo mutations and inbreeding practices may result in new cases in mixed breed and previously unaffected breeds or lines. To date, all of the intrinsic platelet defects in animals demonstrate autosomal recessive inheritance patterns with males and females at equal risk for expression and transmission of the traits. Genetic counseling is warranted after diagnosis of an index patient. When mutation-type is known, molecular diagnostic testing allows accurate carrier detection to prevent production of affected individuals and ultimately reduce breed prevalence of clinically severe traits.

Persistent thrombocytopenia, characterized by the presence of large platelets, but without clinical signs, is characteristic of hereditary macrothrombocytopenia. These patients risk inappropriate and potentially harmful drug treatment. Documentation of a *TUBB1* mutation provides definitive diagnosis for the previously characterized breeds at risk. However, mutations in other genes have been documented to cause macrothrombocytopenia in people[29] and should be considered in the absence of previously discovered mutations.

Definitive diagnosis of hereditary bleeding diatheses due to intrinsic platelet disorders can be based on molecular diagnostic testing, if a breed or familial mutation has previously been defined. Academic and commercial laboratories offer a variety of tests, using either blood samples or cheek swabs to isolate DNA.[68,69] Alternatively, platelet function testing to identify distinct patterns of abnormalities will confirm the presence of a known trait or provide clues to a specific pathway or protein defect. Some point-of-care and remote platelet screening tests can be performed; however, many functional assays require that affected animals be on the premises of the testing facility for analyses within hours of sample collection (see Chapter 79).

Animals with macrothrombocytopenia do not require any special care, and patients with mild functional defects may be maintained comfortably as pets with avoidance of invasive procedures or the use of antiplatelet agents (see Chapters 86, 94, and 100). Transfusion of blood, components, or platelet concentrates may be required to supply functional platelets for surgical prophylaxis, and periodic platelet transfusion may be needed for patients with more severe functional defects to control recurrent hemorrhagic events (see Chapter 100). Administration of 1-desamino-8-D-arginine vasopressin (DDAVP) and antifibrinolytic agents are medical management strategies described for human platelet defects that are applicable for the animal disease counterparts (see Chapters 86 and 94).[19,22]

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CHAPTER 85

Acquired Platelet Dysfunction

BENJAMIN M. BRAINARD

Introduction
Diseases Associated with Platelet Dysfunction
  Renal Disease
  Sepsis, Infectious Disease
  Systemic Inflammatory Response Syndrome Shock
  Hepatic Disease
  Cardiac Disease
  Neoplasia
  Endocrinopathies
Pharmaceuticals that Affect Platelet Function

Acronyms and Abbreviations

AA, arachidonic acid; aPTT, activated partial thromboplastin time; CKD, chronic kidney disease; CT, closure time; DIC, disseminated intravascular coagulation; GP, glycoprotein; HCM, hypertrophic cardiomyopathy; IMHA, immune-mediated hemolytic anemia; LPS, lipopolysaccharide; MPC, mean platelet component; MPV, mean platelet volume; OPA, optical (turbidimetric) aggregometry; PDW, platelet distribution width; PFA-100, platelet function analyzer-100; PRP, platelet-rich plasma; PT, prothrombin time; ROTEM, rotational thromboelastometry, SIRS, systemic inflammatory response syndrome; TEG, thromboelastography; WBPA, whole blood (impedance) aggregometry; VWF, von Willebrand factor.

INTRODUCTION

Acquired disease and drugs may cause platelet hypofunction or hyperfunction, resulting in hemorrhagic or prothrombotic phenotypes, respectively. While mechanistic studies are lacking, veterinary survey studies have described these clinicopathologic effects. Diagnostic tools to assess platelet function are reviewed elsewhere (see Chapter 79). Low shear assays include platelet aggregation in response to specific agonists (typically ADP, collagen, arachidonic acid [AA], or thrombin), performed as optical aggregometry (OPA) using platelet-rich plasma (PRP) or impedance aggregometry using whole blood (WBPA).[52] High shear assays mimic the stress that platelets encounter in flowing blood. The most widely available instrument, the platelet function analyzer-100 (PFA-100, Siemens), aspirates blood through an aperture coated with either ADP and collagen or epinephrine and collagen, and reports the time for aperture occlusion by a platelet plug as closure time (CT).[52] Viscoelastic assessments of coagulation (e.g., thromboelastography [TEG], rotational thromboelastometry [ROTEM]) record the change in viscosity of whole blood as it is transformed from a liquid to a gelid clot. Regardless of functional assay methodology and results, the clinical presentation and ancillary testing such as platelet count and coagulation profile must be considered prior to initiating therapy.

DISEASE CONDITIONS ASSOCIATED WITH PLATELET DYSFUNCTION

Renal Disease

Uremia is associated with platelet hypofunction, but the mechanism is not well defined. One study of 14 uremic dogs identified either decreased or increased OPA relative to healthy dogs, depending on agonist concentration.[78] By contrast, a second study of 22 uremic dogs failed to identify altered function using WBPA.[31] Overall, the decrement in aggregation in the first study was unlikely to be clinically relevant and hyperaggregability may have resulted from elevated fibrinogen,[24,78]

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decreased serum albumin,[89] or low hematocrit.[62] A recent report in 11 dogs with naturally occurring chronic kidney disease (CKD) described prolonged average CT (although values for most animals were within the reference interval) while platelet expression of the von Willebrand factor (VWF) receptor, glycoprotein (GP)Ib, did not vary between CKD and control dogs. The same study described increased expression of platelet P-selectin and integrin αIIbβ3 (GPIIb/IIIa) compared to healthy dogs, suggesting enhanced platelet reactivity.[24] Plasma fibrinogen concentration was also higher in CKD dogs.[24] Other reports support the presence of clinically relevant bleeding or platelet hypofunction in canine patients with chronic and experimental uremia.[54,12,118] Human studies of various renal disorders have identified defects in membrane expression of GPIb and integrin αIIbβ3,[79] impaired thromboxane synthesis, lower platelet content of serotonin and ADP, impaired fibrinogen binding, or soluble inhibitors as causes of decreased platelet function.[102] In veterinary patients, the effects of uremia on platelet function and coagulation likely vary with the type and etiology of renal disease.

Sepsis, Infectious Disease

Many platelet function abnormalities have been described in humans and animals with sepsis. The release of proinflammatory cytokines during initial stages of sepsis increases circulating fibrinogen and results in enhanced platelet function and thrombocytosis. Progression to septic shock may be accompanied by thrombocytopenia secondary to disseminated intravascular coagulation (DIC) (see Chapter 92). Platelets may become hypofunctional with changes in the intravascular milieu (e.g., acidosis, hypothermia, hypocalcemia). In addition to sepsis-triggered DIC, platelet interactions with activated neutrophils and associated neutrophil extracellular trap formation (see Chapter 92) likely contribute to microthrombus formation.[68]

In experimental canine endotoxemia, a transient shortening of CT occurred 30 minutes after IV infusion of *E. coli* endotoxin, despite the development of thrombocytopenia.[125] The dogs receiving high-dose endotoxin displayed a prolonged CT 48 hours after infusion. In a small study of dogs with infection and systemic inflammatory response syndrome (SIRS), WBPA in response to collagen and platelet adherence to collagen in a flow chamber were significantly lower than values for healthy dogs.[29] This difference was more pronounced when platelets were primed with a thrombin receptor agonist. In another study, septic dogs sampled within 24 hours of diagnosis had decreased WBPA.[69] Decreased platelet aggregation occurs in humans with sepsis.[124] While platelet hypofunction alone may not result in a hemorrhagic phenotype, clinical signs of abnormal bleeding may develop when combined with therapies that further compromise hemostasis.

Infection with *Ehrlichia sp*. is associated with thrombocytopenia, but variable bleeding tendencies. In four dogs experimentally infected with *Ehrlichia canis*, circulating antiplatelet antibodies were observed, in addition to thrombocytopenia.[100] Although WBPA decreased concomitantly with thrombocytopenia, aggregation returned to baseline with recovery of platelet count. Notably, changes in some indices of platelet activation (e.g., mean platelet component [MPC] and platelet distribution width [PDW]) suggested that thrombocytopenia was accompanied by increased platelet activation, which might ameliorate thrombocytopenic bleeding. A report of dogs experimentally infected with *Ehrlichia platys* failed to document clinically significant changes in platelet aggregation despite the presence of thrombocytopenia.[34] Other studies of experimental canine ehrlichiosis revealed decreased OPA in the acute stage of disease. Thrombocytopenia in these cases may have influenced OPA.[11] Another report demonstrated decreased OPA in healthy dog PRP incubated with serum from Ehrlichia-infected dogs, suggesting the presence of a platelet-inhibitory antibody or other soluble factor.[44] In dogs with naturally occurring leishmaniasis, OPA may be decreased compared to healthy dogs with equivalent platelet counts.[87] Canine heartworm disease has been associated with platelet activation and increased platelet reactivity,[7] and *Angiostrongylus vasorum* infection in dogs is associated with consumptive thrombocytopenia and variable platelet reactivity.[1]

In horses, exposure of platelets to endotoxin results in activation and degranulation, likely mediated by lipopolysaccharide (LPS)-induced activation of leukocytes.[53,119] Infection with equine infectious anemia virus results in thrombocytopenia, presumably due to platelet activation and clearance, and the remaining platelets are hypofunctional in aggregation assays.[95] Equine herpesvirus also activates platelets, and may support pathologic thrombosis in infected horses.[104] Horses in the prodromal stages of carbohydrate-induced laminitis show platelet activation and increased number of platelet–neutrophil aggregates that, combined with endothelial dysfunction, may contribute to disease pathogenesis.

Systemic Inflammatory Response Syndrome and Shock

A phenomenon of platelet exhaustion resulting from chronic exposure to activated white blood cells and thrombin is believed to develop in some patients with noninfectious systemic inflammatory disease.[50,86] This manifests clinically as platelet hypofunction distinct from the platelet response to sepsis.[124] In addition, platelet hypofunction has been associated with trauma in humans.[17] In dogs with hemorrhagic shock, evidence of platelet dysfunction occurs despite relatively stable circulating platelet concentrations. In an experimental canine study that induced a 20% and then 40% decrease in blood volume and sustained hypotension, decreased WBPA developed and then resolved after autologous blood transfusion.[73]

More commonly, systemic inflammation results in platelet activation, which may support thrombus formation in susceptible patients. Platelet activation and degranulation are detected as decreases in MPC, measured using the ADVIA 120 hematology analyzer (Bayer

Corp.) or increases in the mean platelet volume (MPV) or PDW (see Chapter 78). One retrospective report[101] of dogs with inflammatory disease failed to show significant changes in these parameters; however, another report of 48 dogs with septic peritonitis described increased MPV, plateletcrit, and PDW.[72] Increases in platelet P-selectin expression (basal and poststimulation) and decreased MPC were associated with systemic inflammation and platelet activation in another canine cohort.[81] In dogs with naturally occurring *Babesia rossi* infection, platelet size and activation status were higher than healthy dogs, presumably counteracting hemostatic compromise from thrombocytopenia.[36]

Platelet–neutrophil aggregates in dogs with systemic inflammation may indicate enhanced platelet activation.[21] Neutrophils were more likely to have attached platelets from dogs with SIRS.[21] Dogs with naturally occurring *Babesia rossi* infection have increased circulating platelet–monocyte aggregates.[36] Immune-mediated hemolytic anemia (IMHA) is associated with systemic inflammation, and dogs with IMHA have increased circulating platelet–neutrophil aggregates and platelet P-selectin expression.[91,121] Low MPC (indicating platelet activation) occurs in dogs with IMHA, and lower MPC was associated with improved survival, perhaps indicating a response to inflammation.[127] Platelets of dogs with IMHA have increased tissue factor expression,[46] and platelets may play a role in the viscoelastic hypercoagulability in some dogs with IMHA.[42]

Hepatic Disease

Hepatic disease is associated with abnormalities of secondary hemostasis, as many coagulation factors and inhibitors are synthesized in the liver. Alterations in platelet function and mild to moderate thrombocytopenia have been described in veterinary patients with hepatic dysfunction.[22,58] Thrombocytopenia may be a result of nonovert DIC (see Chapter 92), occult hemorrhage, or an infective agent (e.g., leptospirosis), and may worsen as the disease progresses.[58] Thrombocytopenia in humans with acute hepatic failure appears to be independent of thrombopoietin concentration and due, in part, to platelet autoantibodies,[88] especially in patients with chronic disease. Almost half the dogs with massive hepatocellular carcinoma had platelet counts greater than 500,000/μL, attributed to malignancy-associated increases in thrombopoietin production.[70]

In dogs with congenital portosystemic shunts, impaired WBPA in response to AA and collagen has been described, although ADP-activated aggregation was normal.[123] Another study using WBPA identified similar results but noted increased OPA aggregation to collagen.[57] Impaired or increased platelet aggregation was not reflected in capillary bleeding time or CT.[57] TEG analyses in dogs with acute hepatic failure revealed decreased angle and maximum amplitude (MA), parameters affected by platelet function (although fibrinogen concentration was also decreased).[59] Dogs with acute liver disease and hepatic failure exhibited increased VWF activity, similar to humans with cirrhosis. Increased VWF availability may enhance adhesion of hypofunctional platelets, but platelet hypofunction occurs in people with acute and chronic hepatic disease measured using collagen-binding assays under flow conditions.[71] Proposed mechanisms of platelet hypofunction include disturbed transmembrane signaling, impaired thromboxane production,[65] and depletion of platelet storage pools of ATP and serotonin.[66]

Cardiac Disease

Platelet hypofunction has been demonstrated in dogs with chronic heart disease. Cavalier King Charles Spaniels (CKCS) with subclinical mitral valvular disease had significantly prolonged CT.[115] Evidence of platelet degranulation (e.g., low MPC, increased P-selectin expression) was lacking.[101] Prolonged CT and lower VWF activity were also seen in a cohort of Dachshunds with mild degenerative mitral valve disease.[80] A third report of dogs with cardiac murmurs caused by valvular insufficiency or stenosis also showed prolonged CT.[18] Decreased high-molecular-weight VWF multimers were described in a cohort of CKCS with myxomatous mitral valve disease,[109] and other studies support decreased platelet function in dogs with chronic valvular disease that correlates with severity of the regurgitant jet.[108,110] A single report suggested increased platelet function in CKCS with mitral valve disease.[83]

Platelet activation in cats with hypertrophic cardiomyopathy (HCM) was assessed using flow-cytometric expression of P-selectin in resting and ADP-activated platelets.[107] Cats with severe HCM had increased P-selectin expression in resting and stimulated platelets. Some cats with HCM had exaggerated OPA response to ADP.[45] Another study of cats with severe HCM did not find a difference in CT between healthy cats and those with HCM.[107]

Neoplasia

Many neoplastic diseases are associated with thrombocytopenia, generally attributed to consumptive coagulopathy (see Chapters 81 and 92). Few reports have specifically assessed platelet function. One study described an exaggerated WBPA response for a given dose of agonist in dogs with neoplasia, but did not stratify dogs by tumor type.[77] A report of dogs with lymphoma found that platelet aggregation was decreased at 1 hour following vincristine administration.[37] A longer-term study evaluating platelet function 10 days following vincristine administration did not show an alteration from baseline.[74] Vincristine may cause early release of reticulated platelets from bone marrow, a population with relatively increased reactivity.[43] Neoplasms resulting in hyperglobulinemia or a leukemoid response may result in decreased platelet function, presumably due to crowding or coating of the platelets with globulin molecules. Patients with polycythemia vera (see Chapter 26) or essential

thrombocytosis (see Chapter 82) may also have platelet adhesion abnormalities.[25]

Endocrinopathies

Some canine endocrinopathies have been associated with platelet dysfunction, and occasionally hyperfunction. Thrombocytosis is a common clinical finding in dogs and humans with hyperadrenocorticism. In some humans with hyperadrenocorticism or hypercortisolemia, increased platelet aggregation has been described, and there is evidence of increased thromboxane synthesis[39] and elevations in circulating ultra-large VWF multimers.[15] Although studies of coagulation function have been performed in dogs with naturally occurring hyperadrenocorticism and exogenous hypercortisolemia, definitive characterization of platelet function in these patients is lacking.[28,51,61,84,94] Closure time may be prolonged in dogs with hyperadrenocorticism.[63] TEG analyses of dogs with hyperadrenocorticism generally support increased coagulation function, with an undefined platelet contribution.[61]

Dogs with diabetes mellitus (DM) had increased ADP-stimulated aggregation and ristocetin-induced maximum aggregation, although VWF was not evaluated. Dogs with experimentally induced DM did not have changes in platelet aggregation.[41,60] In humans with type-2 DM, platelets are thought to be hyperreactive, with decreased inhibition compared to platelets of healthy patients receiving equivalent doses of clopidogrel and aspirin.[97] Glycation of platelet surface proteins may contribute to this hyperreactivity, in addition to increased P-selectin expression, impaired prostacyclin and nitric oxide response, and increased intracellular calcium.[30] Although chronic hyperglycemia may enhance platelet hyperreactivity, mechanisms defined in people have not been described in animals. Adverse vascular events in humans with type-2 DM are likely promoted by endothelial dysfunction, oxidative stress, and coronary artery disease.[96]

Dogs with hypothyroidism have smaller platelet size and cats with hyperthyroidism have larger platelet size;[106] however, platelet function in either disease has not been studied. One report of hypothyroid dogs demonstrated decreased circulating VWF concentration;[2] however, another report of dogs with hypothyroidism did not confirm these findings.[85] Human hyperthyroidism is associated with a thrombophilic state, primarily due to procoagulant excess, and no consistent platelet abnormalities.[103]

PHARMACEUTICALS THAT AFFECT PLATELET FUNCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) decrease inflammation through inhibition of cyclooxygenase (COX) enzymes which produce prostaglandins (PG). COX enzymes also catalyze the formation of thromboxane A_2 (TXA_2), a potent platelet agonist and vasoconstrictor. NSAIDs have variable inhibitory activity for COX isoforms,[105] and canine platelets may express both COX-1 and COX-2.[112] Animals treated with NSAIDs may experience platelet hypofunction due to impaired TXA_2 synthesis. Aspirin is an irreversible COX inhibitor, while other NSAIDs demonstrate reversible inhibition. Decreased platelet aggregation or prolonged CT was found in dogs treated with ketoprofen, carprofen, and meloxicam in some reports,[10,41,47,114,82] but not in others.[4,5,32,33,126] A single report described prolonged collagen/epinephrine CT after deracoxib therapy.[82] Horses treated with phenylbutazone, flunixin meglumine, or firocoxib had no changes in OPA or CT, despite decreased TXA_2 metabolite production in response to phenylbutazone or flunixin.[14]

Despite decreases in TXA_2 metabolites after aspirin therapy, some dogs demonstrate no change in platelet function.[55] A similar lack of platelet inhibition has been described in cats[16] and warmblood horses.[93] Although the lack of predicted response has been termed "aspirin resistance," the wide range of observed responses to varying dosages of aspirin brings into question routine use of aspirin as an antiplatelet agent in dogs or cats. Only eight of 24 healthy dogs receiving 1 mg/kg of aspirin PO, q 24h had prolonged CT, while all showed decreased urinary TXA_2 metabolites.[23] Sixteen dogs receiving the same aspirin dosage had variable response in multiple tests of platelet function,[40] while another report of 20 dogs failed to show decreased urinary TXA_2 metabolites.[49] A lower dosage of aspirin (0.5 mg/kg, PO, q 24 h, for 2 days) given to 10 dogs demonstrated variably decreased ADP or collagen-activated aggregation.[99] More recently, a study of six dogs given an escalating dosage of aspirin found that a threshold dose of 2 mg/kg, PO, q 24h reliably decreased OPA and prolonged CT.[76] Aspirin given to horses at 10 mg/kg PO for 5 days did not change ADP or collagen-induced OPA,[8] but another study noted slight prolongations in CT (ADP/collagen).[98] In cats treated with aspirin of varying dosages, platelet function as measured by CT and aggregometry was unaffected, despite demonstrable decreases in TXA_2 metabolites.[16,48]

The pharmacokinetics and pharmacodynamics of clopidogrel (see Chapter 94) have been described in dogs, cats, and horses. Clopidogrel requires hepatic conversion to an active metabolite, which irreversibly binds the platelet P2Y12 ADP receptor. Hepatic biotransformation can be affected by coadministered medications[111] and variability in hepatic P450 activity.[67] Clopidogrel decreases ADP-induced aggregation in dogs,[9] cats,[38,117] and horses,[8,13] and often decreases platelet reactivity. Collagen-induced aggregation in clopidogrel-treated patients is variably decreased. Because clopidogrel is an irreversible inhibitor, restoration of normal platelet function requires release of new platelets, generally 3–5 days after drug discontinuation in dogs,[9] cats,[38] and horses.[8]

Hydroxyethyl starches (HES) and gelatin-based synthetic colloid solutions can result in decreased platelet aggregation and decreased coagulation factor activities. Studies have evaluated high-molecular-weight (e.g., HES 670/0.8, HES 600/0.75) and lower-molecular-weight

(e.g., HES 130/0.4) starches. The second number in the product name is the substitution ratio of the starches and thus the molecular complexity. At in vitro dilutions of 1:5 or lower, HES solutions have no apparent clinically relevant effects on coagulation. In vivo, bolus IV administration (15 mL/kg) to anesthetized dogs caused transient hypocoagulability characterized by prolonged CT and coagulation dysfunction assessed by ROTEM.[90] Similar results have been described in horses following a 5 mL/kg IV bolus of either HES 670/0.75 or HES 130/0.4.[27]

HES solutions are used to treat low oncotic pressure and are generally administered as an IV constant rate infusion (CRI). In hypoalbuminemic dogs administered either 1 or 2 mL/kg/h of HES 130/0.4, IV, for 24 h, no changes in ROTEM parameters or prothrombin time (PT), activated partial thromboplastin time (aPTT), or fibrinogen concentration were seen.[6] Anesthetized dogs given an IV bolus of 15 mL/kg of HES 130/0.4 demonstrated transient (1 hour) prolongation of CT.[90] In dogs with experimental hemorrhagic shock administered 20 mL/kg HES 130/0.4 as an IV bolus, ROTEM and coagulation tests suggested hypocoagulability lasting 3 hour after the bolus.[19] Interestingly, HES did not prolong CT, whereas a treatment group that received 20 mL/kg of a 4% succinylated gelatin solution for resuscitation had significant prolongations of CT through the 3 hour time point. In this report, decreased factor VIII activity and VWF concentration developed with the onset of shock and worsened after fluid, but not whole blood, resuscitation. These decreases were attributed to dilutional effects and not to colloid.[75] Horses treated with a 10 mL/kg IV bolus of 0.9% saline, HES 130/0.4, or HES 600/0.75, developed decreased platelet counts, VWF concentration, and factor VIII by 1 hour after administration. These changes were accompanied by prolongation of CT that resolved by 8 hours in the HES 130/0.4 and saline groups, but remained prolonged in the HES 600/0.75 group for at least 24 hours.[27] These results suggest that small HES molecules have more rapid clearance than larger molecules.[26]

A transient decrease in platelet aggregation and platelet count has been reported for dogs after premedication with acepromazine and atropine,[3] a finding not confirmed in a subsequent study.[20] Few reports have evaluated the effects of anesthetic drugs on platelet reactivity; however, sevoflurane and propofol were associated with decreased aggregation.[64] Antibiotics may have transient effects on platelet aggregation. Tetracycline, cephalothin, and cefmetazole decreased canine platelet responses to ADP,[35,122] while amoxicillin, cephalexin, enrofloxacin, and doxycycline did not.[120] Other penicillin-related drugs may have antiplatelet effects at clinically relevant doses.[56] An in vitro study found no effect on platelet function of intravenous lipid therapy.[116] A recent study suggested that immunosuppressive medications including prednisone, cyclosporine, mycophenolate mofetil, azathioprine, and leflunomide do not have significant effects on platelet function of healthy dogs.[113] This report differs from another that documented increased ADP-induced aggregation in healthy dogs receiving immunosuppressive dosages of prednisone.[92]

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CHAPTER 86

Treatment of Disorders of Platelet Number and Function

MARY BETH CALLAN

Treatment of Disorders of Platelet Number
- Primary Immune Thrombocytopenia
  - Immunosuppression and Immunomodulation
  - Thrombopoietin Receptor Agonists
  - Splenectomy
  - Blood Component Therapy
- Essential Thrombocythemia

Treatment of Disorders of Platelet Function
- Von Willebrand Disease
  - Desmopressin
  - Blood Component Therapy
  - Antifibrinolytic Agents
- Inherited Platelet Dysfunction
  - Blood Component Therapy
  - Desmopressin
  - Antifibrinolytics
- Acquired Platelet Dysfunction
  - Desmopressin
  - Antifibrinolytic Agents

Acronyms and Abbreviations

BMBT, buccal mucosal bleeding time; CRYO, cryoprecipitate; DDAVP, 1-deamino-8-D-arginine vasopressin; EACA, epsilon aminocaproic acid; ET, essential thrombocythemia; FFP, fresh frozen plasma; FWB, fresh whole blood; hIVIG, human intravenous immunoglobulin; ITP, immune thrombocytopenia; JAK, janus kinase; MA, maximum amplitude; PC, platelet concentrate; PFA-100, Platelet Function Analyzer-100; pRBC, packed red blood cells; PRP, platelet-rich plasma; TEG, thromboelastogram; TPO, thrombopoietin; TPO-RA, thrombopoietin receptor agonist; TXA, tranexamic acid; VWD, von Willebrand disease; VWF, von Willebrand factor.

TREATMENT OF DISORDERS OF PLATELET NUMBER

Disorders of platelet number include thrombocytopenia and thrombocytosis (see Chapters 80–82). Primary immune thrombocytopenia (ITP) is considered the most common cause of *severe* thrombocytopenia in dogs, but is diagnosed rarely in cats. Treatment of quantitative platelet defects is often directed at correction of underlying disorders. Guidelines and procedures for platelet transfusion are presented in this chapter (and see Chapters 94 and 101).

Primary Immune-Mediated Thrombocytopenia

Immunosuppression and Immunomodulation

Glucocorticoids are the mainstay of treatment for primary ITP and other immune cytopenias (see Chapters 35 and 36). Adjunctive therapy of ITP includes vincristine and human intravenous immunoglobulin (hIVIG). Vincristine's precise mechanism of action leading to increased platelet counts in patients with ITP is unknown. It has been proposed that vincristine, bound to platelet microtubules, is transported to the spleen, where it inhibits the phagocytic function of splenic macrophages.[1] Vincristine may also stimulate thrombopoiesis.[1] While

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hIVIG has many actions, blockade of Fc receptors on macrophages is considered the mechanism for the observed response in dogs with ITP.[7,43] Both vincristine and hIVIG have been documented to shorten the duration of severe thrombocytopenia in dogs with primary ITP versus prednisone alone.[3,7,44] A comparison of platelet recovery times for dogs treated with single-dose hIVIG (0.5 g/kg IV) versus vincristine (0.02 mg/kg IV) in combination with glucocorticoids revealed no significant difference between treatments.[3] Therefore, vincristine is considered more cost-effective as first-line adjunctive treatment for initial management of canine primary ITP. Although single-dose vincristine is generally well tolerated, perivascular sloughing due to extravasation is a severe potential complication. Moreover, since the majority of dogs with primary ITP respond to glucocorticoids alone within 7 days,[7,44] adjunctive treatment may not be appropriate for all cases.

Beyond the comparison of vincristine and hIVIG, prospective, controlled studies comparing efficacy of adjunctive immunomodulatory agents in treatment of dogs with primary ITP are lacking. Retrospective studies and case series of canine primary ITP have described use of azathioprine,[22,39,41,48] cyclosporine,[13,39,41,48] and mycophenolate mofetil,[13,39,48] in combination with glucocorticoids (with or without vincristine); however, these studies are not designed to determine efficacy. A case series describing treatment with mycophenolate mofetil as a single agent in five dogs with presumptive primary ITP found that all dogs achieved a complete remission and had no evidence of relapse during a median follow-up of 16 months (range, 5–32 months).[54]

The optimal duration of treatment for dogs with primary ITP is unknown, but many dogs are empirically managed with tapering doses of immunosuppressive drugs over 4–6 months while monitoring for relapse.[39] Reported rates of relapse (typically defined as platelet count <150,000/μL after prior increase to within reference interval) for dogs with ITP vary widely, ranging from 9 to 47%.[25,39,41,48,52] Differences in treatment regimens and monitoring protocols likely contribute to such variability. A retrospective study comparing relapse rates among 45 dogs with ITP prescribed immunosuppressive therapy for a finite period of time (median, 137 days) or indefinitely (median, 640 days), found no difference, with relapse rates of 15% and 18.9% in the finite and indefinite treatment groups, respectively.[48] These results suggest no clinical benefit to continuing indefinite immunosuppression after initial diagnosis of primary ITP.[48]

While glucocorticoids are the mainstay treatment of primary ITP in dogs, oral prednisolone was deemed effective in only five of nine cats in two case series.[6,53] Other immunosuppressive agents administered included dexamethasone, cyclosporine, and chlorambucil, though there are insufficient cases to assess efficacy.[6,53] In a four-cat case series, each had a chronic course with frequent relapses and long-term immunosuppression was required for three cats that survived to discharge.[6]

Thrombopoietin Receptor Agonists

In addition to platelet destruction, ITP is characterized by suppressed platelet production (see Chapter 80).[38] Thrombopoietin receptor agonists (TPO-RAs), including romiplostim and eltrombopag, have been approved for treatment of chronic primary ITP in humans (see Chapter 80).[2] These agents bind to the TPO receptor c-Mpl, thereby increasing megakaryocyte growth and differentiation.[2] Thrombotic complications are potential adverse events of TPO-RA-induced thrombocytosis. A variable clinical response to TPO-RAs has been observed in humans with chronic ITP; nonresponders have higher endogenous TPO levels than responders.[2]

There is limited information on the use of TPO-RAs in veterinary medicine. Five dogs, including one with primary ITP, two with primary immune-mediated hemolytic anemia and ITP (Evans syndrome), and two with secondary ITP associated with ehrlichiosis, were treated with romiplostim at 3–5 μg/kg SC once weekly, after one or more relapses while receiving conventional immunosuppressive therapy.[28] An increased platelet count was observed within 3–6 days of administration of romiplostim in four of five dogs, all of which achieved complete remission within 2 weeks with no adverse effects.[28] The maintenance romiplostim dosage was decreased in all four dogs (2–3.4 μg/kg/week). The mean length of treatment was 13.8 weeks.[28] While these results are promising, high cost may limit romiplostim use in veterinary patients.

Splenectomy

Splenectomy has traditionally been a second-line therapy for relapsing or refractory primary ITP in humans, with reported 5-year response rate of 60–70%. More recently, the use of TPO-RAs and rituximab, an anti-CD20 antibody, has resulted in a tendency to avoid or delay splenectomy, despite the newer agents having lower response and higher relapse rates than splenectomy.[29] Major complications associated with splenectomy in humans include increased risk of bacterial infections, sepsis, and thrombosis.[29]

Anecdotal experience suggests that splenectomy may be an effective option for dogs with relapsing primary ITP; however, controlled studies are lacking. Among fourcase series, splenectomy was performed in 22 dogs with presumptive primary immune-mediated hematologic diseases, including 16 dogs with ITP only, three dogs with both ITP and immune-mediated hemolytic anemia, and three dogs with both ITP and immune-mediated neutropenia.[18,25,51,52] Fourteen of 22 dogs achieved remission or maintained a stable platelet count >60,000/μL postsplenectomy, and medications were discontinued in eight dogs. Thorough screening for occult vector-borne pathogens should be performed before splenectomy, since it is particularly difficult to clear Babesia spp., and other such infections after splenectomy.

Blood Component Therapy

Transfusion may be required in dogs with ITP experiencing severe mucosal bleeding, especially hemorrhage into the gastrointestinal tract. In such cases, packed red blood cell (pRBC) transfusions are indicated to provide additional oxygen-carrying support, and to enhance hemostasis. In a study evaluating a bleeding assessment tool (DOGiBAT—see Chapter 80) to standardize characterization of the severity of bleeding in dogs with primary ITP, 13 of 34 dogs (38.2%) received blood products during hospitalization, with pRBCs the most common blood product administered.[31] The admission platelet count was not correlated with transfusion, duration of hospitalization, or survival to discharge, indicating that platelet count alone does not consistently predict clinical bleeding.[31] Therefore, a "platelet transfusion trigger," i.e., a platelet count threshold to administer prophylactic platelet transfusions, is not appropriate for dogs with ITP. Furthermore, transfused platelets may be rapidly destroyed in dogs with ITP, rendering platelet transfusions of questionable benefit. However, in dogs with ITP experiencing uncontrolled or life-threatening bleeding (e.g., bleeding into the brain or lungs), platelet transfusions might provide short-term hemostasis despite negligible platelet count increments post-transfusion.

In clinical practice, fresh whole blood (FWB) is often the most readily available platelet product, but other options include platelet-rich plasma (PRP), platelet concentrate (PC), cryopreserved PRP/PC, and lyophilized platelets (see Chapter 101). In a prospective clinical trial of 37 thrombocytopenic dogs (27 primary ITP) randomized to receive canine lyophilized platelets or fresh PC, active bleeding was unchanged immediately post-transfusion in 18 of 22 dogs receiving lyophilized platelets and in all dogs receiving fresh PC.[14] In a retrospective evaluation of cryopreserved canine PC transfusions to thrombocytopenic dogs (28 of 43 with ITP), there was no difference in clinical bleeding or patient survival compared to a control group that did not receive any platelet products.[36] While not conducted as efficacy studies, cryopreserved and lyophilized platelets appeared to be well tolerated by thrombocytopenic dogs.[14,36]

Essential Thrombocythemia

Thrombocytosis (see Chapter 82) is frequently an incidental CBC abnormality, but marked thrombocytosis associated with hemorrhage or thrombosis may warrant treatment. Reactive thrombocytosis, even with platelet counts >1,000,000/μL, does not typically cause clinical signs. Resolution of reactive thrombocytosis involves treating the underlying disease (e.g., inflammation, infection, neoplasia, iron deficiency).

Essential thrombocythemia (ET), a chronic myeloproliferative disorder of clonal proliferation of megakaryocytes, is rare in animals (see Chapter 82). Case reports of probable ET in dogs and cats describe the use of radiophosphorus and hydroxyurea.[4,23,49] In human medicine, cytoreductive therapy is warranted only in patients with high-risk disease, defined as a history of thrombosis or advanced age (>60 years) with janus kinase (JAK)2 mutations. The first-line drug of choice is hydroxyurea and second-line drugs are interferon-α and busulfan.[50] In addition, low-dose aspirin has been recommended for humans with low- to high-risk disease to alleviate vasomotor (microvascular) disturbances associated with ET.[50] Use of ruxolitinib or other JAK2 inhibitors is not recommended as first-line therapy.[50] The molecular basis of ET in dogs and cats has not yet been elucidated and might differ from that in humans, thus preventing any benefit of JAK2 inhibition.

TREATMENT OF DISORDERS OF PLATELET FUNCTION

Disorders of platelet function include von Willebrand disease (VWD) (see Chapter 83), hereditary thrombopathia, or intrinsic platelet dysfunction (see Chapter 84), and acquired platelet function defects associated with various systemic disorders and medications (see Chapter 85).

Von Willebrand Disease

Medical management of VWD is aimed toward controlling spontaneous (e.g., epistaxis, hematuria) or post-traumatic or postoperative bleeding and supporting surgical hemostasis. While dogs with type 2 and type 3 VWD inevitably have a severe bleeding tendency, the phenotype among dogs with type 1 VWD is variable. Some type 1 VWD dogs have marked reduction in plasma von Willebrand factor (VWF) concentration, but no apparent bleeding tendency. Therefore, the need for prophylactic treatment, particularly with blood component therapy, should be assessed on a case-by-case basis.

Desmopressin

Desmopressin acetate (1-deamino-8-D-arginine vasopressin [DDAVP]), a synthetic analog of the neurohypophyseal hormone vasopressin, has been used to control bleeding in a variety of canine hemostatic disorders, but most often for VWD. DDAVP causes release of VWF from endothelial cell storage granules, referred to as Weibel–Palade bodies. A study evaluating the effects of DDAVP (1 μg/kg SC) to dogs with type 1 VWD found only modest increase in mean plasma VWF concentration (baseline, 10%; 1 hour post-treatment, 17%) but evidence of improved hemostatic function assessed by Platelet Function Analyzer (PFA)-100 and shortening of buccal mucosal bleeding time (BMBT).[11] DDAVP has been reported to be ineffective in controlling bleeding in patients with type 3 VWD, since it acts by releasing intracellular stores of VWF and these patients do not synthesize VWF protein.[5]

DDAVP is available as a sterile solution (4 μg/mL) for intravenous administration and a nasal spray (100 μg/mL)

that can be administered subcutaneously to dogs. The nasal spray is used more frequently due to lower cost. A recommended dose is 1 µg/kg SC, administered not more than once daily due to the risk of antidiuretic hormone effects of water retention and hyponatremia, as well as failure to elicit VWF release with repeated administration. DDAVP should be administered 30 minutes prior to surgery to assist surgical hemostasis.

Blood Component Therapy

Blood products containing VWF include FWB, fresh frozen plasma (FFP), and cryoprecipitate (CRYO), the latter representing the blood component of choice (see Chapters 83 and 100). Since CRYO contains a concentrated amount of VWF compared to FFP, CRYO is more easily administered and avoids volume overload. One of the main indications for CRYO or FFP is to control spontaneous or traumatic/surgical bleeding not amenable to topical therapies (e.g., compression, cautery, gel foam) or not responsive to DDAVP. Due to the short half-life of plasma VWF (approximately 12 hours), CRYO or FFP transfusions may need to be administered every 8–12 hours to control severe bleeding. In addition, CRYO or FFP can be administered prophylactically before surgery, particularly in dogs with a known bleeding tendency or for surgical procedures in which small volume hemorrhage could have devastating consequences (e.g., cervical ventral slot surgery). Sufficient CRYO or FFP (see Chapter 100 for dosage guidelines) should be given to ensure rapid increase in plasma VWF levels for effective platelet adhesion. However, prophylactic administration of CRYO or FFP is not always required, as DDAVP may be sufficient to prevent surgical bleeding in some dogs. In VWF-deficient dogs without a bleeding history that receive only DDAVP preoperatively, CRYO or FFP should be available in case excessive bleeding is observed during or after surgery. Similarly, blood-type-compatible and cross-match-compatible pRBCs should be available in the event of excessive blood loss.

Feline VWD is rare, with only two published case reports of type 3 VWD. Epistaxis was controlled in one cat with administration of FFP. Feline CRYO is not prepared routinely due to limited need and technical challenges associated with processing small volumes of FFP.[5]

Von Willebrand disease has been reported uncommonly in horses. Transfusion of fresh plasma (4 L) to an affected Thoroughbred mare effectively controlled epistaxis and periparturient hemorrhage.[42] However, a single preoperative transfusion of fresh plasma (2 L) did not prevent abnormal bleeding after surgical repair of an umbilical hernia and castration of an affected foal, suggesting that more aggressive transfusion support might be necessary to control surgical hemorrhage.[42] Use of DDAVP to manage VWD in horses has not been reported and the cost of the drug may be prohibitive in large animals.

Antifibrinolytic Agents

The antifibrinolytic agents epsilon aminocaproic acid (EACA) and tranexamic acid (TXA) are synthetic lysine analogs that reversibly bind to plasminogen, preventing plasmin from binding to fibrin, thereby slowing the rate of fibrinolysis. Antifibrinolytics are used to control bleeding due to VWD in humans, with suggested management including TXA with or without DDAVP for minor bleeding and TXA plus VWF concentrate for major bleeding.[37] Antifibrinolytics are typically administered until bleeding is controlled, or for 5–7 days postoperatively.[37]

While there are no published studies evaluating efficacy of antifibrinolytics for canine VWD, clinical experience suggests that TXA and EACA can be used as adjunctive therapy. In an in vitro model of hyperfibrinolysis, both TXA (10–20 mg/kg IV or 15–20mg/kg PO) and EACA (20–100 mg/kg IV or 100 mg/kg PO) improved clot strength and decreased fibrinolysis in a dose-dependent manner in blood samples from healthy dogs 1–4 or 1–6 hours after administration of EACA or TXA, respectively.[10,40] Although optimal dosing for dogs has not yet been determined,[33] pharmacokinetic and in vitro fibrinolytic studies[10,40] suggest dosing recommendations for TXA of 15–20 mg/kg PO or IV and for EACA dosing at 50–100 mg/kg PO or IV, both given q 6–8 hours.

In a retrospective study evaluating the safety of TXA in 68 dogs with bleeding due to a variety of causes, vomiting was the only observed adverse effect.[27] In experimental studies, vomiting was observed within 5 minutes of receiving intravenous doses >10 mg/kg.[26,27,40] Tranexamic-acid-induced vomiting is thought to result from activation of the tachykinin neurokinin 1 receptor.[26] Pretreatment with maropitant, a neurokinin 1 antagonist, could help prevent nausea. In a retrospective study of 122 bleeding dogs treated with EACA, no adverse events were reported; however, its efficacy is currently unlcear.[15]

Inherited Platelet Dysfunction

Variable bleeding tendencies are associated with inherited intrinsic platelet disorders (see Chapter 84). Guidelines from the United Kingdom Haemophilia Centre Doctors' Organisation (UKHCDO) for management of bleeding associated with inherited thrombopathias in humans include use of antifibrinolytics to control minor spontaneous bleeding (e.g., epistaxis) and prevent excessive bleeding associated with dental extractions and surgical procedures. Antifibrinolytic therapy is begun the day before a scheduled procedure and continued for 5–7 days, or longer if needed.[8] Addition of DDAVP is considered if antifibrinolytics alone are ineffective. Platelet transfusions are recommended for severe platelet disorders and when other agents have failed.

Blood Component Therapy

Animals with hereditary thrombopathias experiencing severe, uncontrolled, or life-threatening bleeding require

platelet transfusions. In addition, prophylactic platelet transfusions should be considered in patients with a known bleeding tendency and documented (or suspected) thrombopathia undergoing surgery. Fresh PRP administered to a Great Pyrenees with Glanzmann thrombasthenia immediately before an elective ovariectomy provided effective surgical hemostasis.[9] Canine cryopreserved PC has been administered prophylactically to dogs with platelet procoagulant deficiency (Scott syndrome) prior to elective neutering and for management of nonsurgical hemorrhage, mainly epistaxis.[24] Since cryopreserved platelets externalize phosphatidylserine, they may offer a theoretical advantage to fresh platelets in promoting thrombin generation in these patients.[24]

Desmopressin

Administration of DDAVP has been associated with shortening of bleeding time or reduced bleeding tendencies in people with some forms of inherited intrinsic platelet dysfunction, such as platelet storage pool disease (platelet granule defect) and Bernard–Soulier syndrome (lack of platelet GPIb receptor), but not in Glanzmann thrombasthenia (lack of platelet GPIIb/IIIa receptor).[17,32] The mechanisms underlying improved hemostasis in such conditions remain unclear. It has been proposed that DDAVP improves platelet function via increased formation of procoagulant "COAT" platelets (referring to combined agonists, COllagen And Thrombin, that induce this response in vitro and the resultant coating of the platelet surface by a layer of adhesive and procoagulant proteins).[12]

Controlled studies evaluating efficacy of DDAVP in improving hemostasis in animals with inherited intrinsic platelet disorders are lacking. In light of the beneficial hemostatic effect of DDAVP observed in humans with a variety of hereditary platelet function disorders and its excellent safety profile in dogs, it is reasonable to consider a trial with DDAVP (1 μg/kg SC administered not more than once daily) in dogs with suspected or confirmed inherited thrombopathia.

Antifibrinolytics

Antifibrinolytics have been used in dogs with inherited thrombopathias in conjunction with blood component therapy, thus complicating assessment of their beneficial effect. Tranexamic acid (10 mg/kg IV q8h for a total of four doses) was administered postoperatively to seven German Shepherds with Scott syndrome that had received cryopreserved PC in preparation for elective ovariohysterectomy or castration.[24] Five of seven dogs developed mild to moderate incisional bruising and swelling that did not require additional therapy, whereas two dogs developed postoperative hemorrhage that required additional platelet transfusions.[24] Epsilon aminocaproic acid (30 mg/kg IV q8h) and fresh PRP were administered to a Greater Swiss Mountain dog heterozygous for a *P2RY12* ADP receptor mutation that developed a hemoabdomen post ovariohysterectomy, with successful control of the bleeding.[20] Similar to DDAVP, TXA and EACA are well tolerated by dogs and could be considered as adjunct therapy for management of bleeding in dogs with hereditary thrombopathias, recognizing that efficacy is uncertain in this situation.

Acquired Platelet Dysfunction

In patients with acquired platelet dysfunction (see Chapter 85), treatment and reversal of the underlying disease is the most effective approach to managing the resultant bleeding tendency. For example, in patients with severe kidney disease, hemodialysis to remove uremic toxins and administration of darbepoetin or pRBC transfusions for anemia should improve hemostasis. However, correction of the underlying disease is not always possible or does not yield timely results, thus platelet transfusions may be necessary to achieve hemostasis. Medical management with DDAVP and antifibrinolytic agents may be appropriate, based on human reports.

Desmopressin

Administration of DDAVP has been associated with a shortening of bleeding time or an improvement in bleeding tendencies noted in people with acquired platelet dysfunction due to aspirin ingestion, renal disease, and liver disease.[17,32] Studies evaluating efficacy of DDAVP in animals with acquired platelet dysfunction are lacking, though small case series in dogs have described shortening of prolonged BMBTs associated with aspirin administration,[16,46] chronic liver disease,[46] and chronic monocytic ehrlichiosis.[21] Furthermore, excessive intraoperative bleeding was not observed in three dogs with aspirin-induced platelet dysfunction treated with DDAVP before surgery for intervertebral disc disease,[16] and surface bleeding observed in dogs with ehrlichiosis subsided within 1 hour of DDAVP administration.[21]

Antifibrinolytic Agents

Antifibrinolytic agents may be used as adjunct therapy in people with acquired platelet dysfunction. Tranexamic acid reduced circulating plasminogen concentration, decreased fibrin/fibrinogen degradation products, shortened bleeding time, and improved platelet aggregation and secretion in people with severe chronic renal failure.[34] A study evaluating low-dose TXA (administered orally 5 days per week for 3 months) in chronic hemodialysis patients demonstrated improved platelet function as assessed by PFA-100 and increased blood hemoglobin levels compared to baseline.[45] However, use of TXA in patients with chronic kidney disease is controversial, as it is excreted primarily through the kidneys. Failure to adjust dosage has caused neurotoxicity (seizures, blurred vision, myoclonus), which resolved within a few days of discontinuing therapy.[30]

The use of antifibrinolytics to manage bleeding caused by acquired platelet dysfunction has not been evaluated in veterinary medicine. Both TXA and EACA have been administered to dogs with bleeding associated with neoplasia, surgery, trauma, liver failure, anticoagulant rodenticide intoxication, cyclophosphamide-induced hemorrhagic cystitis, and *Angiostrongylus vasorum* infection.[19,27,35,47] The efficacy of antifibrinolytics in ameliorating bleeding in most of these conditions is unclear, though TXA and EACA appeared to be well tolerated, with no thrombotic complications observed.

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SECTION VII
Hemostasis

CHAPTER 87

Overview of Hemostasis

MAUREEN A. McMICHAEL

Biochemistry of Coagulation
- Coagulation Cascade Enzymatic Reactions
- Coagulation Complexes (Cofactors and Enzymes)
- Inhibition of Coagulation Enzymes
- Membrane Surface Interactions

Coagulation Proteins that Generate Thrombin
- Contact Pathway (Kallikrein–Kinin Pathway)
  - Factor XII
  - Prekallikrein
  - Kininogens
- Extrinsic (Tissue Factor) Pathway
  - Tissue Factor
  - Factor VII
- Intrinsic Pathway
  - Factor XI
  - Factor IX
  - Factor VIII
- Common Pathway
  - Factor X
  - Factor V
  - Prothrombin
- Coagulation Inhibitors
  - Tissue Factor Pathway Inhibitor
  - C1-Inhibitor
  - Antithrombin
  - Protein C Pathway
  - Miscellaneous Inhibitors

Cells that Participate in Hemostasis
- Endothelial Cells
  - Antithrombotic Properties of Endothelium
  - Prothrombotic Properties of Endothelium
  - Other Properties of Endothelial Cells
- Platelets
- Tissue-Factor-Bearing Cells
- Microvesicles
- The Role of Cell Surface Membranes

Cell-Based Model of Thrombin Generation
- Initiation
- Amplification
- Propagation

Thrombin
- Feedback Regulation of Coagulation
- Platelet Activation
- Fibrin Formation
- Activation of Factor XIII

Fibrinogen and Fibrin Clot Structure

Fibrinolysis
- Plasminogen
- Plasminogen Activators
  - Tissue-Type Plasminogen Activator
  - Urokinase Plasminogen Activator
- Fibrinolysis Inhibitors
  - Plasminogen Activator Inhibitor 1
  - α_2-Antiplasmin
  - Thrombin-Activatable Fibrinolysis Inhibitor
  - Miscellaneous Inhibitors

Hemostasis and Inflammation
- Effects of Hemostasis on Inflammation
- Effects of Inflammation on Hemostasis

Conclusion

Acronyms and Abbreviations

α_1-PI, α_1-protease inhibitor; α_2-MG, α_2-macroglobulin; α_2-AP, α_2-antiplasmin; AMP, adenosine monophosphate; APC, activated protein C; AT, antithrombin; C1-INH, C1-inhibitor; C4BP, C4-binding protein; DDAVP, desamino-8-D-arginine vasopressin; EPCR, endothelial protein C receptor; ER, endoplasmic reticulum; FV/FVa, factor V/activated factor V; FVII/FVIIa, factor VII/activated factor VII; FVIII/FVIIIa, factor VIII/activated factor VIII; FIX/FIXa, factor IX/activated factor IX; FX/FXa, factor X/activated factor X; FXI/FXIa, factor XI/activated factor XI; FXII/FXIIa,

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factor XII/activated factor XII; FXIII/FXIIIa, factor XIII/activated factor XIII; FDP, fibrin degradation products; FpA, fibrinopeptide A; FpB, fibrinopeptide B; Gla, gammacarboxyglutamic acid; GPI, glycosylphosphatidylinositol; HC, heparin cofactor II; HK, H-kininogen; HSPG, heparan sulfate proteoglycan; LPS, lipopolysaccharide; LRP, low-density lipoprotein receptor-related protein; MP, microparticle; MW, molecular weight; NO, nitric oxide; PAF, platelet-activating factor; PAI-1, plasminogen activator inhibitor-1; PARs, protease-activated receptors; PC, protein C; PChol, phosphatidylcholine; PCI, protein C inhibitor; PEth, phosphatidylethanolamine; PGI_2, prostacyclin; PK, prekallikrein; PLG, plasminogen; PS, protein S; PSer, phosphatidylserine; PZ, protein Z; sctPA, single-chain tissue plasminogen activator; scuPA, single-chain urokinase plasminogen activator; serpin, serine protease inhibitor; TAFI/TAFIa, thrombin-activatable fibrinolysis inhibitor/activated-thrombin-activatable fibrinolysis inhibitor; TF, tissue factor; TFPI, tissue factor pathway inhibitor; TM, thrombomodulin; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor; VWF, von Willebrand factor; ZPI, protein-Z-dependent protease inhibitor.

Hemostasis is a vital protective mechanism that prevents blood loss by sealing sites of injury in the vascular system. Hemostasis must be controlled, however, so that blood does not coagulate within the vasculature. Uncontrolled coagulation restricts normal blood flow, resulting in tissue hypoxia. Hemostasis consists of a tightly controlled and well-balanced interplay among a large number of cellular and protein participants. Endothelial cells lining the vasculature, cells outside the vasculature, and platelets all play important roles. Hemostatic proteins include inactive precursors (zymogens) that are converted to active enzymes, regulatory proteins (cofactors) that enhance the functionality of their corresponding coenzymes, and inhibitors that interfere with protein function through a variety of mechanisms (see Table 87.1).

Physiologic coagulation does not occur within the vasculature because of the nonreactive properties of the resting cellular participants, and because the majority of hemostatic proteins circulate in an inert or zymogen form. The initiation of coagulation in response to injury depends on the exposure of extravascular components that are not present within the bloodstream under physiologic conditions. Exposure of these extravascular participants initiates an explosive cascade of cellular activation, changes in cell-surface properties, and generation of active enzymes that produce a stable clot. Some of the cells and enzymes also trigger vascular wall responses, inflammatory pathways, and immune-defense mechanisms. Coagulation also initiates fibrinolysis, the process by which the clot is removed to restore blood flow. Ultimately, coagulation triggers the cell migration and proliferation that promote healing.

BIOCHEMISTRY OF COAGULATION

Coagulation Cascade Enzymatic Reactions

The clotting cascade consists of a series of enzymatic reactions. Protein participants circulate in the blood mostly as inert precursors, or zymogens. Zymogens are activated by limited proteolysis to form active enzymes. With the exception of thrombin, all of these enzymes have limited activity unless they bind to their protein cofactor and a procoagulant membrane surface. Upon complex assembly on a suitable membrane surface, the enzyme's bioactivity is markedly exaggerated to as much as several hundred–thousandfold.

Each cascade reaction is initiated when the active enzyme complex binds to its substrate, resulting in cleavage of the substrate to generate the next enzyme in the series. In many cases, positive feedback occurs because a downstream enzyme activates an upstream zymogen back, thereby markedly amplifying the generation of downstream enzymes from minimal activation of an upstream zymogen. Negative feedback is also a common feature in the coagulation cascade, with downstream enzymes generating inhibitors that downregulate activity of upstream enzymes. The generation of a blood clot is carefully controlled through the complex interplay of the initial trigger, amplification reactions, and inhibitors.

Coagulation Complexes (Cofactors and Enzymes)

The majority of coagulation enzymes are serine proteases with homology to trypsin, the prototype enzyme of this class. Serine proteases cleave after arginine residues. Other coagulation proteins have no enzymatic capabilities, rather they play regulatory roles in enhancing enzymatic activity. These proteins circulate in inactive forms, called procofactors. A procofactor must be cleaved by the appropriate enzyme to result in a fully active cofactor. Proteolytic activation of zymogens to enzymes and procofactors to cofactors can take several forms (Figure 87.1).

Many of these proteins contain disulfide bonds between two cysteine residues. Enzymatic cleavage of an activation site that lies between the cysteine residues will convert the single-chain protein to a two-chain protein of identical molecular weight, without removing any amino acids. When the activation site does not lie between disulfide bond cysteine residues, cleavage of a single site will result in two different protein fragments. The smaller, nonfunctional fragment is the activation peptide, while the remaining fragment is the fully activated enzyme or cofactor.

Some of the proteins contain multiple activation sites. If both lie between disulfide-bridged cysteine residues, then an activation peptide is released and a two-chain enzyme is produced. The order in which the target sites are cleaved may result in inactive intermediates, or partially or fully active intermediate enzymes. Complete cleavage of all of the sites will generally result in multiple fragments.

TABLE 87.1 Hemostatic proteins, factors, cofactors, and inhibitors.

Functional classification	Abbreviation	Current/previous names	Characterization
Extrinsic pathway	TF	Tissue factor/thromboplastin	Cofactor
	FVII	Factor VII/proconvertin	Zymogen
	FVIIa	Factor VIIa	Enzyme
Contact pathway	FXII	Factor XII/Hageman factor	Zymogen
	FXIIa	Factor XIIa	Enzyme
	PK	Prekallikrein/Fletcher factor	Zymogen
	K	Kallikrein	Enzyme
	HK	H-kininogen/Williams–Fitzgerald–Flaujeac factor, High molecular weight (MW) kininogen	Cofactor
Intrinsic pathway	FXI	Factor XI/plasma thromboplastin antecedent	Zymogen
	FXIa	Factor XIa	Enzyme
	FIX	Factor IX/Christmas factor	Zymogen
	FIXa	Factor IXa	Enzyme
	FVIII	Factor VIII/antihemophilic factor	Procofactor
	FVIIIa	Factor VIIIa	Cofactor
Common	FX	Factor X/Stuart–Prower factor	Zymogen
	FXa	Factor Xa	Enzyme
	FV	Factor V/labile factor	Procofactor
	FVa	Factor Va	Cofactor
	Pro	Prothrombin/factor II	Zymogen
	Fg	Fibrinogen/factor I	Substrate
	Fb	Fibrin	Final product
	FXIII	Factor XIII	Zymogen
	FXIIIa	Factor XIIIa	Enzyme
Coagulation inhibitors	α_1-PI	α_1-Protease inhibitor/α_1-antitrypsin	
	AT	Antithrombin/antithrombin III	Serpin
	HC	Heparin cofactor II	Serpin
	TFPI	Tissue factor pathway inhibitor/extrinsic pathway inhibitor	Kunitz inhibitor
	PC	Protein C	Zymogen
	APC	Activated protein C	Enzyme, inhibitor
	PS	Protein S	Cofactor
	TM	Thrombomodulin	Cofactor
	PZ	Protein Z	Cofactor
	ZPI	Protein-Z-dependent protease inhibitor	Enzyme
Fibrinolysis	PLG	Plasminogen	Zymogen
	PLM	Plasmin	Enzyme
	scuPA	Single-chain urokinase plasminogen activator/Prourokinase	Zymogen (slight enzyme)
	tcuPA (uPA)	Two-chain urokinase plasminogen activator/urokinase	Enzyme
	sctPA	Single-chain tissue-type plasminogen activator	Enzyme
	tctPA (tPA)	Two-chain tissue-type plasminogen activator	Enzyme
Fibrinolysis inhibitors	α_2-AP	α_2-Antiplasmin/plasmin inhibitor	Serpin
	PAI-1	Plasminogen activator inhibitor-1	Serpin
	TAFIa	Thrombin-activatable fibrinolysis inhibitor/carboxypeptidase U, B, or R	Enzyme
Nonspecific inhibitors	C1-INH	C1-inhibitor	Inhibitor
	α_2-MG	α_2-Macroglobulin	Inhibitor
	PN1	Protease nexin-1	Inhibitor
	PCI	Protein C inhibitor/plasminogen activator inhibitor-3	Serpin

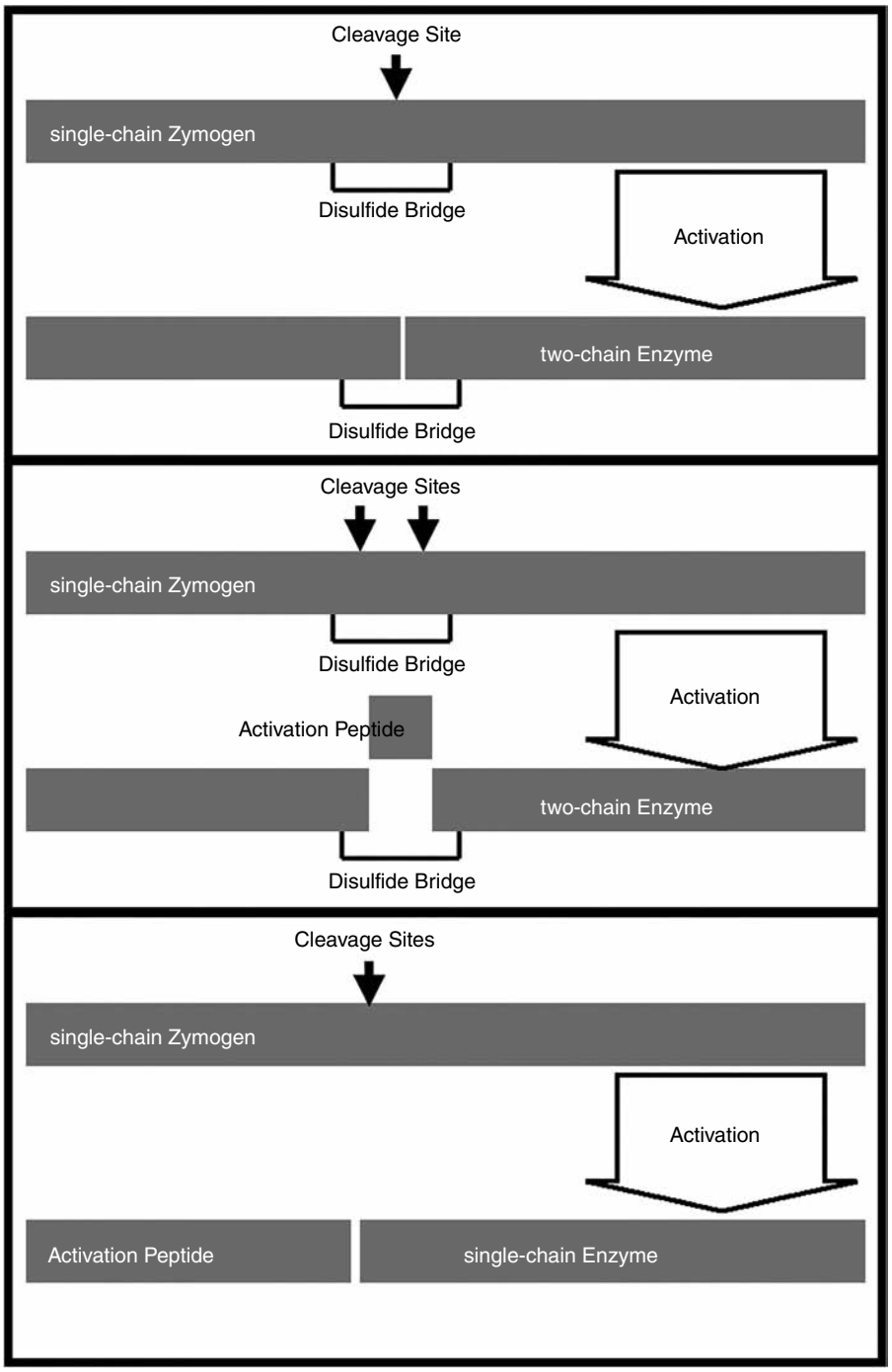


FIGURE 87.1 Enzymatic activation of coagulation proteins. Most coagulation proteins circulate as inactive, single-chain polypeptides containing disulfide bridges between nonadjacent cysteine residues. Depending on the location of the disulfide bridges and the cleavage site (or sites), enzymatic cleavage may result in a two-chain activated protein of identical size to the precursor, a two-chain activated protein (smaller than the precursor) and a released activation peptide, or a single-chain activated protein (smaller than the precursor) and a released activation peptide.

Fully functional enzymatic complexes often consist of both an enzymatic subunit (the serine protease) and a regulatory subunit (the cofactor).

Inhibition of Coagulation Enzymes

Coagulation and fibrinolysis enzymes are inhibited through several mechanisms: enzyme cleavage, active site blockade, stable complex formation, and substrate modification (Figure 87.2).[43]

Enzymatic inhibitors act by cleaving the target protein, cutting it into dysfunctional fragments. An example of this enzymatic inhibition is the cleavage of the cofactor activated factor V (FVa) by the serine protease, activated protein C (APC).

Active site blockade, a common mechanism of pharmacologic inhibitors, is also employed by naturally occurring inhibitors. Small molecule inhibitors may fit directly into an enzymatic site, thereby limiting substrate access.

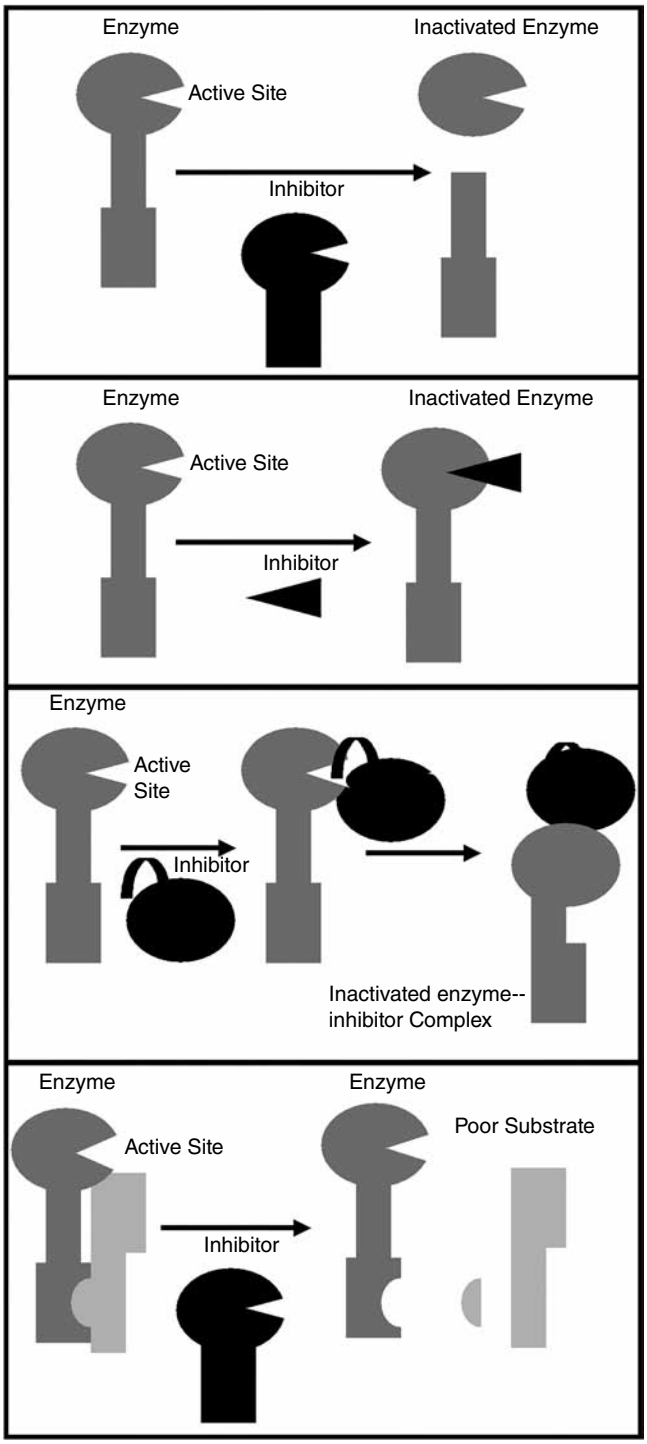


FIGURE 87.2 Coagulation enzyme and cofactor inhibition. Inhibitors work through a variety of different mechanisms: (1) Activated coagulation proteins can be inhibited by enzymes that cleave the target protein, damaging the structure and resulting in an inactivated protein. (2) Active site inhibitors bind the enzymatic site of the target enzyme, preventing the protease from cleaving its target protein. (3) Other inhibitors, such as serpins, display a "bait" peptide that looks like a substrate for the target enzyme. When the enzyme binds to and attempts to cleave the bait, the inhibitor changes in structure, resulting in a shift in position and structure of the target enzyme, which inactivates the enzyme into a stable complex with the inhibitor. (4) Some inhibitors modify the substrate rather than the target enzyme. These changes usually decrease binding interactions between the enzyme and the substrate, slowing or eliminating the ability of the enzyme to cleave its substrate.

Other inhibitors act through enzyme binding. The target enzyme and inhibitor form a stable complex that permanently inactivates the enzyme. An example of this mechanism is inhibition of thrombin by antithrombin-heparin. Antithrombin (AT) is a member of the serpin (serine protease inhibitor) class of inhibitors. These inhibitors employ a "suicide-substrate" mechanism. They contain a small peptide loop that acts as "bait" that mimics substrates of the target enzyme. Binding of the target enzyme to the bait results in serpin structural

modifications that shift the position and conformation of the enzyme to form a stable complex.

A few inhibitors work by changing the nature of the substrate, so that the enzyme cannot bind effectively to its target site. Thrombin-activatable fibrinolysis inhibitor (TAFI) cleaves fibrin residues such that plasmin binding is impaired and plasmin-mediated fibrinolysis is delayed.

Membrane Surface Interactions

The rate of coagulation is profoundly affected by the presence of appropriate membrane surfaces for enzymatic reactions. Membrane binding enhances enzyme activity because localization to a membrane surface helps properly align the participating proteins. Tissue factor (TF) is the only coagulation protein that is permanently attached to the membrane surface.[40] Some coagulation proteins (factors VII, IX, X [FVII, FIX, FX], prothrombin, proteins C, S, Z [PC, PS, PZ]) contain gammacarboxyglutamic acid (Gla) residues that enable protein binding to a membrane surface via interaction with calcium and negatively charged phospholipids. Calcium binding of the Gla domains of these proteins requires that the glutamic acid residues are carboxylated via intrahepatic post-translational modifications involving the vitamin K cycle (Figure 87.3). Incompletely carboxylated Gla proteins do not properly bind calcium and are unable to assemble on an activated membrane surface. The importance of carboxylation in membrane binding is dramatically illustrated by the profound adverse impact of vitamin K antagonists such as warfarin (see Chapter 89). The requirement of calcium for membrane binding explains the ability of calcium chelators (e.g., citrate and EDTA) to prevent activation of many coagulation enzymes.

Some cofactors (factors V and VIII [FV and FVIII]) also have regions that interact with phospholipids, allowing fully functional enzymatic complexes to assemble on a membrane surface. The mechanism of membrane binding is less well-described than Gla protein binding, but involves the stereochemical configuration of the phosphatidylserine (PSer) head group.[24,26]

COAGULATION PROTEINS THAT GENERATE THROMBIN

Previous coagulation models divided coagulation into the extrinsic, intrinsic, and common pathways (Table 87.1, Figure 87.4). The extrinsic pathway leads to generation of activated factor X (FXa) by the complex of TF and activated factor VII (TF–FVIIa). The terminology "extrinsic" pathway arose from the requirement for TF (an extravascular, cell-bound protein) as a clotting trigger. The intrinsic pathway proceeds with the contact activation of factor XII (FXII), followed by the cascading activation of the downstream protease factor XI (FXI), then FIXa–FVIIIa, and ultimately results in FXa generation. This pathway was termed the "intrinsic" pathway due to the fact that it did not require the addition of any extravascular trigger and appeared to be intrinsic to blood. The common pathway consists of the formation of the FXa–FVa complex, which activates prothrombin to generate thrombin. Thrombin then cleaves fibrinogen to fibrin, which spontaneously polymerizes into an insoluble fibrin gel.[9] These pathway classifications have been useful for in vitro laboratory evaluation, but such divisions do not occur in vivo.

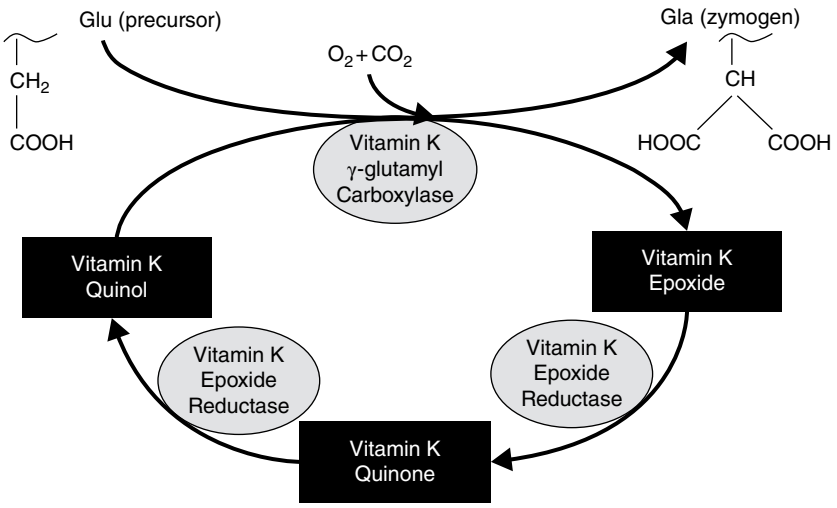


FIGURE 87.3 The vitamin K cycle. This cycle is necessary for hepatic production of fully functional vitamin-K-dependent coagulation proteins. These proteins (prothrombin, FVII, FIX, FX, PC, PS, PZ) are produced as precursors containing glutamic acid residues. The enzyme vitamin K γ-glutamyl carboxylase modifies these residues to γ-carboxyglutamic acid (Gla). The zymogens containing Gla residues are capable of binding Ca2+ to assemble on membrane surfaces and form active enzyme complexes. As a result of the carboxylase reaction, the vitamin K is converted to an epoxide, and must be regenerated via the activity of vitamin K epoxide reductase. Warfarin and its derivatives interfere with vitamin K epoxide reductase, preventing recycling of vitamin K epoxide to vitamin K quinol.

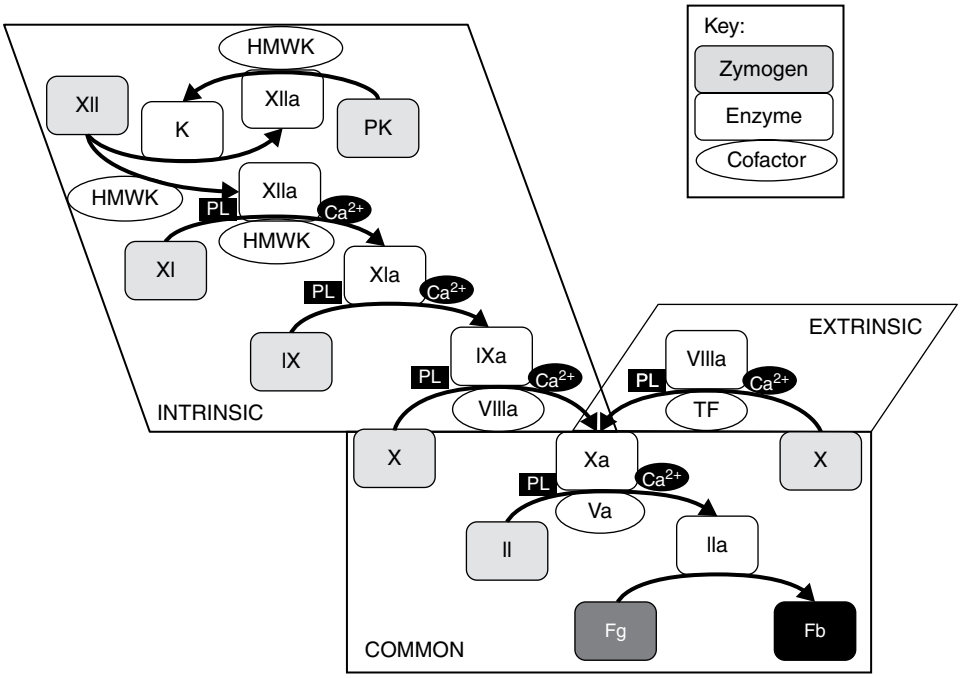


FIGURE 87.4 The cascade model of fibrin formation. This early model divided the coagulation system into separate, seemingly redundant pathways (extrinsic and intrinsic) either of which could result in the generation of FXa. The common pathway consisted of formation of the prothrombinase complex (FXa–FVa) and resulted in generation of thrombin, followed by cleavage of fibrinogen to form fibrin. Many of the enzymes and enzymatic complexes require calcium (Ca2+) and binding to active membrane surfaces (PL) for full activity. For simplicity, inhibitors of the various enzymes and feedback activation of procofactors to cofactors have been omitted. See Table 87.1 for abbreviations.

Contact Pathway (Kallikrein–Kinin Pathway)

The contact pathway consists of the zymogens FXII (Hageman factor) and prekallikrein (PK), and the cofactor high-molecular-weight kininogen (H-kininogen, HK). Activation of the two zymogens occurs spontaneously (autoactivation) upon assembly of the proteins on a suitable negatively charged surface.[8] Because FXIIa can cleave downstream coagulation proteases, contact activation ultimately results in generation of thrombin and therefore formation of a fibrin clot. Consequently, contact activation (i.e., creation of the enzymes FXIIa and kallikrein) occurs whenever blood comes in contact with an artificial surface. Contact activation always occurs, to some extent, when blood is removed from the vascular system. The contact pathway is the source of blood's apparent "intrinsic" clotting potential, and the reason blood coagulates when collected into a syringe or tube in the absence of anticoagulants. The amount of FXIIa and kallikrein generated depends on the negative charge of the artificial surface. Therefore, blood clots relatively slowly in plastic, more quickly in glass, and most rapidly when in contact with a strong negative charge. Note that contact activation is *not* calcium-dependent, and consequently is not abolished by collection of blood into calcium chelators such as citrate or EDTA. Activation of the contact pathway may influence viscoelastic tests that utilize a holding period, such as thromboelastography or thromboelastometry. An observed shift toward hypercoagulability during sample holding was most pronounced for equine blood samples, followed by canine, and then human blood samples, suggesting a hierarchy in contact pathway activation.[47,51] The results of this study illustrate species variability in the contact pathway (see Chapters 88 and 95).

Traditional coagulation cascade models depict the contact pathway and the extrinsic pathway as independent and redundant generators of FXa, and subsequent thrombin and fibrin production (see Figure 87.4). Current coagulation models indicate that the contact pathway, while an important feature of in vitro coagulation, is irrelevant for in vivo hemostasis. The evidence to support this concept includes comparative genomics data that reveal loss or inactivation of the FXII gene in cetaceans and non-mammalian vertebrates (see Chapter 95). In addition, many mammalian species (e.g., humans, mice, cats, and dogs) with inactivating FXII or PK mutations have no bleeding tendency.[41]

Research suggests that the contact pathway plays a role in pathologic thrombus formation.[17,19,41] The physiologically relevant negatively charged surface that allows contact activation to influence coagulation in vivo has not been identified, although RNA,[25] polyphosphate,[52] and misfolded proteins[30] have been proposed to serve this function.

Contact activation has a defined role, however, in several physiologic processes. Contact activation on negatively charged phospholipid cell membranes is primarily responsible for bradykinin generation. The contact pathway has additional anti-adhesive, profibrinolytic, proinflammatory, and antithrombotic properties, and is involved in angiogenesis (Figure 87.5).[8]

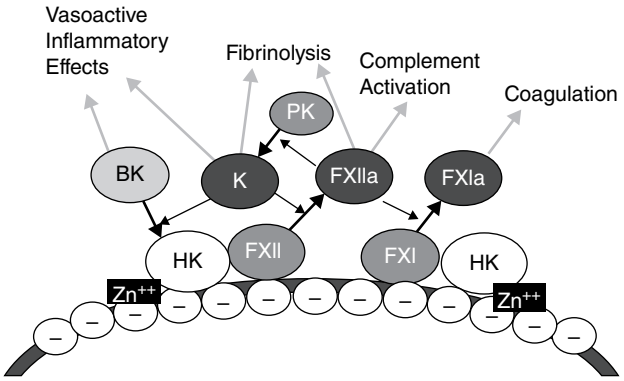


FIGURE 87.5 The contact pathway. This involves the assembly of FXII and K on a negatively charged surface, resulting in autoactivation of zymogen FXII to the enzyme FXIIa. FXIIa can then activate PK to K, and K can activate back more FXII to FXIIa. K also cleaves HK to generate BK, while FXIIa can activate FXI to FXIa. The major products of contact activation have important roles unrelated to coagulation, in inflammation, vasoactive regulation, and fibrinolysis. See Table 87.1 for abbreviations.

Factor XII

Factor XII is produced in the liver and circulates as a single-chain molecule with molecular weight of approximately 80 kDa. Cleavage by either kallikrein or plasmin generates a two-chain active enzyme (FXIIa) composed of a light and heavy chain. The heavy chain contains two sites that bind to negatively charged surfaces, and the light chain is a serine protease. Autoactivation to FXIIa may occur when FXII binds to a negatively charged surface, especially in the presence of Zn^{2+}. Prekallikrein, HK, and FXI are FXIIa's primary coagulation substrates; however, it also activates the complement pathway and cleaves single-chain urokinase plasminogen activator (scuPA) into its two-chain form. C1-inhibitor (C1-INH) is the major inhibitor of FXIIa.[8,48]

Prekallikrein

Prekallikrein is a single-chain molecule of about 86 kDa expressed in many tissues, with highest levels in the liver. Approximately 75% of PK circulates bound to HK. Conversion of PK to kallikrein by FXIIa occurs on a negatively charged surface when HK is also bound. This generates a light chain (containing the serine protease) and a heavy chain. Autoactivation of PK at a different cleavage site results in a less-active enzyme, β-kallikrein. Kallikrein's primary substrates are FXII and HK. Like FXIIa, kallikrein also cleaves scuPA. Kallikrein is primarily inhibited by C1-INH and α_2-macroglobulin (α_2-MG). Kallikrein is also inhibited by protein C inhibitor (PCI) and, when bound to HK, by heparin–AT.[8]

Kininogens

High-molecular-weight kininogen (H-kininogen) and low-molecular-weight kininogen are multidomain, distinct protein products of the same gene produced by alternative splicing. Both proteins contain an identical heavy chain (domains 1, 2, and 3) and domain 4. The light chain of HK contains domains 5 and 6, while the light chain of its low-molecular-weight form contains a different domain 5 and no domain 6.

Kininogens have a multitude of functions unrelated to coagulation. Domains 1, 2, and 3, respectively, inhibit atrial natriuretic peptide, prevent calpain-mediated platelet aggregation, and prevent thrombin binding to platelets. Upon cleavage by kallikrein, domain 4 releases the critical vasoactive peptide bradykinin. Domain 5 of HK contains the binding site for charged surfaces, has antiadhesive properties affecting protein and cell binding to neutrophils and endothelial cells, and also influences apoptosis. Domain 6 is the binding site for kallikrein and affects neutrophil activation. Endothelial cells, granulocytes, and platelets contain HK and express binding sites for HK on their resting membrane surface. Platelet activation results in dramatic increase in surface HK, due to release and increased expression of HK-binding sites. Zn^{2+} is absolutely required for binding of HK to membrane surfaces.[8]

Extrinsic (Tissue Factor) Pathway

Tissue Factor

Tissue factor, also known as thromboplastin, CD142, and factor III, is a glycosylated single-chain polypeptide of approximately 29.5 kDa. It is synthesized in a variety of cells, and expressed on the cell surface as an integral membrane protein. The extracellular portion of the molecule is responsible for interaction with coagulation proteins. The transmembrane domain anchors TF to the cell surface and is required for procoagulant activity. A small cytosolic domain is dispensable for coagulation functions, but is involved with intracellular signaling in TF's role as a cell-surface receptor.[38,40]

In physiologic circumstances, TF is expressed by adventitial cells surrounding blood vessels larger than capillaries, differentiating skin keratinocytes, and by other epithelial cells, particularly in mucous membranes and organ capsules. This distribution is consistent with TF's function as a protective "hemostatic envelope" whose role is instant initiation of coagulation. Under pathologic conditions, cytokines and other inflammatory mediators upregulate TF expression on other cell types. Endothelial cells, vascular smooth muscle cells, monocytes, and granulocytes may express TF in disease states. Tissue factor is also found in atherosclerotic plaques and is expressed by some neoplastic cells. Based on the lack of reports of TF deficiency in humans or animals, and the lack of viability of mice when the TF gene is deleted, TF expression appears to be essential for life.[40]

Tissue factor serves as the regulatory subunit of the TF–FVIIa enzymatic complex. Cell-surface TF, unlike most cofactors, fully functions as a cofactor without proteolysis. Although FVIIa is a weak enzyme alone, the TF–FVIIa enzyme complex is the most potent activator of coagulation. Consequently, TF–FVIIa is the first enzyme in the clotting cascade.

Tissue factor is primarily sequestered outside the vasculature to prevent intravascular coagulation; however, TF antigen has been detected at low levels in flowing blood. The source and role of this "blood-borne" TF are currently a matter of intense investigation. Blood-borne TF is thought to be associated primarily with monocytes. In addition, monocytes can release microvesicles (MVs) which are tiny blebs of the cell membrane whose expression profiles mimic their cell type of origin. Microvesicles from TF-bearing monocytes also express TF on the cell membrane. These shed microvesicles can be transferred to other cell types, thereby complicating differentiation of the cell type of origin of cell-surface TF. Tissue factor appears to accumulate in thrombi and may play a role in thrombus propagation. Circulating TF appears to be of limited procoagulant activity under normal circumstances. Some investigators believe that circulating TF is "encrypted" and must be cleaved by an enzyme for activity. Others believe that circulating TF is not fully active because the membrane surface on which it resides is not an appropriate procoagulant membrane.[38,40] Sphingomyelin, a phospholipid on the outer leaflet of the cell membrane, appears to play an important role in maintaining TF in the encrypted state. Researchers recently demonstrated de-encryption of TF after hydrolysis of sphingomyelin.[57]

Factor VII

Factor VII is a glycosylated single-chain polypeptide with a molecular weight of approximately 50 kDa. It is a vitamin-K-dependent protein produced in the liver and contains 10 Gla residues. It has the shortest half-life of the Gla proteins, so that FVII levels decrease rapidly if the vitamin K cycle or liver function is impaired. Factor VII binding to TF and to membrane surfaces via its Gla domain are calcium-dependent interactions. The FVII zymogen is converted to the enzymatically active FVIIa by proteolysis of a single peptide bond resulting in a two-chain molecule of identical size. Factor VIIa can be generated by a variety of downstream proteases, including FIXa, FXa, FXIIa, FVII activating protease, thrombin, and plasmin. Factor VII bound to TF can also be autoactivated by TF–FVIIa. The most important physiologic FVIIa activator has yet to be definitively determined.[40]

Factor VIIa is unique among coagulation enzymes in that it circulates with a long plasma half-life of approximately 2 hours (compared to other serine proteases whose enzyme half-lives are measured in seconds). Approximately 1% of total circulating FVII antigen is active FVIIa, although interindividual FVIIa levels vary considerably.[39] The intriguing question of how and where circulating FVIIa is generated remains unanswered. Factor IXa may be an important contributor of FVIIa generation, as patients deficient in FIX (hemophilia B) have very low levels of FVIIa.[58] Factor VIIa levels are higher in lymph than plasma, suggesting that some FVIIa may be generated outside the vasculature and returned to the bloodstream via lymphatic circulation.[36]

Because some FVII circulates as FVIIa, exposure of blood to TF as a result of injury leads to formation of both TF–FVII and TF–FVIIa complexes. The initial TF–FVIIa formation and its autoactivation are believed to generate sufficient enzymatic activity to trigger the clotting cascade via cleavage of the primary TF–FVIIa substrates, i.e., FIX and FX.[40] The TF–FVIIa complex is often referred to as the extrinsic tenase when its target substrate is FX. Deficiency of FVII is associated with a highly variable, but potentially severe bleeding tendency.[33]

Free plasma FVIIa is not inactivated by any plasma protease inhibitor, hence its long half-life. The TF–FVIIa complex is inhibited by the tissue factor pathway inhibitor (TFPI)–FXa complex, and by heparin–AT (albeit very slowly).[40] The inhibition of TF–FVIIa by heparin–AT is unique, in that heparin–AT binding to FVIIa releases a stable FVIIa–AT complex and regenerates fully functional TF. Consequently, high FVIIa–AT levels may be an indicator of systemic TF exposure.[33]

Intrinsic Pathway

The intrinsic cascade primarily occurs on the activated platelet surface. Platelet activation is a critical component of the intrinsic tenase complex (FIXa–FVIIIa). Upon activation, platelets expose many (approximately 6000 per platelet) binding sites for FIXa as well as 1000–2000 each for the cofactor, FVIIIa, and the substrate FX. Colocalization of FIXa, FVIIIa, and FX on the platelet surface increases the rate of FXa generation by many millionfold.

Factor XI

Factor XI is an approximately 160-kDa protein that is produced in the liver. Its structure is unique among coagulation proteins as a disulfide-linked dimer of two identical polypeptide chains. Factor XI circulates in plasma complexed with HK, which is required for FXI binding to negatively charged surfaces. Prothrombin can substitute for HK as a cofactor for binding to platelet surfaces.

Factor XI can be activated by FXIIa, FXIa, and thrombin in calcium-dependent reactions. Cleavage results in a 160-kDa enzyme with two disulfide-linked heavy chains and two active-site-containing light chains. Because FXII deficiency does not cause a bleeding tendency, it is likely that thrombin (rather than FXIIa) is the physiologically relevant enzyme generating FXIa. The GPIbα subunit of the platelet GPIb/IX/V complex binds FXI, localizing it and thrombin on the platelet for efficient cleavage. After activation, FXIa remains surface-bound where it cleaves its substrates, FXI and FIX. Factor IX is FXIa's preferred substrate.

Inhibition of FXIa depends on its localization. Plasma inhibitors include its major inhibitor, α_1-protease inhibitor (α_1-PI), and C1-INH, α_2-antiplasmin (α_2-AP), AT, protease nexin-1, plasminogen activator inhibitor-1 (PAI-1), and PCI. Platelet-bound FXIa is protected from

α_1-PI inactivation. When bound to the endothelial cell surface, protease nexin-2, in concert with surface heparan sulfate proteoglycans (HSPGs), strongly inhibits FXIa. This differential inhibition may serve to restrict FXIa-mediated cleavage of FIX to the platelet surface.

Factor IX

Factor IX's structure and size (approximately 55 kDa) are similar to FVII. Factor IX is a glycosylated single-chain polypeptide, produced in the liver. Post-translational carboxylation of 12 residues by the vitamin K cycle is required for normal Gla domain function and consequently for full-membrane-binding capability.[2]

Tissue factor–FVIIa or FXIa activate FIX bound (in the presence of calcium) to a procoagulant membrane surface. Cleavage of two FIX sites releases a 35-residue activation peptide, resulting in a two-chain enzyme of approximately 45 kDa. The catalytic domain is in the heavy chain portion of the molecule. The presence of detectable levels of the FIX activation peptide in blood suggests that coagulation is ongoing, but controlled by inhibitory pathways.[2] The primary substrate for FIXa is FX. The FIXa–FVIIIa complex on a suitable membrane surface (usually the platelet surface) in the presence of calcium is a powerful generator of FXa. Either FIX or FIXa may bind to endothelial cells, via specific receptors (collagen IV). Binding to the endothelial surface supports activation of FX by FIXa–FVIIIa in the absence of platelets. Factor IXa alone (without its cofactor FVIIIa) is able to activate FVII in the absence of TF. FIXa is primarily inhibited by AT–HSPG, although at a relatively slow rate compared to AT's inhibition of FXa or thrombin.

Factor VIII

Factor VIII synthesis and secretion are complex. Two distinct interactions involving metal ions (copper and calcium/manganese) are vital for normal FVIII structure and function. The primary site of FVIII production is unclear, although the liver and the reticuloendothelial system are strongly implicated. Factor VIII is synthesized as a single-chain protein containing three A domains, one B domain, and two C domains, with multiple free cysteine residues and disulfide bonds. After translation, FVIII is translocated to the endoplasmic reticulum (ER), for modification. Factor VIII is retained within the ER by two protein chaperone systems, immunoglobulin-binding protein and calnexin/calreticulin. Improperly folded FVIII is extracted from the ER and degraded. Factor VIII then transits to the Golgi apparatus for further processing, which includes cleavage at two sites. The resulting protein consists of a heavy chain (160 or 200 kDa) and a light chain (80 kDa), which are not connected to one another unless bound in a complex with von Willebrand factor (VWF) (see Chapter 83).[26]

Binding to VWF enables proper association of the two FVIII chains and markedly improves its intracellular and plasma stability. The ratio of the two proteins is maintained at one molecule of FVIII to 50–100 subunits of VWF. Although somewhat species-dependent, VWF deficiency is associated with lower levels of circulating FVIII, and infusion of desamino-8-D-arginine vasopressin (DDAVP) to increase circulating VWF levels elicits a concomitant increase in FVIII. Some FVIII is stored with VWF in storage granules, such as Weibel–Palade bodies of endothelial cells and α-granules of platelets.

Factor VIII can be dissociated from VWF by exposure to negatively charged phospholipids. Thrombin cleavage of FVIII at multiple sites activates FVIII to FVIIIa and releases it from VWF. Factor VIII can be activated by FXa, although the maximal activation by thrombin is greater than FXa.

Factor VIIIa interacts with PSer on membrane surfaces by stereoselective recognition of the PSer head group via a phospholipid-binding site in the light chain. Factor VIIIa also interacts with two platelet receptors, GPIb and $\alpha_{IIb}\beta_3$. Platelet binding of FVIII is inhibited by VWF. The FIXa–FVIIIa enzyme/cofactor complex assembles in a calcium-dependent manner on procoagulant phospholipid surfaces, and is referred to as intrinsic tenase, because its target substrate is FX. Factor VIIIa is inherently labile due to spontaneous dissociation of its subunits; however, FIXa binding stabilizes FVIIIa and delays decay. Factor VIIIa is cleared from the circulation by low-density lipoprotein receptor-related protein (LRP), a common cell-surface receptor found on a variety of cell types. Proteolytic cleavage by APC and FXa also inactivates FVIIIa.[26]

Common Pathway

Factor X

Factor X is a Gla protein of approximately 59 kDa. It is a glycosylated single-chain polypeptide requiring post-translational carboxylation of 11 glutamic acid residues. Factor X is activated to FXa by either extrinsic tenase (TF–FVIIa) or intrinsic tenase (FIXa–FVIIIa) on a suitable membrane surface in the presence of calcium. The activation is due to a single cleavage site and results in a 46-kDa enzyme.

Factor Xa forms the prothrombinase complex with its cofactor FVa. Factor Xa is strongly inhibited by AT–HSPG and by TFPI. It is also inhibited by the enzyme/cofactor protein-Z-dependent protease inhibitor (ZPI)/PZ. Inhibition by ZPI/PZ is calcium- and phospholipid-dependent.

Factor V

Factor V circulates in plasma as a glycoprotein of approximately 300 kDa. Portions of the molecule are very similar in structure to FVIII. Like FVIII, FV contains a copper ion, and requires post-translational modifications and molecular chaperones for successful cellular trafficking. Approximately 20% of FV in blood is contained within platelet α-granules. The plasma half-life of FV in human beings is fairly long (approximately 13 hours).[24]

Factor V is activated to FVa by cleavage at two sites that releases a large activation peptide, and a resultant

two-chain cofactor of about 160 kDa. Activation is primarily due to thrombin cleavage, but FXa can also activate FV when both are bound to a procoagulant membrane surface. Activation by FXa is less efficient than activation by thrombin. However, FV released from platelets is somewhat more susceptible to FXa activation than plasma FV.[24]

Factors Xa and FVa form an enzyme/cofactor complex, referred to as the prothrombinase complex, in the presence of calcium on PSer-containing membrane surfaces. While FVa binding to the membrane is not calcium-dependent, the binding of FX and prothrombin to the membrane is calcium-dependent, but of low affinity. Binding of FVa to a negatively charged membrane surface markedly increases the affinity of FXa for FVa, and enhances formation of the complex. Platelets and microparticles primarily provide this membrane surface in vivo.[24]

Proteolysis of FVa by APC results in its inability to bind prothrombin, and therefore functional loss of the prothrombinase complex. Inactivation of FVa by APC occurs more rapidly on endothelial cells than on activated platelet surfaces.[13,24]

Prothrombin

Prothrombin is a 72-kDa glycoprotein synthesized in the liver that (like other Gla proteins) undergoes vitamin-K-dependent post-translational carboxylation of 10 glutamic acid residues. Prothrombin is the second most abundant coagulation protein in plasma, after fibrinogen.[23]

In addition to the Gla domain, prothrombin contains two kringle domains and a serine protease precursor domain. Prothrombin cleavage at two distinct sites by prothrombinase generates its most abundant active form, α-thrombin. The first in vivo cleavage site is generally between the A and B chains of its serine protease domain to form the important intermediate, meizothrombin. Meizothrombin contains all the domains of the prothrombin molecule, with a fully active enzyme site, but it is less active toward fibrinogen and platelets than α-thrombin. After the second cleavage, prothrombin fragments 1 (containing the Gla domain and kringle 1) and 2 (containing kringle 2 domain) are removed. The A and B chains of the much smaller (36.7 kDa) α-thrombin molecule remain. Under some conditions, the order of cleavage is reversed, releasing the membrane-binding Gla domain prior to activation of the protease.[23]

Coagulation Inhibitors

Tissue Factor Pathway Inhibitor

Tissue factor pathway inhibitor is the key regulator of the extrinsic pathway. It is a Kunitz-type inhibitor, with three Kunitz domains, synthesized by endothelial cells and expressed on their surface. Alternative splicing creates two forms: TFPIα and TFPIβ. Approximately 15% of the blood-borne TFPI circulates in the active form, TFPIα, with the remainder carboxy-terminally truncated and bound to lipoproteins. Alternatively spliced TFPIβ is attached to the endothelial cell surface via interactions between the C terminal region and a glycosylphosphatidylinositol (GPI) anchor.[41] Cell-surface-associated heparan sulfates also play a role in the binding of TFPI to the endothelial cell.[1,34]

Platelet α-granules also contain TFPIα that is released upon platelet activation.[34] Polyphosphate released from platelet dense granules profoundly abrogates TFPI inhibition.[52] Intravenous heparin administration causes release of TFPI from the endothelial cell surface and a marked increase in plasma TFPI.[3] Free TFPI is cleared from the circulation primarily by the liver and kidneys.

TFPI is a slow tight-binding inhibitor that inactivates TF, FVIIa, and FXa. The inhibition of FXa can be overcome by high FXa generation. The TFPI–FXa complex is a strong inhibitor of TF–FVIIa, binding via the first Kunitz domain. The second Kunitz domain of TFPI binds to FXa; however, the FXa–FVa complex is protected from inhibition by TFPI, particularly if the substrate prothrombin also binds.[35] The third Kunitz domain regulates PS binding as PS serves as a TFPI cofactor, enhancing its inhibitory function.[46,54] In the absence of FXa, TFPI is only a weak inhibitor of TF–FVIIa.

Endocytosis and degradation of the quaternary complex (TFPI–FXa–TF–FVIIa) are mediated by cell-surface-associated LRP.[1]

Inherited deficiency of TFPI has not been described, suggesting that complete lack of this inhibitor is incompatible with life.

C1-Inhibitor

C1-Inhibitor, an acute phase protein, is the largest member of the serpin family and its main function is to inhibit the complement system. It has an unusual structure for this class of proteins, containing a typical serpin domain and an additional N-terminal domain of ill-defined structure and function. It is a relatively slow inhibitor of its target proteases, but C1-INH is potentiated by glycosaminoglycans such as heparin and heparan sulfates.[56]

The C1-INH is the major inhibitor of the contact pathway, acting on FXIIa and kallikrein, and the downstream protease FXIa. Hereditary deficiency of C1-INH has no impact on hemostasis. Rather, it causes excess episodic production of bradykinin, producing the disease phenotype hereditary angioedema.[56]

Antithrombin

Antithrombin is the prototype serpin; however, it is a relatively inefficient inhibitor of its target enzymes. The "bait" portion of the AT molecule is available only if AT binds to its cofactor. This cofactor is a specific pentasaccharide sequence that is found on approximately 30% of pharmaceutical unfractionated heparin molecules and on endothelial-bound HSPG molecules. Upon AT–pentasaccharide binding, the bait loop becomes available as a target for the enzyme and

inhibitory complex formation. Antithrombin's inhibitory activity is enhanced up to several thousandfold by interaction with heparins, but the impact varies with heparin structure and the target protease. Pentasaccharide alone is sufficient for AT inhibition of some enzymes, while longer heparin molecules are required for inhibition of others. In particular, AT inhibition of thrombin requires, in addition to the specific pentasaccharide sequence, heparin molecules of at least 18 sugar units to interact with thrombin.

Antithrombin molecules in circulation have limited inhibitory capabilities in the absence of the necessary pentasaccharide. Endogenous activation of AT occurs when it binds to heparans on the surface of the endothelial cell. Additionally, endothelial cell surface thrombomodulin (TM) can also bind AT, which enhances AT inhibition of thrombin approximately eightfold.[4,55]

In addition to thrombin, AT–heparin has broad inhibitory activity against a variety of serine proteases. These include FXIIa, FXIa, FXa, kallikrein, FIXa, and FVIIa. The primary targets are FXa and thrombin. Platelet-bound thrombin (via $GPIb_\alpha$) and thrombin bound to the fibrin clot or fibrin degradation products (FDPs) are refractory to AT–heparin inhibition. By contrast, inhibition of FVIIa requires that the enzyme be bound to its cofactor (TF) on a cell surface, and kallikrein is more readily inhibited when bound to HK on a cell surface.

Antithrombin bound to a target protease forms a stable complex with long half-life. The complexes are primarily cleared in the liver by LRP. Inherited or acquired AT deficiency is associated with development of pathologic thrombosis (see Chapter 91).[4]

Protein C Pathway

The PC pathway involves a complex interplay among endothelial cell-surface molecules, thrombin, PC, and PS. Activated PC is an essential regulator of thrombin generation due to its inactivation of the critical cofactors, FVa and FVIIIa. The thrombotic disorders in people attributed to inherited and acquired PC deficiency demonstrate its important anticoagulant action.

Protein C is an approximately 62-kDa vitamin-K-dependent protein, synthesized in the liver, containing a Gla domain with nine glutamic acid residues. It circulates as a two-chain zymogen that is activated by thrombin (or meizothrombin) to APC. Cleavage at two sites results in removal of a 12-residue activation peptide and formation of an approximately 56-kDa two-chain enzyme, APC. Plasmin activation also produces limited amounts of APC. As a Gla-containing protein, PC activation is calcium-dependent and markedly enhanced by negatively charged phospholipid surfaces, in particular phosphatidylethanolamine (PEth). Thrombin alone is a poor activator of PC unless it is bound to TM.[12]

Thrombomodulin is an endothelial transmembrane molecule that interacts with thrombin to influence its substrate specificity. Thrombomodulin inhibits thrombin's interaction with fibrinogen and protease-activated receptors (PARs), and markedly enhances the ability of thrombin to cleave PC. Cellular expression of TM is modulated by a large number of factors. It is upregulated in response to cyclic AMP, thrombin, vascular endothelial growth factor, and platelet factor 4. It is downregulated by inflammatory mediators (e.g., tumor necrosis factor, interleukin-1, endotoxin), hypoxia, transforming growth factor β, and neutrophil activation.[12]

Protein S is an approximately 69-kDa vitamin-K-dependent protein with a Gla domain that interacts with membranes. Protein S is the cofactor for APC-mediated cleavage of FVa and FVIIIa, and for TFPI's inhibition of FXa.[46] Approximately 60% of circulating PS is bound to a large (540 kDa) member of the complement system, C4-binding protein (C4BP). C4-binding protein is an acute phase reactant and therefore increases in inflammatory conditions. Protein S complexed with C4BP is unable to provide APC cofactor activity. Activated platelets and neutrophils contain proteases that cleave and inactivate PS.[11,12]

Endothelial protein C receptor (EPCR) is a transmembrane protein found primarily on arterial endothelium. The EPCR can substitute for negatively charged phospholipids in promoting activation of PC. It binds directly to PC via the Gla domain, anchoring it to the cell surface. The APC–EPCR complex is unable to bind PS and does not have anticoagulant activity, but may interact with other receptors, potentially contributing to the anti-inflammatory effects of APC.[12,13] Once APC is produced and dissociates from thrombin–TM or EPCR, it assembles with PS on a procoagulant membrane surface and rapidly inactivates FVa and FVIIIa. A mutation at one of the APC cleavage sites on FV ("FV Leiden") confers resistance to APC inactivation. This common hereditary prothrombotic disorder in people has not been identified in animals. Activated PC cleaves FVa more efficiently on endothelial surfaces than on platelets,[42] and FVIIIa is resistant to APC inactivation when bound to VWF. The anticoagulant activity of APC is highly species-specific.[12]

The PC pathway appears to have a major role in linking inflammation and coagulation, in part due to the ability of APC to downregulate thrombin generation. Additionally, APC alters gene expression in endothelial cells via activation of membrane surface PARs, resulting in anti-apoptotic and anti-inflammatory effects. Activated PC–PS also inactivates an important inhibitor of fibrinolysis (PAI-1).[12] Activated PC has a relatively long half-life of about 15 minutes. It is inhibited by α_1-PI, PCI, and α_2-MG.

Miscellaneous Inhibitors

Heparin cofactor II (HC) is a 66-kDa serpin with some similarities to AT. It requires binding to proteoglycans for full function, but the mechanism differs from that of AT. Heparin cofactor II is strongly activated by binding to dermatan sulfate and has a narrow spectrum of activity restricted to thrombin inhibition. Although HC–thrombin complexes are detectable in vivo, HC deficiency is not associated with increased risk of

thrombosis. Roles for HC in inflammation and wound healing, rather than in regulation of coagulation, have been suggested.[4]

ZPI is a 72-kDa serpin that strongly inhibits FXa when bound with its cofactor, PZ, on a phospholipid surface in the presence of calcium. Protein Z is a 62-kDa vitamin-K-dependent protein that binds to the membrane via its Gla domain. Thrombomodulin also acts as a cofactor for ZPI. Factor XIa is also susceptible to ZPI inactivation, in a heparin-dependent reaction that does not require calcium or phospholipids.

α_1-Protease inhibitor, PCI, and α_2-MG are inhibitors that act on a variety of serine proteases including the serine protease coagulation factors. α_1-Protease inhibitor is a 52-kDa molecule, synthesized primarily in the liver, whose major physiologic role is the protection of alveolar tissue from proteolytic damage by enzymes such as neutrophil elastase. It is an acute phase reactant, and is upregulated by inflammatory mediators.[15]

Protein C inhibitor is a 53-kDa serpin that inhibits APC, FXa, FXIa, kallikrein, thrombin, thrombin–TM, urokinase plasminogen activator (uPA), and tissue plasminogen activator (tPA). Its inhibitory activity is enhanced by heparin and it is involved in modulating vascular permeability, tissue regeneration, proteolysis in the kidney, and tumor cell invasion.[53]

α_2-Macroglobulin is a large, 725-kDa inhibitor of matrix metalloproteinases that works through a unique mechanism. The target enzyme cleaves an α_2-MG "bait" region to produce a conformational change that provides sites for covalent enzyme attachment. The enzyme binds and is entrapped by the inhibitor, with resultant α_2-MG–enzyme complexes internalized and degraded via LRP binding.

CELLS THAT PARTICIPATE IN HEMOSTASIS

Endothelial Cells

The endothelium, once considered an inactive barrier between the blood and the subendothelial tissues, is now recognized as a highly active organ. The endothelium has numerous functions, including regulation of coagulation and fibrinolysis, local control of vascular tone, systemic regulation of blood pressure, interaction with lipoprotein metabolism, production of angiotensin-converting enzyme, presentation of histocompatibility antigens, and interaction with leukocytes. The majority of endothelial cells reside in capillary beds due to the surface area distribution of the vascular system. Most interactions between the endothelium and flowing blood occur in capillaries, facilitated by the high ratio of endothelial cell surface to blood volume.

Antithrombotic Properties of Endothelium

Healthy endothelium plays a primary role in hemostasis by preventing coagulation in flowing blood (Figure 87.6). Consequently, disease conditions that affect endothelial function may lead to pathologic thrombus formation (see Chapter 91).

Nonactivated, resting endothelial cells have anticoagulant properties, in part due to proteins expressed on their surface, and via the glycocalyx that does not support coagulation reactions. Heparan sulfate proteoglycans are glycosaminoglycans expressed by endothelial cells on the luminal surface, in contact with flowing blood. Much larger abluminal stores of HSPG act as a reservoir. Antithrombin bound to HSPGs (at pentasaccharide sequences) is then able to inactivate its serine

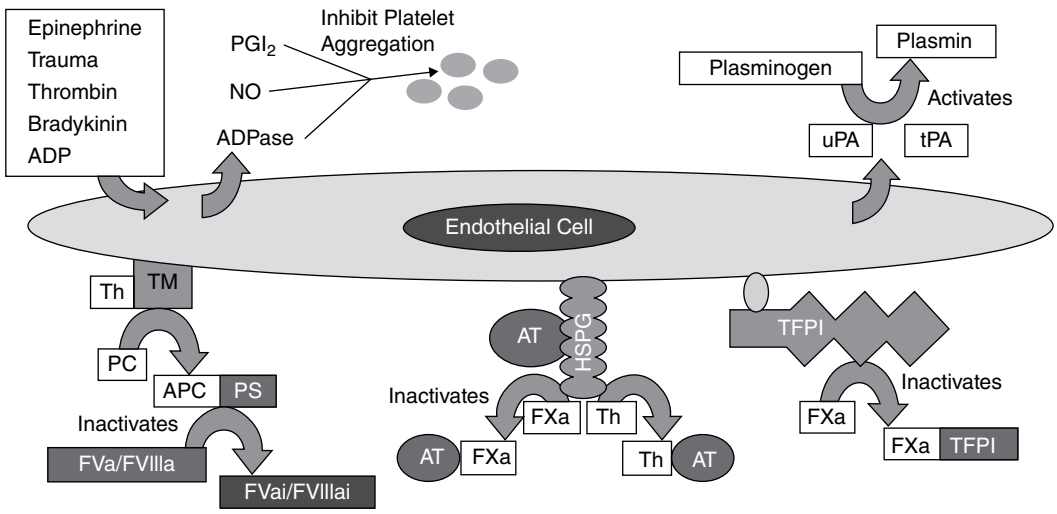


FIGURE 87.6 Anticoagulant properties of endothelial cells. Endothelial cells are thromboresistant under normal physiologic conditions. They express adenosine diphosphatase (ADPase) and synthesize and release prostacyclin (PGI_2) and nitric oxide (NO), leading to inhibition of platelet aggregation. They also produce urokinase (uPA) and tissue-type (tPA) plasminogen activators, which activate plasminogen to plasmin, the enzyme that lyses fibrin clots. Endothelial cells express thrombomodulin (TM), heparan sulfate proteoglycans (HSPG), and tissue factor pathway inhibitor (TFPI) on their membrane surface, which collectively inhibit generation of thrombin (Th). TM acts as a cofactor for activation of the protein C (PC) pathway; HSPG acts as a cofactor for antithrombin (AT) inhibition of FXa; TFPI inhibits FXa and TF–FVIIa.

protease targets (e.g., thrombin, FXa). Resting endothelial cells also express TM. Thrombin, once bound to TM, demonstrates anticoagulant rather than procoagulant properties. Thrombomodulin-bound thrombin is unable to cleave fibrinogen or activate PARs, and the TM–thrombin complex rapidly activates PC. Expression of TM is hundredfold higher in capillary endothelium compared to endothelium in the major vessels. Thrombin circulating in large vessels is therefore quickly removed by TM binding as blood passes through capillaries. Lastly, TFPI on the endothelial cell surface prevents thrombin generation by acting as an upstream inhibitor of FXa and TF–FVIIa.

Prothrombotic Properties of Endothelium

Endothelial cells store VWF in granules called Weibel–Palade bodies. This protein has important roles in FVIII biology, and in mediating platelet adhesion and aggregation (see Chapter 83). Endothelial cells express a specific G-protein-coupled receptor for thrombin. Thrombin binding to this receptor initiates a number of procoagulant and proinflammatory events. Intracellular signaling initiates endothelial secretion of VWF and P-selectin from Weibel–Palade bodies, nitric oxide (NO) release, and phospholipase A_2 activation that results in platelet-activating factor (PAF) production. Activated endothelial cells express intracellular adhesion molecule 1, generate interleukin-1, and secrete both plasminogen activators and PAI-1.

Other Properties of Endothelial Cells

Endothelial cells synthesize NO in response to a variety of stimuli including thrombin, histamine, ATP, bradykinin, and acetylcholine. Nitric oxide inhibits platelet activation and causes vasodilation. Thrombin and other stimuli also induce endothelial cell synthesis of prostacyclin (PGI_2), a platelet inhibitor that also maintains vascular relaxation.[8]

Platelets

Platelets are a fundamental reactant of normal hemostasis. Activated platelets adhere to the site of injury, secrete molecules necessary for coagulation and for wound healing, and form platelet aggregates that obstruct the site of injury to prevent further blood loss. Platelets provide the primary membrane surface for thrombin generation, allowing for formation of a platelet-rich fibrin clot (see Chapters 76 and 77).

Tissue-Factor-Bearing Cells

As previously discussed, exposure of TF on a TF-bearing cell is the physiologically relevant trigger for in vivo coagulation. Tissue-factor-bearing cells are primarily outside the vasculature, particularly in the adventitia, skin, mucous membranes, and organ capsules. Under pathologic conditions, leukocytes, endothelial cells, and neoplastic cells may also express TF.

Microvesicles

MVs are intact vesicles surrounded by membranes. They range in size from 2–20% of the size of a red blood cell (i.e., 0.1–1 μm diameter) and arise from activated or apoptotic cells. The term "microvesicle" may be used to describe ectosomes shed from the plasma membrane, apoptotic particles arising from the cytoplasm, and exosomes discharged upon release of intracellular multivesicular bodies. Cytokines (such as tumor necrosis factor and interleukin-6), thrombin, shear stress, and hypoxia can stimulate MV formation. Under normal conditions, MVs in circulation appear to be derived primarily from endothelial cells, platelets, and monocytes, but in certain disease states, MVs may arise from granulocytes and erythrocytes. Once released, MVs bind to specific receptors on the target cell type and, in some cases, fuse with the target cell membrane. Transfer of bioactive materials (e.g., lipids, proteins, mRNA, miRNA) occurs via release of MV components into the target cell. Fusion of MVs with target cell membranes makes it difficult to define and differentiate cells that form and release MVs versus cells that bind and express them. The quantity of circulating MVs is increased in certain illnesses such as diabetes mellitus, sepsis, and cardiovascular disease and may contribute to pathologic coagulation in a variety of disorders.[37] Shed MVs may also influence the composition and quality of stored blood products. In a study of stored canine packed RBCs, MVs were significantly higher in non-leuko-reduced products, increasing 5.5-fold during the 35-day storage period.[21]

Microvesicles may express cell-surface proteins similar to those found on their parent cell (e.g., ultralarge VWF multimers on endothelial cell-derived MVs, TF on monocyte-derived MVs). When co-expressed with procoagulant molecules (e.g., Pser), MVs provide a surface for coagulation reactions. The contribution of MVs to normal and pathologic hemostasis is currently under active investigation.[27,37,44]

The Role of Cell Surface Membranes

All cells are surrounded by a lipid membrane bilayer, which contains a large number of membrane surface proteins. In most mammalian cells, membrane bilayers consist primarily of cholesterol and phospholipids with neutral head groups such as phosphatidylcholine (PChol), sphingomyelin, sugar-linked sphingolipids, and PEth. The membranes also contain phospholipids with negatively charged head groups, primarily PSer. In the resting state, PEth and PSer are sequestered on the membrane's inner surface. This membrane asymmetry is essential and tightly controlled by lipid transporters. Enzymes referred to as "flippase" actively transport PSer from the external to the internal leaflet, while "floppase" refers to the transport of PChol in the opposite direction. These ATP-dependent enzymes maintain cell membrane asymmetry in the resting state.

Cell injury or activation stimuli that produce a sustained increase in intracellular calcium induce an

incompletely characterized "scramblase" activity that shuffles phospholipids between the membrane leaflets. This results in the appearance of PSer and PEth on the external membrane surface.[44,59] The scramblase responsible for rapid platelet PSer externalization has been identified as an anoctamin family transmembrane protein, TMEM16F (see Chapters 77 and 84).[29] Although the mechanisms of coagulation on activated membrane surfaces are not fully understood, it is known that membrane-surface PSer markedly (often by a factor of thousands to hundreds of thousands) increases the rate of coagulation reactions. The presence of PEth enhances the properties of PSer-expressing membranes, so that less PSer is required for maximum speed when PEth is present. It has been proposed that PEth aids in grouping PSer into clusters that support preferential binding of coagulation proteins.[40,61] Under normal physiologic conditions, cells do not express these procoagulant phospholipids on their outer membrane. As a consequence, thrombin production is limited to cell surfaces in an injured area that have been triggered to externalize PSer (and PEth). The ability of cells to control the composition of their membrane surface constitutes a powerful mechanism to localize coagulation reactions.[61]

CELL-BASED MODEL OF THROMBIN GENERATION

Our current understanding of hemostasis incorporates the role of cells. This model suggests that in vivo coagulation occurs in distinct overlapping phases that require the participation of two different cell types: TF-bearing cells and platelets.

Initiation

All evidence to date indicates that TF is the relevant initiator of coagulation in vivo. Cells expressing TF are generally localized outside the vasculature, which prevents initiation of coagulation under normal flow circumstances with an intact endothelium. Some circulating cells (e.g., monocytes) and MVs may express TF on their membrane surface, but this TF under normal conditions is thought to be inactive or "encrypted."[31,40]

Upon injury, flowing blood is exposed to a TF-bearing cell and FVIIa rapidly binds to TF.[39,40] The TF–FVIIa complex then activates additional TF–FVII to TF–FVIIa, which generates small amounts of FIXa and FXa. Although it occurs slowly, FV can be activated directly by FXa. The resultant enzyme and cofactor (FXa, FVa) form the prothrombinase complex, which subsequently cleaves prothrombin to generate a small amount of thrombin on the surface of TF-bearing cells. Any FXa that dissociates from the membrane surface is rapidly inactivated by either TFPI or AT–HSPG. Factor Xa, therefore, is effectively restricted to the surface of the TF-bearing cell on which it was generated. However, FIXa is not inhibited by TFPI, and much more slowly inhibited by AT than FXa. Consequently, trace amounts of generated FIXa can dissociate and move to the surface of nearby platelets or other cells.[22,45]

Since TF is always expressed in the perivascular space, any FVIIa that leaves the vasculature will bind to TF and potentially initiate coagulation. The gaps in the physiologic endothelial envelope under normal conditions are very small. Most of the upstream coagulation proteins are relatively small, whereas some of the downstream proteins are much larger. These downstream substrates, as well as platelets, are sequestered from the extravascular space. Coagulation progresses beyond initiation (and its generation of trace amounts of thrombin) only when the injury allows platelets and larger proteins to leave the vascular space and adhere to extravascular TF-bearing cells (Figure 87.7a).[22,45]

Amplification

The small amount of thrombin generated in the initiation phase is available to activate platelets that have leaked from the vasculature at the site of injury. Thrombin binding to platelet surface receptors causes extreme changes in the surface of the platelet, resulting in shape change, shuffling of membrane phospholipids to create a procoagulant membrane surface, and release of granule contents that provide additional "fuel for the fire." Platelet granules contain a large number of proteins and other substances which include raw materials for clotting reactions, agonists to induce further platelet activation, and calcium. Calcium may induce membrane externalization and clustering of PSer, and promotes binding of coagulation proteins to the activated membrane surface. In addition to activating platelets, thrombin generated in the initiation phase cleaves FXI to FXIa and activates FV to FVa on the platelet surface. Thrombin also cleaves VWF from FVIII and activates FVIII to FVIIIa (Figure 87.7b).[22,45]

Propagation

The release of platelet granule contents recruits additional platelets to the site of injury. The propagation phase occurs on the surface of these platelets. Expression of ligands on their surface results in cell–cell interactions that lead to platelet aggregation. Factor IXa generated by TF–FVIIa in the initiation phase can bind to FVIIIa (generated in the amplification phase) on the platelet surface. Additional FIXa is generated due to cleavage of FIX by FXIa (generated during amplification on the platelet surface). The intrinsic tenase complex (FIXa–FVIIIa) assembled on the activated platelet surface rapidly generates FXa. Since FXa formed on TF-bearing cells is rapidly inhibited in plasma, the majority of FXa in the propagation phase is generated directly on the platelet surface by the intrinsic tenase complex. The platelet-surface-generated FXa then rapidly binds to FVa (generated in the amplification phase), thus forming an active prothrombinase complex. The prothrombinase complex then rapidly and efficiently cleaves prothrombin resulting in a burst of thrombin. When enough thrombin is generated with enough speed to cleave fibrinogen, a clot forms (Figure 87.7c).[22,45]

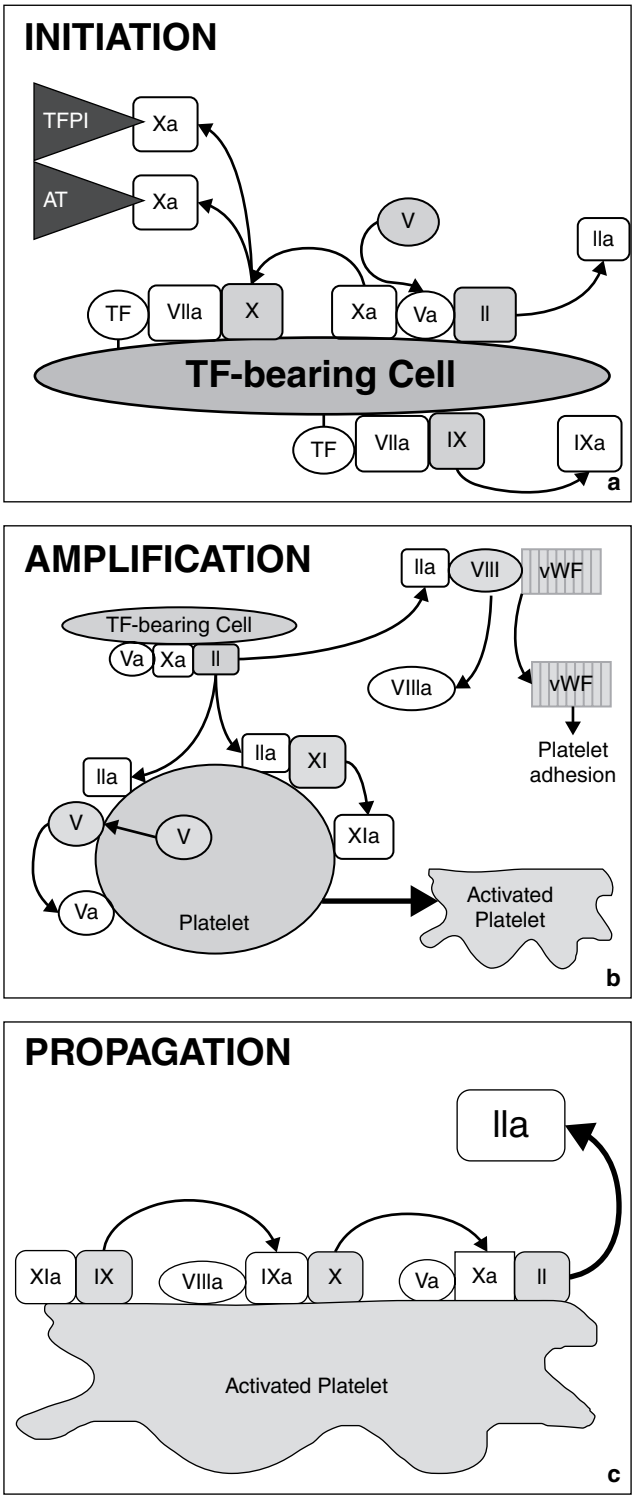


FIGURE 87.7 Cell-based model of fibrin formation. In this model, thrombin generation occurs in overlapping phases. (a) Initiation phase: This phase occurs on the TF-bearing cell and is initiated when injury exposes the TF-bearing cell to the flowing blood. It results in the generation of a small amount of FIXa and thrombin that diffuse away from the surface of the TF-bearing cell to the platelet; (b) Amplification phase: In the second phase, the small amount of thrombin generated on the TF-bearing cell activates platelets, releases VWF, and leads to generation of activated forms of FV, FVIII, and FXI; (c) Propagation phase: In the third phase, the various enzymes generated in earlier phases assemble on the procoagulant membrane surface of the activated platelet to form intrinsic tenase, resulting in FXa generation directly on the platelet surface. Prothrombinase complex forms and results in a burst of thrombin generation directly on the platelet. See Table 87.1 for abbreviations.
(**Source:** From Smith[50] © 2009 John Wiley & Sons)

THROMBIN

Thrombin is generated from prothrombin by cleavage at two activation sites. Thrombin is the terminal coagulation protease responsible for fibrin formation. Thrombin generation, however, is not the end of coagulation. Many steps vital to normal hemostasis occur temporally *after* generation of a fibrin gel. Approximately 5% of the prothrombin that will be cleaved has been converted to thrombin at the time of fibrin gel formation, as detected in routine plasma-based clotting assays.[32]

Free thrombin has an extremely short plasma half-life of less than 15 seconds due to rapid inhibition by AT–HSPG.[11] Thrombin bound to the fibrin clot, however, is both enzymatically active and protected from inhibition. In addition to its function in cleavage of fibrinogen to fibrin, thrombin has many additional roles in coagulation and fibrinolysis, as well as functions in cell regulation (Figure 87.8).[32]

Feedback Regulation of Coagulation

Thrombin provides positive feedback to the process of coagulation by activating a number of upstream proteases and cofactors. These include FVII, FXI, FVIII, and FV. Thrombin bound to endothelial surface TM acts primarily to inhibit coagulation via activation of the PC anticoagulant pathways. Moreover, TM binding inhibits the interactions between thrombin and fibrinogen, limits thrombin activation of platelet PARs, and promotes activation of the fibrinolytic mediators, TAFI and scuPA. Through these feedback mechanisms, thrombin influences the quantity and speed of its own generation, and the amount of fibrin deposition and degradation.

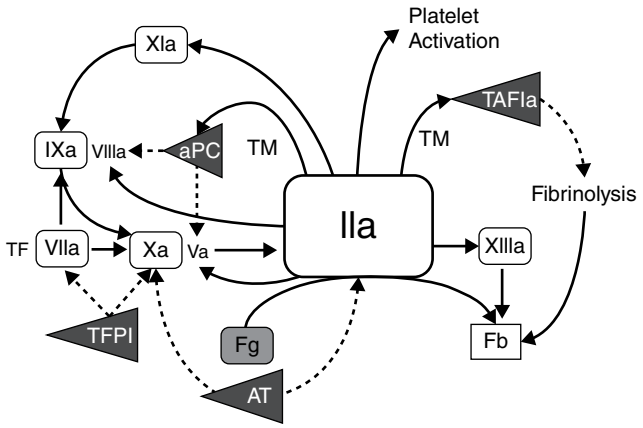


FIGURE 87.8 Central role of thrombin. Thrombin plays a central role in hemostasis, acting as both a procoagulant and an anticoagulant. Procoagulant thrombin is generated in small quantities during the initiation phase (which occurs on the TF-bearing cell), then in larger quantities on the platelet surface in the propagation phase. Thrombin is responsible for platelet activation, which provides the procoagulant surface needed for the burst of thrombin generation during propagation. Thrombin cleaves fibrinogen to fibrin, resulting in clot formation. Thrombin also impacts clot structure through activation of FXIII, which cross-links fibrin, and activation of the fibrinolysis inhibitor, TAFI. Thrombin becomes anticoagulant via thrombomodulin-mediated activation of the protein C system. See Table 87.1 for abbreviations. (**Source:** From Smith[50] © 2009 John Wiley & Sons)

Platelet Activation

Thrombin, along with many other serine proteases, can bind and cleave PARs. These cell-surface-associated G-coupled proteins are found on platelets, endothelial cells, smooth muscle cells, leukocytes, and other cell types. Cleavage by thrombin untethers a portion of the PAR molecule that self-inserts into the receptor, thereby initiating intracellular signaling. This PAR-mediated signaling is a major mechanism by which thrombin activates platelets (see Chapter 77). Thrombin also binds to the platelet GPIb/IX/V complex via a binding site in GPIbα, although how this binding mediates platelet activation is not clearly understood.[10]

Fibrin Formation

The primary function of thrombin is conversion of fibrinogen to fibrin. Thrombin cleaves two short peptides from the fibrinogen molecule, fibrinopeptide A (FpA) and fibrinopeptide B (FpB), thus exposing binding sites that interact with pre-existing sites on other fibrin molecules. The interaction between multiple fibrin molecules results in spontaneous polymerization into an insoluble fibrin gel.

Activation of Factor XIII

Factor XIII (FXIII) is a 320-kDa protein that consists of five chains arising from transcription of two different genes. Half of the circulating pool of functional FXIII is free in plasma, and the remainder is released from platelet α-granules. Factor XIII cleavage by thrombin releases an activation peptide, resulting in a 312-kDa active enzyme. Thrombin cleavage of free FXIII is slow; however, polymerized fibrin acts as a cofactor, markedly increasing the rate of FXIIIa generation. Consequently, FXIIIa is generated primarily after a critical weight of fibrin has polymerized.

Factor XIIIa is a transglutaminase whose primary function is cross-linkage of fibrillar fibrin. This activity is calcium-dependent, and critical for normal clot strength and stability. Factor XIIIa also cross-links fibronectin to fibrin and to collagen, which influences anchoring of the clot to the vessel wall. Additionally, FXIIIa has roles in cell adhesion, angiogenesis, and tissue repair.

FIBRINOGEN AND FIBRIN CLOT STRUCTURE

Fibrinogen is a soluble 340-kDa dimer consisting of three pairs of disulfide-bridge-linked polypeptides. It is the most abundant coagulation protein in plasma and is

abundant in platelet α-granules. In addition to the role of fibrinogen as a precursor to fibrin, fibrinogen mediates intracellular interactions between platelets in conjunction with thrombospondin (see Chapters 76 and 77).

Thrombin cleaves fibrinogen at two sites, releasing FpA and FpB. Upon FpA release, soluble fibrin monomer spontaneously assembles into an insoluble dimer. This polymerization occurs because removal of FpA exposes a binding site "A knob" that interacts with a complementary binding "hole" on separate fibrin molecules. Initial polymerization produces a dimer with a half-staggered structure. Dimers continue to associate to form longer, two-fibrin-wide strands, called protofibrils.[3,20] Subsequent removal of FpB allows for lateral aggregation of protofibrils of increasing width via the "B knob" interacting with its complementary binding site. Protofibrils are stabilized by the same A-a and B-b knob–hole interactions that allowed for initial dimer association. Protofibril growth continues, with branch points developing where multiple fibrin monomers interact laterally. The resulting fibrin polymer is a mesh-like network (Figure 87.9).[3,20]

Thrombin-mediated release of fibrinopeptides from fibrinogen is neither calcium-dependent nor membrane-dependent. However, fibrinogen contains several calcium-binding sites. Calcium influences fibrin assembly, enhancing the rate and extent of lateral protofibril association. Consequently, calcium influences the structure of the mature fibrin clot.[3,20] As the fibrin network forms, some thrombin is bound into the developing mesh via specific binding sites on the two molecules. Fibrin binding is important in activation of FXIII, and clot-bound thrombin also acts as a cofactor that accelerates fibrin assembly.[3,18,20]

FIBRINOLYSIS

Fibrin clot formation prevents hemorrhage, but the clot must be removed for later restoration of blood flow. Clot dissolution is referred to as fibrinolysis. The fibrinolytic system is normally quiescent due to the relative abundance of fibrinolysis inhibitors, and the requirement for fibrin as a cofactor for many of its processes. Although the primary function of fibrinolysis is removal of fibrin, many components of the system are involved in tissue-repair processes, such as cell adhesion, migration, and proliferation.

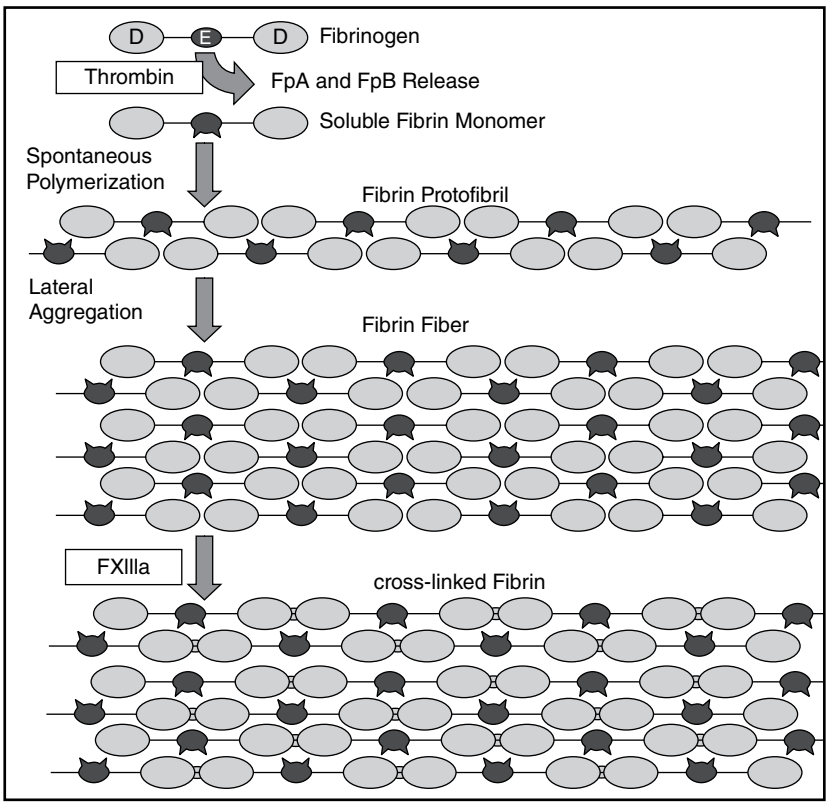


FIGURE 87.9 Fibrin formation. Fibrinogen consists of a central E domain connected by coiled chains to two lateral D domains. Thrombin cleaves fibrinogen to fibrin monomer by removing two short peptides from the E domain (fibrinopeptides A and B) exposing binding sites in the E domain. These exposed sites then interact with corresponding binding sites on the D domains of other fibrin molecules, resulting in spontaneous polymerization into a fibrin protofibril. Multiple protofibrils laterally polymerize, forming a fibrin fiber. FXIII is activated to FXIIIa by thrombin. FXIIIa creates cross-links between D domains of contiguous fibrin monomers. (**Sources:** Blomback and Bark[3]; Hantgan and Lord[20])

Plasminogen

Plasminogen (PLG) is a single-chain 92-kDa glycoprotein synthesized primarily in the liver. It circulates in plasma with a long half-life of approximately 2 days. Its complex structure consists of five kringle domains and a serine protease domain. Several of the kringle domains bind aminocarboxylic acids (such as α_2-aminocaproic acid and tranexamic acid) that are used as pharmacologic fibrinolysis inhibitors (see Chapter 94).

Plasminogen activators transform plasminogen to plasmin by cleavage at a single site, resulting in a two-chain molecule with an N-terminal glutamic acid residue (Glu-plasmin). In vitro, plasmin cleaves other plasmin molecules to remove part of the N-terminus, leaving an N-terminal lysine residue (Lys-plasmin). This additional cleavage probably does not occur in vivo due to rapid neutralization of free plasmin by inhibitors. The half-life of free plasmin is extremely short (0.1 seconds). Fibrin is a strong modulator of PLG activation. It enhances PLG activation due to binding and colocalization of PLG and its activators to the terminal lysine residues of fibrin monomers. Partially degraded fibrin exposes additional lysine sites, enhancing PLG activation through a positive feedback loop. Aminocarboxylic acids interfere with PLG binding to the lysine residues of fibrin. The fibrinolysis inhibitor, TAFI, acts by removing lysine residues, thereby limiting available PLG-binding sites.

The PLG molecule contains within its kringle domains the antiproliferative protein angiostatin. Tumor-mediated proteolysis or proteolysis by macrophages releases this fragment from plasmin. Angiostatin also inhibits endothelial cell and smooth muscle proliferation and migration, and induces apoptosis.[5]

Plasmin degrades polymerized fibrin to form heterogeneous fragments referred to collectively as FDPs. The D-dimer fragment is produced by degradation of the linked region between D domains of adjacent fibrin molecules. This linkage is created exclusively by FXIIIa; therefore, the presence of D-dimers indicates that polymerized fibrin was subject to cross-linking.

Plasminogen Activators

Mammals produce two physiologic PLG activators, tPA and uPA, named for the source from which they were originally isolated (tissue and urine). Some bacteria and vampire bats also produce unique plasminogen activators, distinct from tPA or uPA.[5]

Tissue-Type Plasminogen Activator

Tissue-type plasminogen activator is a 68-kDa serine protease glycoprotein produced and secreted by endothelial cells in response to a variety of triggers, including bradykinin, histamine, acetylcholine, α-adrenergic agents, and PAF. Very little tPA circulates in the bloodstream and most is bound to its major inhibitor, PAI-1. Free tPA and tPA–PAI-1 complexes are rapidly removed from circulation by binding to endothelial cell and hepatocyte receptors. The α_2-MG–LRP interaction is a major clearance mechanism.

Endothelial cells secrete tPA as a single-chain molecule (sctPA) that is a functional enzyme; however, its activity is weak in the absence of fibrin. Plasmin cleaves sctPA to form the two-chain mature enzyme form (tPA). Fibrin binds to either single-chain or two-chain tPA. The protease activity of fibrin–tPA is strong and highly specific for cleavage of PLG to form plasmin.[5]

Urokinase Plasminogen Activator

Urokinase plasminogen activator is a 54-kDa serine protease glycoprotein synthesized by fibroblast-like cells, epithelial cells, monocytes, and endothelial cells. It is secreted as a single-chain molecule (scuPA) with less than 1% protease activity of the two-chain mature enzyme, uPA. Plasmin, FXIIa, and kallikrein cleave scuPA to form uPA.[5] Monocytes express an important cellular receptor for scuPA (urokinase plasminogen activator receptor, uPAR). Colocalization of scuPA and uPAR on cell surfaces enhances PLG activation.

Unlike tPA, uPA can activate PLG in the absence of fibrin. It appears that tPA is the major PLG activator within the vasculature, whereas uPA is the major extravascular PLG activator. Consequently, uPA's primary roles include degradation of extracellular matrix in the processes of cell migration, and healing.[5]

Fibrinolysis Inhibitors

Plasminogen Activator Inhibitor-1

Plasminogen activator inhibitor-1, a 52-kDa serpin and acute phase reactant, is the major inhibitor of PLG activators. Elevated plasma levels of PAI-1 are strongly correlated with prothrombotic disease, while PAI-1 deficiencies are associated with bleeding and hyperfibrinolysis.[7] The primary source of plasma PAI-1 is unknown; however, many cell types, including megakaryocytes, endothelial cells, and hepatocytes, synthesize PAI-1. It is produced as an unstable, active form found primarily in blood and tissues, and a more stable latent form present in platelets. Most of the circulating PAI-1 is complexed with vitronectin, which stabilizes PAI-1's active conformation.[60]

The target enzymes of PAI-1 are sctPA, and the two-chain forms of tPA and uPA. When bound to a PLG activator, PAI-1 loses its affinity for vitronectin, but gains affinity for cell-surface LRP. This shift results in rapid clearance of PAI-1 by LRP-mediated internalization. Vitronectin-bound PAI-1 also inhibits thrombin, an activity enhanced by HSPG.[60] In addition to its protease inhibitory activities, PAI-1 mediates vitronectin's adhesive properties and its role in tissue remodeling.[5,60]

α_2-Antiplasmin

α_2-Antiplasmin is a single-chain, 70-kDa serpin that is the primary plasmin inhibitor. It is synthesized in the liver, circulating with a long half-life of approximately

3 days. It also binds to PLG, which interferes with PLG activation due to impaired PLG–fibrin interaction. Plasmin-bound fibrin is relatively resistant to α_2-AP inhibition. Factor XIIIa cross-links some forms of α_2-AP to fibrin, thereby making the fibrin more resistant to lysis.[5]

Thrombin-Activatable Fibrinolysis Inhibitor

Thrombin-activatable fibrinolysis inhibitor is a 60-kDa protein synthesized in the liver and released as a zymogen.[6] Thrombin and plasmin activate TAFI by cleavage at a single site; however, activation is extremely slow. Thrombomodulin-bound thrombin, however, increases the TAFI activation rate more than thousandfold. Activated TAFI inhibits fibrinolysis by removing the C-terminal lysines of polymerized fibrin. These residues are the primary binding site on fibrin for both PLG and its activators; therefore, modification of fibrin by TAFIa makes the fibrin clot resistant to fibrinolysis. Complement cascade proteins and adhesive proteins are secondary targets of TAFIa.

No TAFIa inhibitors have been identified, rather TAFIa appears to be regulated via spontaneous loss of activity. This inherent lability is temperature-dependent. At body temperature, TAFIa has a half-life of about 10 minutes.

Miscellaneous Inhibitors

The nonspecific protease inhibitor, α_2-MG, acts to inhibit plasmin (especially in the absence of free α_2-AP) and also binds to tPA and uPA. Protein C inhibitor and protease nexin-1 also inhibit tPA.

HEMOSTASIS AND INFLAMMATION

The inflammatory and hemostatic systems demonstrate apparent "cross-talk." This communication plays important roles in the healing processes necessary for tissue repair, and in the inflammatory and innate immune response to tissue injury and infection. Cross-talk may be responsible for inappropriate activation of hemostasis associated with systemic inflammation (see Chapter 91).

Effects of Hemostasis on Inflammation

Contact pathway activation produces two enzymes (kallikrein and FXIIa) that activate complement and induce neutrophil activation, and stimulate monocytes to downregulate Fc receptors and release inflammatory cytokines. Additionally, cleavage products of HK have effects on cellular adhesion, cellular proliferation, and apoptosis.[14,28,49]

The TF–FVIIa complex expressed on monocytes and endothelial cells activates cell surface PARs. This stimulation induces PAR-mediated signaling and increased expression of inflammatory cytokines (e.g., interleukin-1 and interleukin-6, tumor necrosis factor) and expression of chemotaxis proteins.[14,28,49]

Thrombin is chemotactic for macrophages and neutrophils, stimulates selectin expression that promotes neutrophil adhesion to endothelial cells, and activates endothelial cells to produce PAF, a potent neutrophil agonist. Thrombin activation of platelets releases CD40 ligand, which can induce TF formation and increase cytokine production. Thrombin also increases endothelial permeability.[14,28,49]

The inflammatory response is attenuated by APC's enzymatic action on PAR-1, which downregulates TF expression. Soluble EPCR, released by metalloproteinases from the endothelial cell surface, inhibits neutrophil adhesion. Downregulation of TM expression increases endothelial intracellular signaling pathways responsible for production of proinflammatory cytokines, and limits activation of TAFI, adversely affecting clearance of some complement proteins.[14,28,49]

Fibrin can directly stimulate production of proinflammatory cytokines by monocytes, and release of chemokines by endothelial cells and fibroblasts.[14,28,49]

Effects of Inflammation on Hemostasis

Inflammatory mediators, especially components of the complement system, induce transmembrane phospholipid movement, resulting in expression of a procoagulant surface. The acute phase response results in a shift in the balance of protein synthesis. Proteins whose synthesis increases include fibrinogen, PLG, tPA, uPA, C1-INH, C4BP, PS, vitronectin, PAI-1, and α_1-PI. Proteins whose synthesis is decreased include FXII, AT, and PC.[16,28]

Inflammatory cytokines downregulate the expression of EPCR and TM on the endothelial cell surface. This results in less activation of APC, and consequently less inactivation of FVa and FVIIIa. Proinflammatory cytokines also induce expression of TF by cell types that do not express this antigen under resting conditions. Proinflammatory cytokines also downregulate expression of HSPG on the endothelial cell surface, which decreases the anticoagulant activity of AT.[14] Neutrophil elastase cleaves a variety of coagulation and fibrinolysis proteins, including AT, PC, and TM.[28]

CONCLUSION

Our understanding of hemostatic pathways, and their regulation in health and disease, is continually evolving. The discovery of polyphosphate's in vivo activation of the contact pathway launched a new era of research into the differences between physiologic hemostasis and pathologic thrombosis. Studies demonstrating that lipopolysaccharide (LPS) induces TF expression on monocytes and endothelial cells, and their shed microvesicles, now confirm the role of the TF pathway as a trigger of both pathologic and physiologic hemostasis. Undoubtedly, these discoveries will lead to the further development of new models to help unravel the complexity of hemostasis.

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CHAPTER 88

Laboratory Testing of Coagulation Disorders

MARJORY B. BROOKS

Sample Collection and Processing for Coagulation Testing
Clotting Time Tests
  Assay Methodologies
    Activated Partial Thromboplastin Time
    Prothrombin Time
    Reptilase Time
    Russell's Viper Venom Time
    Thrombin Time
Fibrinogen and Procoagulant Assays
  Fibrinogen Assays
  Procoagulant Factor Assays
    Contact Pathway Factors
    Intrinsic Pathway Factors
    Extrinsic and Common Pathway Factors
  Factor XIII Assays
  Thrombin Generation Assays
Anticoagulant Assays
  Antithrombin
  Thrombin–Antithrombin Complex
  Proteins C and S
  Pathologic Inhibitors: Factor Inhibitors and Lupus Anticoagulants
  Anticoagulant Drugs: Unfractionated and Low-Molecular-Weight Heparins
  Anticoagulant Drugs: Vitamin K Antagonists and Direct Oral Acting Anticoagulant Drugs
Fibrinolytic Pathway Assays
  Plasminogen Assays
  Plasminogen Activators: Tissue Plasminogen Activator and Urokinase
  Fibrinolysis Inhibitors
    Antiplasmin
    Plasminogen Activator Inhibitor-1
    Thrombin-Activated Fibrinolysis Inhibitor
  Euglobulin Lysis Time
  Turbidimetric Fibrinolysis Assays
  Viscoelastic Fibrinolysis Assay
  Fibrin/Fibrinogen Degradation Products and D-Dimer Concentration
Whole Blood Assays
  Viscoelastic Assays
  Microfluidic Flow Assays

Acronyms and Abbreviations

APC, activated protein C; aPTT, activated partial thromboplastin time; AT, antithrombin; BU, Bethesda unit; DIC, disseminated intravascular coagulation; DOACs, direct oral acting anticoagulants; ETP, endogenous thrombin potential; FDP, fibrin/fibrinogen degradation products; INR, international normalized ratio; ISI, international sensitivity index; mAU, milli-absorbance units; OD, optical density; OHP, overall hemostasis potential; PAI-1, plasminogen activator inhibitor-1; PIVKA, proteins induced by vitamin K absence or antagonism; PROVETS, Partnership on Rotational ViscoElastic Standardization; PT, prothrombin time; PT-Fg, prothrombin-derived fibrinogen; RVVT, Russell's viper venom time; SERPIN, serine protease inhibitor; TAT, thrombin–antithrombin complex; TCT, thrombin clotting time; TEG, thromboelastograph; TGA, thrombin generation assays; tPA, tissue plasminogen activator; UFH, unfractionated heparin.

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The coagulation cascade encompasses a series of rapid, cell-membrane-associated enzymatic reactions that culminate in a burst of thrombin. Thrombin then cleaves peptide fragments from plasma fibrinogen to form an insoluble, fibrin clot. Vascular patency is later restored, as the fibrinolytic pathway gradually degrades mature fibrin. While these processes are intricately related and tightly regulated in vivo, laboratory tests of coagulation and fibrinolysis typically follow a reductionist approach by evaluating single components in purified assay systems. New technologies facilitate more "global" assessments; however, traditional tests remain relevant for interpreting global assays and guiding patient management.

SAMPLE COLLECTION AND PROCESSING FOR COAGULATION TESTING

Careful blood sample collection and processing are critical for ensuring that assay results accurately reflect in vivo hemostatic function.[55,60] The key steps for sampling include atraumatic venipuncture and withdrawal of an exact volume of blood directly into tubes or syringes containing anticoagulant. With few exceptions, buffered citrate-based anticoagulants are utilized for coagulation and fibrinolysis tests. Blood is typically collected at a ratio of one part citrate (3.2% or 3.8% citrate) to nine parts blood. Marked deviations from this ratio will influence test results; excess anticoagulant can dilute factors and delay reaction rates, whereas inadequate anticoagulant risks factor activation, depletion, and clot formation. An adjusted citrate volume to accommodate high hematocrit (>55%) is recommended for some human tests: 0.00185 × (100-Hct) × volume of blood drawn = adjusted citrate volume.[61]

Citrate blood samples should be held at room temperature until centrifugation or use in whole blood assays.[1,55] Separation of plasma from whole blood should be performed as soon as possible after collection. A single centrifugation of 2500 g × 20 minutes to generate platelet-poor plasma is generally sufficient for routine coagulation testing. However, double centrifugation to reduce residual platelets and large microparticles in the supernatant plasma may be required for specialized hemostasis testing.[12,60,99] Most hemostatic proteins are stable and retain activity for prolonged (at least 1 year) storage at temperatures at or below −70 °C.[111] Storage at −20 °C is adequate for short-term (weeks) storage and factors from domestic species have been shown to retain activity under refrigeration for several days in transfusion units.[108,113]

CLOTTING TIME TESTS

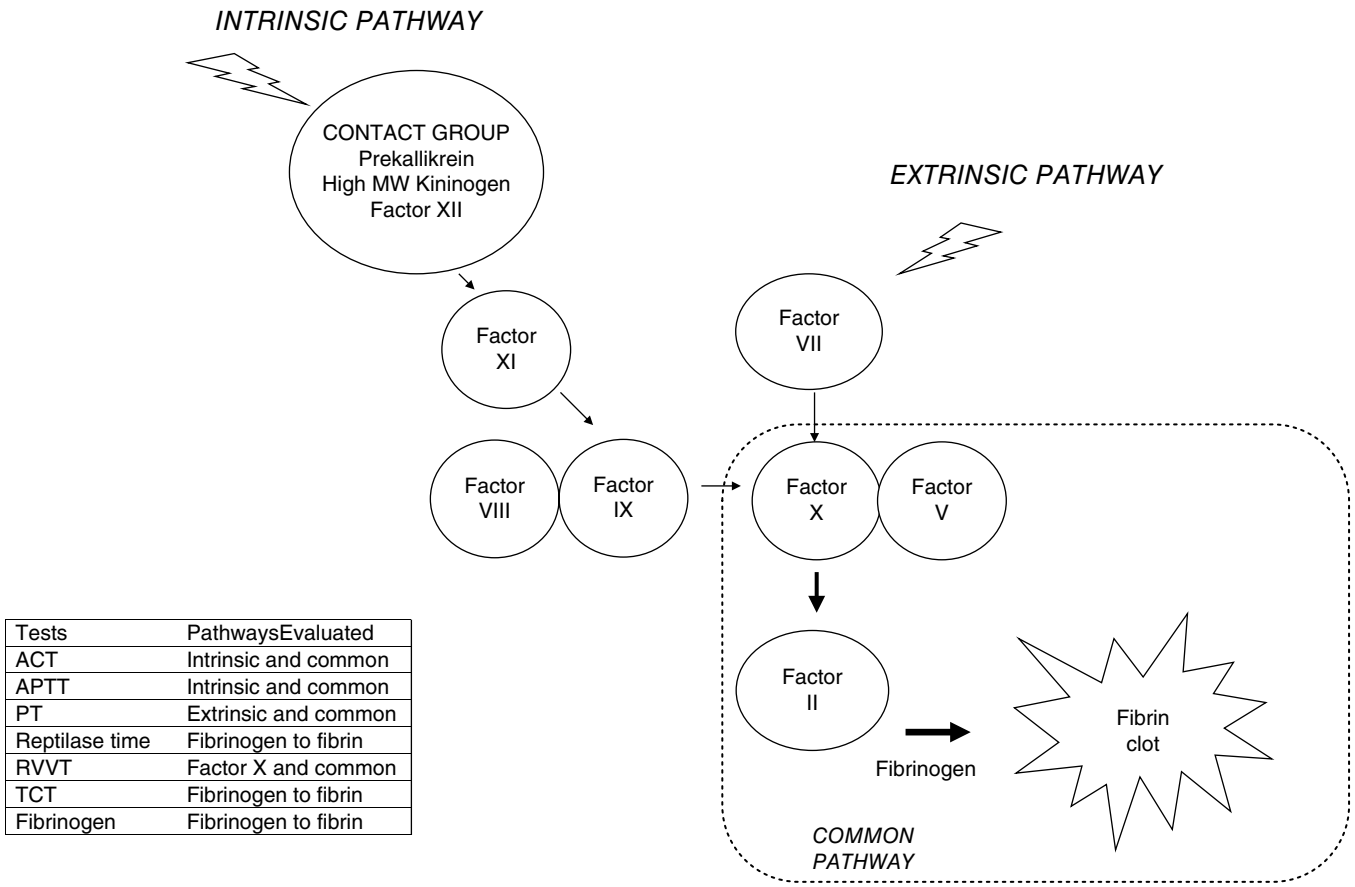
Clotting time tests are functional bioassays that measure the time for clot formation in a reaction mixture containing the test sample and a "trigger" reagent. These reagents sequentially activate distinct series of coagulation factors, or pathways, of the coagulation cascade (Figure 88.1). The activated partial thromboplastin time (aPTT) and prothrombin time (PT) are often performed in tandem as a coagulation panel for initial screening to detect procoagulant factor deficiencies.[53] While traditionally performed on plasma samples in a testing laboratory, point-of-care instruments to measure aPTT and PT on canine or feline whole blood samples are now widely available for veterinary practice.[24,112] The kinetics of fibrin formation and the relative activities of coagulation factors and inhibitors vary among species.[36,48,77,90] Assay systems optimized for human coagulation testing may require modifications for animal testing, and different reagent and instrument combinations yield different results.[5,88] Species-specific methodologies, reference intervals, and same-species controls may be needed to appropriately interpret test results.

Assay Methodologies

The formation of an insoluble fibrin clot is the endpoint for all clotting time tests; however, coagulation instruments utilize a variety of endpoint detection methods.[3,21,58,75,92,102] The BBL Fibrometer, an early semi-automated coagulation instrument, detected clot formation in an electromechanical system based on change in conductivity due to fibrin strands connecting a stationary and fixed metal electrode. Several mechanical instruments employ magnetic sensors to monitor changes in movement of a steel ball, as viscosity increases due to fibrin formation. Photo-optical coagulometers detect changes in plasma optical density (OD), as fibrin polymerization increases light scatter. Nephelometry, a modification of photo-optical detection, measures increase in forward-angle and side-angle light scatter as fibrin forms. Photo-optical methods can be adapted to provide continuous readings and a dynamic clot "signature" or waveform analysis.[50,88] Mechanical endpoint coagulometers are not subject to interference from lipemia, bilirubinemia, or hemolysis and are generally able to use smaller sample volumes and detect weaker clot endpoints with shorter lag times than photo-optical methods.

Activated Partial Thromboplastin Time

The trigger reagent for the aPTT assay contains phospholipid and negatively charged particles.[53] Upon contact with these particles, factor XII undergoes a conformation change and autoactivation, and then participates in reciprocal activation of prekallikrein in an amplification loop referred to as the contact pathway (Figure 88.2). Coagulation complexes assemble during preincubation of the aPTT reaction mixture; the addition of calcium then drives activation of factor XI and the remaining intrinsic pathway factors (factors IX, VIII) and the common pathway factors [factors X, V, and thrombin (factor II)]. The aPTT is insensitive to factor VII deficiency, but is prolonged by deficiencies of any other factor (Figure 88.1). Similarly, the activated clotting time is a point-of-care whole blood assay triggered by contact pathway activation. The



Tests	PathwaysEvaluated
ACT	Intrinsic and common
APTT	Intrinsic and common
PT	Extrinsic and common
Reptilase time	Fibrinogen to fibrin
RVVT	Factor X and common
TCT	Fibrinogen to fibrin
Fibrinogen	Fibrinogen to fibrin

FIGURE 88.1 Coagulation pathways and clotting time tests. The clotting time endpoint for each screening test relates to the activities of individual factors within coagulation pathways. Abbreviations: ACT, activated clotting time; aPTT, activated partial thromboplastin time; PT, prothrombin time; RT, reptilase time; RVVT, Russell's viper venom time; TCT, thrombin clotting time.

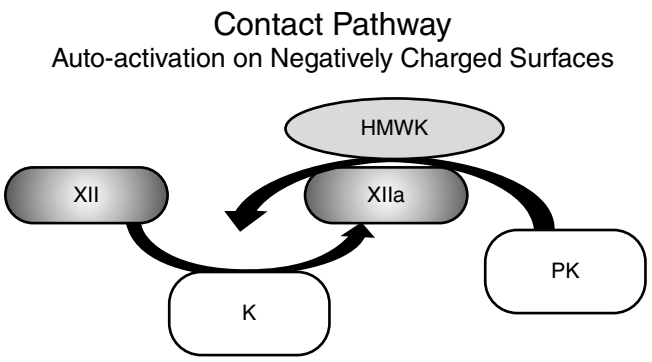


FIGURE 88.2 The contact pathway of coagulation. The contact pathway involves reciprocal activation of two proteases that assemble on negatively charged surfaces. A cofactor, high-molecular-weight kininogen (HMWK), participates in the process of transforming zymogen factor XII (XII) to its active form factor XIIa (XIIa) and prekallikrein (PK) to its active form kallikrein (K).

assay system lacks exogenous phospholipid, however, and is more susceptible to non-factor-specific influence of platelet count, hematocrit, and plasma protease activity.

Prothrombin Time

The PT trigger reagent contains tissue factor, phospholipids, and calcium and initiates coagulation through activation of factor VII.[53] The source of tissue factor and phospholipid composition vary among reagents, including traditional "thromboplastins" derived from brain extracts and recombinant, lipidated tissue factor reagents. The PT assay is sensitive to factor VII deficiency, and deficiencies of the common pathway factors (Figure 88.1). The international normalized ratio (INR) represents a data transformation of the PT result that accounts for differential reagent composition based on the manufacturers' supplied international sensitivity index (ISI).[53,55] The INR simplifies comparison of results among testing laboratories and is calculated using the formula: INR = [patient PT/mean normal PT]^{ISI}. In human medicine, the INR is often used to gauge the severity of coagulopathy and the anticoagulant intensity of coumadin therapy. Depending on disease indication, coumadin-target INR values typically range from 2 to 3, with values >4 indicating risk for excessive anticoagulation.[54,107]

Vitamin K deficiency results in a combined functional deficiency of factors II, VII, IX, and X (see Chapter 89).

The noncarboxylated (des-carboxyl) forms of these proteases accumulate in plasma and are collectively referred to as proteins induced by vitamin K absence or antagonism (PIVKA). A commercial bovine thromboplastin reagent (Thrombotest, Nyegaard & Co.) was developed as a sensitive clotting time test to monitor vitamin-K-deficiency states, and has been used for this purpose in veterinary clinical and toxicologic studies.[15,49] The assay, however, is nonspecific and is prolonged for patients deficient in any of the extrinsic or common pathway factors, regardless of vitamin K status. While Thrombotest is now used infrequently in human medicine, immunologic assays, specific for descarboxyprothrombin (PIVKA-II), are used as biomarkers of human hepatocellular carcinoma.[45]

Reptilase Time

The reptilase time is a functional test of fibrin formation based on the specific cleavage of fibrinopeptide A from fibrinogen.[64,75,102] The reptilase reagent is isolated from *Bothrops atrox* snake venom and contains no added calcium. The reptilase time is influenced by the concentration and conformation of fibrinogen (Figure 88.1), and is typically used to characterize hypofibrinogenemia/dysfibrinogenemia. Reptilase time is not affected by the presence of heparin or direct thrombin inhibitory drugs.

Russell's Viper Venom Time

Russell's viper venom (RVV) is a direct activator of factor X. Combined with phospholipid and calcium, RVV triggers coagulation through the common pathway (Figure 88.1). The RVV reagent demonstrates multispecies reactivity and is useful to detect vitamin-K-dependent coagulopathy in birds in the absence of an avian-source thromboplastin (see Chapter 95).[80] The dilute RVV time (dRVVT) is a modified assay using limiting dilution of venom and phospholipid to enhance assay sensitivity to the presence of inhibitors.[64,69]

Thrombin Time

The thrombin time, also referred to as thrombin clotting time (TCT), is triggered by the addition of a human or bovine thrombin reagent that directly cleaves fibrinopeptides A and B from fibrinogen (Figure 88.1). The TCT is a functional test of fibrinogen, sensitive to fibrinogen deficiency and dysfunction, fibrinogen inhibitors, and to the presence of heparin and direct thrombin inhibitory drugs.[54,75,102]

FIBRINOGEN AND PROCOAGULANT ASSAYS

Fibrinogen is a glycoprotein that circulates at high concentration in plasma, with values in most species ranging from 150 to 450 mg/dL (1.5–4.5 gm/L).[3,58] This high concentration allows detection of fibrinogen using physicochemical and viscoelastic methods, in addition to more sensitive immunologic and functional tests.[59,75,102] By contrast, coagulation factors are enzymes and coenzymes that circulate at much lower concentrations and are typically measured in functional assays with clotting time or colorimetric endpoints.[101,109] The use of same-species assay standards is ideal, and may be required to account for inherent species differences in hemostatic proteins, clotting times, and relative affinities of antibody reagents.[36,48,81,93]

Fibrinogen Assays

Heat precipitation assays provide semi-quantitative estimates of fibrinogen and are primarily used as screening tests to detect hyperfibrinogenemia.[59] The assays are typically performed on EDTA blood spun in microhematocrit tubes. Fibrinogen content represents the differential in plasma total protein by refractometry, measured before and after heat treatment.

Total clottable fibrinogen and gravimetric methods are performed by adding an excess of thrombin to plasma in a reaction lacking calcium but containing fibrinolysis inhibitors (clottable fibrinogen) or in the presence of calcium (gravimetric assay). In both methods, the insoluble clot is physically removed from the reaction mixture. In the clottable fibrinogen assay, the clot is then dissolved in urea for spectrophotometric determination of total protein. For the gravimetric method, the clot is dried and weighed. These assays are unsuitable for use as clinical diagnostic tests, but may be used to prepare fibrinogen standards or in research applications.[21,59]

Clauss fibrinogen assays are widely used in clinical diagnostic laboratories and are performed using commercial thrombin reagents and automated instruments for clot endpoint detection.[3,109] Assays are configured with dilute samples of test plasma and high-concentration thrombin. Clotting time is then converted to fibrinogen concentration by comparison with a calibration curve prepared from a fibrinogen reference standard. The choice of standard thus influences fibrinogen results. Clauss fibrinogen assays are sensitive to the presence of fibrin/fibrinogen degradation products (FDP) that impair fibrin polymerization; however, thrombin reagents often contain heparin inhibitors to allow measurement of fibrinogen in patients undergoing heparin therapy.

Prothrombin-derived fibrinogen (PT-Fg) assays are also based on clot endpoint detection and transformation of clotting time to fibrinogen concentration from a calibration standard.[3,75] These assays differ from Clauss fibrinogen by the use of a thromboplastin trigger reagent and results are more susceptible to variability based on reagent, instrument, and fibrinogen standard. Results of PT-fg and Clauss methods are generally correlated in healthy individuals; however, discrepant values may be found in samples with dysfibrinogenemia, high FDP, and marked hyperfibrinogenemia or hypofibrinogenemia.[59]

Turbidimetric fibrinogen assays monitor change in light scatter resulting from the transformation of soluble fibrinogen into a fibrin gel after the addition of a

thrombin reagent.[92] The assays are typically performed in research settings and can be configured with plasminogen activators to monitor clot formation and fibrinolysis.

Immunologic assays measure fibrinogen protein concentration regardless of its functional properties. Fibrinogen-reactive antibodies can be immobilized on microtiter plates in ELISA format or on microbeads in immunoturbidimetric assay platforms.[102] Fibrinogen is conserved among species, but reactivity of commercial fibrinogen assays must be confirmed for use in animal studies, and assay results will vary depending on calibration standard and antibody cross-reactivity to epitopes on FDP and fibrin.

Procoagulant Factor Assays

The specific procoagulant activities of individual coagulation factors and cofactors are commonly measured in modified aPTT and PT, referred to as "one-stage" assays.[64,99,114] The assays are configured with defined, single-factor-deficient plasmas combined with the test plasma in a series of assay mixtures. The time for clot formation in each reaction is inversely proportional to the deficient factor content of the test plasma. Results are transformed to percentage activity or units/mL based on comparison with clotting times of a plasma standard. Commercial suppliers of single-factor-deficient substrates are limited to human-origin factors (e.g., George King Bio-Medical, Precision Biologics) as are plasma standards containing defined factor activities. The factor activities for animal plasma obtained from these heterologous assay systems must be interpreted in the context of same-species controls. The use of same-species pooled plasma may also provide a more relevant assay standard.

Chromogenic substrate assays measure the specific amidolytic or cofactor activity of coagulation factors based on cleavage of factor-specific peptide substrates.[30,78,99] The test reagents contain purified recombinant or bovine factors and phospholipids, and the test sample provides the missing enzyme or coenzyme required to assemble an active factor complex. In these assays, the rate of color change resulting from substrate cleavage is directly proportional to factor activity in the test sample. Chromogenic assays avoid some of the species variability of clotting time tests; however, specific peptide substrates are not available for all factors, the assays are relatively expensive, and the use of same-species standards is often required to interpret test results.

Immunologic methods are typically reserved for specialized laboratories and research studies, due to the clinical relevance of measuring the functional activity of factors rather than their plasma concentration.[49,64,101] While antimurine factor antibodies and ELISA kits may be commercially available, confirmation of antibody reactivity and specificity for nontarget species are required due to the variability across species in factor sequence and post-translational processing.

Analyses of mass-spectrometry-based proteomics allow identification and quantification of any of the individual hemostatic proteins from any species without the need for species-reactive antibodies; however, these methods are currently limited to research applications.

Contact Pathway Factors

The contact pathway, consisting of serine protease factors (prekallikrein, factor XII) and a cofactor, high-molecular-weight kininogen, initiates coagulation in the aPTT assay system (Figure 88.2). This pathway, however, is not required for physiologic clot formation and the constituent factors are variably present in different classes and species of animals.[77] Non-mammalian vertebrates lack a well-developed contact pathway and cetaceans have no functional factor XII gene. For appropriate species, functional tests can be configured, as modified aPTT assays using human-deficient plasmas and a chromogenic substrate of prekallikrein is also commercially available (Diapharma).

Intrinsic Pathway Factors

Deficiencies of the intrinsic pathway factors (factors VIII, IX, XI) are associated with clinical bleeding diatheses, including the most common hereditary coagulopathies hemophilia A and B (see Chapter 90). Modified one-stage aPTTs are typically used for clinical diagnosis.[64,78,93,109] Chromogenic substrate assays to measure factor VIII coagulant activity are configured as two-stage tests. In the first stage, a reagent containing calcium, phospholipids, and purified factors IX and X is combined with test plasma, which supplies factor VIII. In the second stage, generated factor Xa is measured by cleavage of a factor Xa substrate.[30,99,114] Chromogenic factor VIII assays are often used to measure activity of purified and recombinant proteins, as they are more easily standardized across testing laboratories.[112,114] Molecular diagnostic tests for specific canine breed variants of hemophilia A and B are available (PennGen); however, molecular heterogeneity and frequent de novo mutations underlying these traits limit their utility (see Chapter 90).

Extrinsic and Common Pathway Factors

Deficiencies of thrombin and factors V, VII, and X are typically measured in modified one-stage PT assays, and chromogenic thrombin and factor X substrates are commercially available to configure two-stage assays.[30,109] A molecular diagnostic test to identify a common mutation associated with factor VII deficiency in beagles and several other breeds of dogs is offered through several commercial laboratories (see Chapter 90).

Factor XIII Assays

Factor XIII is a transglutaminase that, upon activation by thrombin, cross-links fibrin. Unlike deficiencies of serine protease factors and cofactors, factor XIII

deficiency does not cause prolongation of any clotting time test. Rather, functional tests are based on evaluation of fibrin clot stability or more specific measures of transglutaminase activity.[98] Screening tests to detect severe deficiency (<5% factor XIII activity) monitor solubility of a clot generated ex vivo from test plasma in concentrated urea or acetic acid solutions.[6,64] Non-cross-linked fibrin is unstable and rapidly dissolves. Quantitative functional assays are based on either detection of ammonia released during a transglutaminase reaction or the incorporation of a labeled amine group into a protein substrate. While generally applicable for animal plasmas, quantitative factor XIII assays are technically complex and not routinely available. As for other factors, immunologic methods require confirmation of antibody cross-reactivity with nonhuman species.

Thrombin Generation Assays

The fibrin endpoint of clotting time tests forms from the action of approximately 10 nM thrombin. This threshold value, however, represents only a small portion of the total thrombin enzymatic potential within a plasma sample. Thrombin generation assays (TGA) are kinetic assays that monitor the cumulative total thrombin formed over time based on the cleavage of a fluorogenic thrombin substrate.[50,99] The TGA reaction is triggered by a recombinant human tissue factor and phospholipid reagent and proceeds through assembly of the tenase and prothrombinase complexes to ultimately generate thrombin (see Chapter 87). The rate of increase and decrease in thrombin concentration over time is calculated from a thrombin calibrator and displayed as a thrombin generation curve or thrombogram (Figure 88.3). The area under the thrombogram profile is a summary of the overall capacity for thrombin production comprising net forces that promote and ultimately inhibit thrombin formation. The assay parameter endogenous thrombin potential (ETP) summarizes this net balance. Additional TGA numeric parameters include lag time, peak thrombin concentration, time to peak, and the total amount of thrombin generated (Figure 88.3). The TGA test principle is broadly applicable across species and two reagent/assay platforms are commercially available [Calibrated Automated Thrombin Generation Assay (CAT), Thrombinoscope; Technothrombin TGA, Technoclone]. Species variability in response to human-optimized trigger reagents and inherent species differences in the contact pathway influence thrombogram parameters such that assay adaptation and same-species controls are often needed to perform TGA in animal plasmas.[12,16,22,90] The use of defined sample collection procedures and centrifugation conditions are also important to standardize the influence of preanalytic processing on TGA parameters.[12,16,83]

ANTICOAGULANT ASSAYS

Deficiencies of physiologic coagulation inhibitors develop in a variety of prothrombotic disease conditions, due to decreased hepatic synthesis, excessive consumption, or renal or intestinal losses (see Chapter 91). By contrast, pathologic inhibitors and anticoagulant drug overdose induce hemorrhagic states. Assays that measure individual anticoagulant proteins and/or gauge anticoagulant intensity are useful to characterize and manage patients with either form of hemostatic imbalance.

Antithrombin

Antithrombin (AT) is a serine protease inhibitor (SERPIN) produced by the liver. It is a major plasma anticoagulant that localizes coagulation to membrane surfaces by inactivating circulating serine protease

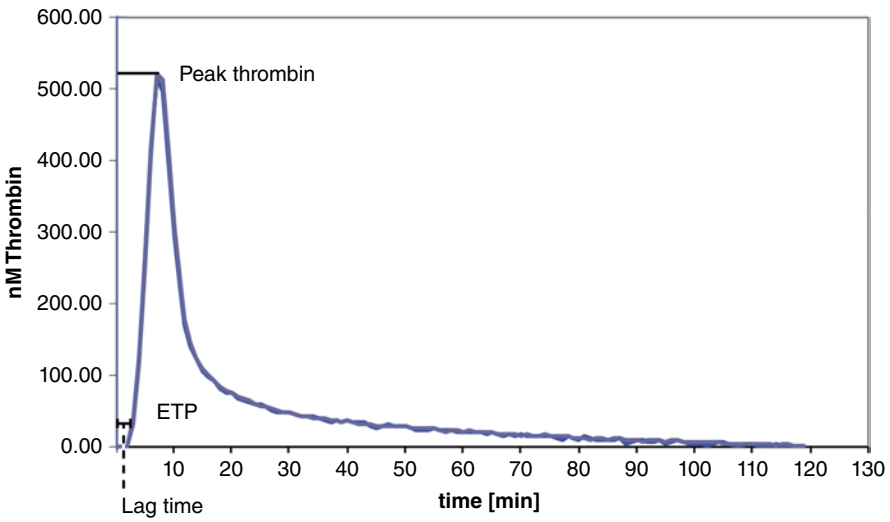


FIGURE 88.3 Thrombin generation curve (Thrombogram). The thrombogram displays the amount of thrombin formed (nM thrombin, Y axis) over a 2-hour reaction time (minutes, X axis). The "lag time" represents the delay for initial thrombin formation after initiation of the reaction, "peak thrombin" denotes maximal thrombin concentration, and "ETP" (endogenous thrombin potential) represents the summation of total thrombin as area under the curve.

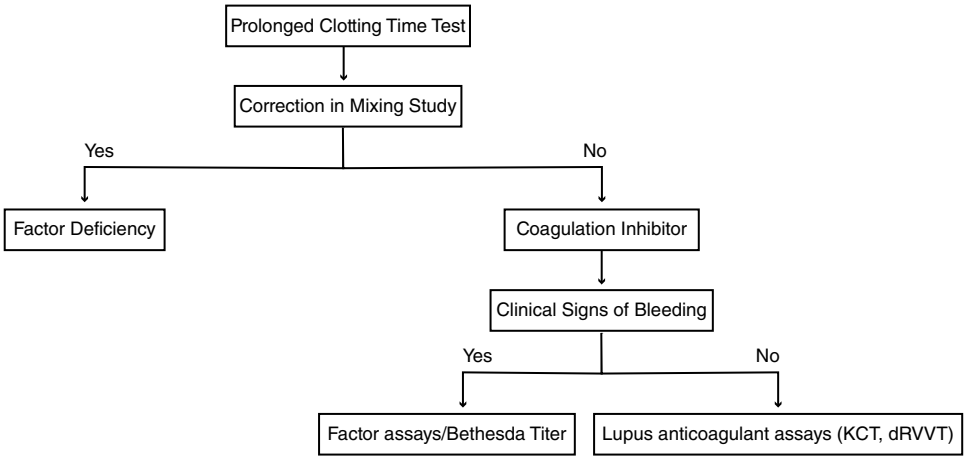


FIGURE 88.4 Diagnostic algorithm for identification of coagulation inhibitors. Abbreviations: KCT, kaolin clotting time; dRVVT, dilute Russell's viper venom time.

factors, particularly factor Xa and thrombin (see Chapter 87; Figure 88.4). The assay principle of functional AT assays is based on inhibition of a purified factor Xa or thrombin reagent. Chromogenic substrate assays are most common for clinical diagnosis and are performed by incubating the test plasma with a factor reagent containing heparin to enhance AT's interaction and inhibition of the target factor.[6,30,109] Absorbance change in the assay mixture is then monitored after addition of a peptide substrate. In these assays, AT activity is inversely related to color change in the assay mixture. Several commercially available chromogenic AT kits, configured as "anti-IIa" assays, have been used in animal studies.[5,39,113]

Thrombin–Antithrombin Complex

Upon binding with thrombin, AT forms a covalent thrombin–antithrombin (TAT) complex with a short plasma half-life of approximately 15 minutes. High circulating TAT complex concentration is an indicator of dysregulated and/or systemic thrombin generation, and often accompanies prothrombotic inflammatory states and disseminated intravascular coagulation (see Chapter 92). Plasma TAT concentration can be measured by sandwich ELISA, configured with an antithrombin capture antibody and anti-AT conjugated detector antibodies.[7] A commercially available TAT assay kit (Enzygnost TAT, Siemens) is configured with cross-reactive polyclonal antibodies and has been used for a variety of species in veterinary studies.[63,81,96]

Proteins C and S

Protein C is a vitamin-K-dependent, serine protease anticoagulant factor that circulates as a zymogen. in vivo. The activation of protein C to a functional protease, activated protein C (APC), occurs on cell membrane surfaces. Once formed, APC inactivates factor Va and factor VIIIa, and also exhibits endothelial cytoprotective effects through thrombin receptor signaling (see Chapter 87).

Functional assays for clinical diagnosis of protein C deficiency are commercially available with either clotting time or chromogenic endpoint.[46] Clot endpoint assays are typically configured as modified aPTT reaction mixtures containing the test plasma, a contact pathway activator, and a snake-venom-derived protein C activator. After recalcification, prolongation of clotting time compared to a calibrated standard indicates relatively high protein C concentration. Chromogenic assays are also configured with venom protein C activators, but contain a specific peptide substrate to directly measure the protease's amidolytic activity. Chromogenic protein C assays have been used in a variety of canine veterinary studies.[32,68,100] Protein C deficiency is a useful biomarker of liver function, with severe protein C deficiency a feature of portosystemic shunting and liver failure[100] (see Chapter 89).

Activated protein C resistance refers to a human prothrombotic condition resulting from ineffective APC-mediated inhibition of factor Va.[82] A point mutation in the gene encoding factor V (referred to as factor V Leiden) is worldwide in distribution and a common risk factor for thrombosis. Assays to detect APC resistance are typically configured as functional, clotting time tests with paired reactions measuring clotting times in the presence or absence of exogenous APC. A veterinary syndrome comparable to APC resistance has not yet been described.

Protein S is a vitamin-K-dependent serine protease that acts as a cofactor to enhance APC's inactivation of factors Va and VIIIa at membrane surfaces. Functional protein S assays are configured as modified aPTT or PT, containing the relevant coagulation triggers with either APC or protein C activators included in the reaction mixture.[62] Adaptation of a commercially available assay system (Diagnostica Stago) to measure protein S activity in dogs has been described; however, the assay's diagnostic utility is unknown.[5]

Pathologic Inhibitors: Factor Inhibitors and Lupus Anticoagulants

Pathologic coagulation inhibitors include antibodies targeting epitopes on individual coagulation factors, or on phospholipid-binding proteins (referred to lupus anticoagulants).[101,109] Antifactor antibodies either directly interfere with factor activity or enhance factor clearance, thereby causing severe factor deficiencies and clinical signs of bleeding.[56] Factor VIII is the most common targeted factor. Transfusion may trigger alloantibody formation; however, antifactor antibodies also develop as an autoimmune phenomenon (see Chapter 89).[33] Paradoxically, lupus anticoagulants are associated with hypercoagulability and signs of arterial or venous thrombosis, and pregnancy loss (see Chapters 89 and 91). Lupus anticoagulants represent a feature of the antiphospholipid syndrome, a primary autoimmune disorder that may also develop in patients with lupus erythematosus.[69]

Factor inhibitors and lupus anticoagulants are typically detected by prolongation of the aPTT screening test (Figure 88.4). A preliminary mixing study can be performed by incubating equal volumes of the test plasma with a pooled normal plasma and repeating the aPTT.[53,109] Persistent prolongation indicates the presence of a coagulation inhibitor, rather than uncomplicated factor deficiency. The specificity of antifactor antibodies is then further characterized by performing individual coagulant activity assays. By convention, the titer of factor inhibitory antibodies is defined in Bethesda units (BU).[103] Bethesda unit assays are clotting time tests configured with dilutions of patient plasma combined with equal volumes of pooled normal plasma for a 2-hour incubation at 37 °C. The coagulant activities of these mixtures, and an incubation mixture containing equal volumes of normal plasma with buffer, are then measured in one-stage assays. After calculation of residual activity of the sample dilutions versus the incubation mixture, values are data transformed to BU/mL. The antibody titer sufficient to inhibit 50% of the factor activity in normal plasma is defined as 1 BU/mL. While the original BU method has been refined, including a common "Nijmegen modification" to further standardize reaction mixture pH and protein content, the assay principle is unchanged and BU are still used to manage hemophilia A patients.[56]

Lupus anticoagulants are defined in clotting time tests configured with low concentration of phospholipid, such as the kaolin clotting time (KCT) or dilute RVVT[69,97] (Figure 88.4). The presence of antiphospholipid-binding antibodies in the test plasma impairs assembly of active coagulation complexes, thus delaying clot endpoint formation. Samples with prolonged KCT or dRVVT are then assayed in a 1:1 mixture with normal plasma to confirm that prolongation persists and was not due to factor deficiency. In human antiphospholipid syndrome, autoantibodies targeting cardiolipin or beta-2 glycoprotein often accompany lupus anticoagulant antibodies, and immunologic detection of these antibodies enhances diagnostic sensitivity and specificity. Lupus anticoagulant assays are readily adapted for animal plasmas, and adaptation of immunologic assays to detect anti-cardiolipin antibodies has also been described.[66,72] Prevalence of the antiphospholipid antibody syndrome appears low in the canine thrombotic syndromes studied to date.[66,86]

Anticoagulant Drugs: Unfractionated and Low-Molecular-Weight Heparins

The biologic activity of unfractionated heparin (UFH) is highly variable among patients, and high-dose UFH requires monitoring for individualized dose adjustments.[54] Traditionally, point-of-care activated clotting time (ACT) or aPTT were used to gauge UFH anticoagulant intensity with a target for prolongation of 1.5–2.5 times the control value. Refinements of this target include nomogram adjustments based on patient weight, pretreatment clotting times, and reagent sensitivity.[53]

Measurements of factor Xa inhibitory activity (anti-Xa) represent an alternate and more readily standardized method to monitor heparin therapy. These assays are also suitable to monitor newer low-molecular-weight heparins that do not prolong clotting times at therapeutic levels (see Chapter 94).[54] Chromogenic assays are widely used in human studies and are applicable for veterinary studies.[2,67] The assays are configured with a purified factor Xa reagent, and after incubation with test plasma, a peptide substrate of factor Xa is added to the reaction mixture. Heparin levels in the plasma are inversely related to color change. The assays are configured with calibrated standards containing defined concentration of either UFH or low-molecular-weight heparins.[11] Human anti-Xa therapeutic targets are approximately 0.3–0.70 U/mL for UFH, with a range of approximately 0.5–1.5 U/mL for low-molecular-weight heparin.[54,107] Clinical trials to define safe effective targets in veterinary studies are limited (see Chapter 94).

Anticoagulant Drugs: Vitamin K Antagonists and Direct Oral Acting Anticoagulant Drugs

Coumadin, a vitamin K antagonist, was the major oral anticoagulant drug used in human medicine for more than 50 years. Similar to monitoring UFH with the aPTT, the PT is used to ensure appropriate coumadin dosage and prevent major bleeding.[6,53] The INR is a data transformation of the PT based on thromboplastin composition and the testing laboratory's PT reference interval. Target INR values between 2 and 3 indicate appropriate anticoagulant intensity for many human thrombotic disorders.[54] The risk of hemorrhage and need for frequent monitoring and individualized dose adjustments have limited the use of coumadin in veterinary medicine (see Chapter 94).

Newer direct oral acting anticoagulants (DOACs) have begun to replace coumadin in human medicine. When used at therapeutic dosages in people, the DOACs do not reliably prolong routine clotting time tests.[26,27] Apixaban and rivaroxaban are DOACs that specifically inhibit factor Xa. Anti-Xa assays configured

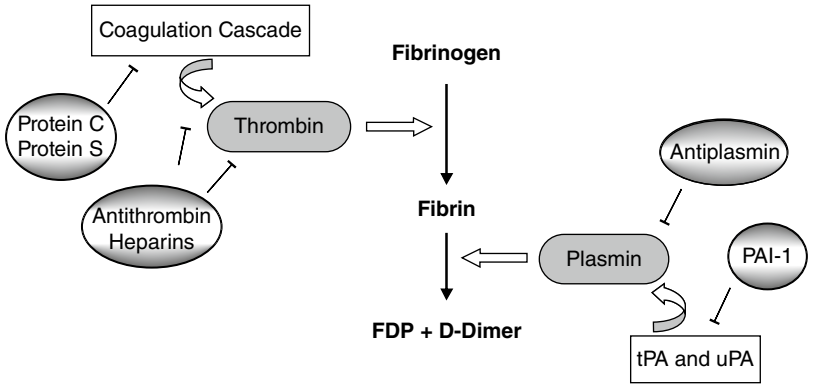


FIGURE 88.5 Regulation of fibrin formation and degradation. The coagulation cascade reactions generate thrombin, which acts on fibrinogen to form insoluble fibrin. Proteins C and S are major inhibitors of the procoagulant factors that generate thrombin. Antithrombin inhibits thrombin and the other procoagulant serine proteases. Endogenous heparins and heparin drug therapy greatly enhance antithrombin's inhibitory activity. Plasmin is the major protease that degrades fibrin. The zymogen plasminogen is activated by tissue plasminogen activator (t-PA) and urokinase (u-PA), whose actions are inhibited by plasminogen activator inhibitor-1 (PAI-1). Antiplasmin is the major circulating inhibitor of plasmin.

with apixaban or rivaroxaban standards can be used to monitor these drugs, and have shown excellent correlation with spectrophotometric assays in veterinary pharmacokinetic studies.[19,25,87]

FIBRINOLYTIC PATHWAY ASSAYS

Plasmin is the major protease responsible for fibrinolysis. Its zymogen, plasminogen, undergoes activation to plasmin primarily on the surface of fibrin fibrils through the action of tissue plasminogen activator (tPA) or urokinase (Figure 88.5). Clinical signs of bleeding develop in patients with dysregulated or "hyperfibrinolytic" syndromes, whereas delayed or impaired fibrinolysis is associated with signs of thrombosis.[17] Although the fibrinolytic pathway is less complex than the coagulation cascade, simple screening tests analogous to the aPTT and PT are lacking.[41,102] Quantitative measurements of circulating fibrin degradation products are the most widely used assays to evaluate fibrinolysis. Functional assays of specific pathway reactants and more global pathway assays are performed primarily in referral or research laboratories. Mass-spectrometry-based proteomics offers an alternate method for quantitation of fibrinolytic pathway proteins when cross-reactive antibodies are not available.

Plasminogen Assays

Functional, plasminogen activity assays have been adapted for veterinary clinical and research applications.[51,68] Commercially available kits are configured as chromogenic assays, including a peptide substrate of plasmin, and a plasminogen activator to transform plasminogen in the test sample to the active enzyme, plasmin. Human kits typically contain streptokinase as a plasminogen activator. A clinical study of fibrinolysis in dogs with babesiosis reported use of a chromogenic plasminogen assay configured with a streptokinase activator.[47] Streptokinase, however, has been reported to be an ineffective plasminogen activator for many species. An assay modification of plasma acidification, followed by neutralization and urokinase activation, has been described for measuring equine plasminogen,[106] and has been applied to other species, including dogs and rats.[13,52]

Plasminogen Activators: Tissue Plasminogen Activator and Urokinase

Two serine protease enzymes, tPA and urokinase, are the major plasminogen activators. Under physiologic conditions, these proteases act on the surface of fibrin formed within the vascular space (tPA) or extravascular tissues (urokinase). Free tPA and urokinase are rapidly cleared by complex formation with plasminogen activator inhibitor-1 (PAI-1). Assays to measure circulating levels of these activators are primarily performed in research settings. A functional assay has been described based on in vitro formation and lysis of fibrin with the time for lysis monitored by change in turbidity.[57] The assay is performed in microtiter plates by combining a mixture of fibrinogen, thrombin, and plasminogen and then adding test sample as the source of plasminogen activator. The lysis time of the test sample is then compared with a reaction configured with a streptokinase reagent representing a defined potency plasminogen activator. A canine-specific ELISA (MyBioSource) was used to measure urokinase concentration in a case series of dogs with cancer.[79] The reported mean concentration for healthy dogs (0.19 ng/mL) was not significantly different from that of the cancer patients.

Fibrinolysis Inhibitors

Antiplasmin

Antiplasmin (alpha-2-antiplasmin or serpin F2) is the major plasma inhibitor of free and fibrin-bound

plasmin. Antiplasmin is a SERPIN family protein that inactivates plasmin by forming plasmin–antiplasmin complexes.[20] Commercially available chromogenic assays of human antiplasmin activity have been used in clinical studies of fibrinolysis in horses, cats, and dogs.[18,52,68,105] The assays are configured with a human plasmin reagent that is first incubated with the test plasma, prior to addition of a plasmin substrate. Color change in the assay mixture is inversely related to plasma antiplasmin activity. Immunologic assays to measure human and murine antiplasmin concentration are available from several sources, as are human plasmin–antiplasmin complex kits. Use of these assays has not been described in companion animal studies.

Plasminogen Activator Inhibitor-1

The SERPIN family inhibitor PAI-1 (or serpin E1) is a major regulator of fibrinolysis. Circulating levels of PAI-1 are often increased in prothrombotic conditions including sepsis, obesity and metabolic syndrome, and neoplasia (see Chapter 91). It is synthesized primarily by endothelial cells and is also released from platelets and other cell types, including adipose tissue. A functional test of canine PAI-1 activity has been described based on its inhibitory action on an exogenous tPA reagent.[110] The assay is configured as a two-step chromogenic assay, wherein test plasma (containing PAI-1) is first reacted with a human recombinant tPA reagent to allow formation of the SERPIN/protease complex. In the second step, a recombinant human plasminogen reagent and a plasmin substrate are added to the reaction mixture. Free tPA generates plasmin, thus the rate and final absorbance change in the reaction are inversely related to PAI-1 content of the test plasma. Dogs with hyperlipidemia were found to have increased plasma PAI-1 activity compared with healthy dogs; however, PAI-1 activity was labile in plasma stored at 4 °C. Functional assays to detect rat or human PAI-1 are also commercially available (Molecular Innovations).[13] These assays are configured with microtiter plates coated with urokinase that will interact with PAI-1 in the test sample. The amount of bound PAI-1 is then detected immunologically with antibodies directed against human or rat PAI-1.

Thrombin-Activated Fibrinolysis Inhibitor

Thrombin-activated fibrinolysis inhibitor (TAFI) is a non-serpin, carboxypeptidase that acts at endothelial surfaces upon its activation by thrombomodulin-bound thrombin (see Chapter 87). Active TAFI cleaves plasminogen-binding residues from fibrin polymers, thus slowing fibrinolysis and facilitating clot stabilization. A commercially available, chromogenic assay has been used to measure TAFI activity in canine plasma.[43] The assay is configured with a thrombin–thrombomodulin reagent to activate TAFI in the test plasma, and a specific TAFI chromogenic substrate. Healthy dogs had TAFI activities ranging from approximately 32 to 71% of a human calibration standard, whereas the highest values (>200%) were found in dogs with neoplasia and thrombocytopenia.

Euglobulin Lysis Time

The euglobulin lysis time is a qualitative screening test of fibrinolysis that measures the time for in vitro dissolution of a fibrin clot.[41,91] The term "euglobulin fraction" refers to hemostatic proteins that precipitate from plasma under conditions of low pH and low temperature. This fraction contains procoagulant factors, fibrinogen, plasminogen, and plasminogen activators, but is largely depleted of fibrinolysis inhibitors, including antiplasmin, alpha-2-macroglobulin, TAFI, and PAI-1. The euglobulin lysis time test is performed by diluting the test plasma in a cold 1% acetic acid solution, followed by incubation at 4 °C, and cold centrifugation to precipitate the euglobulin fraction. After removal of the supernatant (containing inhibitors), the precipitate is resuspended in buffered saline and recalcified, with or without addition of thrombin, to initiate coagulation. The coagulation reaction is held at 37 °C and monitored over time for clot lysis. While originally performed in glass tubes with visual inspection, more recent modifications of the assay are performed in microtiter plates with spectrophotometric monitoring of OD to generate absorbance curves and numeric parameters of lysis time and lysis rate.[41] Human euglobulin fractions lyse over the course of hours, ranging from approximately 3–10 hours in one study of healthy adults. This broad range complicates interpretation of results to differentiate enhanced or impaired fibrinolysis from normal variation. In addition, plasma fibrinogen content influences clot stability, and lysis times have been shown to correlate with plasma fibrinogen levels.[57]

Turbidimetric Fibrinolysis Assays

Plasma-based assays, configured in microtiter plates containing exogenous coagulation and fibrinolysis activators, have been developed as global measures of hemostatic balance.[4] Adaptations of one of these assays, referred to as "overall hemostasis potential" (OHP), have been described for dogs and rats.[12,23,42] Initial fibrin formation and polymerization, followed by later fibrinolysis, is monitored as changes in turbidity of the reaction mixture, detected as absorbance change at 405 nM. Paired reactions, containing either a coagulation activator alone or coagulation activator with tPA, are each performed in replicates and the output absorbance change over the course of 1 hour is displayed as superimposed curves (Figure 88.6). Numeric parameters derived from these curves include: delay in time for first deflection from baseline, overall coagulation potential (area under the coagulation curve), maximal OD of the coagulation curve, and OHP (area under the lysis curve). First descriptions of the human OHP assay were

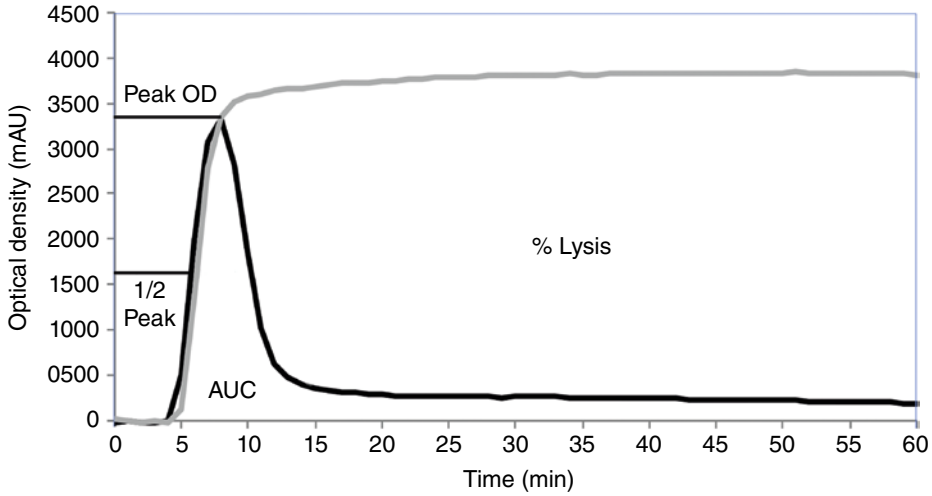


FIGURE 88.6 Microplate fibrinolysis assay tracings. Graphic overlays represent the change in absorbance (optical density, milli-absorbance units (mAU) on the Y axis versus reaction time (minutes) on the X axis. The dark line represents a lysis reaction containing a coagulation trigger reagent and tissue plasminogen activator, and the light gray line represents a reaction containing a coagulation trigger alone. The maximal and half-maximal changes in optical density are indicated by the parameters "peak OD" and "½ peak." The area under the lysis curve (AUC) and the difference in AUC between the two curves (% lysis) summarize overall hemostasis potential and coagulation potential, respectively.

configured with thrombin as coagulation activator, with subsequent modifications in thrombin dose, addition of platelet phospholipids, and tissue factor trigger reagents. Similarly, refinements in the composition and concentration of coagulation and fibrinolysis activators are described for the animal assays.

Viscoelastic Fibrinolysis Assay

Viscoelastic methods have also been developed to monitor clot formation, clot stabilization, and clot lysis. Thromboelastographic (TEG) assays, configured with coagulation activators and tPA, have been described to study drug effects in vitro and in pharmacodynamic studies in dogs and horses.[14,31,105] A plasma-based assay, referred to as clot life span analysis, has also been described for mechanistic studies of human thrombotic disorders.[73] The assay has been adapted for use in human studies of fibrinolysis inhibitors, in an equine pharmacodynamic study of a hemostatic herbal treatment, and in studies of snake venom and antidotes in dogs and several other species.[44,71] The clot life span analysis assay is performed in TEG reaction cups in reaction mixtures containing a coagulation activator (kaolin, celite, or tissue factor) with a tPA reagent. Coagulation is then initiated by the addition of calcium chloride. The TEG software generates standard numeric parameters including rate of coagulation, clot firmness, and lysis time, and displays a qualitative tracing. For clot life span analyses, additional TEG coagulation kinetic parameters related to elastic modulus (dyne/cm^2) and derived curves are included in the data compilation (Figure 88.7).

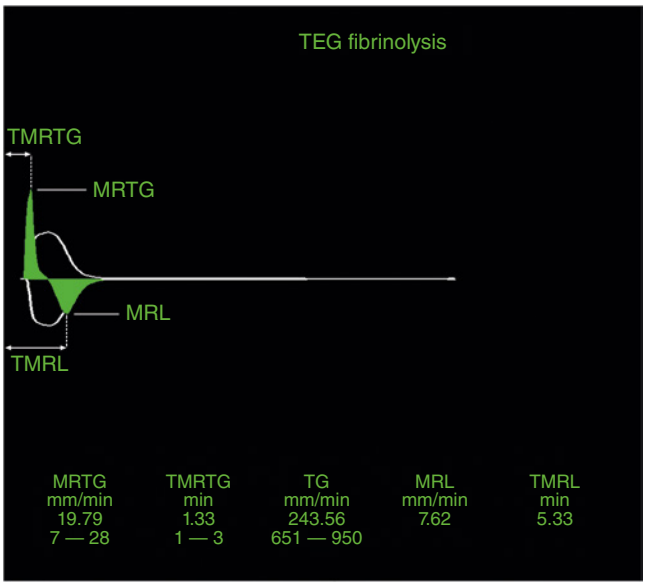


FIGURE 88.7 Clot life span model thromboelastographic variables. The data collected from cynomolgus monkey plasma treated with a coagulation activator [Innovin, Siemens (tissue factor reagent)] and fibrinolysis activator [Alteplase, Genentech (tissue plasminogen activator)] are displayed. The white tracing depicts rapid clot formation (symmetric upward and downward deflections from baseline) and complete lysis is represented by return to baseline. The green fill represents elastic modulus velocity variables of the same sample. MRTG = maximum rate of thrombus generation (dyne/cm^2); TMRTG = time to maximum rate of thrombus generation (minutes); TG = calculated thrombus generation (mm/minute); MRL = maximum rate of lysis (mm/minute); TMRL = time to maximal rate of lysis (minutes).

Fibrin/Fibrinogen Degradation Products and D-Dimer Concentration

The surface of fibrin thrombi is the major activation site of plasmin, resulting in plasmin cleavage of fibrin polymers and release of a series of degradation peptides, also referred to as fibrin split products.[41] Under pathologic conditions of hyperplasminemia, fibrinogen degradation and release of fibrinogen fragments may also occur. Fibrinogen and fibrin degradation products are collectively referred to as FDP. D-dimer fragments are the terminal products of plasmin's degradation of cross-linked fibrin and are generated only in the process of fibrinolysis. High concentrations of D-dimer, therefore, are markers of thrombin-mediated fibrin formation and secondary fibrinolysis (see Chapters 91 and 92).

A semi-quantitative latex slide agglutination test (Thrombo-Wellcotest, Thermo Fisher) has been widely used in clinical veterinary studies of disseminated intravascular coagulation (DIC).[95] The assay requires special collection tubes containing a coagulation activator and proteolysis inhibitor, and can be performed on blood or urine samples. The anti-FDP reagent is a polyclonal antibody raised against purified fibrinogen fragments D and E. More recently, commercially available semi-quantitative and quantitative D-dimer assays have been evaluated to aid in animal DIC diagnosis.[8,94] While D-dimer testing is included in diagnostic algorithms to exclude pulmonary thromboembolism or deep vein thrombosis in defined human patient populations,[6,109] cutoff values with similar diagnostic utility for animals are lacking (see Chapter 91).

WHOLE BLOOD ASSAYS

Cell-based models of hemostasis emphasize the importance of cell membrane surfaces in promoting and regulating coagulation (see Chapter 87). Assay techniques designed for whole blood samples attempt to capture the contribution of blood cells and hemostatic proteins for "global" assessment of overall hemostatic balance. Similar to other functional coagulation assays, preanalytic variables and reagent composition influence test results. Moreover, test interpretation often requires knowledge of platelet count, hematocrit, and plasma hemostatic protein content, particularly fibrinogen concentration.

Viscoelastic Assays

Thromboelastography (TEG®, Haemonetics), rotational thromboelastometry (ROTEM®, Instrumentation Laboratory), and microviscometry (Sonoclot, Sienco) are viscoelastic assays with associated software designed to measure and display the kinetics and tensile properties of clot formation in whole blood. These instruments monitor changes in clot strength and stability that occur beyond the time of initial fibrin formation. In addition to qualitative tracings, the instruments' software performs direct measurements and derived calculations that describe features of clot formation and subsequent clot lysis. The principles of operation and output parameters vary among these instruments[35] (Figures 88.8 and 88.9). These instruments are often placed in medical emergency room or operative or settings, and used to guide transfusion requirements for complex trauma patients and cardiovascular and transplant surgical procedures. The instruments require accurate pipetting skills and careful technique for operation. A handheld viscoelastic monitor (VCM™, Entegrion) designed for patient-side testing of non-anticoagulated blood has recently become available for human hemostasis monitoring, with a similar device (VCM Vet™) marketed for veterinary patients.

Thromboelastography has been the most widely used viscoelastic assay for clinical veterinary studies in the United States. A multi-institution veterinary panel [Partnership on Rotational ViscoElastic Standardization (PROVETS)] to standardize thromboelastography and thromboelastometry identified several sources of variation among testing centers, and confirmed the

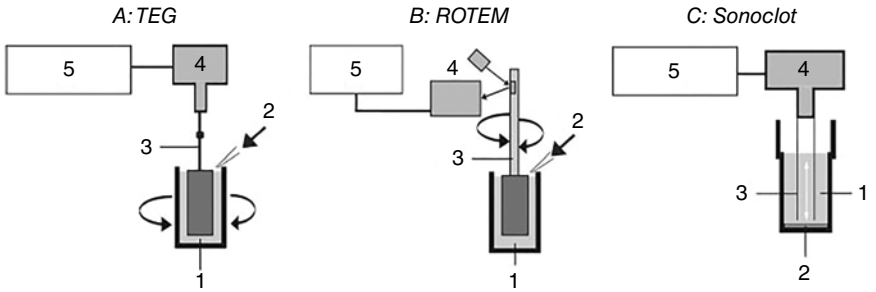


FIGURE 88.8 Principles of viscoelastic coagulation devices. (a) Thromboelastograph (TEG): 1 = rotating cup with blood sample; 2 = coagulation activator; 3 = pin and torsion wire; 4 = electromechanical transducer; 5 = data processing; (b) Rotational thrombelastometry (ROTEM): 1 = cuvette with blood; 2 = activator added by pipetting; 3 = pin and rotating axis; 4 = electromechanical signal detection by light source and mirror mounted on axis; 5 = data processing; (c) Sonoclot: 1 = blood sample in cuvette; 2 = cuvette containing activator; 3 = disposable plastic probe; 4 = probe oscillation motion in blood sample mounted on electromechanical transducer head; 5 = data processing. (**Source:** Ganter and Hofer[35])

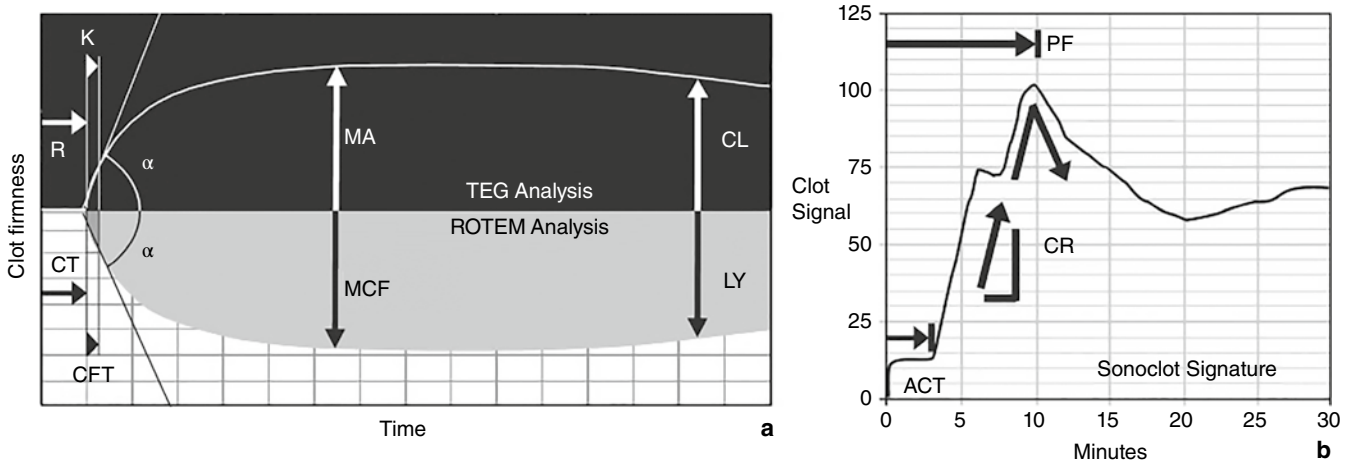


FIGURE 88.9 Sample output of viscoelastic coagulation devices. (a) Left (upper) side: thromboelastograph (TEG) tracing: "R" reaction time; "K" kinetics; "alpha" slope between R and K; "MA" maximum amplitude; "CL" clot lysis. Left (lower) side: rotational thrombelastometry (ROTEM) tracing: "CT" clotting time; "CFT" clot formation time; "alpha" slope of tangent at 2 mm amplitude; "MCF" maximal clot firmness; "LY" lysis; (b) Right side: Sonoclot Signature: "ACT" activated clotting time; "CR" clot rate; "PF" platelet function. (**Source:** Ganter and Hofer[35])

inherent biologic variability of viscoelastic testing.[37] The same group developed evidence-based guidelines for use of TEG® and ROTEM®. Consensus recommendations included practices to minimize preanalytic variation, development of instrument-specific assay configurations, and use of in-house reference intervals. The panel also identified areas of uncertainty for future research.[38]

Unlike traditional clotting time tests, viscoelastic monitors allow characterization of disease conditions of procoagulant excess (hypercoagulability) and defects of enhanced or delayed fibrinolysis. Thromboelastographic features of hypercoagulability have been described in dogs with immune-mediated hemolysis, protein-losing nephropathy, and neoplasia,[39,40,104] and in horses with acute gastrointestinal disease.[29] Conditions associated with hyperfibrinolysis include canine acute hemoabdomen,[32] hepatopathies,[34] and equine gastrointestinal diseases.[105] A modification of whole blood TEG® to include reactions containing tPA identified hyperfibrinolysis in dogs with acute hemoabdomen.[32] To date, clinical studies utilizing the handheld viscoelastic monitor have not yet been published.

The limitations common to all whole blood viscoelastic assays include the influence of hematocrit, platelet count, blood viscosity, and fibrinogen content on assay parameters such that interpretation of test results requires consideration of all of these parameters.[74] Low hematocrit and high fibrinogen are consistently associated with "hypercoagulable" TEG tracings; however, conditions within the in vitro reaction cup may not reflect in vivo hemostatic balance.[10,65] These features complicate the use of viscoelastic assays as clinical diagnostic tests. Nevertheless, viscoelastic assays have shown utility for serial patient monitoring, pharmacodynamic studies, and in mechanistic studies of hemostatic disorders.[35,38,50]

Microfluidic Flow Assays

In addition to fluid phase and cellular reactants, physiologic hemostasis is coordinated and regulated by blood flow properties. Microfluidic devices have been developed to study thrombus formation in whole blood under controlled conditions of shear stress.[9,84] This technology allows for low sample volume, and varying matrix compositions of the device walls to capture different aspects of platelet adhesion and activation, and fibrin polymerization in a cellular milieu under flow. Microfluidic chambers are also able to incorporate endothelial cell surfaces and model vascular stenosis. While primarily designed for research settings, commercialized devices have been developed for biomedical and clinical studies. The Total Thrombus-Formation Analysis System (T-TAS) is a novel whole blood microfluidic device that combines microscopic imagery and pressure sensors.[85] The instrument's output includes qualitative analyses of thrombus formation that generate time/pressure graphs and numeric parameters related to time for thrombus occlusion of the microcapillary chamber (Figure 88.10). The assay system is configured with disposable chips whose capillaries are coated with either collagen alone to assess platelet contribution, or with collagen and thromboplastin to assess coagulation. The shear rates are varied to mimic either arterial or venous flow conditions. Among human applications, the T-TAS device has been used in pharmacodynamic studies of antiplatelet and anticoagulant drugs, and monitoring recovery from cardiovascular procedures.[85] Preliminary studies indicate that the T-TAS system is capable of monitoring thrombus formation in canine samples, and demonstrates aberrant thrombus formation in dogs with hereditary bleeding disorders (Figure 88.11).

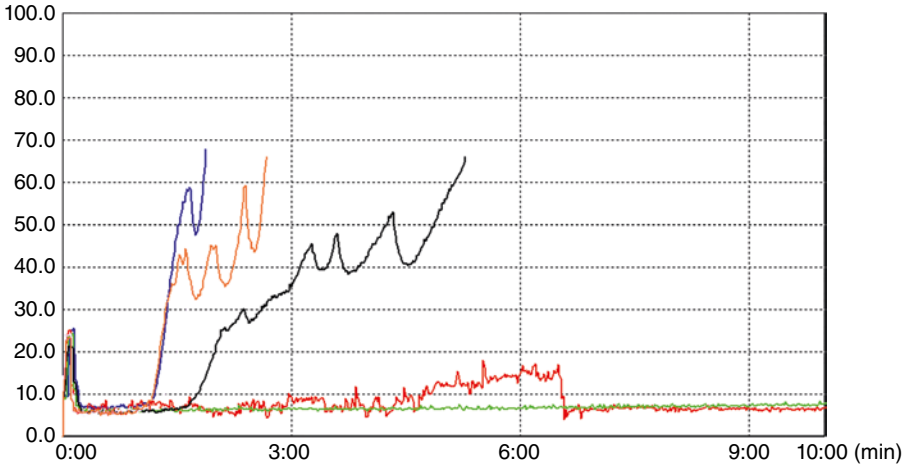


FIGURE 88.10 Total Thrombus-Formation Analysis System (T-TAS) pressure waveform output. Flow pressure waveform tracings demonstrating change in pressure [Y axis, kPa (kilopascal)] over time (X axis, minutes) of canine blood containing varying concentrations of von Willebrand factor. Note delay in occlusive thrombus formation or failure of thrombus formation depicted as slow pressure increment or lack of pressure increment. Blue line: Von Willebrand factor concentration = 100%. Orange line: Von Willebrand factor concentration = 40%. Black line: Von Willebrand factor concentration = 20%. Red line: Von Willebrand factor concentration = 0%.

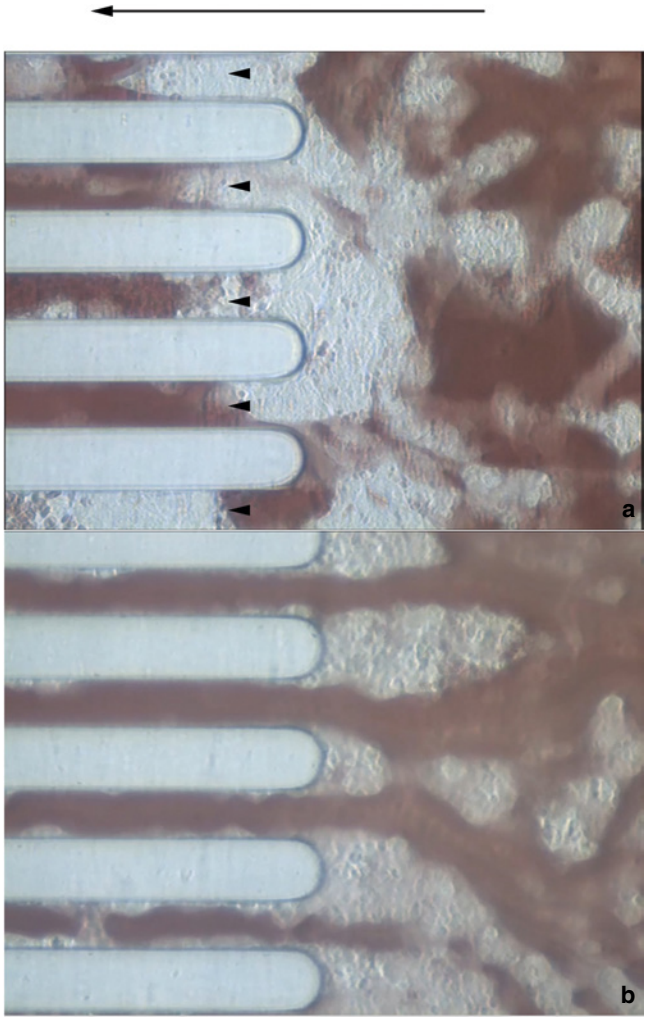


FIGURE 88.11 Total Thrombus-Formation Analysis System (T-TAS) microscopic imaging. Platelet thrombus formation in canine whole blood on collagen-coated microchip in the flow chamber. Arrow indicates direction of flow. Shear rate = 1500 s^{-1}. (a) Control dog, arrow heads indicate occluding thrombi; (b) Type 3 Von Willebrand disease; note the absence of occluding thrombi.

Continued technological advances in miniaturization, 3D fabrication, imaging, and data management will lead to the next generation of coagulation instruments.[70,84] These devices will help develop predictive models of physiologic and pathologic thrombus formation, and ultimately improve clinical diagnosis and treatment of hemostatic disorders.

ONLINE RESOURCES

Coagulation Testing, Test Principles, and Methodologies:

https://captodayonline.com/productguides/instruments/coagulation-analyzers-january-2019.html?product_list_limit=all – CAP Today. Searchable product guide to coagulation instrument manufacturers.

http://eclinpath.com/ – eClin Path. On-line textbook on veterinary clinical pathology.

http://mghlabtest.partners.org/coagbook/handbook.htm – Massachusetts General Hospital Laboratory Handbook. Lists of coagulation assays, methodologies and assay principles.

https://www.vet.upenn.edu/research/academic-departments/clinical-sciences-advanced-medicine/research-labs-centers/penngen/tests-worldwide – PennGen Database. Searchable database of genetic testing laboratories for hereditary disease in dogs and cats

http://www.practical-haemostasis.com/index.html – Practical Haemostasis.com. Medical education site providing background of hemostasis assay methodologies, principles, and test interpretation exercises.

https://doctorlib.info/medical/hematology/45.html – Rodak's Hematology, Hemostasis and Coagulation instrumentation. Summary of assay principles and lists of coagulation instruments and manufacturers.

Commercial Sources of Coagulation Instruments and Reagents

https://www.stago-us.com/ – Diagnostica Stago, Inc.

https://diapharma.com/products/hemostasis/ – Diapharma Hemostasis.

http://www.haemonetics.com/en – Haemonetics

https://www.helena.com/coagulation.html – Helena Laboratories.

https://www.instrumentationlaboratory.com/us/en/product-line-hemostasis – Instrumentation Laboratory

https://mol-innov.com/ – Molecular Innovations,

https://www.siemens-healthineers.com/en-us/hemostasis – Siemens Healthineers.

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CHAPTER 89

Acquired Coagulopathies

MARJORY B. BROOKS and ARMELLE DE LAFORCADE

Pathogenetic Classification of Acquired Coagulopathies
- Coagulation Factor Deficiencies
  - Synthetic Failure
  - Activation Defects
  - Factor Consumption and Dilutional Coagulopathies
- Coagulation Inhibitors
- Fibrinolysis Defects

Clinical Evaluation
- History
- Physical Examination
- Quick Assessment and Point-of-Care Tests
- Coagulation Panel

Specific Disorders
- Liver Disease
- Vitamin K Deficiency
- Disseminated Intravascular Coagulation
- Dilutional Coagulopathy and Traumatic Coagulopathy
- Coagulation Inhibitors
  - Factor Inhibitory Antibodies
  - Antiphospholipid Syndrome Inhibitors
  - Anticoagulant Overdose

Acronyms and Abbreviations

ACOTS, acute coagulopathy of trauma and shock; APLA, antiphospholipid-protein antibody; APS, antiphospholipid syndrome; aPTT, activated partial thromboplastin time; AT, antithrombin; DIC, disseminated intravascular coagulation; DOAC, direct oral anticoagulant; FDP, fibrin degradation product; KH_2, vitamin K hydroquinone; KO, vitamin K epoxide; PIVKA, proteins induced by vitamin K absence/antagonism; PT, prothrombin time; TCT, thrombin clotting time; TEG, thromboelastography; t-PA, tissue plasminogen activator; UFH, unfractionated heparin.

Clinical signs of hemorrhage are usually the result of a breach in vascular integrity that overwhelms an individual's normal hemostatic response. For these cases, diagnosis and management are directed at defining the site and cause of blood vessel injury. A subset of patients, however, have abnormal bleeding due to impaired hemostasis. Thrombocytopenia (see Chapters 80 and 81) and acquired coagulopathies are the most common hemostatic defects encountered in clinical practice. An index of suspicion and consistent use of screening tests early in the diagnostic workup are key to recognition and effective management of acquired coagulopathies.

PATHOGENETIC CLASSIFICATION OF ACQUIRED COAGULOPATHIES

Acquired coagulopathies discussed in this chapter include disease processes that delay or prevent fibrin clot formation or increase the rate of fibrinolysis (Table 89.1).

Coagulation Factor Deficiencies

Coagulation factor activation generates a local burst of thrombin that transforms soluble plasma fibrinogen into an insoluble fibrin clot. Quantitative and/or functional

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TABLE 89.1 Pathogenesis of acquired coagulopathies.

Mechanism	Disease syndromes	Associated factor defects
Synthetic failure	Liver disease: severe failure and markedly reduced functional hepatic mass	Most coagulation factors low but relative retention of factor VIII activity Hypofibrinogenemia and dysfibrinogenemia Antithrombin and protein C low
	Portosystemic shunting	Protein C low
Vitamin-K-dependent activation defect	Cholestasis, intestinal malabsorption, vitamin K antagonists, neonates	Factors II, VII, IX, and X low Protein C low
Excessive rate of consumption	Overt (hemorrhagic) disseminated intravascular coagulation, envenomation	Complex pattern of increased and decreased factor activities, hypofibrinogenemia Antithrombin low Hypofibrinogenemia, variable deficiency of other factors
Coagulation inhibitors	Alloimmune and autoimmune factor inhibitors Phospholipid-protein antibodies (associated with thrombosis) Anticoagulant overdose	Single-factor deficiency (factor VIII deficiency most common) Coagulation factor activities normal Heparins: Serine protease factors neutralized (especially factor X, factor II) DOACs: specific factor Xa or factor IIa inhibition
Hyperfibrinolysis	Disseminated intravascular coagulation, neoplasia, infectious agents, liver disease, prostate surgery, traumatic hemorrhage	Complex factor deficiencies High plasminogen activators Hypofibrinogenemia

deficiencies of coagulation factors influence the rate and amount of thrombin produced (see Chapter 87). The common acquired factor deficiencies, unlike hereditary coagulopathies, are characterized by combined deficiencies of multiple clotting factors.

Synthetic Failure

The liver is the sole or primary source of all the serine protease procoagulant and anticoagulant factors (i.e., factors II, VII, IX, X, XI, and XII, and proteins C and S) and other critical hemostatic proteins including factors V and XIII, antithrombin (AT), antiplasmin, and plasminogen. Liver disease is associated with complex abnormalities, with combined and variable deficiencies of procoagulant and anticoagulant proteins.[2,34] Liver failure, however, typically manifests as a hemorrhagic diathesis. A lack of hepatic synthetic capacity results in quantitative coagulation factor deficiencies, first detectable by decreased activities of factors with short plasma half-lives (e.g., factor VII, half-life of 6 hours). Plasma fibrinogen, as an acute phase reactant, is often normal or elevated in patients with inflammatory and cholestatic liver disease. Diseased hepatocytes, however, may produce a dysfunctional, "nonclottable" fibrinogen due to abnormal alpha chain conformation and high sialic acid content that impairs fibrin formation and polymerization. The development of hypofibrinogenemia in patients with liver disease is an indicator of severely compromised synthetic functional reserve, associated with poor prognosis.[2]

Activation Defects

Vitamin K is essential for the post-translational processing of the prothrombin group of coagulation factors (factors II, VII, IX, X) and the anticoagulants, proteins C and S. All of these factors require the addition of gamma-carboxyglutamic acid residues in order to interact with calcium, bind to membrane surfaces, and form active enzyme complexes (see Chapter 87). Vitamin K participates in the carboxylation reaction through oxidation of vitamin K hydroquinone (KH_2) to vitamin K epoxide (KO). The epoxide form of vitamin K is continually recycled to replenish KH_2 by the enzyme vitamin K epoxide reductase (Figure 89.1).

Coagulation factors synthesized in the absence of vitamin K circulate in plasma as inactive, descarboxy precursors referred to as proteins induced by vitamin K absence or antagonism (PIVKA). While PIVKA are sensitive indicators of vitamin K deficiency or antagonism, PIVKA assays are specialized tests thus limiting their clinical utility.[3,25] By contrast, the prothrombin time (PT) assay is widely available as a point-of-care or routine coagulation screening test that is commonly used to detect vitamin-K-dependent coagulopathies.

Factor Consumption and Dilutional Coagulopathies

Factor consumption can outpace hepatic synthesis in animals with severe systemic inflammation and other disease processes that trigger overt disseminated intravascular coagulation (DIC). The resultant coagulation factor depletion causes clinical signs of hemorrhage (see

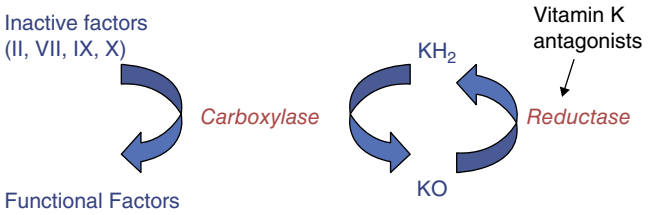


FIGURE 89.1 Vitamin K cycle. A vitamin-K-dependent carboxylase simultaneously converts vitamin K quinol (KH2) to vitamin K 2,3-epoxide (KO) as it transforms glutamate to functional gamma carboxyglutamate residues on factors II, VII, IX, and X. Vitamin K 2,3-epoxide is then reduced back to a quinol by vitamin K epoxide reductase. This enzyme is inhibited by coumarin-type anticoagulants.

Chapter 92). Systemic amyloidosis in people is associated with variable factor X deficiency, although the phenomenon has not been described in animals. Amyloid fibrils are believed to adsorb factor X and facilitate its rapid plasma clearance.[12] The bleeding tendency of amyloidosis is multifactorial; due to amyloid tissue infiltration and deposition in the microvasculature, and potentially exacerbated by severe factor X deficiency (e.g., values below 25%). Many snake venoms contain proteases that activate clotting factors and/or directly cleave fibrinogen, resulting in systemic factor depletion and defibrination in a consumptive process.[19]

Chronic hemorrhage does not result in factor deficiencies in animals with normal liver function; however, severe acute hemorrhage may be associated with a complex coagulopathy including factor depletion. Aggressive fluid resuscitation may also induce dilutional coagulopathy.[5,30] Factor dilution develops secondary to fluid shifts from the interstitial and intracellular space into the intravascular space. These shifts are triggered by reduced intravascular hydrostatic pressure and are compounded by administration of large volumes of intravenous fluids. Activation of coagulation secondary to trauma-induced tissue factor expression also contributes to factor consumption. Massive transfusion is defined as replacement of more than one blood volume within a 24-hour time interval or one-half of a blood volume within 6 hours.[15,30] Thrombocytopenia, acidosis, hypothermia, and hypocalcemia (due to citrate-based anticoagulants in blood products) further contribute to hemostatic compromise associated with aggressive transfusion of blood products.[15,30]

Coagulation Inhibitors

The classification of coagulation inhibitors includes antibodies (usually IgG) directed against specific coagulation factors.[28] The antibodies bind to and directly inhibit factor activity and/or speed the rate of factor clearance. Inhibitory alloantibodies develop in patients with hereditary factor deficiencies after transfusion exposure to the "foreign" factor antigen. Factor VIII inhibitory antibodies develop in approximately 20% of people with severe hemophilia A (factor VIII deficiency).[28,29] Alloimmunization and development of factor VIII and IX inhibitors has also been described in dogs with hemophilia A and B, respectively.[27] Autoantibodies directed against coagulation factors represent a loss of tolerance and can also develop in patients without hereditary factor defects, regardless of prior transfusion.[14] Factor VIII is the most commonly implicated factor and the resultant coagulopathy is referred to as "acquired hemophilia." The epitope specificities of factor VIII alloantibodies and autoantibodies generally overlap, with most binding to the protein's light chain C domain. Acquired factor V inhibitors have been recognized in people exposed perioperatively to topical bovine thrombin and fibrin glues.[18] These products contain trace amounts of bovine factor V, sufficient to induce cross-reactive antibody production, especially in multiply exposed patients.

The term "lupus anticoagulant" describes antibodies that prolong in vitro clotting times by blocking the interaction of coagulation factors with phospholipid reagents.[21] The immunogenic epitopes are actually phospholipid-binding proteins and a more accurate term for these inhibitors is antiphospholipid-protein antibodies (APLAs).[10] Paradoxically, people with APLA develop signs of thrombosis, rather than hemorrhage. Although their prothrombotic action is not fully defined, APLAs are believed to displace natural anticoagulants on cell surfaces and induce exposure of procoagulant phospholipids in the vascular space.

Pharmacologic anticoagulants include drugs that alter factor synthesis or directly inhibit factor activity, and heparin compounds that act indirectly to enhance antithrombin's ability to neutralize active factors. Traditionally, unfractionated heparin (UFH) was the sole anticoagulant used widely in clinical veterinary practice; however, dosage guidelines for low-molecular-weight heparins have recently been published for dogs and cats (see Chapter 94). While the antiplatelet drugs clopidogrel and aspirin are most often used for oral antithrombotic therapy, newer direct oral anticoagulants (DOACs) that interact directly with activated factor II and factor X are now under investigation (see Chapter 94). Finally, high plasma concentration of fibrin degradation products (FDPs) may act as coagulation inhibitors by interfering with normal fibrin polymerization.

Fibrinolysis Defects

Accelerated fibrinolysis causes hemorrhage by degrading fibrin clots before vascular repair is complete. Disease syndromes that enhance plasminogen activation, decrease plasminogen inhibitors, or combine these factors produce a hyperfibrinolytic state.[39] Hyperfibrinolysis, mediated by high tissue plasminogen activator (t-PA), develops in people infected with the agents causing Rocky Mountain spotted fever, African swine fever, and dengue fever, and secondary to extensive tissue trauma.[13] A syndrome of hyperfibrinolysis, referred to as acute coagulopathy of trauma and shock (ACOTS), has been described in people with severe traumatic hemorrhage and shock. In a large prospective trauma trial, early treatment with fibrinolytic inhibitors was found to significantly reduce the risk of

death due to bleeding.[4] In a potentially related syndrome, dogs with spontaneous hemoperitoneum experienced accelerated fibrinolysis that correlated with the severity of shock.[8] Hemostatic defects in dogs with hepatic disease vary, reflecting the complexity of their state of "rebalanced hemostasis." Studies using viscoelastic testing to investigate hemostatic alterations in dogs with hepatic disease have documented hyperfibrinolytic changes in up to 40% of affected dogs.[16] Certain neoplastic diseases are associated with hyperfibrinolysis due to specific elaboration of plasminogen activators by tumor cells (e.g., acute promyelocytic leukemia) or in the context of a DIC process (see Chapter 92). The prostate and uterus are rich in t-PA and urokinase plasminogen activators. Neoplasia and surgery involving these organs risk hemorrhagic complications due to local hyperfibrinolysis resulting in acute hemorrhage or delayed rebleeding.

CLINICAL EVALUATION

The initial evaluation of animals with signs of hemorrhage should aim to differentiate blood loss caused by vessel injury or vascular disease from a systemic failure of normal hemostasis (i.e., bleeding diathesis). The location and nature of hemorrhage and prior history of abnormal hemorrhage or underlying disease conditions provide clues to guide subsequent laboratory evaluation. Some animals with acquired coagulopathies do not manifest overt hemorrhage at initial presentation. Detection of factor deficiency in these cases requires an index of suspicion and consistent inclusion of coagulation screening tests early in the diagnostic workup.

History

A history of recurrent, episodic hemorrhage is suggestive of a hereditary hemostatic defect, rather than an acquired coagulopathy (see Chapters 83, 84, and 90). Hemostasis questionnaires have been developed for medical studies with the goal of simplifying history gathering and generating bleeding "scores" for standard comparison among trials.[31] A brief questionnaire with specific questions on hemostasis is also useful early in the clinical workup of animals suspected of having a bleeding disorder. Pertinent questions include: Has this animal ever had spontaneous bleeding from the nose, mouth, urinary tract, or noticeable bleeding when teething? Has this animal ever had a surgical procedure, dentistry, or traumatic injury, and were there any bleeding complications? Are you aware of any bleeding problems in relatives or in this breed? Is this animal receiving any medication or dietary supplements? Does this animal have any ongoing medical problems? Additional questions regarding housing conditions and potential access to drugs and toxins are useful; however, a negative exposure history does not preclude toxicity.

Physical Examination

The physical examination should define focal or multifocal sites of hemorrhage and detect evidence of gross and subtle hemorrhage. Funduscopic examination and careful inspection of mucous membranes and non-haired skin may reveal petechiae and ecchymoses, suggestive of platelet or von Willebrand factor defects or vasculopathies. Large vessel (arterial/venous) injury typically causes obvious blood loss from localized sites of vessel injury. Animals with severe coagulopathies may develop spontaneous hemorrhage into any body cavity or potential space, producing effusion, mass effect, or hematoma formation. Simultaneous hemorrhage from several sites, including prolonged hemorrhage from venipuncture or catheter sites, is evidence of severe coagulopathy. The possibility of an acquired coagulopathy should be included in the differential for any patient with unexplained hemorrhage, effusion or mass lesion, or disease conditions associated with factor deficiencies (see Table 89.1). Routine coagulation screening of these cases will minimize delays in instituting appropriate management.

Quick Assessment and Point-of-Care Tests

The initial assessment of any patient suspected of having a hemostatic defect should include platelet count or platelet estimate (see Chapter 78) and a point-of-care coagulation test. Point-of-care instruments are now available to perform clotting time tests in dogs and cats using whole blood samples. A clinical study of coagulopathies in dogs found similar diagnostic utility comparing results of laboratory and point-of-care coagulation panels.[35] These instruments have largely replaced the activated clotting time for rapid detection of moderate to severe factor deficiencies. Viscoelastic monitors provide a global assessment of hemostasis that incorporates the contribution of cellular elements and procoagulant and anticoagulant plasma proteins (see Chapter 88).[11] The thromboelastography (TEG) analyzer is the viscoelastic instrument used most widely to study coagulopathies in dogs.[27,37] This instrument provides a visual tracing and numeric parameters reflecting the rate and strength of fibrin formation. Clinically significant coagulopathies are expected to cause prolonged initiation times and diminished tracing amplitude. Evidence of hypocoagulability, in either simple or sophisticated point-of-care tests, may require follow-up with specific coagulation panel assays (and possibly more detailed factor and anticoagulant assays) for definitive diagnosis of acquired coagulopathies.[11]

Coagulation Panel

The screening tests to detect coagulopathies include activated partial thromboplastin time (aPTT), PT, thrombin clotting time (TCT), and fibrinogen concentration (see Chapter 88). The pattern of abnormalities in these screening tests reflects the deficient factor or factors (Figure 89.2).

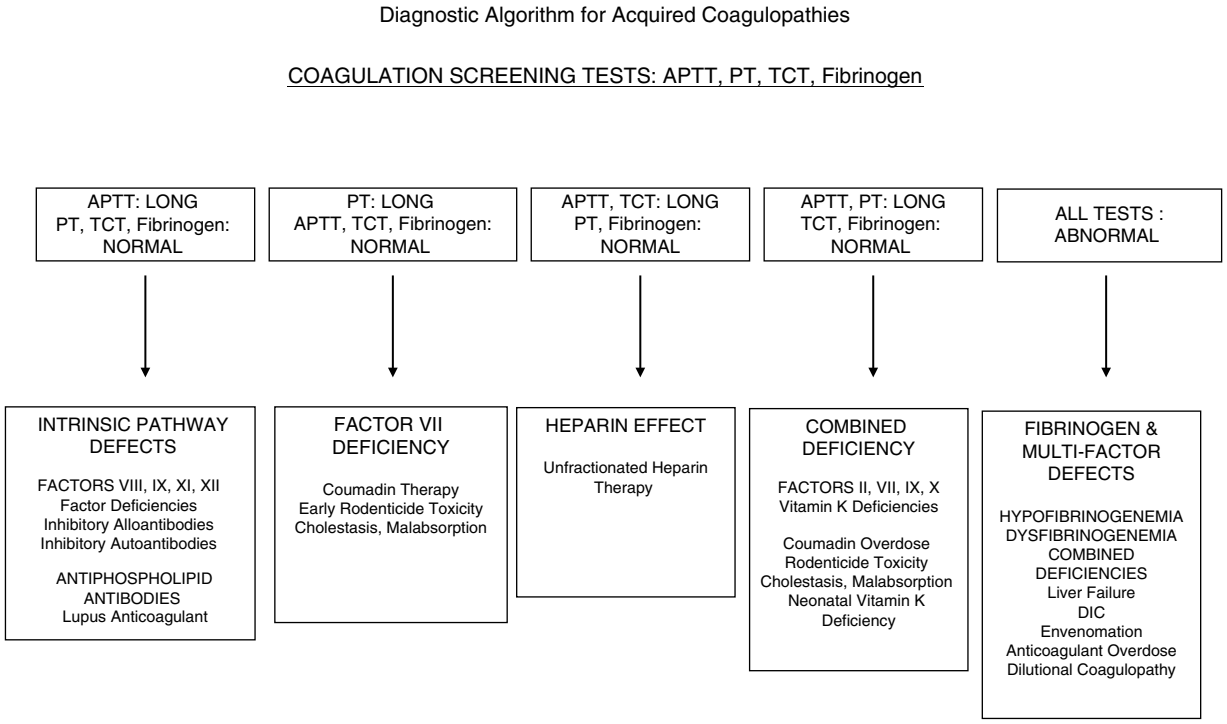


FIGURE 89.2 Coagulation screening test algorithm for acquired coagulopathies.

Coagulation panel results, combined with clinical presentation, are often sufficient for management of acquired coagulopathies. Complex cases may require specific analyses of individual coagulation factors, anticoagulant proteins, drugs, or measures of fibrinolysis to better define pathogenesis or facilitate case management. Collection of pretreatment samples of citrated plasma is critical for timely and accurate diagnosis of coagulopathies, and clinicians must bear in mind that the validity of all hemostasis testing depends on appropriate sampling techniques (see Chapter 88).

SPECIFIC DISORDERS

Liver Disease

Animals with hepatobiliary disease demonstrate variable signs of coagulopathy, ranging from spontaneous bleeding to subclinical prolongation of in vitro clotting times.[3,20,33,16] Liver disease causes major alterations in many hemostatic mechanisms, including platelet and endothelial cell reactivity, procoagulant and anticoagulant protein production and function, dysregulation of fibrinolysis, and complications arising from portal hypertension and splenomegaly. These changes often act as opposing procoagulant and anticoagulant forces, resulting in variable net bleeding risk (rebalanced hemostasis). Clinically severe hemorrhage is seen primarily in animals with fulminant or end-stage hepatic failure, or those with concomitant DIC. Other forms of liver disease may cause relatively milder bleeding tendencies, albeit sufficient to impair surgical hemostasis for animals undergoing biopsy or hepatobiliary surgery.

Marked prolongation of coagulation screening tests (aPTT, PT, TCT) and hypofibrinogenemia indicate high risk of spontaneous and postsurgical hemorrhage, and are overall poor prognostic signs. Mild prolongation or the absence of abnormal clotting times, however, does not rule out the risk of procedure-related bleeding. Newer hemostasis tests, such as endogenous thrombin potential and thromboelastography, are designed to assess net procoagulant and anticoagulant forces. Thromboelastography has demonstrated hyperfibrinolysis in dogs with both acute and chronic hepatic disease. In dogs with acute hepatic impairment, hyperfibrinolysis becomes more pronounced with disease severity.[16,9] The utility of these tests for predicting bleeding risk in patients with liver disease is under clinical investigation.[11] A preliminary study has shown that measurement of protein C activity may be useful as a biomarker of liver function and hepatoportal blood flow in dogs.[33] The finding of low protein C activity helps differentiate portosystemic vascular anomalies from microvascular dysplasia, and is a useful predictor of clinical outcome when combined with traditional biochemical assays.

Management strategies for coagulopathy of liver disease include prophylactic and "rescue" therapies. Prophylactic administration of vitamin K_1 is often included with specific and supportive medical therapy for liver disease. Preoperative vitamin K_1 administration is indicated for animals with cholestatic disorders and/or prolonged aPTT and PT (see Chapter 94). Current trends in human medicine favor

more restrictive use of prophylactic transfusion therapy.[2] Fresh frozen plasma is transfused to patients with liver failure and signs of hemorrhage to supply active procoagulant and anticoagulant factors (see Chapters 94 and 100). The need for preoperative transfusion is generally assessed on an individual patient basis in human and veterinary medicine. People undergoing liver transplantation invariably require transfusion and algorithms based on thromboelastographic parameters have been developed to guide the use of blood components in this patient population.[11]

Vitamin K Deficiency

Vitamin K deficiency is a common cause of coagulopathy, resulting from inadequate intestinal absorption or impaired intrahepatic recycling.[3,20,7,22] Biliary obstruction, intrahepatic cholestasis, chronic oral antibiotic administration, and infiltrative bowel disease may all reduce vitamin K absorption. Hemorrhagic disease of the newborn refers to vitamin K deficiency in infants within the first few days to weeks of life. Hemorrhage is attributed to inadequate placental transfer of vitamin K and inadequate hepatic recycling.[17] Coumadin (warfarin) and second-generation anticoagulant rodenticides (e.g., brodifacoum) act by irreversibly blocking the activity of epoxide reductase, the critical hepatic vitamin K recycling enzyme (see Figure 89.1). Severe coagulopathies due to anticoagulant rodenticide toxicity are commonly encountered in small animal practice, have been reported in equine practice, and are increasingly recognized in wild birds (see Chapter 95). Factor deficiency and resultant signs of a bleeding diathesis develop over a period of several days post ingestion of anticoagulant poisons as vitamin K stores become depleted. Signs of hemorrhage may be obvious as external blood loss, or may be nonspecific as pleural or abdominal effusion or mass lesions anywhere in the body due to hematoma formation. Treatment delays often lead to death from hemorrhagic shock, or respiratory or central nervous system compromise.

Prolongation of both the aPTT and PT screening tests, with normal TCT and fibrinogen, are the hallmarks of hemorrhage caused by severe vitamin K deficiency (Figure 89.2). Specific factor analyses reveal that deficiencies of factors II, VII, IX, and X cause this characteristic coagulation profile. Therapeutic dosages of the anticoagulant coumadin, while no longer recommended as a first-line anticoagulant, cause only mild deficiencies of the vitamin-K-dependent factors that should not cause prolongation of the aPTT. Coumadin monitoring to ensure adequate, but not excessive dosage, is based on PT analyses (see Chapter 94). In human medicine, the international normalization ratio (INR) is used to account for variability in PT reference ranges among testing laboratories. An INR target value between 2 and 3 indicates appropriate anticoagulant intensity for many human thrombotic disorders.

Management of vitamin K deficiency requires replacement therapy with vitamin K_1 (phytonadione), rather than vitamin K_3 (menadione). Vitamin K therapy is often given empirically pending results of pretreatment coagulation assays, because synthesis of sufficient active factors to correct the coagulopathy requires at least 12 hours from the start time of initiating vitamin K supplementation. Transfusion is also indicated for patients with severe signs at presentation (see Chapter 100).

Disseminated Intravascular Coagulation

Microvascular thrombosis is the characteristic feature of DIC; however, signs of a severe bleeding diathesis dominate some case presentations. Dogs with DIC secondary to neoplasia, sepsis, or heatstroke may develop consumptive coagulopathies, with coagulation factor and fibrinogen depletion, and concomitant increase in FDP and D-dimer (see Chapter 92). Hemorrhagic, overt DIC is often transfusion-resistant, requiring high-volume plasma replacement to sustain hemostasis (see Chapter 100).

Dilutional Coagulopathy and Traumatic Coagulopathy

The term dilutional coagulopathy describes a hemostatic defect that develops after high-volume fluid resuscitation. Acute traumatic coagulopathy secondary to shock and hypoperfusion occurs in up to one of four human trauma patients and is considered a negative prognostic indicator in people with severe trauma. Concomitant acidemia, hyperfibrinolysis, and hypothermia further exacerbate coagulopathy in these patients. Administration of high-volume fluid therapy exacerbates traumatic coagulopathy. The effect of crystalloids versus colloids in promoting a dilutional coagulopathy has been extensively studied, with colloids demonstrating a greater dilutional effect than crystalloids and a negative effect on clot stability.[24] Clinical studies in people also reveal that fibrinogen deficiency generally develops early in the course of hemodilution and signs of inadequate hemostasis manifest at fibrinogen concentration below 50–100 mg/dL (0.5–1.0 g/L).[30] A retrospective study of massive transfusion in dogs noted prolonged aPTT and PT in half the cases.[15] Correction of hemostatic abnormalities is associated with improved survival in people, and factor replacement in the form of fresh frozen plasma is typically administered after recognition of coagulopathy.[13]

Coagulation Inhibitors

Factor Inhibitory Antibodies

Pathologic antibodies that bind to and inhibit coagulation factor activity cause clinical signs of hemorrhage. Antibodies targeting factor VIII are a well-recognized complication of factor replacement therapy for people with hemophilia A, and factor IX inhibitory antibodies occasionally develop in hemophilia B patients. Patients with severe factor deficiency (i.e., ≤1% factor activity) are at highest risk for inhibitor development.[28] Case reports have described factor VIII or IX inhibitor formation in

dogs with hemophilia A or B, respectively.[26,1] Clinical signs of coagulopathy due to factor inhibitory antibodies also occur as an autoimmune phenomenon, with factor VIII the most commonly reported target antigen.[14] Underlying conditions associated with acquired factor VIII inhibitor development in people include systemic lupus erythematosus, rheumatoid arthritis, drug hypersensitivity, inflammatory bowel disease, malignancy, and the postpartum state. Although apparently rare in animals, an acquired coagulopathy due to factor VIII inhibitors has been described in a Thoroughbred mare.[38] Factor VIII inhibitors (alloantibodies and autoantibodies) often cause severe coagulopathies characterized by spontaneous hemorrhage refractory to standard replacement therapy.

Factor VIII or IX inhibitors are detected by specific prolongation of the aPTT that is not corrected by mixing patient plasma with normal plasma. The Bethesda unit (BU) assay is used to quantify the titer of inhibitory antibodies. Values below 5 BU/mL are considered low titers.[28,29] Active bleeding in patients with low BU titers often responds to transfusion of high-dose factor replacement therapy. Long-term management of high-titer factor inhibitors consists of specific therapy of underlying disorders (for autoantibody patients) and immune-modulating or immunosuppressive therapy.[29]

Antiphospholipid Syndrome Inhibitors

The antiphospholipid syndrome (APS) is recognized as a thrombotic risk factor in people. The syndrome typically develops in patients with autoimmune disorders and is characterized by the production of APLAs that prolong in vitro clotting time.[21] Long aPTT that fails to correct in a mixing study is compatible with the presence of APLA, and more specialized clotting time tests (e.g., dilute Russell's viper venom time) and quantitative immunoassays to detect antibodies directed against lipid-binding proteins are used as confirmatory assays.[10] Inhibitory antibodies were proposed as the cause of long clotting times in a case report of canine immune-mediated hemolytic anemia (IMHA) and thrombosis; however, the prevalence and clinical relevance of APLA and APS in animals are not well-defined.[32]

Anticoagulant Overdose

Thrombotic syndromes and indications for antithrombotic therapy are increasingly recognized in veterinary medicine (see Chapters 91 and 94). The growing use of anticoagulant therapy raises the possibility of iatrogenic coagulopathy due to drug overdose. Unfractionated heparin remains a widely used anticoagulant. The pharmacokinetic profile of UFH is complex due to its extensive protein and cell-binding and dose-dependent half-life (see Chapter 94).[6,23,36] Since hemorrhage is a major risk of high-dose UFH therapy, individual patient monitoring is required to prevent excessive anticoagulant effect. The aPTT is the most readily available coagulation screening test sensitive to therapeutic UFH levels. Adjustment of UFH dose to prolong aPTT to 1.5–2 times pretreatment values is the traditional method for guiding UFH therapy to prevent overdosage. Clinical signs of hemorrhage or marked prolongation of aPTT (i.e., >2.5 times baseline) are indications for discontinuing UFH therapy. Due to its short (2–3 hour) plasma half-life, hemorrhage is usually transient. People with major hemorrhage due to UFH overdosage are infused with protamine sulfate, a basic polypeptide that binds tightly to UFH and immediately neutralizes its anticoagulant activity.[36] Protamine administration causes systemic hypotension in dogs; therefore, transfusion therapy is used to control life-threatening hemorrhage caused by excessive UFH anticoagulant effect (see Chapter 100).

Newer anticoagulants do not consistently prolong clotting time tests at therapeutic levels. Laboratory assays that specifically detect anticoagulant inhibition of activated factor X (anti-Xa assays) are suitable, however, to monitor low-molecular-weight heparins, UFH, and the DOACs targeting factor Xa (e.g., rivaroxaban, apixaban). Dosing guidelines and appropriate anti-Xa target ranges for the various drugs are not yet established, but are undergoing investigation for a variety of thrombotic syndromes (see Chapter 94).

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ON-LINE RESOURCES

1. **Comparative Coagulation Laboratory**. Animal coagulation, anticoagulant, and fibrinolysis assays. https://www.vet.cornell.edu/animal-health-diagnostic-center/laboratories/comparative-coagulation
2. **Massachusetts General Hospital**. Overview of assays to aid in diagnosis of coagulopathies. http://www2.massgeneral.org/pathology/coagbook/handbook.htm

CHAPTER 90

Hereditary Coagulopathies

MARJORY B. BROOKS

Hereditary Coagulation Factor Deficiencies
X-Linked Factor Deficiencies: Hemophilia A and B
  Clinical Diagnosis of Hemophilia
  Treatment of Hemophilia
  Genetics
Autosomal Factor Deficiencies
  Fibrinogen Deficiency
  Prothrombin Deficiency
  Factor VII Deficiency
  Factor X Deficiency
  Factor XI Deficiency
  Factor XII and Contact Factor Deficiencies
  Combined Deficiencies of the Vitamin-K-Dependent Factors
Uncharacterized Factor Deficiencies

Acronyms and Abbreviations

ACT, activated clotting time; aPTT, activated partial thromboplastin time; coagulation factors: FII, factor II; FVII, factor VII; FVIII, factor VIII; FIX, factor IX; FX, factor X; FXI, factor XI; FXII, factor XII; FXIII, factor XIII; DIC, disseminated intravascular coagulation; DSH, domestic shorthair; HMWK, high-molecular-weight kininogen; PIVKA, proteins induced by vitamin K absence/antagonism; OMIA, Online Mendelian Inheritance in Animals; OMIM, Online Mendelian Inheritance in Man; PK, prekallikrein; PT, prothrombin time; PTA, plasma thromboplastin antecedent; RVVT, Russell's viper venom time; SINE, short interspersed nuclear element; TCT, thrombin clotting time; VKOR, vitamin K epoxide reductase.

HEREDITARY COAGULATION FACTOR DEFICIENCIES

Hereditary coagulopathies arise from mutations within genes required for synthesis or processing of active coagulation factors. The bleeding tendency associated with each trait depends on the physiologic role of the deficient factors in fibrin clot formation and the degree of factor deficiency. Hemophilia A is the most common hereditary coagulopathy in human beings and animals, due in part to a high de novo mutation rate in the coagulation FVIII gene (*F8*).[2,13] New factor deficiencies, however, can arise within any breed population and breed frequencies of known defects may vary over time. The general breeding strategies to limit propagation of hereditary factor defects include exclusion of individuals expressing a bleeding tendency, avoidance of repeat matings that produce affected individuals, and familial screening (especially dams, sires, siblings) of affected patients to identify others with quantitative or functional factor deficiencies. In addition to the review of defects presented in this chapter (Table 90.1), public access databases, such as Online Mendelian Inheritance in Man and Online Mendelian Inheritance in Animals (OMIM, OMIA), provide information on hereditary factor deficiencies in animals. The PennGen website provides an online searchable database of veterinary genetic testing laboratories that offer diagnostic tests to identify hereditary defects, including bleeding disorders, in domestic dogs and cats.

X-LINKED FACTOR DEFICIENCIES: HEMOPHILIA A AND B

Hemophilia A (OMIM 306700) and hemophilia B (OMIM 306900) are distinct X-linked bleeding disorders caused by functional or quantitative deficiencies of FVIII and

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TABLE 90.1 Hereditary factor deficiencies.

Factor deficiency	Coagulation screening test results		Species (breeds)
	Abnormal	Normal	
Fibrinogen	TCT,[a] fibrinogen,[a] all tests[b]	aPTT,[a] PT[a]	Dogs (Bichon Frise, Borzoi, Collie), cats (DSH), goat (Saanen), sheep (Leicester)
Factor II (prothrombin)	aPTT, PT	TCT, fibrinogen	Boxer
Factor VII (proconvertin)	PT	aPTT, TCT, fibrinogen	Dogs (Alaskan Klee Kai, Beagle, Deerhound, Malamute, Schnauzer), cats (DSH)
Factor VIII (hemophilia A)	aPTT	PT, TCT, fibrinogen	Dogs (any breed, mixed breeds, German Shepherd, Golden Retriever), cats (any breed, DSH), horse (any breed), sheep
Factor IX (hemophilia B)	aPTT	PT, TCT, fibrinogen	Dogs (any breed, mixed breeds), cats (any breed, DSH), horse
Factor X (Stuart–Prower factor)	aPTT, PT	TCT, fibrinogen	Dogs (Cocker Spaniel, Jack Russell Terrier), cats (DSH)
Factor XI (plasma thromboplastin antecedent (PTA) deficiency, hemophilia C)	aPTT	PT, TCT, fibrinogen	Dogs (English Springer Spaniel, Kerry Blue Terrier), cats (DSH, Maine coon)
Factor XII (Hageman factor)	aPTT	PT, TCT, fibrinogen	Dogs (Miniature Poodle, Shar Pei), Cats (DSH, DLH, Himalayan, Maine Coon, Siamese)
Contact pathway (prekallikrein) deficiency	aPTT	PT, TCT, fibrinogen	Dogs (German Shorthaired Pointer, Husky, others)
Combined vitamin-K-dependent factor deficiency (factors II, VII, IX, X)	aPTT, PT	TCT, fibrinogen	Dogs: Labrador Retriever Cats: Devon Rex

[a] Mild fibrinogen deficiency;
[b] Severe fibrinogen deficiency.
aPTT = activated partial thromboplastin time; PT = prothrombin time; TCT = thrombin clotting time.

FIX, respectively.[13] Although both genes (*F8* and *F9*) map to the long arm of the X chromosome, they are inherited independently. FIX (a serine protease enzyme) and FVIII (its coenzyme) play critical roles in the amplification phase of coagulation by participating in formation of the "tenase" enzyme complex (see Chapter 87).

Clinical Diagnosis of Hemophilia

Hemophilia A and B are diagnosed almost exclusively in males because of the sex-linked, recessive inheritance and expression pattern of these traits. Carrier (heterozygous) females are not at risk for abnormal bleeding due to the presence of one functional *F8* or *F9* allele. Signs of hemophilia include lameness due to hemarthrosis, intramuscular and subcutaneous hematoma formation, and prolonged hemorrhage from even minor wounds and from gingiva at tooth eruption sites (Figure 90.1). Severe hemophilia typically manifests within the first few months of life. Severely affected patients are at risk for spontaneous and fatal hemorrhage, whereas milder forms of hemophilia may become apparent only after surgery or trauma.

Hemophilia A is much more common than hemophilia B in all species, with an incidence of one to two cases per 10,000 male births (in human populations). Unlike many hereditary diseases in animals, hemophilia is not restricted to a single breed or inbred line. Cases of hemophilia A and B have been identified in mixed breed dogs and more than 50 different purebred lines.[2,3,5] Both traits have been reported in domestic and purebred cats[4,11,16] and horses,[6,27] and hemophilia A has been characterized in sheep.[1]

The laboratory diagnosis of hemophilia is based on results of coagulation assays. Both forms of hemophilia cause specific prolongation of intrinsic pathway screening tests (activated clotting time [ACT] and activated partial thromboplastin time [aPTT]), but do not affect tests of the extrinsic/common pathways or fibrinogen (see Table 90.1). Definitive diagnosis and



FIGURE 90.1 Clinical signs of hemophilia. Left: Intramuscular hematoma formation in the lateral thigh muscles of a German Shepherd with hemophilia B. Note distal limb swelling due to edema, venous obstruction, and extravasated blood. Right: Subcutaneous hematoma overlying the frontoparietal muscles of a mixed-breed puppy with hemophilia A.

differentiation of hemophilia A from hemophilia B requires specific measurements of FVIII and FIX coagulant activities (FVIII:C, FIX:C). Clinical severity generally relates to residual factor activity. In human medicine, severe hemophilia is characterized by FVIII:C or FIX:C < 2% of normal, whereas factor activities of 2–20% are associated with moderate to mild hemophilia. Factor assay values are derived from comparisons of patient plasma with the activity of plasma standards. Reaction kinetics of coagulation enzymes vary among species; therefore, animal factor activity assays are best performed using same-species plasma standards (see Chapter 88).

Treatment of Hemophilia

Transfusion is the primary means for controlling or preventing hemorrhage in animals affected with hemophilia A or B (see Chapter 94 and Section VIII). Fresh frozen plasma supplies both FVIII and FIX, whereas cryoprecipitate is a more specific product for replacement of FVIII, and cryosupernatant supplies FIX.[28] The transfusion interval for hemophiliacs varies depending on residual factor activity, and clinical features such as patient size, activity, the nature of hemostatic insult, and other factors influencing hemostasis. Maintenance of patients with severe hemophilia (<2% FVIII:C, FIX:C) can be difficult due to recurrent hemorrhage and short transfusion intervals. The most severely affected animals may be euthanized due to poor quality of life, or experience fatal bleeds before 1 year of age. Gene transfer therapy using adeno-associated viral vectors has been successful in correcting the bleeding tendency of dogs with hemophilia A and B, with sustained duration of factor expression in some cases for more than 1 year.[7,26] Inhibitory antibody formation and immune response directed against the viral vector were observed complications, and the cost and complexity of current gene transfer therapies limit its availability to research settings.

Genetics

New cases of hemophilia arise from frequent de novo mutations in *F8* and *F9*. Once present, these mutations are typically transmitted by asymptomatic carrier dams; however, males with mild forms of hemophilia may survive to adulthood and widely propagate the trait if they are bred (Figure 90.2). Males are accurately diagnosed as affected or clear of hemophilia based solely on FVIII and FIX assays. Obligate carrier females are then identified by pedigree review as the dams and daughters of the affected males. The status of suspect carrier females is difficult to classify with factor assays, however, because values overlap for carrier and clear females at the low end of the normal range. Pedigree review and preliminary molecular genetic analyses of hemophilia indicate that unique *F8* or *F9* mutations are likely causative for hemophilia in different breeds and families.[2,3,7,9,21,26] Distinct *F9* mutations have been identified in each unique breed variant of canine and feline hemophilia B. The discovery of hemophilia A causative mutations is more challenging because of *F8*'s large size and complex organization. Mutation detection through targeted gene sequencing, however, has been successful in identifying unique mutations in several breeds, including distinct point mutations in a Boxer, a German Shepherd, and a mixed-breed dog, and a complex inversion in a research colony derived from an Irish Setter.[7,9,21] Although direct mutation detection is the most accurate means of hemophilia carrier detection, this strategy requires knowledge of the causative mutation within each family. An alternate, indirect strategy applicable to

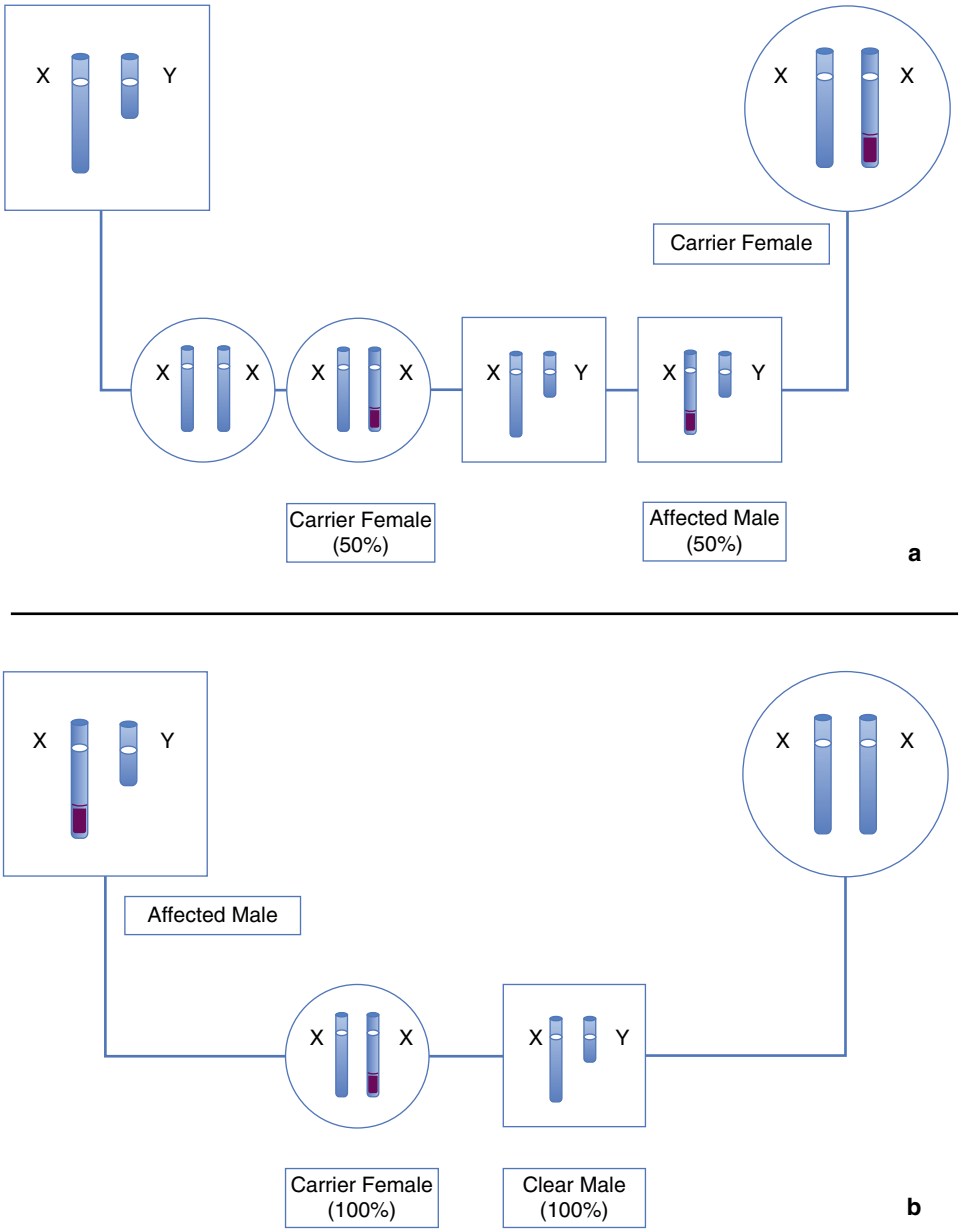


FIGURE 90.2 Inheritance of hemophilia A and B. (a) Propagation of hemophilia by a carrier female. If a carrier female is bred, on average one-half of her daughters and one-half of her sons will inherit her mutant gene (depicted as a purple bar on the X chromosome); (b) Propagation of hemophilia by an affected male. Males with mild forms of hemophilia may survive to reproduce. If bred, they transmit a mutation to all their daughters and none of their sons.

any family is based on utilizing DNA markers linked to *F8*. This strategy has proven feasible for hemophilia A carrier detection in sheep and dogs.[1,5]

AUTOSOMAL FACTOR DEFICIENCIES

All of the remaining factor deficiencies identified in animals (fibrinogen, FII, VII, X, XI, contact pathway, and combined factor defects) are less common than hemophilia, and are typically restricted to a few breeds or families (Table 90.1). These autosomal defects are typically recessive traits. In this form of inheritance, males and females express and transmit the traits with equal frequencies, affected individuals are homozygous for the disease-causing mutation, and heterozygous carriers do not express a bleeding tendency (Figure 90.3).

Fibrinogen Deficiency

Fibrinogen is the plasma precursor of fibrin and also supports intraplatelet bridging in the process of aggregation. Fibrinogen consists of three polypeptide chains (A-α, B-β, and γ), coded for by three closely linked genes. Mutations in any of these genes can impair fibrinogen function or secretion.

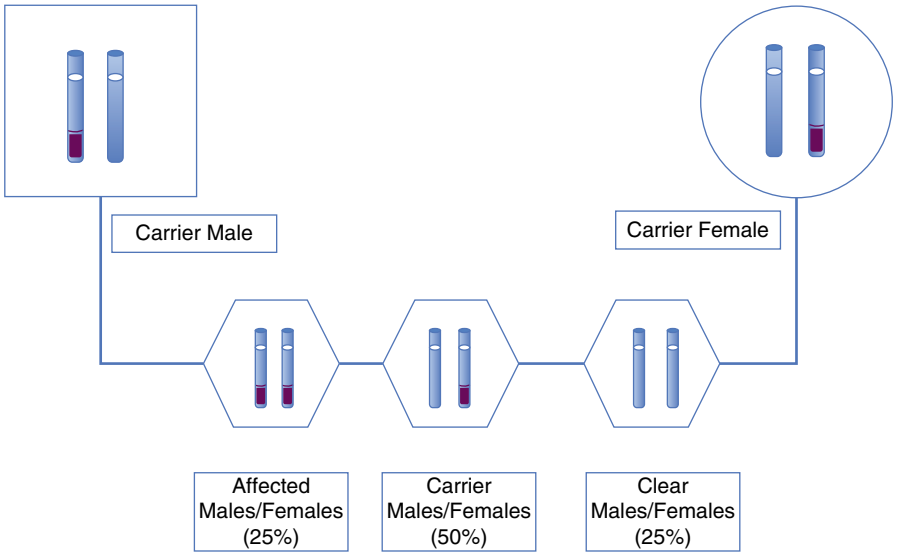


FIGURE 90.3 Inheritance of autosomal recessive factor deficiencies. Mutant genes (depicted as purple bars on an autosome) are transmitted from dam and sire to male and female offspring with equal frequencies. On average, one-quarter of the offspring will be homozygous (affected with the defect), one-half of the offspring will be asymptomatic heterozygous carriers, and one-quarter of offspring will inherit no mutant gene and therefore be clear of the trait.

Many distinct hereditary fibrinogen defects have been identified in people, broadly classified as quantitative deficiencies (i.e., afibrinogenemia and hypofibrinogenemia) and functional defects (dysfibrinogenemia and hypodysfibrinogenemia) (OMIM 202400). Afibrinogenemia is rare and causes a severe bleeding diathesis. Signs of dysfibrinogenemias are variable, ranging from severe hemorrhage to thrombosis. Hereditary fibrinogen defects have been identified in several breeds of dogs, and in cats, goats, and sheep (Table 90.1).[12,33] Reported manifestations include severe, spontaneous hemorrhage and hemarthrosis. Milder forms are characterized primarily by post-traumatic and postsurgical hemorrhage.

Fibrinogen deficiency affects formation of a fibrin clot endpoint in all clotting time tests (Table 90.1). The thrombin clotting time (TCT) is the most sensitive of these screening tests to detect mild fibrinogen deficiencies. A combination of functional (clottable) fibrinogen assays and quantitative methods, such as ELISA, is required for definitive diagnosis of hypofibrinogenemia/dysfibrinogenemia and afibrinogenemia (see Chapter 88).

Hereditary fibrinogen defects are much less common than acquired hypofibrinogemia, which often develops secondary to hepatic synthetic failure or consumption in a disseminated intravascular coagulation (DIC) process (see Chapters 89 and 92). Unlike acquired disorders, hereditary fibrinogen deficiencies are rarely associated with concomitant thrombocytopenia, or deficiencies of other coagulation factors. The absence of clinical and laboratory signs of hepatic failure or DIC further aids in differentiating hereditary from acquired fibrinogen defects.

Cryoprecipitate is the treatment of choice for fibrinogen defects, as it supplies high-concentration fibrinogen in low-volume transfusion. Fresh frozen plasma is an alternate component (see Chapter 100). Transfusion is indicated for patients with active hemorrhage, or for preoperative prophylaxis. The human dosage recommendation is transfusion to a target of 50–100 mg/dL fibrinogen.[28] Fibrinogen's half-life of 2–4 days is relatively long compared with most factors; thus, fewer transfusions at longer intervals are often sufficient to support hemostasis.

Fibrinogen defects in people are mutationally heterogeneous and display autosomal dominant and recessive inheritance patterns. Afibrinogenemia is usually a recessive trait caused by mutations in the fibrinogen A-α chain. Hypofibrinogenemias/dysfibrinogenemias have been described in patients with mutations in all three fibrinogen genes. With pending pedigree studies and molecular analyses, it is likely that animals will display the same variety of disease gene, mutation location, and mutation type found in human patients.

Prothrombin Deficiency

Hereditary FII (prothrombin) deficiency is uncommon, with fewer than 100 cases reported in human beings and isolated cases in a few breeds of dog (OMIM 176930).[12] Prothrombin is the zymogen of thrombin, the serine protease factor that transforms fibrinogen to fibrin. Thrombin also plays a central role in platelet activation, inflammation, and wound healing. Knockout mice lacking the prothrombin gene are nonviable and all naturally occurring human (and animal) cases have some residual thrombin activity.

Clinical signs of hemarthrosis and post-traumatic hemorrhage are generally seen in patients with residual thrombin activities below 10% of normal. Prothrombin deficiency causes prolongation of the aPTT and prothrombin time (PT) (and proteins induced by vitamin K

absence/antagonism [PIVKA]) screening tests, but does not affect the TCT or fibrinogen assay (Table 90.1). Differential diagnoses for this pattern of screening test abnormalities include deficiency of a single common pathway factor (i.e., FII, FV, FX) and combined deficiency of all vitamin-K-dependent factors (FII, FVII, FIX, FX). The finding of specific deficiency of FII coagulant activity (FII:C), with normal activity of the other factors, provides definitive diagnosis of prothrombin deficiency (see Chapter 88).

Fresh frozen plasma and plasma cryosupernatant supply FII. The half-life of FII is approximately 3 days, and transfusion to attain target FII:C of 20–40% is the recommended human guideline.[28]

The expression of FII deficiency in people is autosomal recessive; affected patients are homozygous with residual FII:C ranging from 2% to 20%. Heterozygotes have FII:C of approximately 50% and are clinically asymptomatic. Although new mutations could arise, FII deficiency is uncommon and rarely recognized in any breed at this time.

Factor VII Deficiency

Trace amounts of activated FVII (FVIIa) bind to membrane-bound tissue factor at sites of vascular injury. This FVIIa/tissue factor complex plays a central role in the initiation phase of coagulation. Hereditary FVII deficiency is often associated with a clinically mild or inapparent bleeding diathesis, suggesting that sufficient residual FVII remains for active complex assembly. The trait has been identified in people and several different breeds of dogs (OMIM 227500).[8,18]

Canine FVII deficiency is best characterized in Beagles.[8] Factor-VII-deficient Beagles rarely experience abnormal bleeding, even after surgery or trauma. By contrast, FVII deficiency in Malamutes is typically associated with more severe signs including hematoma formation, prolonged post-traumatic bleeding, and fatal hemorrhage.[2] Clinical severity may vary among individuals within a single breed. The index case of FVII deficiency in Alaskan Klee Kai experienced prolonged post-traumatic hemorrhage, whereas other related FVII-deficient dogs had undergone major surgery with no complications.[18]

Factor VII deficiency causes an isolated prolongation of the PT (and PIVKA) screening tests, with normal aPTT, TCT, and fibrinogen (Table 90.1). Hereditary FVII deficiency is differentiated from vitamin-K-dependent coagulopathies as a single factor, rather than a combined multifactor defect. In most canine and human kindreds, FVII-deficient patients have some residual FVII activity with FVII coagulant activity (FVII:C) generally ranging from 1% to 10% of normal. More detailed laboratory characterization of residual FVII includes measurement of FVII antigen and levels of circulating FVIIa (see Chapter 88).

Most FVII-deficient patients are transfusion-independent; however, those with severe forms can be transfused with fresh frozen plasma or cryosupernatant to supply FVII. Factor VII has a short plasma half-life of approximately 3–4 hours; therefore, transfusion intervals of 8–12 hours may be needed to sustain hemostasis after initial stabilization (see Chapter 100).

Factor VII deficiency is a recessive trait. A missense mutation in exon 5 of *F7* was first identified in FVII-deficient Beagles, and subsequently found to cause FVII deficiency in Alaskan Klee Kai and Deerhounds.[8,18] Heterozygous carriers of the mutation generally have FVII:C values intermediate between affected and clear dogs. Direct mutation detection is preferable for carrier detection because factor activities overlap between carrier and clear individuals. Mutations causative for FVII deficiency in other breeds or species have not yet been identified.

Factor X Deficiency

Factor X and its cofactor, FV, play a central role in generating thrombin. The factors assemble on cell membrane surfaces to form the prothrombinase complex, which rapidly converts prothrombin to the active enzyme, thrombin (see Chapter 87).

An absolute lack of FX is an apparently lethal trait; however, partial FX deficiency has been identified in human beings, dogs, and a domestic shorthair (DSH) cat (Table 90.1; OMIM 227600).[2,10,15] The defect is a relatively severe bleeding diathesis in these species, with signs of spontaneous epistaxis, hematoma formation, and severe hemorrhage post-trauma or surgery.

Factor X deficiency causes prolongation of the aPTT, PT, PIVKA, and Russell's viper venom time (RVVT) screening tests; however, TCT and fibrinogen are normal (see Chapter 88). Clotting time in the RVVT is initiated by direct activation of FX, independent of the intrinsic or extrinsic pathways. The differentials for this pattern of abnormalities include vitamin-K-dependent coagulopathy, and isolated deficiencies of FX, FV, or FII. Definitive diagnosis is based on the specific finding of low FX coagulant activity (FX:C). Acquired FX deficiency has been reported in people with systemic amyloidosis, attributed to adsorption of FX onto amyloid fibrils. This phenomenon has not been documented in animals.

Plasma products for replacement of Factor X include fresh frozen plasma and cryosupernatant (see Chapter 100). After initial control of active hemorrhage, subsequent transfusions can be given at relatively long (q12–24 hours) interval due to the long half-life of FX of 40 hours.

Missense and nonsense mutations are the most common mutation types causing human FX deficiency; however, specific FX mutations have not yet been discovered in animals. The trait demonstrates a recessive inheritance pattern in people and animals. Familial testing in dogs and cats revealed severe deficiency (FX:C < 10%) in clinically affected patients and moderate factor deficiencies (20–50% FX:C) in obligate carrier parents.

Factor XI Deficiency

Thrombin activates FXI to form FXIa in the early initiation phase of coagulation. This reaction takes place most efficiently on the surface of activated platelets,

where FXIa then generates FIXa and promotes assembly of the tenase coagulation complex (see Chapter 87).

Factor XI deficiency has been identified in people, dogs, cattle, and cats (Table 90.1; OMIM 264900).[12,20,23,31] The defect is clinically variable in all species, but typically causes a mild bleeding tendency. Spontaneous hemorrhage is rare and affected patients are often diagnosed because of prolonged hemorrhage or abnormal rebleeding after surgery or trauma. Clinical severity does not correlate as closely with residual factor activity as most other factor deficiencies; however, clinical expression of a bleeding tendency is more likely to occur in patients with FXI activities (FXI:C) below 30% of normal.

Factor XI deficiency causes specific prolongation of the aPTT, with normal PT, TCT, and fibrinogen (Table 90.1). Hemophilia A and B cause the same screening test abnormalities; however, specific coagulation factor analyses define isolated FXI deficiency (see Chapter 88).

Transfusion of fresh frozen plasma and cryosupernatant will supply FXI (see Chapter 100). Transfusion is usually required only for surgical or post-traumatic hemostasis. After an initial preoperative or loading transfusion, FXI half-life of approximately 50 hours allows repeat transfusions to be given at 12–24-hour intervals. Although their use has not been reported in animals, antifibrinolytic agents and topical fibrin sealants have been used successfully in human FXI-deficient patients (see Chapter 94).

An exonic short interspersed nuclear element (SINE) insertion has been identified in FXI-deficient Kerry Blue Terriers and insertions in exons 3 and 9 have been described in FXI-deficient Holstein and Japanese cattle, respectively.[23,30] Homozygous cattle have marked FXI deficiency (FXI:C < 5%), whereas affected Kerry Blue Terriers and DSH cats had relatively moderate reduction with FXI:C of approximately 2–15%.

Factor XII and Contact Factor Deficiencies

The contact group of coagulation factors includes FXII, prekallikrein (PK; Fletcher factor), and high-molecular-weight kininogen (HMWK). Contact factors interact in vitro to initiate coagulation on artificial surfaces through activation of FXI. However, these factors are not required for fibrin clot formation in vivo and deficiency of these factors does not cause a bleeding diathesis (see Chapter 87). The physiologic activities of the contact group factors are now believed to include regulation of blood pressure, cellular proliferation, angiogenesis, and inflammation. Early reports of FXII deficiency in people suggested an increased risk of systemic thrombosis; however, subsequent studies have not confirmed this phenomenon.

In addition to human beings, deficiencies of the contact pathway factors have been identified in dogs, cats, and horses (Table 86.1; OMIM 234000,229000, 228960).[4,12,14,19,24,32] Factor XII deficiency, in particular, is a common factor deficiency found in DSH and purebred cats.[4,24]

Contact pathway deficiencies are characterized by markedly prolonged aPTT (often >120 seconds), with normal PT, TCT, and fibrinogen. A prolonged aPTT that shortens by increasing activation time is a common characteristic of PK-deficient plasmas. Specific deficiencies of the contact group factors can be diagnosed based on functional assays of FXII, PK, and HMWK (see Chapter 88). Due to its high prevalence, FXII deficiency should be considered a likely cause of specific prolongation of aPTT in cats. Deficiency of FXII and other contact pathway factors causes persistent prolongation of aPTT, but is not an indication for transfusion.

FXII and contact factor deficiencies are recessive traits. Cats homozygous for FXII deficiency have residual FXII:C values of approximately 2–10%, whereas heterozygous carriers generally have moderate reduction in activity with residual FXII:C from 20% to 40%.[17] The high prevalence of FXII deficiency in cats has recently been attributed to the presence of two distinct mutations segregating in domestic and purebred cats.[24]

Combined Deficiencies of the Vitamin-K-Dependent Factors

Factors II, VII, IX, and X are serine protease enzymes that require post-translational γ-carboxylation in order to form active complexes with their coenzymes and substrates. Defects in the hepatic vitamin K reductase or carboxylase enzymes impair vitamin K recycling and cause coagulopathy due to a persistent vitamin K deficiency state.

Familial combined deficiency of FII, VII, IX, and X is a rare recessive trait in people and has been identified in Devon Rex cats, sheep, and in Labrador Retrievers (Table 90.1; OMIM 277450 and 607473).[17,25,29] Clinical signs range from moderate to severe coagulopathy, with reports of spontaneous hematoma formation, prolonged hemorrhage after surgery, and some fatal hemorrhage.

Combined deficiency of the vitamin-K-dependent factors causes prolongation of the aPTT and PT (and PIVKA) screening tests, but does not affect TCT and fibrinogen. Specific coagulation factor assays confirm deficiencies of FII, VII, IX, X, with normal activities of the non-vitamin-K-dependent factors. Since acquired vitamin K deficiency is common (see Chapter 89), initial diagnostic workup should rule out exposure to vitamin K antagonists, liver disease, or malabsorptive disorders. After exclusion of acquired disorders, specific analyses of hepatic microsomal vitamin K epoxide reductase (VKOR) and carboxylase enzyme activities further define the biochemical basis of a hereditary trait.

Patients with severe hemorrhage at presentation may require transfusion for rapid replacement of active factors (see Chapter 100). After an initial lag time of 1–2 days, case reports describe normalization of aPTT and PT and control of clinical signs of hemorrhage in response to oral vitamin K_1 at approximately 2.5 mg/kg per day. Long-term maintenance of hemostasis has been reported at reduced daily dosages (e.g., 1 mg/kg/day) given at 3-day intervals.

Familial studies to date in all species reveal a recessive inheritance and expression pattern; however,

causative mutations in disease genes have not yet been identified.

Uncharacterized Factor Deficiencies

Hereditary deficiencies of FV, FXIII, and antiplasmin have been reported as rare hemorrhagic defects in people, with an estimated case incidence of <1 per million (OMIM 612309; 134570; 262850). The traits are autosomal recessive and cause clinically severe bleeding diatheses. Factor V deficiency causes prolongation of the aPTT and PT screening tests; however, identification of FXIII and antiplasmin deficiencies requires specific functional analyses of the corresponding proteins. While not yet recognized in animals, these defects should be included in the differential for severe hereditary coagulopathy after ruling out more common disorders.

A syndrome of prolonged and potentially life-threatening hemorrhage within 1–4 days of surgery has been described in racing Greyhounds. Anecdotal reports describe similar signs in other Sighthound breeds. To date, coagulation assays and screening tests of primary hemostasis have not revealed the underlying cause. Prophylactic treatment with antifibrinolytic agents was shown to significantly decrease prevalence of postoperative bleeding in a randomized treatment trial of Greyhounds undergoing elective surgery, suggesting that more in-depth evaluation of fibrinolytic pathways is warranted.[22] The relationship between expression of a bleeding diathesis and the vasculitic syndrome also found in Greyhounds also remains unknown (see Chapter 93).

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ON-LINE RESOURCES

1. **OMIM** Online Mendelian Inheritance in Man. Catalogue of human genetic disorders, https://omim.org/
2. **OMIA** Online Mendelian Inheritance in Animals. Catalogue of animal genetic disorders, https://omia.org/home/
3. **PennGen Database**. Searchable database of genetic testing laboratories for hereditary disease in dogs and cats, https://www.vet.upenn.edu/research/academic-departments/clinical-sciences-advanced-medicine/research-labs-centers/penngen/tests-worldwide
4. **Comparative Coagulation Laboratory**. Animal hemostasis testing and coagulation factor assays, https://www.vet.cornell.edu/animal-health-diagnostic-center/laboratories/comparative-coagulation

CHAPTER 91

Thrombotic Disorders

ERICA BEHLING-KELLY and ROBERT GOGGS

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  Mesenteric Vein Thrombosis
  Cerebrovascular Accidents
  Coronary Thrombosis

Acronyms and Abbreviations

APC, activated protein C; aPTT, activated partial thromboplastin time; AT, antithrombin; ATE, aortic thromboembolism; CAT, calibrated automated thrombogram; CM, cardiomyopathy; COX, cyclooxygenase; CP, cancer procoagulant; CT, computerized tomography; CTPA, computerized tomography pulmonary angiography; cTn, cardiac troponin; DIC, disseminated intravascular coagulation; DVT, deep vein thrombosis; EKG, electrocardiograph; EPCR, endothelial protein C receptor; ESL, endothelial surface layer; ET-1, endothelin; HAC, hyperadrenocorticism; HCM, hypertrophic cardiomyopathy; IL, interleukin; IMHA, immune-mediated hemolytic anemia; MODS, multiple organ dysfunction syndrome; MPs; microparticles NETs, neutrophil extracellular traps; NO, nitric oxide; NT-proBNP, N-terminal pro-B-type natriuretic peptide; PA, pulmonary artery; PAI-1, plasminogen activator inhibitor-1; PGI_2, prostacyclin; PLE, protein-losing enteropathy; PLN, protein-losing nephropathy; PS, phosphatidylserine; PT, prothrombin time; PTE, pulmonary thromboembolism; ROTEM, rotational thromboelastometry; SIRS, systemic inflammatory response syndrome; TAT, thrombin–antithrombin complex; TE, thromboembolism; TEG, thromboelastography; TF, tissue factor; TFPI, tissue factor pathway inhibitor; TM, thrombomodulin; TNF, tumor necrosis factor; tPA, tissue plasminogen activator; TXA_2, thromboxane A_2; VWF, von Willebrand factor.

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INTRODUCTION

Thrombosis is the partial or complete obstruction of a blood vessel by a thrombus, or blood clot. Thromboembolism (TE) refers collectively to obstruction due to local thrombus formation (primary thrombosis) or translocation from a distant site (embolism). Thromboembolism contributes to morbidity and mortality through blood flow obstruction, release of humoral factors from thrombi causing end-organ damage, and ultimately multiple organ dysfunction syndrome (MODS). Signs of TE range from clinically inapparent events to organ failure and death, depending on the site and extent of vascular occlusion, the degree of collateral circulation, and the compensatory reserves of affected organs. Early recognition maximizes therapeutic options, since interventions such as thrombolysis are time-sensitive. This chapter and the treatment guidelines in Chapter 94 include recommendations published under the auspices of the American College of Veterinary Emergency and Critical Care.[66]

PATHOPHYSIOLOGY

Models of cell-based hemostasis (see Chapter 87) describe a complex interplay among cellular and plasma factors that participate in physiologic and pathologic thrombus formation. Platelets, endothelium, and coagulation proteases combined with newly recognized elements, such as neutrophil extracellular trap (NET) formation[73] and hypofibrinolysis, all contribute to TE.[185]

The three risk factors of Virchow's triad: endothelial damage and dysfunction, blood flow abnormalities, and hypercoagulability still underpin our understanding of pathologic thrombosis and identify at-risk patients.[33] A single factor may predominate (Table 91.1); however, multiple abnormalities often act synergistically to imbalance hemostasis and favor thrombus formation.

TABLE 91.1 Virchow's triad categorization.

Criterion	Example diseases or disorders
Vascular injury	Trauma
	Catheterization
	Inflammation
	Neoplastic invasion
	Parasitic damage
	Plaque deposition, atherosclerosis, amyloidosis
Stasis or turbulent flow	Hypoperfusion, hypovolemia, cardiac insufficiency, distributive
	Blood vessel compression
	Immobility
	Hyperviscosity, dehydration, polycythemia, leukemia, hyperglobulinemia, hyperfibrinogenemia
	Decreased RBC deformability, spherocytosis
Hypercoagulability	Platelet hyperaggregability, increased activation, decreased negative modulation
	Excessive activation of coagulation
	Decreased natural anticoagulant activity, AT deficiency, protein C deficiency, TFPI deficiency
	Hypofibrinolysis, decreased plasminogen activators, increased plasminogen activator inhibitors

Endothelial Dysfunction

Endothelial damage and dysfunction may promote thrombosis or reflect the consequences of TE. Healthy endothelium supports blood flow through maintenance of an anticoagulant surface, including release of vasodilators and platelet inhibitors, and by enhancing fibrinolysis.[93] The anticoagulant actions of endothelial cells include cell membrane expression of endothelial protein C receptor (EPCR) and thrombomodulin (TM) that localize anticoagulant reactions, and release of tissue factor pathway inhibitor (TFPI), a potent plasma anticoagulant.[93] Endothelial cells secrete vasoactive mediators including endothelin-1 (ET-1), nitric oxide (NO), and prostacyclin (PGI_2).[218,135] Endothelial cells also express CD39, a nucleoside triphosphate diphosphohydrolase, that hydrolyzes the nucleosides ADP and ATP, thereby reducing local concentrations of platelet and endothelial cell activators.[98] The endothelium modulates fibrinolysis though release of tissue plasminogen activator (tPA) and its specific serpin family inhibitor, plasminogen activator inhibitor-1 (PAI-1).

The endothelial surface is lined by the glycocalyx, a carbohydrate-rich matrix of proteoglycans and glycoproteins that protects the cell surface, repels negatively charged blood cells, and generates a boundary layer through adsorption of plasma proteins and water. This endothelial surface layer (ESL) is the mechanical transducer of the physical shear stress induced by blood flow, and is the site of action of the anticoagulant proteins antithrombin (AT), TM, and proteins C and S.[47] Damage to the glycocalyx and disruption of the ESL contribute to the pathology of sepsis, trauma, ischemia-reperfusion injury, and diabetes mellitus, and the prothrombotic nature of these conditions.[137,161,180,26] In disease processes such as sepsis and severe pancreatitis, high concentrations of proinflammatory cytokines such as IL-1 and IL-6 lead to expression of tissue factor (TF) on the surfaces of activated monocytes and endothelial cells. This intravascular TF expression promotes thrombin generation.[27] In addition, reduced venous wall shear stress stimulates endothelial cell release of P-selectin and von Willebrand factor (VWF), further promoting thrombus formation.[219]

Abnormal Blood Flow

Blood flow abnormalities include stasis, turbulent flow created by cardiac valve degeneration, congenital cardiac anomalies, indwelling vascular devices, and the flow microenvironments downstream of venous valves.[19] Disease processes characterized by blood flow abnormalities include shock, cardiac disease, vascular anomalies, hyperviscosity, and cancer.

Reduced venous blood flow and stasis reduce wall shear stress. These conditions modulate endothelial cell phenotype through alterations in shear-stress-responsive transcription factors resulting in an imbalance of prothrombotic and antithrombotic protein expressions.[216] Recirculation zones in dilated cardiac chambers and downstream from venous valves are often characterized by low shear conditions and long blood-dwell times that increase the likelihood of interactions between procoagulant proteins and activated platelets.[197]

High shear stress associated with vascular stenoses also increases endothelial cell, platelet, and procoagulant protein interactions,[77] and induces flow-dependent platelet aggregation.[142] Shear-dependent expression of platelet P-selectin and formation of monocyte–platelet aggregates suggest that that these cells undergo activation when traversing stenotic regions.[220] Shear rate also affects fibrin monomer formation and polymerization by affecting the rates of delivery of procoagulant proteins and removal of coagulation end products.[139] High shear depletes local fibrin concentrations, thereby limiting thrombus growth, whereas low shear enables clot propagation. The structure of the fibrin meshwork affects clot stability and susceptibility to fibrinolysis.[205]

Endothelial cells are also subjected to blood pressure, the force per unit area acting perpendicular to the vessel wall. High pressure affects endothelial function through mechanical damage and increase in circulating microparticle (MP) levels and TF exposure.[158] This mechanism is believed to contribute to the thrombotic risk of systemic hypertension, particularly cerebrovascular accidents.[62]

Hypercoagulability

Hypercoagulability describes a state of altered hemostatic balance favoring thrombus formation that may result from increases in coagulation factor concentrations, inappropriate platelet activation, reductions in the availability of endogenous anticoagulants, or a lack of fibrinolysis (Table 91.1).[8] Heritable thrombophilias, thus far only described in people, represent a risk factor, but rarely are the sole cause of thrombosis.[36]

Excessive, unbalanced activation of coagulation often results from intravascular TF expression or massive exposure of extravascular TF to the vascular space. Heterogeneity across vascular beds in terms of endothelial cell prothrombotic and antithrombotic properties produces a variable tissue distribution of TE among patients, often related to their underlying disease.[168] Intravascular TF expression can be induced by lipopolysaccharide and inflammatory cytokines, particularly IL-1, IL-6, and tumor necrosis factor (TNF)-α.[117] This TF expression on monocytes, endothelial cells, and leukocyte and platelet-derived microparticles may initiate systemic thrombin generation. Some neoplastic cells express TF constitutively,[214] while others produce a protease, cancer procoagulant (CP), that directly activates factor X.[20]

Thrombosis may represent a protective response against pathogen invasion, a process termed immunothrombosis.[81] Dysregulated or excessive immunothrombosis, however, promotes TE in diseases characterized by intense inflammation such as severe trauma, sepsis, heat stroke, and hemolytic anemia. The innate immune system, including neutrophils, monocytes, and dendritic cells, can initiate and accelerate fibrin formation and activate platelets. The process of NETosis refers to neutrophils' release of chromatin and granule protein meshworks called "neutrophil extracellular traps."[155,13] Excessive NETosis appears detrimental to the host.[32] Histones within NETs may act as damage-associated molecular patterns that initiate apoptosis, cytotoxicity, and systemic inflammation via toll-like receptor signaling pathways.[24] Prothrombotic properties of NETs include contact pathway activation through intravascular expression of a polyanionic surface,[119] DNA–histone integration into fibrin clots, thus impairing clot dissolution,[24] and cell-free DNA competition with plasmin on fibrin, resulting in decreased clot lysis.[49] Cell-free DNA also accelerates the inactivation of tPA by PAI-1.[119]

Factors that promote platelet activation include stimulation by inflammatory cytokines and tumor cells, cytokine or cell-mediated release of platelet agonists, and hypoalbuminemia-mediated upregulation of thromboxane A_2 (TXA_2).[42,130] Agonist-triggered release of platelet polyphosphate directly activates factor V and may initiate contact-pathway-mediated thrombosis.[181,221] Finally, platelet inhibition is attenuated by endothelial damage, which decreases availability of prostacyclin, NO, and CD39.

Hypercoagulability may result from decreased synthesis, increased loss, or excess consumption of anticoagulant proteins. Increased glomerular permeability in protein-losing nephropathy (PLN) permits selective loss of low-molecular-weight plasma proteins including AT.[178] Pathologic AT consumption occurs in conditions of increased thrombin generation, such as disseminated intravascular coagulation (DIC) or massive TE. Drugs such as l-asparaginase may also lower plasma AT.[100] Acquired protein C deficiencies develop in association with sepsis, malignancy, hepatic or cardiac failure, pancreatitis, DIC, colic, laminitis, and postoperatively.[50,4,107,193] Low protein C activity in the context of portosystemic shunting in dogs is not associated with an increase in TE.[195] By contrast, recurrent TE developed in a foal with primary protein C deficiency.[46] Acquired TFPI deficiency has been related to hypercholesterolemia, and may represent a risk factor for TE[93]

Hypofibrinolysis, often mediated by upregulation of PAI-1, may result in relative hypercoagulability.[883] PAI-1 is an acute phase protein that complexed with subendothelial fibronectin has inhibitory action on activated protein C (APC) generation. High PAI-1 has been associated with increased TE risk in people with atherosclerosis, sepsis, diabetes mellitus, and post trauma and surgery.[83] Although fibrinolytic defects are relatively ill-defined in animals, hypoplasminogenemia has been reported in traumatized dogs and in horses with strangulating obstructions.[131,157] Increased PAI-1 has been described in hyperlipidemic dogs,[217] and increased α2-antiplasmin activities have been reported in septic foals.[4]

DISEASES ASSOCIATED WITH THROMBOSIS

While causal relationships are difficult to establish, numerous retrospective studies of veterinary patients with TE have identified comorbidities hypothesized to represent hypercoagulable states. Moreover, the high frequency of thrombotic complications in patients with specific disease processes supports disease-induced TE. The common diseases associated with thrombosis in dogs, cats, and horses are summarized in Tables 91.2, 91.3, and 91.4, respectively.

Immune-Mediated Hemolytic Anemia (IMHA)

Thromboembolism is a major cause of mortality in canine IMHA, with incidence at necropsy ranging from 30% to 80%.[17521] The pulmonary system is the most common, but not exclusive site of TE.[67,21] Although inflammatory mediators likely activate coagulation,[117] the pathogenesis of IMHA-associated TE is not completely understood. Potential prothrombotic mechanisms include increased TF expression,[154] platelet activation,[206] generation and release of procoagulant microparticles,[95] and the formation of NETs.[87,115] Common laboratory abnormalities in dogs with IMHA include hyperfibrinogenemia, high D-dimer, and low AT.[21] Multiple groups have documented hypercoagulability in whole blood thromboelastographic parameters in dogs with IMHA.[53,68,179] Two studies found that a lack of hypercoagulability (relative hypocoagulability) was a negative prognostic indicator.[68,179]

Protein-Losing Nephropathy and Enteropathy

An association between canine PLN and venous and/or arterial TE has been described in many studies, with incidence rates from 6% to 42%.[28,41,164,72] The pulmonary vasculature is most commonly affected,[2811289164] followed by aortic thromboembolism (ATE).[28,212,211] Urinary AT loss plays a major role in mediating the hypercoagulability of PLN, and markers of thrombin activation and platelet aggregation increase with even modest urinary protein loss.[72] Concomitant hyperfibrinogenemia, hypercholesterolemia, high FVIII, and increased PAI-1 are hypothesized to contribute to TE risk. Three prospective studies of dogs with PLN identified features of hypercoagulability based on thromboelastography (TEG) and AT.[41,116,209] Hypoalbuminemia (i.e., serum albumin concentration below 2 g/dL) is associated with enhanced canine platelet aggregability.[178,72]

Protein-losing enteropathy (PLE) is typically due to chronic intestinal inflammation, with malabsorption and protein loss into the gut lumen. Analogous to inflammatory glomerulonephritis and PLN,[116] intestinal inflammation with protein loss promotes a hypercoagulable state.[71] Clinically, PLE has been associated with portal vein[163] and pulmonary TE; however, splenic vein[85] and aortic thrombosis are also described.[84,25]

Pancreatitis

Canine studies of pancreatitis describe hypercoagulability[79,151], and overt TE involving the splenic vein[114,76,11476] portal vein,[163,210,109163210109] and aorta.[211,9921199] Pancreatitis is

TABLE 91.2 Canine thrombotic syndromes.

	Sites of thromboembolism										
Condition	**Pulmonary**	**Portal**	**Hepatic venous**	**Vena caval**	**Splenic**	**Renal**	**Mesenteric**	**Aortic/ iliac**	**Myocardial**	**Cerebrovascular**	**Deep dermal**
Protein-losing nephropathy	X	X	X	X	X	X	X	X	X	X	
Neoplasia	X	X		X	X			X	X	X	
IMHA	X	X	X	X	X	X			X		
Necrotizing pancreatitis	X	X			X			X	X		
Hyperadrenocorticism	X				X			X			
Corticosteroid therapy	X	X		X	X			X		?	
Infective endocarditis	X		X		X	X		X	X	X	X
Dirofilariasis	X				X			X		X	X
Cardiac diseases	X		X	X	X			X			
Atherosclerosis								X		X	
Sepsis	X	X		X	X				X	X	
Diabetes mellitus	X							X			
Protein-losing enteropathy								X			
Trauma									X	X	
Surgical procedures	X				X						
Parvoviral enteritis					X						
Spirocerca lupi								X			

Cardiac diseases: valvular disorders, cardiomyopathy, myocardial failure, atrial and vena cava anatomic anomalies.

TABLE 91.3 Feline thrombotic syndromes.

	Sites of thromboembolism										
Condition	Pulmonary	Portal	Hepatic venous	Vena caval	Splenic	Renal	Mesenteric	Aortic/ iliac	Myocardial	Cerebrovascular	Deep dermal
Neoplasia	X	X						X			
Cardiomyopathy	X					X	X	X		X	
Necrotizing pancreatitis	X	X									
IMHA	X										
Corticosteroid therapy	X										
Sepsis/SIRS	X										
Protein-losing nephropathy	X										
Protein-losing enteropathy	X										
Hepatic lipidosis	X										
Feline infectious peritonitis	X										
Bacterial pneumonia	X										
Encephalitis	X										
Heartworm disease	X										
Congenital portosystemic shunt		X									

TABLE 91.4 Equine thrombotic syndromes.

	Sites of thromboembolism											
Condition	Pulmonary	Jugular vein catheter site	Portal	Hepatic	Vena caval	Splenic	Renal	Mesenteric	Aortic/ iliac	Myocardial	Cerebrovascular	Peripheral vasculature
Neoplasia			X									
Cardiac				X			X			X	X	
Immune-mediated hemolytic anemia	X											
Sepsis/systemic inflammatory response syndrome (SIRS)	X	X					X		X			X
Severe gastrointestinal disorders (endotoxemia)	X	X		X			X	X			X	X
Bacterial pneumonia	X	X										
Presumed verminous arteritis								X	X	X		

also associated with venous TE in cats.[173,165] The high frequency of comorbidities, however, complicates risk attributable to pancreatitis alone.[114] Experimental studies in animals demonstrate platelet hyperaggregability, hyperfibrinogenemia, increased PAI-1 activity, and decreased tPA activity.[52] Pathogenic mechanisms of hypercoagulability include release of TF and inflammatory cytokines from damaged pancreatic tissue and inflammation-mediated hypofibrinolysis.[189,94,134] Pancreatitis also induces phospholipase A2, thereby increasing platelet-activating factor and cytokine production. High-plasma free fatty acids and hypoalbuminemia inhibit synthesis of PGI_2 and promote TXA_2 release, respectively, thus enhancing platelet reactivity. Septic peritonitis or acute pancreatitis may reduce hepatic, pulmonary, and splenic TM expression,[96] and reduce AT.[85]

Hypercortisolism

Hyperadrenocorticism (HAC) and corticosteroid therapy are considered risk factors for TE in dogs. Many retrospective case series have described thrombotic states or overt TE associated with glucocorticoid therapy in dogs.[150,112,89,163,114,211,76,150,163,210,114,108] Glucocorticoid therapy, typically associated with portal vein TE, occurs in dogs with cancer, immune-mediated disease, and pancreatitis. Various retrospective studies and case series suggest that canine HAC is associated with TE, but most lack a control group.[213,112,89,163,114,76,162,11181,94] Thus, the relative contribution of comorbidities remains ill-defined. Oral prednisone administration to healthy dogs has been reported to decrease AT, enhance platelet aggregation, and increase coagulation factors, fibrinogen, clot strength, and thrombin generation.[167,166,147,57] Proposed mechanisms for steroid-mediated hypercoagulability include hypercholesterolemia, hypofibrinolysis (due to increased PAI-1 and α2-antiplasmin), hyperfibrinogenemia, and procoagulant excess.[150] In contrast to dogs, glucocorticoid therapy is rarely associated with feline venous thrombosis or TE.[145,165,173]

Neoplasia

Neoplasia is a common underlying disease in retrospective studies of canine TE,[112,163,114,211,109] and TE is commonly documented in populations of dogs with cancer.[126,207] Multiple mechanisms promote TE, including release of procoagulant substances and inflammatory cytokines by malignant cells, and direct interaction among tumor cells, endothelial cells, platelets, and monocytes.[20,42] Human and animal tumor tissues demonstrate constitutive TF expression and release of CP. Acquired APC resistance and anticoagulant deficiencies are prothrombotic forces in some neoplastic disorders. Contributory factors include vascular invasion by tumor cells, immobility, venous catheterization, corticosteroid therapy, surgery, and chemotherapy.[42] Enhanced or impaired fibrinolysis may develop secondary to neoplasia, with resultant signs of hemorrhage or thrombosis. The balance between plasminogen activators and inhibitors may also play a role in tumor metastasis. Tumor cell production of TNF and IL-1β induces endothelial cell TF and PAI-1 expression, and downregulates TM expression. Tumor cells also release vascular endothelial growth factor, which induces macrophage expression of TF, increases microvascular permeability, and promotes tumor angiogenesis. Tumor cells, cell extracts, and membrane fragments promote platelet aggregation in vitro, and tumor cell injections cause in vivo platelet aggregation in experimental animals.[20,42] Viscoelastic testing demonstrates hypercoagulability in dogs with neoplasia.[2,102,202] The underlying mechanisms may include overexpression of circulating TF,[214] thrombocytosis,[202] platelet hyper-reactivity,[129] hyperfibrinogenemia,[202] and reduced AT[104] or endogenous fibrinolytic potential.[185]

Sepsis

Sepsis is a common trigger of microvascular thrombosis and DIC in animals, including overt venous and arterial TE.[150,157,112,173,163,211,76,109] The pathogenesis of sepsis-related TE is complex, mediated by inflammatory cytokines, hemostatic proteins, leukocytes, platelets, and endothelial cells and influenced by the underlying infection.[117,124] Features of hypercoagulability identified in dogs and people with sepsis include reduced protein C and AT,[104,106] hyperfibrinogenemia, increased thrombin-activatable fibrinolysis inhibitor,[88] TM downregulation,[96] and platelet hyper-reactivity.[118]

Cardiovascular Disease

Feline cardiac disease, in particular hypertrophic cardiomyopathy (HCM), is associated with left atrial thrombus formation and ATE.[182,169] The cumulative risks of ATE in cats with HCM at 1 year, 5 years, and 10 years were 3.5%, 9.5%, and 11.3%, compared to 0.0%, 0.4%, and 0.7% in healthy cats.[59] A positive association was found between renal infarction, HCM, and ATE.[80] Reported incidence rates of TE secondary to cardiomyopathy (CM) range from 12% to 50%.[182,169] In addition, feline cardiac disease accompanied by spontaneous echocontrast, left atrial (LA) dilation, reduced LA function, and low flow velocities may portend ATE and death.[182,174,153] However, feline cardiac disease does not appear to be a risk factor for venous TE. In addition to abnormal blood flow, cats with spontaneous echocontrast or atrial thrombi demonstrated laboratory criteria of hypercoagulability including high-plasma thrombin–antithrombin complexes (TAT) and D-dimers.[189] Endothelial disruption, platelet hyper-reactivity, and endothelial dysfunction secondary to metabolic imbalance may further promote TE.[182,127]

Plasma indices of hypercoagulability (i.e., hyperfibrinogenemia, high TAT, and low AT and APC) have been described in dogs with cardiac disease.[193] However, only a single study linked cardiac disease in dogs to overt TE,[108] a finding not confirmed by others.[212,213,76] By contrast, canine infective endocarditis and heartworm disease are frequently associated with TE.[112,89,190]

Although atherosclerosis is rare in animals, aortic and cerebrovascular TE have been reported in hypothyroid and diabetic dogs with atherosclerosis.[211,90,184] Mechanisms promoting TE include endothelial disruption by atherosclerotic plaques, hypercholesterolemia, and a prothrombotic lipoprotein profile.

Diabetes Mellitus

Thromboembolic disease is an uncommon complication of diabetes mellitus in dogs, and most cases have concomitant prothrombotic conditions.[211,162] Loss of insulin's modulating effects on platelet activation and decreased endothelial synthesis of PGI_2 and NO combine to potentiate platelet reactivity.[171] Hyperlipidemia can occur concurrently with diabetes mellitus, thus exacerbating TE risk.[184] In people, hyperglycemia correlates with increased plasma PAI-1, a relationship not identified in dogs.[217]

Hepatobiliary Disease

The hepatobiliary system plays crucial roles in normal hemostasis (see Chapters 87 and 89). The liver synthesizes almost all procoagulant and anticoagulant factors, and removes desialylated, aged platelets through the activity of the Ashwell–Morell receptor.[74] The biliary system is essential for vitamin K absorption required for effective carboxylation of factors II, VII, IX, and X. Liver dysfunction is associated with complex coagulopathies[60,159] and risk for TE.[146] Hemostatic balance in these patients is influenced by procoagulant deficiency, deficiencies of anticoagulant and profibrinolytic factors such as AT, proteins C and S, and plasminogen,[196] and variable increases in von Willebrand factor.[121] Canine hepatobiliary diseases have been associated with portal vein TE[163,210,165] and other sites.[210,211,76]

Endovascular Devices and Extracorporeal Circuits

Blood clot formation in the lumen of venous and arterial catheters is a daily occurrence in veterinary practice,[201] and often necessitates device removal. The thrombogenicity of different catheter materials varies.[31,30,6] The increased risk of thrombosis associated with long-term catheterization can be reduced by heparin or citrate instillation,[110,23] or managed by local tPA infusion.[111] Retrospective studies of thrombosis identify current or recent intravenous catheterization as a risk factor, among comorbidities.[150,112,89,145] TE solely due to endovascular devices such as catheters, vascular access ports, or cardiac pacing wires is rare.[299] Prosthetic cardiac valves (mechanical or biologic) are associated with a much greater likelihood of thrombosis and TE, necessitating long-term anticoagulation.[3,148]

Extracorporeal circuits used for cardiopulmonary bypass, intermittent hemodialysis, continuous renal replacement therapy, plasmapheresis/plasma exchange and extracorporeal CO_2 removal, or membrane oxygenation may predispose patients to TE. Exposure of blood to extracorporeal circuits initiates a well-recognized consumptive coagulopathy phenomenon,[7] but also carries the risk of clotting of the circuit and TE.[203]

Equine Thrombotic Disease

Vascular injury is a defined (or presumptive) cause of equine large- and small-vessel thrombosis (Table 91.4). Jugular vein catheter thrombosis and thrombophlebitis are relatively common in the horse, and reported risk factors include endotoxemia, hypoproteinemia, salmonellosis, and antidiarrheal and antiulcer therapy.[157] Parasitic endovascular migration and parasite-induced intestinal vasculopathy and TE cause acute and chronic colic, and signs of lameness in foals with aortic, iliac, and distal limb TE. Distal limb TE has also been reported secondary to sepsis and gastrointestinal inflammation.[143] Equine infectious agents cause small-vessel TE, including equine-viral-arteritis-induced panarteritis, and vasculitis due to disseminated aspergillosis (see Chapter 93). Severe gastrointestinal disease and resultant endotoxemia are often associated with renal microvascular TE. Microvascular thrombosis in the dermal lamina may play a role in the development of carbohydrate-induced laminitis. As in dogs and cats, pulmonary thromboembolism (PTE) is likely under-recognized in horses. In a small case series, endotoxemia-induced hypercoagulability was considered an underlying cause of equine PTE,[144] and reports describe toxin-induced hemolysis and pneumonia as primary disorders in horses with PTE.[14,122] Cardiac disease is an uncommon cause of TE in horses.

LABORATORY DIAGNOSIS OF THROMBOSIS

The complex pathogenesis of hypercoagulability and its associated disease states complicates TE diagnosis. Test strategies include assays measuring "global" or net hemostatic forces and scoring systems combining routine tests and clinical parameters (see Chapter 92). Establishing clinical utility of new and traditional diagnostic tests ultimately requires demonstrable improvement in patient outcomes, including minimizing hemorrhagic complications of antithrombotic therapy.

Coagulation Profile

The routine coagulogram (activated partial thromboplastin time [aPTT], prothrombin time [PT], and fibrinogen) screens for risk of hemorrhage due to coagulation factor deficiency (see Chapter 88). Although variable shortening or prolongation of aPTT and PT is often found in patients with TE, these changes are nonspecific and rarely aid in diagnosis or management.[183] Hyperfibrinogenemia, however, is an independent risk factor for TE in people,[199] and is a common finding in canine IMHA, feline CM, and sepsis.[4,157,175,189] The predictive value of fibrinogen determinations for animal TE has yet to be established.

Antithrombin

Antithrombin loss promotes hypercoagulability in conditions such as PLN and PLE.[85] While risk of TE has been attributed to AT values below 60%,[72] there is little empiric data in animals to define a threshold value.

D-Dimer

D-dimer is a distinct fibrin degradation product formed by plasmin's proteolysis of cross-linked fibrin (see Chapter 87). Latex-agglutination and immunoturbidimetric assays are the most common; however, human-specific antibodies in commercially available assays may demonstrate reduced reactivity with canine D-dimers.[133] Discordant results among assays were found in a canine DIC study.[188] Quantitative D-dimer assays are generally sensitive, but nonspecific and have proven diagnostic utility for ruling out PTE and predicting mortality in human thrombotic syndromes.[187,192] Comparable data are lacking for animals, with studies revealing low predictive value for canine PTE,[48] nonspecific increases in D-dimer in dogs with hemoabdomen,[141,56] and high D-dimer unrelated to overt TE in dogs with cancer.[2]

Thrombin–Antithrombin Complex

Free thrombin in plasma is neutralized by the formation of an irreversible 1:1 complex with AT. The plasma concentration of the TAT is an indirect measure of in vivo thrombin generation. High TAT is evidence of a hypercoagulable state and TAT has been used to characterize thrombotic syndromes in dogs, cats, and horses (see Chapter 92).

Thrombin Generation

Thrombin generation assays measure the in vitro capacity of plasma coagulant factors to generate thrombin over time. These assays use fluorogenic thrombin substrates to measure the kinetics of thrombin generation and provide numeric parameters and qualitative "thrombograms."[133] They are considered "global tests" that reflect the balance of coagulation activators and inhibitors. The calibrated automated thrombogram (CAT) refers to a thrombin generation assay platform and associated software that has recently been evaluated in healthy dogs,[177] and for heparin monitoring,[1] however, clinical utility for diagnosing TE has not been established.

Viscoelastic Testing

TEG (Figure 91.1), rotational thromboelastometry (ROTEM), and Sonoclot measure the viscoelastic properties of a nascent fibrin clot in whole blood.[70,92] These point-of-care tests generate qualitative tracings depicting clot formation and breakdown, and numeric values summarizing clot strength and stability. The assays are not interchangeable[128] and subject to activator-dependent variability.[37] Thromboelastography has been used in human patients to test for hypocoagulability and hypercoagulability, monitor anticoagulants, guide transfusion, and predict postoperative TE. Thromboelastographic features of hypercoagulability have been described in dogs with neoplasia, DIC, IMHA, parvoviral enteritis, PLN, PLE, renal failure, hyperadrenocorticism, and deracoxib therapy.[41,103,131,68,179,116]

The diagnostic utility of viscoelastic testing remains unproven and many dogs without TE have evidence of hypercoagulability.[67] Tests are influenced by hematocrit, fibrinogen, and platelet count.[125] Recent guidelines have been published to reduce the variability associated with viscoelastic testing and improve diagnostic utility.[55,37]

Examination of Platelets and Microparticles

Platelet assays include measurements of size, aggregability, and expression of activation markers; complex analyses are usually performed to diagnose bleeding disorders (see Chapter 79). While considered a gold standard measure of platelet function, aggregometry is not useful for clinical characterization of TE.

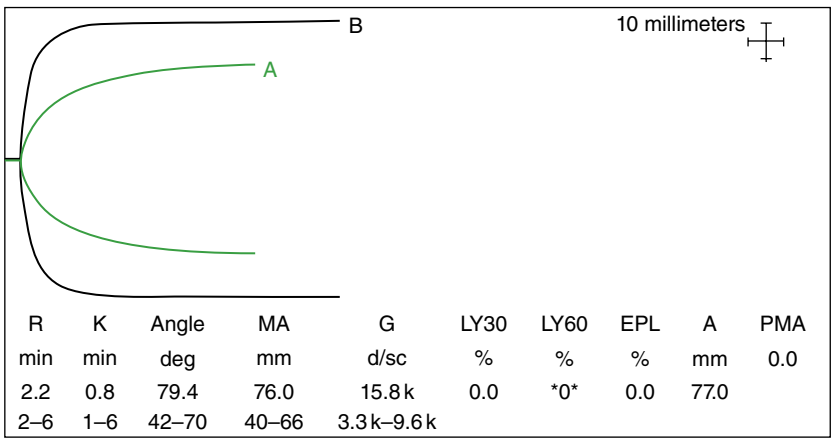


FIGURE 91.1 Tissue-factor activated TEG of a healthy dog and a dog with hyperadrenocorticism. (A) Healthy dog demonstrating normal TEG tracing. (B) Dog with hyperadrenocorticism demonstrating parameters of hypercoagulability: shortened clot formation time (K), increased angle (α), maximum amplitude (MA), and clot elasticity (G) values.

Automated Platelet-Sizing Parameters

Automated hematology analyzers measure several parameters of platelet function.[5,136] Mean platelet component concentration, a measure of refractive index, is linearly related to platelet density. Activated (i.e., degranulated) platelets have relatively low density compared to non-degranulated platelets. Mean platelet component distribution width measures the uniformity in activation status. Mean platelet component correlated with P-selectin expression in dogs with inflammatory disease;[136] however, additional studies are needed to clarify its diagnostic utility for TE.

Platelet Flow Cytometry

Flow cytometry is a method to evaluate platelet size, granularity, expression of activation markers, and presence of bound fibrinogen.[136,206] Exposure of P-selectin on the platelet outer membrane surface is an indicator of platelet degranulation and a common marker of platelet activation (Chapter 79). High platelet P-selectin expression has been demonstrated in clinical studies of dogs with inflammatory disease and IMHA.[136,206]

MPs are small (0.1–1 μm) membrane vesicles shed from apoptotic or activated cells, and cytometric assays to detect MP in a variety of species have been described.[35,186,101] Prothrombotic MPs bear externalized phosphatidylserine (PS) and express tissue factor.[15,149] Platelet-derived microparticles drove in vitro thrombin generation in a canine study.[78] In a clinical study of canine IMHA, some dogs had increased TF-bearing MP and PS activity, but MP numbers were no different between diseased and healthy dogs.[95] Similarly, cats with asymptomatic hypertrophic cardiomyopathy and healthy cats were found to have no difference in numbers of platelet-derived MPs.[101]

Assays to Quantify Neutrophil Extracellular Traps

Markers of NETosis include cell-free DNA, neutrophil-derived proteins, and protein–DNA complexes.[61,155] A recent study described detection of extracellular DNA in canine blood samples;[101] however, the diagnostic utility of NET assays requires further investigation.

CLINICAL DIAGNOSIS OF THROMBOSIS

The clinical signs of TE are highly variable, depending on anatomic site and inciting disease. Validated clinical scoring systems do not exist for animals, rather the diagnosis of TE is based on a characteristic clinical syndrome, such as feline ATE, or imaging that defines the presence of a thrombus.

Pulmonary Thromboembolism

Establishing a definitive diagnosis of PTE is challenging.[140] The most common signs include dyspnea, tachypnea, and depression, while coughing, hemoptysis, cyanosis, syncope, collapse, and sudden death are less frequent. Auscultation may reveal harsh lung sounds and crackles, or no abnormalities. Signs compatible with backward or forward heart failure may be present. Arterial blood gas abnormalities due to PTE may include hypoxemia, hypocapnia, and an increased A-a gradient.[89] While not a sensitive or specific test, relative hypoxemia represents the extent of thromboembolic occlusion and may predict poor response to oxygen therapy due to intrapulmonary shunting.[893438]

Imaging: Ultrasonography, Radiography, Echocardiography

Bedside ultrasound and echocardiography may reveal pulmonary parenchymal disease, denoted by the appearance of B-lines, comet-tail artifacts, and/or evidence of right heart dysfunction, dilation, or tricuspid regurgitation. Abnormalities are typically present in thoracic radiographs; however, their absence should not rule out a diagnosis of PTE.[89,145,58,39,40] Pulmonary infiltrates are focal or multifocal, with alveolar or alveolar–interstitial patterns.[58] Other abnormalities include uneven vessel diameter, pulmonary arterial enlargement, lobar vein or artery attenuation, pleural effusion, and right-sided cardiomegaly.[173,58,132] Hyperlucent pulmonary regions denoting zones of reduced blood flow distal to sites of vascular occlusion are pathognomonic for PTE, but rare.[58,208]

Echocardiographic abnormalities that have been identified in small animals with PTE include right atrial and ventricular enlargement, paradoxical septal motion, pulmonary hypertension, and thrombi in the heart or pulmonary artery (PA).[89,150,89,16,200] A recent prospective study of at-risk dogs revealed pulmonic and tricuspid regurgitation as the most common signs.[67]

Imaging: Computerized Tomographic Pulmonary Angiography

Rapid, multislice spiral computerized tomography pulmonary angiography (CTPA) is considered the definitive diagnostic test of PTE.[69] These studies combine rapid, spiral thoracic computerized tomography (CT) with bolus injection of contrast media to ensure peak enhancement of the pulmonary arteries.[75] The most advanced (256 slice) scanners are capable of scan rates up to 458 mm/s, which enables imaging within 1 second exposure times, thereby avoiding the problems of respiratory or cardiac motion and contrast drainage. Diagnostic criteria for PTE using CTPA are well-described.[215] A normal CTPA study essentially rules out PTE as the cause of the patient's respiratory distress, unless the index of suspicion is very high.[160] Multislice CTPA has been described in dogs using 16-slice[75] and 64-slice scanners,[43] including protocols to maximize PA contrast enhancement.[123] Multislice CT angiography has also been used to detect PTE in experimental dogs[191] and to identify a descending aortic thrombus secondary to spirocercosis in a parasitized dog.[97] A recent canine IMHA study confirmed the diagnostic utility of CTPA with a 16-slice scanner to identify PTE, using a sedation-only restraint protocol.[67]

Cardiac Biomarkers

In humans, echocardiographic RV dysfunction secondary to PTE is associated with elevated cardiac troponin (cTn) concentrations.[86] Peak cTnI levels develop at 8 hours post embolism in people and point-of-care tests are available to detect the protein.[54,17] In dogs with PTE secondary to dirofilariasis, cTnI values were significantly higher than controls,[22] and dogs with experimental PTE had increased cTnI levels proportional to the severity of occlusion.[198] While high cTn is not specific to PTE, it may assist in gauging PTE severity and risk of death.[91] Similar to cTNI, increases in N-terminal-pro-B-type natriuretic peptide (NT-proBNP) are related to severity of RV dysfunction,[152] and adverse outcome in people with PTE.[170] Although clinical studies are lacking, a recent canine experimental study indicated that NT-proBNP and atrial natriuretic peptide were significantly increased in dogs with severe pulmonary arterial hypertension secondary to PTE.[82]

Feline Aortic Thromboembolism

Feline aortic TE is a common, devastating condition with reported survival-to-discharge rates of approximately 30%.[182,113,169] The inciting cause is usually HCM. Non-HCM cases are attributed to other cardiac disease, or rarely neoplasia.[173,182] Up to 90% of afflicted cats have cardiomegaly, and heart failure, found in 50–70% of cases,[113] is a negative prognostic indicator.[182] Thrombi likely form in the left atrial appendage, atrium, or ventricle, with embolization to the aortic trifurcation ("saddle thrombus") causing hind limb blood flow obstruction. Less frequent sites of embolization include the renal, iliac, or brachial arteries. Acute obstruction of the left ventricle or proximal aorta may cause sudden death. The consequences of ATE include physical occlusion of the vessel and embolus-induced vasoconstriction that disrupts collateral flow.[172] Aortic occlusion increases left ventricular afterload, which may precipitate or exacerbate heart failure.

The diagnosis of ATE is usually based on characteristic history and clinical signs.[182] Thermal imaging will detect alterations in peripheral perfusion.[156] Blood samples from an affected limb have lower pH and glucose and higher lactate and CO_2 values than unaffected areas.[99] Serum biochemical profiles may reveal nonspecific abnormalities reflecting muscle ischemia and stress, and will identify hepatopathy, hyperkalemia, and uremia in patients with concurrent renal vascular TE. Many cats are dyspneic due to pulmonary edema or pleural effusion secondary to congestive heart failure, and may have a heart murmur or gallop rhythm and crackles on auscultation. Thoracic ultrasound typically reveals multiple B-lines,[120,204] and potentially pleural effusion. A brief focused echocardiography assessment to check the ratio of the LA to aorta (which should be <1.6) may identify cardiac disease. Caudal abdominal ultrasound, or less commonly angiography, can reveal the occlusion site. Measurement of cardiac troponin and NT-proBNP can also help assess cardiovascular function.[10]

Canine Aortic Thromboembolism

Canine ATE is less common than the feline syndrome, and most cases have chronic, insidious histories suggestive of in situ thrombus formation rather than embolization. Canine ATE has been reported in association with PLN, PLE, neoplasia, HAC, cardiac disease (infective endocarditis, heartworm, cardiomyopathy, valvular disorders), and localized vascular injury (e.g., *Spirocerca lupi*).[212,190,97] Clinical signs depend on the site, extent, and rapidity of occlusion, and collateral circulation. Prognosis generally depends on the underlying disease process.[97]

Biochemical profiles often reveal high creatine kinase and aspartate aminotransferase; however, biochemical abnormalities may assist in identifying a specific disease process. Glucose differentials >30 mg/dL between venous blood in the affected limbs and in central venous blood samples were highly sensitive and specific for ATE.[97] Definitive diagnosis of ATE is achieved via imaging.[109] Abdominal ultrasonography is usually informative, especially when combined with color flow Doppler assessment and additional techniques to assess blood flow include angiography, magnetic resonance imaging, and thermography.

Vena Cava Thrombosis

Cranial vena cava thrombosis is often accompanied by jugular vein thrombosis in patients with indwelling vascular devices such as central venous catheters or transvenous cardiac pacing leads.[29138] Most patients have a predisposing condition such as immune-mediated disease, sepsis, protein-losing disease, or cancer.[150] The most common clinical signs are edema of the head and neck, dyspnea, and pleural effusion. Jugular vein thrombi may be palpable, or ultrasonography with color flow Doppler may confirm the diagnosis. Thrombosis of the caudal vena cava is most commonly associated with pheochromocytoma and adrenocortical tumors.[12,65] These patients may present with signs of adrenal HAC, or develop ascites, abdominal pain, gastrointestinal signs, hypertension, and tachyarrhythmias due to pheochromocytoma. In patients with an adrenal mass, abdominal ultrasound is 80% sensitive and 90% specific for detection of caval thrombosis,[105] but differentiating tumor invasion from solitary blood clot is challenging, and CT angiography may help.

Portal Vein Thrombosis

Portal vein thrombosis is most common in older, large breed dogs and older domestic short-haired cats. The clinical signs resulting from portal vein thrombosis relate to speed of onset. Acute thrombosis is typically associated with signs of hypovolemic shock, vomiting, and abdominal pain, whereas ascites is suggestive of chronic thrombosis. Case reports of feline portal vein thrombosis identified liver disease as the primary disease process, with

three of six cases having congenital portosystemic shunts.[165] Most dogs with portal vein thrombosis have liver disease, with neoplasia, immune-mediated disease, and sepsis also described.[163,210] Portal vein thrombosis is typically diagnosed by abdominal ultrasound or CT angiography.

Splenic Vein Thrombosis

Splenic vein thrombosis is uncommon, but has been reported in dogs and cats with neoplasia, corticosteroid administration, pancreatitis, and immune-mediated disease.[114] Case reports typically describe splenic vein thrombosis as an incidental finding during abdominal ultrasound of older, large breed dogs. Infrequent clinical signs include splenomegaly, ascites, and abdominal pain. Concurrent portal vein thrombi may be present in approximately 20% cases. Isolated splenic vein thrombosis rarely requires treatment.

Mesenteric Vein Thrombosis

Mesenteric vein thrombosis is also uncommon. One case series described mesenteric vein thrombosis on postmortem exam of three dogs with concurrent portal vein thrombosis.[210] A second case report described a 4-year-old Cocker Spaniel with anorexia and vomiting and intestinal intussusception.[176] Techniques to identify mesenteric thrombosis include ultrasound, CT angiography, and direct visualization at surgery.

Cerebrovascular Accidents

Ischemic stroke is increasingly recognized with the advent of advanced diagnostic imaging in veterinary practice.[62,63,64] Thrombi are rarely identified in the cerebral vasculature and in most cases risk factors for human TE are absent.[62] Neurolocalization of signs reflect the area of vascular ischemia and infarction. The most commonly affected brain regions are the cerebellum and telencephalon.[64] Associated conditions include hypertension, HAC, diabetes mellitus, and cancer.

Coronary Thrombosis

Dogs are often used as models for evaluating luminal prosthetic stents and in preclinical studies of antithrombotic agents. However, spontaneous myocardial infarction appears to be extremely rare in dogs and cats, sometimes only identified post mortem.[45] Clinical signs are nonspecific and may be attributed to cardiac disease or underlying disease such as neoplasia, PLN, or IMHA. In one study, chronic cardiac disease was the most common associated disorder, with echocardiographic signs of systolic dysfunction and atrial fibrillation as the most common electrocardiograph (EKG) abnormality.[51] Coronary angiography by direct catheterization is feasible for definitive diagnosis in dogs, but CT angiography is more widely available.[44]

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CHAPTER 92

Disseminated Intravascular Coagulation*

TRACY STOKOL

Primary Disease Conditions
Pathogenesis
  Initiation of Thrombin Generation
  Amplification of Thrombin Generation
    Decreased Inhibition
  Progression/Maintenance of Thrombin Generation
    Hemostasis-Inflammation Cross Talk
    Endothelial and Tissue Injury
  Fibrinolysis
Diagnosis of DIC
  Overt DIC
  Clinical Diagnosis of Overt DIC
  Laboratory Criteria of Overt DIC
  Nonovert DIC
  Scoring Schemes for DIC
Treatment of DIC
  Replacement of Coagulation Factors and Platelets
  Anticoagulant Therapy
  Antifibrinolytics
  Future Treatment Approaches

Acronyms and Abbreviations

APTT, activated partial thromboplastin time; AT, antithrombin; CT, computerized tomography; DAMPs, damage-associated molecular patterns; DIC, disseminated intravascular coagulation; ECs, endothelial cells; EPCR, endothelial protein C receptor, FDPs, fibrin(ogen) degradation products; EVs, extracellular vesicles; FV/FVa, factor V/activated factor V; FVII/FVIIa, factor VII/activated factor VII; FVIII/FVIIIa, factor VIII/activated factor VIII; FIX/FIXa, factor IX/activated factor IX; FX/FXa, factor X/activated factor X; FXI/FXIa, factor XI/activated factor XI; FXIII, factor XIII; HMGB1, high-mobility group box-1 protein; IMHA, immune-mediated hemolytic anemia; ISTH, International Society on Thrombosis and Haemostasis; LPS, lipopolysaccharide; NETs, neutrophil extracellular traps; PAI-1, plasminogen activator inhibitor-1; PAR, protease-activated receptor; PS, phosphatidylserine; PT, prothrombin time; SSC, Scientific and Standardization Committee; TAFI, thrombin-activatable fibrinolysis inhibitor; TCT, thrombin clotting time; TF, tissue factor; TFPI, tissue factor pathway inhibitor, tPA, tissue plasminogen activator.

Our understanding of hemostasis has undergone a paradigm shift over the last 10 years, shedding new light into the pathogenesis of disseminated intravascular coagulation (DIC). Hemostasis is now recognized not only as the process that stops hemorrhage after blood vessel injury, but as an integral part of innate immunity. Fibrin and platelet thrombi entrap pathogens and recruit leukocytes, thereby promoting immune clearance. Moreover, platelets have bactericidal capabilities. Together these actions lead to the concept of "immunothrombosis."[18] In this context, DIC can be thought of as a variant of immune dysfunction, where hemostasis is no longer localized to the site of pathogen invasion or vessel wall injury, but rather progresses to a systemic, overexuberant reaction to pathogen-associated or damage-associated molecular patterns (DAMPs). Typical manifestations of DIC include microvascular thrombosis (in arterioles, venules and capillaries), with mixed thrombi consisting of fibrin, platelets, leukocytes, and extruded nuclear DNA, histones, and DNA–histone complexes (nucleosomes) and mitochondrial DNA, with few erythrocytes. Dysregulated and unopposed

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TABLE 92.1 Characterization of overt and nonovert DIC.

	Nonovert DIC	Overt DIC
Alternate names and subtypes	Compensated DIC, chronic DIC, subclinical DIC, pre-DIC, asymptomatic DIC	Uncompensated DIC, acute DIC, fulminant DIC, consumptive coagulopathy
Pathophysiology	Procoagulant excess opposed by coagulation inhibitors, partial compensation modulates fibrin deposition	Decompensated hemostasis with fibrin deposition throughout the microvasculature. Platelet and factor depletion may develop. Can be hyperfibrinolytic, hypercoagulable/hypofibrinolytic, or hypocoagulable
Clinical features	Primary inciting disease, subclinical or tissue thrombosis	Primary inciting disease with some or all of the following: Systemic microvascular thrombosis, pulmonary thromboembolism, multiorgan dysfunction syndrome, hemorrhage from single or multiple sites
Screening test abnormalities	No screening test abnormalities and/or progressive fall in platelet count and/or progressive increase in screening clotting times, FDPs and D-dimers	Some or all of the following (generally 3 out of 6): Thrombocytopenia, prolonged screening coagulation tests (APTT, PT, TCT), hypofibrinogenemia, low AT activity, high FDPs or D-dimer
Tests of prethrombotic state	Evidence of thrombin generation (either by-products of thrombin generation or activity): Prothrombin fragment 1+2, thrombin–antithrombin complex, activated protein C–protein C inhibitor complex, fibrinopeptides A & B More rapid kinetics of thrombin or fibrin generation: Endogenous thrombin potential, thromboelastography/elastometry	Not applicable

activation of hemostasis in DIC can also result in cleavage, inactivation, inhibition and consumption of coagulation factors and platelets, manifesting as hemorrhage due to a hypocoagulable state. The shift in the DIC pendulum toward thrombosis or hemorrhage is governed by the inciting disease and whether procoagulant, antifibrinolytic, anticoagulant, or fibrinolytic forces dominate. The complex and intricate balance among these opposing forces changes dynamically and unpredictably within and between affected patients, complicating recognition and treatment of DIC. With the goals of standardizing terminology and improving diagnosis, the Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH) defined DIC as "an acquired disorder characterized by excessive thrombin generation with loss of localization and endothelial dysfunction that can result in organ injury." Scoring schemes and guidelines to subclassify DIC into nonovert and overt phases were also described (see Table 92.1).[69] The clinical impact of DIC is exemplified by studies that show DIC, particularly in the overt phase, increases the likelihood of mortality in humans[2,22] and animals.[4,10,17,24,74]

PRIMARY DISEASE CONDITIONS

Disseminated intravascular coagulation has been documented in most domestic animals, except for camelids. It is not a primary disorder, but is always induced by an underlying disease (see Table 92.2). In dogs and cats, cancer and systemic inflammation (e.g., sepsis, pancreatitis, immune-mediated hemolytic anemia [IMHA]) are the most common initiating diseases.[4,20,24,66,74] Endotoxemia (secondary to strangulating, inflammatory or ischemic gastrointestinal disorders) and sepsis are the main causes of DIC in adult horses and neonatal foals, respectively.[3,10,67] Similarly, DIC is most often recognized in ruminants with endotoxemia or septic inflammation (metritis, mastitis).[6,31,32]

PATHOGENESIS

Disseminated intravascular coagulation represents a disease continuum. At first, activation of hemostasis is kept in check by natural inhibitors. If activation is excessive (akin to a dysregulated immune response) such that regulatory mechanisms are overwhelmed or if regulatory mechanisms are inhibited by the primary disease, DIC progresses to a decompensated disorder characterized by systemic microvascular thrombosis and potentially, a consumptive coagulopathy.[71] Although disease- and species-dependent differences influence the initiation and clinical course of DIC, the consistent pathogenic mechanism maintains excessive thrombin generation through dysregulated activation of coagulation.[22,69,71] For ease of understanding, the DIC continuum can be separated into three stages: initiation, amplification, and progression with maintenance of thrombin generation.[71]

Initiation of Thrombin Generation

Since DIC represents normal hemostasis gone awry, the aberrant exposure of tissue factor (TF), the physiologic

TABLE 92.2 Inciting diseases and pathophysiologic mechanisms of DIC in animals.

Disease categories and specific disorders	Potential mechanisms
Infections: Bacterial sepsis (Gram-positive and Gram-negative bacteria, including Leptospira; rickettsia [Rocky Mountain spotted fever]), viruses, protozoa (Babesia), parasites (Angiostrongylus)	Induced TF expression on monocytes (and ECs): Secondary to LPS and proinflammatory cytokines, with shedding of PS- and TF-expressing EVs
	Contact pathway activation (FXII): From bacterial polyphosphates and release of DAMPs (cell-free DNA) from tissue injury and inflammation (NETosis). Histones can activate FXI in a platelet-dependent manner
	Endothelial injury: Exposure of extravascular TF, induction of TF on ECs, release of DAMPs
Neoplasia : Solid tumors, such as hemangiosarcoma, carcinomas (e.g., mammary); hematopoietic neoplasia, such as lymphoma, acute myeloid leukemia	Cancer cell expression of TF: Constitutive or induced by hypoxia, cytokines, or cell death
	Induced TF expression on noncancer cells: Tumor-secreted or proinflammatory cytokines stimulate TF expression on monocytes, fibroblasts, and ECs
	Expression/secretion of procoagulants: Cancer procoagulant (a vitamin-K-dependent cysteine protease), mucin, upregulation of factor V receptor, factor XIII-like activity, platelet activators
	Shedding of TF- and PS-bearing EVs from tumor cells, hematopoietic cells, other cell types
	Tissue injury/inflammation: NETosis, release of DNA/histones, exposure of subendothelial TF
	Chemotherapy-induced changes: Tumor lysis, myelotoxicity causing inflammation, release of DAMPs
Inflammation/necrosis: Trauma, IMHA, pancreatitis, heat stroke, hepatitis, vasculitis, gastric dilatation-volvulus, strangulating obstructions, and inflammatory gastrointestinal disorders	Proinflammatory cytokines and release of DAMPs
	Endothelial injury
	Tissue injury/necrosis/apoptosis: Exposure of TF, shedding of TF- and PS-enriched EVs by apoptotic cells, NETosis
	Release of procoagulant proteases (e.g., trypsin)
Intravascular hemolysis: IMHA, acute transfusion reactions, insect/snake bites.	Endothelial injury
	Erythrocyte procoagulant activity: RBC membrane-associated elastase, PS expression (eryptosis), shedding of PS-enriched EVs
Envenomation	Snake venom proteases: Direct coagulation factor activation and fibrinogen cleavage, phospholipase-induced tissue injury/cell lysis

DAMPs, Damage-associated molecular patterns; ECs, endothelial cells; EVs, extracellular vesicles; LPS, Lipopolysaccharide; PS, phosphatidylserine; TF, tissue factor.

trigger of hemostasis, often initiates DIC (Table 92.2).[22,25] Tissue factor (tissue thromboplastin, factor III) is a transmembrane protein whose expression is normally restricted to interstitial perivascular fibroblasts and smooth muscle cells. Some extravascular TF may be prebound to its ligand, factor VII (FVII), leading to the concept of an "idling" hemostatic system, poised for activation on exposure to plasma.[29]

Under physiologic conditions, exposure of extravascular unbound TF to plasma FVII or exposure of FVII-prebound TF to its plasma substrate, FX, occurs only at focal sites of endothelial injury. In DIC triggered by massive tissue trauma, widespread endothelial injury exposes large amounts of extravascular TF. In conditions such as viral infections, intravascular hemolysis, and vasculitis, generalized endothelial injury exposes extravascular TF and/or induces TF expression on damaged endothelium. Proinflammatory cytokines also contribute to TF expression on endothelial cells (ECs) in these latter disorders (see in the following text and Table 92.2).[23,26,70]

In the absence of endothelial injury, DIC may be initiated by both the extrinsic and "contact" pathways of coagulation (see Table 92.2 and Figure 92.1). With the extrinsic pathway, proinflammatory cytokines induce TF on the surface of intravascular cells, particularly monocytes, and likely ECs. This is thought to be the main mechanism of initiation of DIC in bacterial sepsis and many nonseptic inflammatory states.[22,68,70] Pro-oncogenic mutations may induce high constitutive expression of TF on cancer cells,[76] thereby promoting cancer-associated thrombosis and DIC.

Activation of FXII in the contact pathway of coagulation may also trigger DIC in sepsis, tissue injury, inflammatory states, and cancer. Factor XII can be activated by negatively charged polyphosphates and alarmins or DAMPs, such as cell-free or extracellular DNA.[22,49,51,70] Long-chain polyphosphates are found in acidocalcisomes of bacteria and cancer cells and are particularly potent activators of FXII.[47,51,57] Localized release of short-chain polyphosphates from activated platelets may also contribute to excessive thrombin generation.[49]

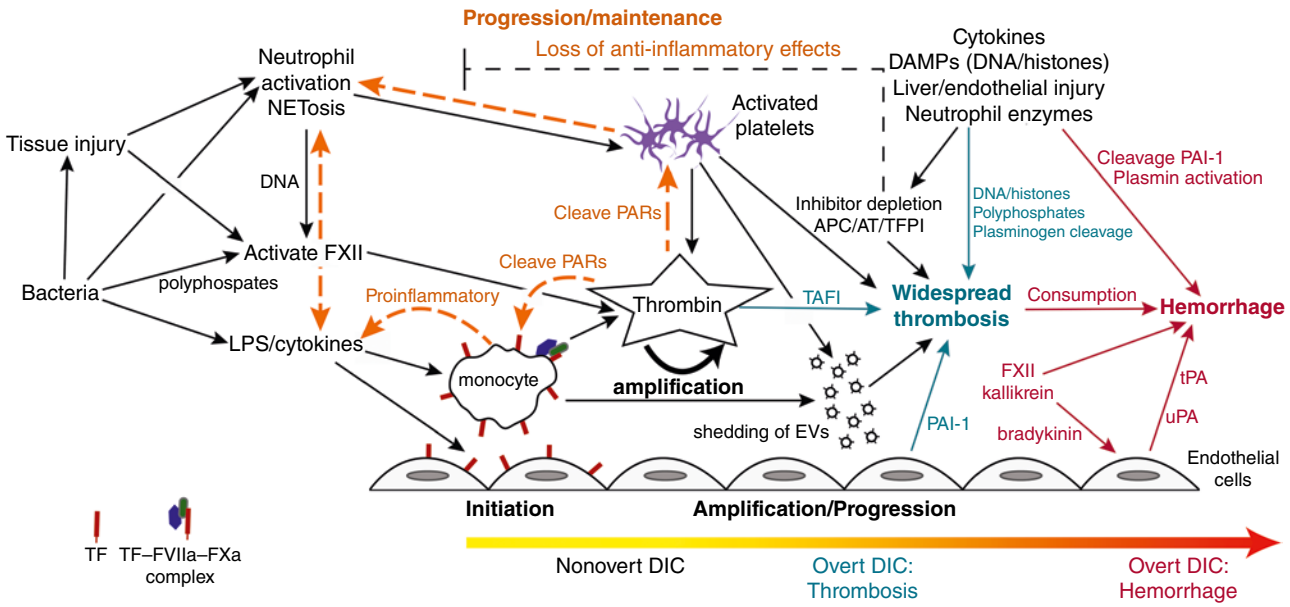


FIGURE 92.1 The DIC continuum. Disseminated intravascular coagulation is typically initiated by lipopolysaccharide (LPS)-induced or proinflammatory-cytokine-induced upregulation of tissue factor (TF) expression on monocytes and likely endothelial cells (ECs) in sepsis due to Gram-negative bacteria. Tissue factor can also be expressed and released by other cells, including platelets and eosinophils, but monocytes are thought to be the main source of TF in bacterial sepsis. In cancer, constitutive expression of TF on cancer cells and induction of TF on monocytes by proinflammatory cytokines are the likely trigger of DIC (not shown). Monocyte- or cancer-surface TF, FVIIa, and FXa form an active complex, which generates thrombin. Bacteria, via release of long-chain polyphosphates, or induction of tissue injury, inflammation, or NETosis, can also initiate DIC through activation of FXII. Bacteria can also activate platelets, promoting release of short-chain polyphosphates and procoagulant activity, via toll-like receptors (particularly TLR4, not shown). Endothelial injury, via release of long strings of von Willebrand factor, can capture and activate circulating platelets in conditions associated with endothelial injury (not shown). Release of damage-associated molecular patterns (DAMPs, e.g., cell-free DNA) from injured tissues (including ECs), inflammatory cells (via NETosis), or cancer can similarly trigger DIC via FXII and potentially other mechanisms (e.g., upregulation of TF via HMGB1). In early, nonovert DIC, inhibitors such as activated protein C (APC), antithrombin (AT), and tissue factor pathway inhibitor (TFPI) control the activated hemostatic system. If inhibitory mechanisms become overwhelmed, then thrombin amplifies its own production, simultaneously inhibiting fibrinolysis via thrombin-activatable fibrinolysis inhibitor (TAFI), resulting in microvascular thrombosis (progression/dissemination). This is now the hypercoagulable or thrombotic phase of overt DIC. Procoagulant extracellular vesicles (EVs) shed from thrombin-activated platelets and other cells, as well as DNA in extracellular traps, provide a negatively charged surface that supports systemic coagulation complex assembly, which is no longer held in check by inhibitors, which are rendered deficient or dysfunctional from DAMPs, liver injury, proinflammatory cytokines, and neutrophil proteases. Systemic thrombin production is also mediated by distant endothelial injury and dysfunction from microvascular thrombi, inflammatory mediators, and histones. Inhibition of fibrinolysis via endothelial release of plasminogen activator inhibitor-1 (PAI-1), bacterial and platelet polyphosphates, DAMPs, and neutrophil protease-mediated cleavage of plasminogen promotes persistence of thrombi. In the maintenance phase, cross talk between activated coagulation factors and platelets, such as through protease-activated receptor (PAR) signaling, potentiates the inflammatory response, including NETosis and release of proinflammatory cytokines from monocytes and other cells. The depletion of anti-inflammatory coagulation inhibitors further facilitates inflammation, thereby maintaining the inflammation–DIC cycle. As coagulation factors are cleaved or consumed (along with platelets), DIC may shift to an overt hemorrhagic or hypocoagulable phase. This phase of DIC may also occur if profibrinolytic forces dominate, such as through PAI-1 cleavage and FXII-, kallikrein-, protease-mediated cleavage of plasminogen to plasmin and release of tissue-type (tPA; stimulated by bradykinin or endothelial injury) or urokinase-type plasminogen activators (uPA).

Cell-free DNA from nuclei or mitochondria and potentially within nucleosomes (complexes of DNA and histones) released from activated neutrophils or damaged cells can also activate FXII, typically in concert with activated platelets.[1,15,22,26,44,62] The expelled DNA entraps bacteria, leading to the term neutrophil extracellular traps (NETs) in a process referred to as NETosis, although other cells (e.g., eosinophils, monocytes) can similarly extrude DNA. The released DNA is found in long strands decorated with histones, nuclear-associated cytokines, such as high-mobility group box-1 protein (HMGB1), and neutrophil enzymes, such as elastase and cathepsin G. NETosis directly links innate immunity to hemostasis as activated coagulation factors and platelets form fibrin strands enclosing the invading pathogen.[1,53] Excess exogenous histones and polyphosphates alone can trigger a DIC-like syndrome in murine models that is inhibited by degradative proteases (e.g., polyphosphatases, DNase) and facilitated by platelets via toll-like receptors.[26,44,49,62] Studies in dogs show that NETs are also procoagulant[34] and high concentrations of cell-free DNA and histones are seen in dogs with IMHA, sepsis, and trauma, suggesting that these DAMPs may have a role in triggering DIC.[35,39–41] As a cautionary note,

TABLE 92.3 Mechanisms of anticoagulant deficiency in DIC.

Mechanism	Primary condition
Decreased hepatic synthesis of AT, protein C, protein S	Hepatic injury (e.g., ischemia, histones), inflammatory cytokines
Decreased activity of TFPI, AT, protein C, protein S	Decreased protein C and TFPI activity due to decreased free protein S (from increased C4-binding protein, a positive acute phase reactant protein which binds protein S) Decreased protein C activation due to downregulation or modulation of thrombomodulin and endothelial protein C receptor by inflammatory cytokines and nuclear material Decreased protein C activation due to cleavage of thrombomodulin and endothelial protein C receptor by neutrophil elastases Decreased AT activity through downregulation of heparin-like glycosaminoglycans on endothelial cells by inflammatory cytokines and histones
Increased consumption of AT, protein C	Rapid clearance of thrombin–antithrombin complexes and activated protein C–protein C inhibitor complexes
Loss of TFPI, AT, protein C, protein S	Vascular leakage (e.g., bradykinin, histones)
Degradation of TFPI, AT, protein C, protein S	Proteases, e.g., neutrophil elastases, bacterial peptides (e.g., omptin Pla), histones (degrade TFPI)

AT, Antithrombin; TFPI, tissue factor pathway inhibitor.

some study results may have been influenced by the presence of procoagulant contaminants in commercial kits used to generate data[65] and the procoagulant activity of DNA and histones is dependent on their interassociation and tertiary structure.[52,53]

Additional DIC triggers include cancer proteases and mucin, or snake venoms that directly activate FX (see Table 92.2).

Initiation of DIC also depends on the ability of naturally occurring inhibitors to regulate and contain the activated hemostatic system. In physiologic hemostasis, the TF–FVIIa–FXa complex is rapidly neutralized by tissue factor pathway inhibitor (TFPI). This inhibitor is primarily expressed on ECs, with its activity enhanced by free protein S and cell-surface heparin and heparin-like glycosaminoglycans.[43,61] In the process of DIC, TFPI is rendered ineffective through a number of mechanisms (see Table 92.3), including the generation of excess TF–FVIIa that overwhelms TFPI's inhibitory capacity.[22]

Amplification of Thrombin Generation

Thrombin is pivotal to DIC progression (see Figure 92.1). In a positive feedback cycle, trace amounts of thrombin, is amplified by intrinsic pathway reactions, generate a massive burst of thrombin.[30,71] The thrombin burst cleaves soluble fibrinogen to form fibrin polymers and activates FXIII to cross-link fibrin. Thrombin simultaneously inhibits fibrin degradation via thrombin-activatable fibrinolysis inhibitor (TAFI). Thrombin generation is further enhanced by activated platelets, which release their intracellular stores of coagulation factors (e.g., factor V and fibrinogen).

The thrombin burst also relies on membrane exposure of phosphatidylserine (PS), a negatively charged phospholipid normally expressed on the inner membrane leaflet of cells. Activated cells exteriorize PS and shed PS-enriched extracellular vesicles (EVs).[30,54,59] Outer membrane or EV-associated PS is the binding site for assembly of coagulation factor complexes, amplifying their activity and protecting them from inhibition.[30] Although they have a short half-life (in order of minutes),[36] EVs expand the surface area for thrombin generation far beyond that of the cells from which they are derived.[64] In physiologic and pathologic hemostasis, activated, procoagulant platelets represent the major source of outer membrane PS; however, other cells, including leukocytes, cancer cells, dying or apoptotic cells, and RBCs, can express PS and shed PS-expressing EVs.[54,59] Activated monocytes, certain cancer cells, and their shed EVs express both PS and TF, thus bringing the initiator and amplifier of thrombin generation in close proximity.[54,59] Neutrophil extracellular traps also may serve as a scaffold for thrombin generation.[1,15,62]

Decreased Inhibition

The generation of thrombin through the coagulation cascade is opposed by the plasma anticoagulants, antithrombin (AT), protein C, and protein S (the cofactor for protein C). Activated protein C (APC) is generated on EC surfaces through the action of thrombin engaged with its receptor, thrombomodulin, in the presence of the endothelial protein C receptor (EPCR). Endothelial surface APC is a potent inhibitor of FVa and FVIIIa, whereas the initiating TF–FVIIa–FX complex is inhibited by TFPI. Nonovert DIC refers to a state of systemically activated hemostasis whose inhibitory mechanisms are stressed, but not overwhelmed (see Table 92.1 and Figure 92.1).[69] With continuing activation of coagulation and ensuing endothelial dysfunction, natural inhibitors may be downregulated or depleted (see Table 92.3), leading to unopposed thrombin generation. Nonovert DIC represents a hypercoagulable condition, characterized by incipient or formed thrombi, particularly if fibrinolysis is inhibited (see Figure 92.1).

Progression/Maintenance of Thrombin Generation

The evolution and progression of DIC from a nonovert to overt phase and persistence of DIC in spite of treatment are believed to result from potentiating interactions between hemostasis and inflammation, and endothelial dysfunction and tissue injury.

Hemostasis-Inflammation Cross Talk

A self-perpetuating cycle develops in DIC, particularly that triggered by inflammatory processes, such as sepsis (see Figure 92.1). Inflammation initiates hemostasis via tissue injury, induced TF expression, and platelet and leukocyte activation with microvesiculation and NETosis. Hemostasis, in turn, fuels inflammation, through the proinflammatory properties of active coagulation factors, including thrombin, FXa, and FVIIa. Activated coagulation factors act as chemotaxins and bind to protease-activated receptors (PARs) on inflammatory cells and ECs, stimulating proinflammatory cytokine secretion and upregulating adhesion molecules, which facilitates continued TF expression, NETosis and platelet activation, and recruitment of inflammatory cells, respectively.[13,68,70] Fibrin and related degradation products can stimulate proinflammatory cytokine release via toll-like receptors.[68,70] Thrombin, FXa, kallikrein, and plasmin can act as C3 and C5 convertases and FXIIa can activate C1q of the classical complement pathway.[37] In turn, classical-pathway-produced and lectin-pathway-produced complement components can activate platelets and ECs, induce TF expression, and act as procoagulant proteases.[37] A C3 convertase inhibitor (compstatin) reduced laboratory findings of DIC in a baboon model of sepsis, revealing the importance of complement in sepsis-associated DIC.[63] Activated platelets can also release proinflammatory cytokines, activate complement, and induce NETosis.[55]

Anticoagulants not only limit hemostasis, but also have anti-inflammatory actions, including degradation or inactivation of inflammatory mediators, such as HMGB1, activated complement components, and histones.[22,70] Activated protein C with its associated cell-surface receptors, thrombomodulin and EPCR, has endothelial cytoprotective functions, inducing signaling cascades that maintain vascular integrity or preserve the glycocalyx.[70] Thus, anticoagulant depletion helps sustain DIC.

Endothelial and Tissue Injury

Healthy ECs secrete potent platelet inhibitors (e.g., prostacyclin, adenosine diphosphate [ADPase]) and express receptors, including PARs, thrombomodulin, and EPCR, which modulate coagulation and inflammation. As a direct consequence of the primary disease or as a sequela to DIC, various factors including proinflammatory cytokines, ischemia, perturbed blood flow (secondary to bradykinin and other vasoactive proteins), histones, neutrophil proteases, activated complement, EVs, and acidosis can result in loss of the normal protective functions of ECs.[8,70] Histones accumulate in specific tissues and induce endothelial and tissue injury, potentially through binding to and causing direct damage to cell membranes.[1,22] Injured or dysfunctional organs can also contribute to DIC perpetuation, via release of DAMPs, TF upregulation, and microvesiculation of necrotic or apoptotic cells.

Fibrinolysis

The balance between antifibrinolytic and profibrinolytic forces may dictate outcome and clinical manifestations of DIC. Multiple factors affect fibrinolysis in DIC, shifting the balance from a profibrinolytic and more hemorrhagic phenotype to an antifibrinolytic or more thrombotic phenotype. In human-sepsis-associated DIC, antifibrinolytic forces dominate, mediated by endothelial injury and inflammatory cytokines (e.g., tumor necrosis factor-alpha) that stimulate plasminogen activator inhibitor-1 (PAI-1) release.[22] Similarly, increased concentrations of PAI-1 that were associated with endotoxemia have been reported in horses with colic,[11] suggesting that impaired fibrinolysis contributes to the typical thrombotic manifestation of DIC in colicky horses. Cell-free DNA, histones, NETs, and short- and long-chain polyphosphates (from bacteria or platelets) promote the formation of strong fibrin clots resistant to fibrinolysis.[1,34,47] Nuclear material also inhibits tissue plasminogen activator (tPA)-mediated conversion of plasminogen to plasmin,[1] whereas polyphosphates act as cofactors for thrombin-mediated activation of TAFI.[56]

Alternatively, stimulation of fibrinolysis can occur in DIC, secondary to increased tPA or urokinase-type plasminogen activator release from ECs, leukocytes, or cancer cells. A profibrinolytic response occurs early in septic humans, but is quickly opposed by the antifibrinolytic actions of PAI-1.[22] In addition, FXIIa and kallikrein promote plasminogen activation and generate the vasoactive reactant bradykinin that is a potent stimulator of tPA release. Histones may also stimulate tPA release. A hyperfibrinolytic bleeding phenotype, most often described in patients with severe trauma, is considered a variant of DIC by some investigators,[72] but may represent a distinct hemorrhagic syndrome.[23] Fibrinolytic pathways vary among species, including higher tPA activity reported for dogs compared with humans.[38] This difference may shift the balance toward a relatively hemorrhagic phenotype in canine DIC.

DIAGNOSIS OF DIC

Every animal with an inciting primary disease should be considered at risk for developing DIC (see Table 92.2). Confirmation of DIC is challenging because animals may be examined at any point of the DIC continuum, clinical signs may be subtle or nonspecific, and individual laboratory tests are variably sensitive and specific for this syndrome. Histopathologic detection of fibrin thrombi in the microvasculature with phosphotungstic acid–hematoxylin staining or immunohistochemical staining is definitive for DIC; however, these methods are variably sensitive.[9,12] Histologic detection of fibrin thrombi has low diagnostic utility not only due to the requirement for tissue samples, but fibrin thrombi lyse rapidly after death.[48] Indeed, there was no correlation between the laboratory diagnosis of DIC and postmortem evidence of fibrin thrombi in one study of 48 horses with gastrointestinal disorders.[9]

Scoring criteria for the diagnosis of overt and nonovert DIC have been developed by the ISTH and British, Italian, and Japanese hemostasis and thrombosis or hematology societies.[69,73] These schemes have allowed for a degree of standardization across studies and, in 2013, the SSC on DIC of the ISTH recommended the use of any grading scheme, albeit different, for diagnosis and treatment of DIC. The SSC on DIC also recommended the use of serial testing (based on moderate quality of evidence).[73] Two studies have evaluated scoring criteria for the diagnosis of overt DIC in dogs and both showed an association between DIC diagnosis and mortality.[24,74] However, variability among studies and inherent species differences demonstrate the need for an evidence-based approach to standardize diagnostic criteria for the diagnosis of DIC and define appropriate criteria for each animal population of interest.

Overt DIC

The term *overt DIC* is applied to patients with clinical manifestations of thrombosis and/or hemorrhage, and is diagnosed by a combination of abnormal screening test results indicating activation of hemostasis, inhibitor depletion, and fibrinolysis.

Clinical Diagnosis of Overt DIC

Documentation of a primary initiating disease is an essential requirement for the diagnosis of DIC in all species (see Table 92.2). In human medicine, DIC may be subclassified into three distinct clinical types—a bleeding type secondary to hyperfibrinolysis (e.g., prostatic cancer, acute promyelocytic leukemia), an organ failure type due to thrombosis from a combination of hypercoagulability and hypofibrinolysis (e.g., bacterial sepsis), and an often lethal, massive bleeding type due to a consumptive hypocoagulability (e.g., obstetrical or surgical complications).[72] In veterinary medicine, organ failure/thrombotic and consumptive bleeding phenotypes are most common. Bleeding typically manifests as epistaxis, petechiae, bruising, prolonged bleeding, or oozing after venipuncture or minor surgical procedures, and spontaneous hematoma formation and body cavity hemorrhage. Antemortem detection of thrombi is relatively difficult, due to unavailability or cost of advanced imaging modalities, such as computerized tomography.[58] Patients with primary inciting diseases should be monitored, therefore, for the adverse effects of thrombosis on organ function, e.g., hypoxia, azotemia, liver injury, or lactic acidosis. In the author's opinion, overt DIC is characterized first and foremost by widespread microvascular thrombosis, which causes hypoxic tissue injury, a major contributor to the high morbidity and mortality of DIC.[5,17] However, the clinical import of systemic thrombosis depends on species, underlying disease, and nature of progression of DIC. For example, hemorrhagic DIC is more common in dogs than in horses or cats; species frequently referred to as having "subclinical" DIC. The reason for these species differences is unclear; however, the naturally high fibrinolytic activity in dogs[38] may be a factor. Since dysregulated fibrinolysis contributes to pathogenesis and clinical manifestations of DIC, further studies of fibrinolysis in animals with DIC are needed. Due to the difficulties with detecting thrombosis and lack of bleeding in many animals with DIC, screening tests to detect overt DIC is recommended in all patients with primary inciting disorders (see Tables 92.1 and 92.2).

Laboratory Criteria of Overt DIC

Diagnosis of overt DIC in animals and people relies upon identifying abnormalities in multiple standard tests of hemostasis, rather than a single laboratory abnormality (see Table 92.1). Traditional criteria for diagnosis of overt DIC in animals include a combination of two or more test abnormalities, specifically thrombocytopenia, prolonged screening coagulation times, such as the prothrombin time (PT), activated partial thromboplastin time (APTT), and thrombin clotting time (TCT), hypofibrinogenemia, low AT activity, and high fibrin(ogen) degradation product (FDPs) or D-dimer concentrations.[4,9,17,20,24,66,67] The apparent diagnostic utility of these tests varies depending on species and stage at presentation. In the last 10 years, these criteria have remained unchanged, although additional tests, such as measurement of thrombomodulin for detection of organ injury, are used in people[72] and clot curve analysis has been evaluated in dogs with DIC, liver failure, biliary obstruction or rupture, and anticoagulant rodenticide toxicosis (revealing some differences in clot curve results between these disorders).[60]

Thrombocytopenia (typically mild to moderate) is a consistent finding in dogs with overt DIC (75–100% sensitivity),[5,66] but not in cats[20] or horses (0–64% sensitivity).[17,67] Of the routine coagulation screening tests, the APTT appears to be more sensitive for detecting DIC in animals than the PT or TCT.[17,20,24,66,67] Hypofibrinogenemia is generally an insensitive indicator of DIC because fibrinogen is upregulated secondary to underlying inflammation, which often accompanies the DIC process or the initiating disease.[17,20,24,66,67] Indeed, normal fibrinogen concentrations in a patient with active inflammation suggest ongoing consumption of fibrinogen balanced by increased production. Low AT activity can be a sensitive test for diagnosis of DIC in dogs (77–90%)[5,66] and horses (83–93%),[17,67] but AT activity is not a reliable indicator of DIC in cats.[7] For markers of fibrinolysis, assays for D-dimer have largely replaced those for FDPs. D-dimer is a sensitive test for DIC in dogs (75–100%),[50,66] but is less sensitive in horses (50% in one study[67]), and has not been adequately evaluated in cats. D-dimer concentrations can be prognostic; horses with gastrointestinal disorders and D-dimer concentrations >1000 ng/mL (MiniQuant immunoturbidimetric assay) were 5 times more likely to die than those with lower concentrations.[10] Red blood cell fragments, such as schistocytes and keratocytes, can be seen in dogs (75%[66]) and cats (8%[20]) with DIC, but have not been reported in horses. The combination of RBC fragments and thrombocytopenia in clinically ill dogs with

relevant diseases warrants additional hemostasis testing for overt DIC.

Viscoelastic-based tests for hemostasis, including rotational thromboelastometry (ROTEM™), thromboelastography (TEG®), and an oscillatory probe method (Sonoclot®), have been described in dogs with overt DIC[4,75] and in horses with gastrointestinal disorders or critically ill neonatal foals.[14,19] In canine studies, using TF-activated clot formation (analogous to a PT), hypocoagulable profiles were seen in 22%[75] and 34%[4] of dogs with DIC and were associated with bleeding,[4] lower platelet counts,[4,75] and higher mortality.[4,75] These findings are consistent with the long-held anecdotal opinion that the hemorrhagic phenotype in dogs is associated with fulminant, "end-stage" consumptive DIC. Horses had variable changes, with some having evidence of hypocoagulability (based on maximum clot strength).[14,19] Together, these studies do not provide strong evidence that viscoelastic-based tests are more informative than routine hemostasis testing to diagnose overt DIC in animals.

Nonovert DIC

In contrast to overt DIC, nonovert DIC is clinically inapparent and the diagnosis depends on laboratory tests indicating an activated, but not overwhelmed, hemostatic system (hypercoagulable).[69] Nonovert DIC also applies to the older term of "chronic DIC," which reflects continuous low grade, but compensated, activation of coagulation (see Table 92.1 and Figure 92.1). Routine clotting-time-based tests are insensitive to this phase because they are configured to detect coagulation factor deficiencies, rather than accelerated coagulation. Nonovert DIC is marked by excessive thrombin generation and tests geared toward detection of excessive thrombin are useful. This might include measurement of TAT complexes, D-dimer, and assays that measure the kinetics and rate of thrombin generation, e.g., thrombin potential assays. Unfortunately, with the exception of D-dimer (which is not specific for DIC), these tests are not available for routine clinical diagnosis. Detection of nonovert DIC in animals thus remains an index of suspicion based on 1–2 abnormal hemostasis results in an animal with a predisposing condition. The diagnostic utility of serial testing, which is recommended in humans for detection of nonovert DIC[69] and monitoring of overt DIC,[73] has not been evaluated in animals.

In veterinary medicine, viscoelastic-based methods were initially thought to be a "breakthrough" enabling us to detect hypercoagulability. Although these assays have detected hypercoagulability in up to 45% of dogs with DIC, results strongly correlate with fibrinogen concentration, particularly when hypercoagulability is based on clot strength.[4,75] In addition, these assays are impacted by sample collection (e.g., timing of analysis, agitation) and methodologic (e.g., choice of activator, definition of hypercoagulability) and patient-related (anemia in particular) variables, which influence results and interpretation.[4,16,21,27,33,45,46] At this time, viscoelastic-based testing cannot be recommended for diagnosis of DIC in animals. Similarly, there is insufficient evidence to warrant the use of viscoelastic hemostasis testing for diagnosis of DIC in humans.[42]

Scoring Schemes for DIC

The ISTH SSC on DIC developed practical systems for the diagnosis of overt and nonovert DIC in people in 2001.[69] For overt DIC, the system is applied to patients with an underlying disorder associated with DIC and derives a summary score from four readily available laboratory tests: platelet count (<100 × 10^6/L: score of 1; <50 × 10^6/L: score of 2), PT (>3 but <6 sec: score of 1, >6 sec: score of 2), fibrinogen (<1 g/L: score of 1), and fibrin-related markers (soluble fibrin, FDPs, or D-dimer, moderate increase: score of 2, marked increase: score of 3). A composite score of 5 or more was compatible with overt DIC and a score of <5 was suggestive of nonovert DIC. For nonovert DIC, scores were given for a disease associated with DIC, major criteria or tests that are globally available including platelet count, PT and fibrin-related markers, and minor criteria or tests that are not offered by most laboratories, including but not limited to AT, protein C, and thrombin–antithrombin complexes. The scoring scheme also incorporated points for progressive deterioration (positive) or improvement (negative) in major test results on serial testing every 24 hours. Several studies have shown that the ISTH score for overt DIC is associated with higher 28-day mortality rates, although this is dependent on the initiating disease.[2,22] Other societies have developed different scoring schemes. While prospective validation of the ISTH nonovert DIC score is lacking, the basic recommendation of the ISTH is to use a scoring scheme to diagnose and determine treatment regimens for DIC.[73]

The ISTH overt DIC scheme was used to develop a multiple logistic regression model for diagnosis of DIC combining expert opinion and laboratory data from 63 serially tested dogs from one institution.[74] A final model was based on the continuous variables of fibrinogen, PT, and APTT and the dichotomized variable, D-dimer (cutoff, 0.5 mg/L, NycoCard assay), with a score of >0.401 being considered as a high probability of DIC. This model was then applied to data obtained from 46 dogs at risk for DIC at a second institution. Compared to the "expert panel," the model identified DIC in 83% of dogs, with a specificity of 77%. Using the model, dogs with DIC were 2.8 times more likely to die versus dogs without DIC. This logistic regression model was subsequently applied to data analyzed retrospectively from 804 dogs in which hemostasis testing was performed at a single institution.[24] In the latter study, the investigators evaluated 4 different schemes for diagnosis of overt DIC and compared scheme performance as predictors of overall mortality (21% in this cohort). The investigators found that traditional criteria of DIC diagnosis, namely, three or more abnormalities in standard test results (based on relevant changes above or below the institution-derived reference intervals), was the most sensitive (73%) and specific method (81%) for predicting mortality in this large cohort of dogs. By contrast, the ISTH score was poorly sensitive (7%), whereas the unmodified multiple logistic regression

model was highly sensitive (98%), but not specific (3%). These studies show that traditional criteria for diagnosis of DIC are still relevant and that further work is needed to identify the optimum tests and test cutoffs to include in a DIC scoring scheme. Furthermore, due to species differences in underlying diseases and diagnostic test sensitivity for DIC, it is unlikely that a single scoring system will apply across species. Ultimately, there is still a need to establish consensus guidelines for diagnosing nonovert and overt DIC in animals with the goals of achieving consistency across studies in patient stratification and assessment of various treatment protocols on defined clinical outcomes.

TREATMENT OF DIC

Affected animals should always be treated for their primary disease with the goal of breaking the DIC cycle. Supportive care aimed at alleviating metabolic and hemodynamic sequelae of DIC (shock, hypoperfusion, and acidosis) helps minimize organ damage, inflammation, and continued activation of hemostasis. Effective treatment modalities beyond primary disease-specific and supportive care remain unproven. Treatment recommendations are generally derived from older human literature and should be applied with the knowledge that they may not be suitable for animals, due to species-specific differences in hemostasis and drug pharmacokinetics and efficacy. Randomized, controlled clinical trials, with collaboration between multiple institutions or practices and consensus on testing criteria and a defined outcome (e.g., 28-day mortality), are urgently needed in veterinary medicine to identify safe and cost-effective DIC treatment strategies. In 2013, the ISTH SSC on DIC issued evidence-based guidelines for treatment of DIC including treatment of the primary disease (moderate evidence), replacement of coagulation factors and platelets, anticoagulant therapy, and antifibrinolytic therapy.[73]

Replacement of Coagulation Factors and Platelets

The fear that transfusion "fuels the fire" of DIC is largely unfounded; however, the recommendation from the ISTH, despite a low quality of evidence, is that replacement of platelets and coagulation factors (in the form of fresh frozen plasma or cryoprecipitate, rather than whole blood) be restricted to actively bleeding patients (platelet count < 50×10^6/L, prolonged PT or APTT by 1.5 times, fibrinogen < 1.5 g/L) or those at risk of bleeding (e.g., surgery, platelet counts 10–20 × 10^6/L). Fresh frozen plasma and cryoprecipitate are recommended to treat prolonged clotting times or hypofibrinogenemia at rates of 15–30 mL/kg.[73]

Anticoagulant Therapy

The ISTH SSC on DIC considers a low quality of evidence for the use of heparin (preferentially low-molecular-weight) to treat patients with DIC and thrombosis, with stronger support for the use of low-dose heparin for prophylaxis of venous thromboembolism in ill, nonbleeding patients with DIC.[73] Without prospective clinical trials, heparin therapy should be used with caution in dogs with DIC, although heparin therapy may be applicable for horses or cats, in which thrombotic sequelae appear to dominate over hemorrhage. Some inhibitor concentrates or recombinant proteins, e.g., TM, have shown some efficacy for reducing mortality in human patients with sepsis.[22,28,73] Unfortunately, comparable products are unavailable for animals.

Antifibrinolytics

Antifibrinolytics are reserved for human DIC patients with evidence of excessive fibrinolysis (e.g., patients with acute promyelocytic leukemia or trauma). A role for antifibrinolytics in the treatment of canine DIC has not been established.

Future Treatment Approaches

Recognition of the complex interplay between inflammation and hemostasis, the discovery of the toxic and procoagulant activities of histones and cell-free DNA, respectively, and renewed interest in the contact pathway as an initiator of DIC through polyphosphates and NETs, hold promise for development of new strategies to interrupt the DIC cycle that will be applicable to all species. While still at the investigational stage, targeted therapy aimed at minimizing NETosis, inhibiting polyphosphates, or inactivating FXIIa or FXIa[22] may provide opportunities to improve DIC outcomes in veterinary and human patients.

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CHAPTER 93

Vascular Diseases

SEAN P. McDONOUGH

Congenital Anomalies and Malformations
  Shunts
    Persistent Ductus Arteriosus
    Persistent Ductus Venosus
    Arteriovenous Fistula
  Telangiectasia
  Varicose Veins
Degeneration of Arteries
  Atherosclerosis
  Vascular Amyloid
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    Uremic Gastropathy
    Medial Calcific Sclerosis
    Metastatic Mineralization
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Hypertrophy of Arteries
  Chronic Hypoxic Pulmonary Hypertension
  Hypertension
    Systemic Hypertension
    Pulmonary Hypertension
    Feline Pulmonary Artery Medial Hypertrophy
    Fibromuscular Dysplasia
Vasculitis
  Viral Vasculitis
    Equine Herpesvirus-1
    Canine Herpesvirus-1
    Malignant Catarrhal Fever
    Equine Viral Arteritis
    Feline Infectious Peritonitis
  Rocky Mountain Spotted Fever
  Verminous Arteritis
    Strongylus Vulgaris
    Heartworm Disease
  Photosensitization Dermatitis
  Immune-Mediated Vasculitis
    Equine Purpura Hemorrhagica
    Canine Juvenile Polyarteritis Syndrome
    Proliferative Arteritis of the Nasal Philtrum of Dogs
    Paraneoplastic Vasculitis
Thrombotic Microangiopathy

Acronyms and Abbreviations

ADAMTS13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13; AVF, arteriovenous fistula; CH, capillary hemangiomatosis; CJPS, canine juvenile polyarteritis syndrome; CNS, central nervous system; DIC, disseminated intravascular coagulation; DV, ductus venosus; EC, endothelial cell; EVA, equine viral arteritis; FECV, feline enteric corona virus; FIPV, feline infectious peritonitis virus; FMD, fibromuscular dysplasia; IFN-γ, interferon-gamma; IL, interleukin; MCF, malignant catarrhal fever; PDA, persistent ductus arteriosus; PAH, pulmonary arterial hypertension; PVOD, pulmonary veno-occlusive disease; SMC, smooth muscle cell; TMA, thrombotic microangiopathy; TTP, thrombotic thrombocytopenic purpura; TNF-α, tumor necrosis factor-alpha.

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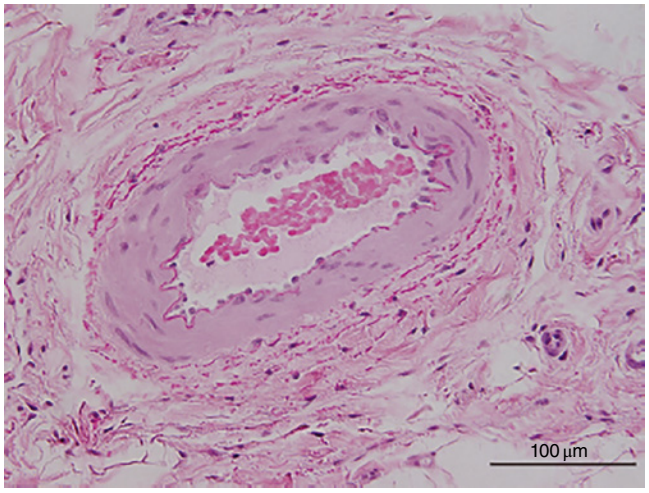


FIGURE 93.1 Cross-section view of a normal muscular arteriole. A single layer of endothelial cells adjacent to the lumen is surrounded by smooth muscle cells and fibroblasts in the media, and an outermost loosely organized layer of adventitial cells. H&E stain; 40×.

Blood vessels contain three concentric layers: intima, media, and adventitia (Figure 93.1). The intima is a single layer of endothelial cells (ECs) that rests on scant subendothelial connective tissue, separated from the media by the internal elastic membrane. The media is composed of smooth muscle cells (SMCs), collagen and elastin fibers, and fibroblasts. The outermost adventitia is a layer of dense fibroelastic connective tissue separated from the media of larger arteries by an external elastic membrane.

Hemodynamic stress evokes a variety of responses from all mural elements.[11,16] Vascular injury induces intimal migration and proliferation of SMCs. Smooth muscle contraction regulates regional blood flow and pressure. ECs have a myriad of functions, including the transfer of molecules across vessel walls and the modulation of blood flow, vascular resistance, hormone metabolism, SMC growth, and inflammatory and immune responses. Moreover, the prothrombotic and antithrombotic properties of ECs play a critical role in regulating hemostasis.

CONGENITAL ANOMALIES AND MALFORMATIONS

Shunts

Persistent Ductus Arteriosus

The ductus arteriosus is the fetal vascular communication between the pulmonary artery and aorta. Hypoplasia or asymmetry of ductal SMCs and aorta-like elastic tissue in the ductus cause failure to contract properly after birth and persistence of a vascular lumen.[2] Persistent ductus arteriosus (PDA) occurs in all species, but is most common in dogs.

Persistent Ductus Venosus

The fetal ductus venosus (DV) allows umbilical and portal venous blood flow to bypass the liver.[34] An abrupt decrease in blood flow and pressure in the umbilical sinus shortly after birth causes retraction and narrowing of the DV orifice, resulting in functional shunt closure. Permanent closure via connective tissue deposition starts within days after birth and is complete by 15–18 days in the dog. Persistent DV may cause hepatic lobular atrophy, reduplication of the hepatic arterioles, and unapparent portal veins in hepatic triads. The associated clinical signs include poor growth and hepatic encephalopathy.

Arteriovenous Fistula

Arteriovenous fistulas (AVFs) are direct arterial–venous communications without intervening capillary beds. AVFs may be congenital or develop from wounds, rupture of arterial aneurysms into adjacent veins, or inflammatory necrosis of vessel walls. AVFs consist of enlarged congested vessels with disorganized arterial walls and thin-walled, dilated venous channels. AVFs typically cause signs of recurrent hemorrhage; however, large AVFs may induce high-output heart failure, and AVF in the central nervous system (CNS) may cause neurologic signs and acute death.[12]

Telangiectasia

Telangiectasias are congenital or acquired exaggerations of preformed vessels. The condition is most common in the liver of cattle, characterized by increased deposition of basement membrane components and fibrosis resulting in hepatocyte atrophy and disruption of sinusoids.[14]

Varicose Veins

Varicosities, most common on the ovary and vulvar lips of horses, are dilated, tortuous veins produced by prolonged increased intraluminal pressure and loss of vessel wall support.

DEGENERATION OF ARTERIES

Atherosclerosis

Atherosclerosis is a chronic inflammatory response initiated by EC injury in the arterial wall, with progression mediated by macrophages, T cells, SMCs, and adjacent extracellular matrix.[37] Atherosclerosis is common in parrots and lesions occasionally develop in the coronary arteries of dogs with hypothyroidism or diabetes mellitus.[9] In early lesions, lipid-laden macrophages, T cells, and small amounts of extracellular lipid accumulate in fatty streaks. Over time, macrophage-derived interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α) recruit leukocytes, produce toxic oxygen species, and elaborate growth factors that stimulate medial SMC

migration, division, and deposition of extracellular matrix. Fatty streaks may evolve into atherosclerotic plaques that protrude into the vessel lumen causing ischemic damage. Focal plaque disruption exposes a central necrotic core of lipid, cholesterol clefts, cellular debris, foam cells, fibrin, variably organized thrombi, and other proteins. These substances are highly thrombogenic, resulting in vascular occlusion and embolization. Atrophy of the media underlying atherosclerotic plaques may lead to aneurysmal dilation and arterial rupture.

Vascular Amyloid

Amyloid is a pathologic protein formed from precursors such as serum-amyloid-associated protein, immunoglobulin light chain, and apolipoprotein A. Abnormal folding of precursors leads to oligomer formation and eventual deposition of fibrils as β-pleated sheets. Histologically, amyloid is an amorphous, acellular eosinophilic extracellular deposit. Amyloid shows a characteristic apple green birefringence under polarized light when stained with Congo red. Vascular amyloid deposition in the media of splenic and cardiac vessels is a common incidental finding in aged dogs, occasionally associated with EC necrosis and obliteration of the vascular lumen. Cerebral amyloid angiopathy in cortical and leptomeningeal vessels is common in aged dogs and cats due to the deposition of β-amyloid produced by SMCs. Apolipoprotein-AI-derived amyloid has been described in pulmonary vessels of old dogs, perhaps due to aberrant metabolism or mutant forms of apolipoprotein AI.

Mineralization

Uremic Gastropathy

Uremic gastropathy occurs most often in dogs with renal disease and occasionally in cats and horses. Vascular damage, presumably induced by a poorly characterized toxic peptide, results in gastric mucosal edema, congestion, and hemorrhage.[21] Medial necrosis, mineralization, and thrombosis of arterioles in the middle and deeper portions of the gastric mucosa may accompany EC injury. Despite vasculopathy, gastric necrosis and ulceration rarely develop.

Medial Calcific Sclerosis

Medial calcific sclerosis (Monckeberg's arteriosclerosis) refers to flat plaques in the media of elastic arteries composed of calcium and phosphorus deposits. Osseous metaplasia with hematopoietic tissue in the intertrabecular spaces may develop at these sites.

Metastatic Mineralization

Metastatic mineralization, due to disturbance of calcium and phosphorus metabolism, occurs in vitamin D toxicity, secondary to chronic granulomatous diseases such as Johne's disease, and in herbivores that consume plants such as *Solanum malacoxylon* or *Cestrum diurnum* that contain 1,25-dihydroxycholecalciferol or related compounds.[17]

Arterial Ruptures

Trauma is the most common cause of arterial rupture in all species. Aortic rupture, from the level of the aortic valve to the brachiocephalic trunk, is best documented in horses,[29] but has been reported in camelids and woodchucks. Degenerative changes of the media, including cyst formation, fragmentation and mineralization of elastin fibers, and accumulation of myxomatous ground substance, have been proposed as risk factors for aortic rupture. Rapid death is usually due to hemopericardium; however, dissection into the myocardium may occur. Additional syndromes in horses include uterine artery rupture and mycotic ulceration of the internal carotid or maxillary artery. Experimental copper deficiency in pigs leads to aortic rupture due to deficiency of the copper-containing enzyme lysyl oxidase. Similarly, lathyrism due to β-aminopropionitrile inhibits lysyl oxidase.

Aneurysms

Aneurysms are localized abnormal dilations of a vessel. True aneurysms consist of multiple layers of the vessel wall, while false aneurysms are hematomas (surrounded by fibrous connective tissue) that communicate with the vessel lumen. Abdominal artery aneurysms are common in Holstein cattle in the Northeastern United States.[13]

HYPERTROPHY OF ARTERIES

Chronic Hypoxic Pulmonary Hypertension

High-altitude disease refers to a syndrome of heart failure in cattle and pigs secondary to chronic hypoxic pulmonary hypertension. The pulmonary vasculature of cattle has a distinctive, well-developed media in small arteries and veins. After initial vasoconstriction, chronic hypoxia induces medial hypertrophy of the small pulmonary arteries and functional obstruction. Hypertrophy and dilation of the right ventricle eventually lead to right heart failure with ventral edema (brisket disease).[24] A mechanism of variable transforming-growth-factor-β1-mediated response to hypoxia has been proposed. This growth factor, elaborated by perivascular neutrophils and mononuclear cells of small pulmonary arteries, appears to stimulate differentiation of vascular fibroblasts into myofibroblasts, a crucial step in hypoxia-induced vascular remodeling.[8]

Hypertension

Systemic Hypertension

Systemic hypertension is most often reported in dogs and cats with chronic renal failure, diabetes mellitus,

hyperthyroidism, hyperadrenocorticism, and chronic anemia.[1] Systemic and local hypertension is reported in horses and cattle with laminitis. Chronic renal disease is typically associated with hyperplastic arteriosclerosis, as interlobular and intralobular renal arteries and afferent arterioles thicken due to medial hypertrophy and adventitial fibrosis. Hyaline arteriosclerosis refers to the deposition of a homogeneous, pale pink hyaline material that obscures mural elements. These changes occur frequently in the nodular arterioles of the spleen of dogs and pigs, and in the uterine and ovarian arteries of pregnant and postpartum animals. Similar changes, but of unknown significance, occur in the intramural coronary arteries and small meningeal and cerebral vessels of dogs. Arteriolar hyaline degeneration is seen in the heart of pigs with vitamin E/selenium deficiency (mulberry heart disease)[23] and edema disease.

Pulmonary Hypertension

Pulmonary hypertension can either cause or result from pulmonary artery disease. Cardiac anomalies that lead to left to right shunting, hypoxic pulmonary vasoconstriction, and pulmonary thromboembolism may increase vascular resistance resulting in pulmonary arterial hypertension, whereas left-sided heart failure causes venous pulmonary hypertension and pulmonary edema. A secondary vasculitis, resulting from EC degeneration and fibrinoid necrosis of the media, often accompanies the acute pulmonary hypertension of PDA. Chronic pulmonary hypertension leads to a spectrum of changes including medial hypertrophy, medial hypertrophy with intimal thickening, and medial hypertrophy with intimal thickening and concurrent plexiform lesions (plexogenic pulmonary arteriopathy). Plexiform lesions, indicative of end-stage damage, consist of small vessel networks spanning the lumen of thin-walled, dilated arteries.

Pulmonary Capillary Hemangiomatosis and Pulmonary Veno-Occlusive Disease Pulmonary capillary hemangiomatosis (PCH) and pulmonary veno-occlusive disease (PVOD) are uncommon idiopathic disorders that cause pulmonary hypertension. The hemodynamics of PCH and PVOD are caused by widespread vascular obstruction that originates either in alveolar capillaries (PCH) or pulmonary venules and small veins (PVOD). It is unknown if PCH and PVOD are two separate diseases or are manifestations of a single disease. Foci of PCH commonly occur in the lungs of dogs with PVOD.[35] Pulmonary capillary hemangiomatosis consists of areas of alveolar hypercellularity due to increased number of capillary ECs. By contrast, PVOD is characterized by severe and widespread remodeling of postcapillary venules and small pulmonary veins and occlusion of the vascular lumen by SMCs.

Feline Pulmonary Artery Medial Hypertrophy

Pulmonary artery medial hypertrophy is a striking vascular lesion in cats.[25] The vascular changes, however, do not cause pulmonary hypertension or right ventricular hypertrophy. Medial changes range from mild thickening to marked hypertrophy and hyperplasia, with intimal proliferation and encroachment on the vascular lumen. Fibromuscular hyperplasia commonly develops in the adjacent pulmonary parenchyma and around alveolar ducts. The cat lungworm, *Aelurostrongylus abstrusus*, produces similar lesions, but most cats with medial hypertrophy are parasite-free.

Fibromuscular Dysplasia

Fibromuscular dysplasia (FMD) is an idiopathic, noninflammatory, nonatherosclerotic segmental vascular wall thickening and central stenotic disease of arteries (and rarely veins).[30] Subtype classification is based on the layer of affected vessel wall, i.e., media, intima, or adventitia. Several canine studies have demonstrated intramyocardial coronary arterial changes similar to intimal FMD. These lesions are often clinically insignificant, but may develop secondary to pathologic conditions such as subaortic stenosis.[5] In the author's experience, segmental FMD characterized by stenosis, intimal thickening, and proliferation of SMC in coronary and atrial arteries may be associated with myocardial necrosis, fibrosis, and sudden cardiac death in dogs.

VASCULITIS

A wide variety of agents cause infectious vasculitis, including viruses, bacteria, chlamydia, rickettsia, fungi, protozoa, and helminths. These agents may directly injure ECs or act indirectly through the elaboration of toxins. Noninfectious vasculitides include hypersensitivity and drug reactions, toxins, and uremia. Although the etiology of vasculitis is often unknown, humoral and cell-mediated immunity are thought to play important roles.[3] Clinical significance varies depending on the number, size, and type of affected vessels, and the presence of thrombosis, ischemia, and infarction.

Vasculitis is characterized histologically by accumulation of leukocytes within and around vessel walls. Fibrinoid change refers to the deposition of a homogeneous or granular material composed of fibrin, immunoglobulins, complement, and platelets, with collagen and SMC degeneration. Inflammatory cells may consist primarily of neutrophils, lymphocytes, or macrophages. The presence of fragmented neutrophil nuclei is classified as leukocytoclastic vasculitis, whereas its absence is classified as nonleukocytoclastic vasculitis.

Viral Vasculitis

Equine Herpesvirus-1

Neurotropic strains of equine herpesvirus type 1 cause an abrupt onset of neurological signs, often due to spinal cord lesions.[36] Grossly, hemorrhagic foci are scattered throughout the brain and spinal cord. Histologic lesions

include lymphocytic infiltrates in the media and adventitia, and EC degeneration.

Canine Herpesvirus-1

Canine herpesvirus-1 infection has been associated with keratitis, encephalitis, abortion, neonatal mortality, and infertility.[26] Serosanguinous pleural and peritoneal effusions are typically accompanied by petechial and ecchymotic hemorrhages scattered throughout the subserosal tissues. Small necrotic foci are found in many organs; however, inflammation and intranuclear inclusions are rare.

Malignant Catarrhal Fever

Malignant catarrhal fever (MCF) is an acute, fulminant vasculitis of cattle, wild ruminants, and occasionally swine. A T-lymphotropic gamma herpesvirus causes Wildebeest-associated MCF in exotic hoofstock, whereas ovine herpesvirus 2 causes sheep-associated MCF in cattle. Signs of MCF include generalized lymphoproliferation and mucocutaneous and cutaneous ulceration. Characteristic vascular lesions consist of focal or segmental perivascular accumulation of herpesvirus-infected CD8+ T cells in many organs.[28] Fibrinoid necrosis may affect a single vascular layer or may be transmural. Although mortality rates are high, survivors develop striking proliferative concentric fibrointimal plaques.

Equine Viral Arteritis

Equine viral arteritis (EVA) is caused by a non-arthropod-borne enveloped positive stranded RNA virus in the order Nidovirales.[4] Panvasculitis develops in many organs, causing clinical signs of edema, congestion, hemorrhage, or abortion due to myometritis. Mild vascular changes consist of perivascular edema with EC hypertrophy and lymphocytic infiltrates, whereas severe lesions include fibrinoid necrosis with marked perivascular and vascular lymphocytic and occasional histiocytic infiltrates. Virus localizes to vascular EC, SMC, and pericytes, but may be found in a variety of epithelial cell types.

Feline Infectious Peritonitis

Feline infectious peritonitis virus (FIPV) is a single-stranded, positive sense, linear RNA virus composed mainly of nucleocapsid, transmembrane, and spike proteins. FIPV evolves from feline enteric corona virus (FECV) in a multistep mutational process. FECV infection generally causes no signs, or mild enteritis. Transition of FECV to FIPV is accompanied by the acquisition of macrophage tropism due to mutations in the C-terminal domain of the spike protein. FIPV infects macrophages and efficiently disseminates when the virus complexes with antibodies against the spike protein, a process referred to as antibody-dependent enhancement. Virus bound to spike-protein-specific antibodies is taken up by Fc-receptor-mediated endocytosis, with subsequent escape of the viral genome into the cytoplasm.[27,32] Most FIPV-infected cats clear the virus, but fecal shedding may persist for several months. Cats that develop clinical signs are typically less than 2 years of age and mount an ineffective cell-mediated immune response characterized by low CD4+ T-cell counts, decreased levels of IL-12 and interferon-gamma (IFN-γ), and increased TNF-α and IL-1β.[18]

Effusive (wet) and noneffusive (dry) FIP represent extremes of a clinical continuum. Effusive vasculitis often manifests as abdominal effusion, with serosal surfaces covered by fibrin and perivascular necrosis and inflammation. Pleural and pericardial effusions are less common. Noneffusive FIP typically affects eyes, CNS, kidneys, liver, pancreas, and intestines. Microscopically, varying proportions of lymphocytes, macrophages, plasma cells, and neutrophils accumulate in and around venules. Fibrin deposition correlates with vasculitis severity, while vascular degeneration and thrombosis lead to parenchymal necrosis. Noneffusive FIP clinically resembles lymphosarcoma, steatitis, mycotic infection, and toxoplasmosis; however, histopathology provides the definitive diagnosis of FIP.

Rocky Mountain Spotted Fever

Rocky Mountain spotted fever is caused by *Rickettsia rickettsii*, a Gram negative bacterium transmitted by tick vectors including *Dermacentor variabilis*, *Dermacentor andersoni*, and *Amblyomma americanum*.[33] Rickettsia replicate within ECs of small blood vessels in a wide variety of mammals, but dogs most often develop disease. Clinical signs include myalgia/arthralgia, lymphadenomegaly, dyspnea, conjunctivitis, edema, vomiting, diarrhea, and petechial or hemorrhagic diathesis. Acute meningoencephalitis, often affecting the vestibular system, may be seen as well as inflammation of retinal vessels. Necrotizing vasculitis of small veins, capillaries, and arteries is accompanied by perivascular lymphocytic infiltrates. EC necrosis and inflammation lead to increased vascular permeability, edema, and multifocal hemorrhage and thrombosis.

Verminous Arteritis

Strongylus Vulgaris

Strongylus vulgaris is the most serious of the large and small equine strongyles.[7] Larvae are unable to penetrate the internal elastic lamina and therefore migrate within the vascular intima. After reaching the cranial mesenteric artery, larvae undergo 3–4 months of development before molting and returning to the cecum and colon walls. Tortuous intimal tracks, consisting of fibrin, necrotic cellular debris, and mixed inflammatory cells are eventually covered by ECs and converted into fibroelastic thickenings. Clinical signs develop secondary to occlusive thrombi, often in the cranial mesenteric artery, but also in the aorta, renal arteries, and celiac artery. Affected vessels may develop saccular or

fusiform aneurysms due to weakening of the wall. Cecal and colic artery thromboembolism, pressure on abdominal autonomic plexuses, and release of toxins from dead larvae induce colic.

Heartworm Disease

Heartworm disease caused by *Dirofilaria immitis* is common in dogs, but occurs in other mammals including domestic and wild felids, wild canids, ferrets, sea lions, muskrats, and horses. Mosquitoes of the genera *Aedes*, *Culex*, and *Anopheles* transmit infective third-stage larvae that migrate through connective tissues to veins, ultimately maturing in the heart 3–4 months later. Adult worms reside primarily in the pulmonary arteries and right ventricle, but may extend into the right atrium and vena cava in heavy infestations. Pulmonary veins in the caudal lung lobes often develop fibromuscular intimal proliferation, resulting in pulmonary hypertension and right heart failure. Worm larvae, adults, and an intracellular bacteria *Wolbachia* induce eosinophilic and neutrophilic endoarteritis, and rugose myointimal proliferation. Adulticide therapy may exacerbate thromboembolism, pulmonary hypertension, and induce granulomatous reactions.

Photosensitization Dermatitis

Photosensitization dermatitis occurs in animals that ingest plants containing photodynamic agents or drugs (type I), have an inability to metabolize heme pigments (type II), or have liver disease that impairs excretion of phylloerythrin, a degradation product of chlorophyll (type III). Type III, or hepatogenous photosensitization, is associated with cholestatic liver disease. Gross lesions occur in lightly colored areas of skin exposed to sunlight, with histopathologic findings of fibrinoid vascular degeneration, dermal edema, and epidermal coagulation necrosis.[15]

Immune-Mediated Vasculitis

Among the various immune mechanisms causing vasculitis, antigen–antibody complexes formed in the circulation (type II hypersensitivity) or locally (type III hypersensitivity) are most common. Complexes precipitate in vascular walls and bind to neutrophils via Fc or C3b receptors, thereby triggering inflammation, proinflammatory cytokine and lysosomal enzyme release, and generation of oxygen-derived free radicals. Complement activation leads to neutrophil and monocyte recruitment and increased vascular permeability. Immune complex injury results in vascular wall necrosis and fibrinoid changes. Lesions may resolve as immune complexes are catabolized.

Equine Purpura Hemorrhagica

Equine purpura hemorrhagica occurs in 1–2% of horses after acute infection with *Streptococcus equi*.[22] Deposits of streptococcal M protein complexed with IgA or IgM damage ECs, and induce systemic leukocytoclastic vasculitis. Head and hind leg edema is accompanied by petechia on mucosal and serosal surfaces. Serious sequelae include immune complex glomerulonephritis and infarction of skeletal muscle, skin, gastrointestinal tract, pancreas, and lungs, similar to the human syndrome Henoch–Schönlein purpura.

Canine Juvenile Polyarteritis Syndrome

Canine juvenile polyarteritis syndrome (CJPS) occurs primarily in young Beagles, but has been documented in other breeds.[6,31] Affected dogs have a sudden onset of fever, anorexia, and a hunched stance, with frequent remissions and relapse. Small to medium muscular arteries in the dura mater and leptomeninges of the cervical spinal cord, extramural coronary arteries, and cranial mediastinum develop segmental vasculitis and perivasculitis. Lymphocytes, macrophages, and plasma cells encircle the vessel and often expand into an eccentric nodule, with neutrophils predominating in acute lesions. Fibrinoid change and rupture of the elastic lamina, and fibrin thrombi, hemorrhage, and necrosis of adjacent tissues characterize severe lesions. CJPS has many features of an immune complex syndrome of children, Kawasaki disease, including high serum IgA, low peripheral T cells and high B cells, mononuclear activation, antineutrophil cytoplasmic antibodies, and suppressed mitogenic response. Other causes of canine systemic vasculitis include leishmaniasis and drug toxicities.

Proliferative Arteritis of the Nasal Philtrum of Dogs

Proliferative arteritis of superficial and deep dermal blood vessels restricted to the nasal philtrum is seen infrequently in large breed dogs. SMC proliferation, accompanied by fibrin thrombi, occludes the vascular lumen. The resulting ischemia causes ulceration and potentially fatal hemorrhage.[20]

Paraneoplastic Vasculitis

Paraneoplastic leukocytoclastic vasculitis with variable degrees of necrosis is occasionally seen in association with lymphosarcoma.[19]

Thrombotic Microangiopathy

Thrombotic microangiopathy (TMA) is a clinical syndrome characterized by a variable combination of fever, thrombocytopenia, microangiopathic hemolytic anemia with schistocytes, neurologic deficits, and renal failure.[38] The causes of TMA in people are diverse. External triggers include viruses, bacterial Shiga toxins (hemolytic uremic syndrome), endotoxin, antibodies, and drugs. Thrombotic thrombocytopenic purpura (TTP) is caused by either an inherited or acquired deficiency of the enzyme a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13), which cleaves very large von Willebrand factor multimers and prevents inappropriate platelet aggregation and microvascular thrombosis. Regardless of cause, widespread hyaline thrombi form in the microcirculation

leading to platelet consumption and organ dysfunction. Although disseminated intravascular coagulation (DIC) also causes widespread thrombosis in the microvasculature, DIC is pathophysiologically distinct from TMA. Activation of the coagulation cascade resulting in factor depletion and fibrin degradation is typical of DIC, but uncommon in TMA.

Cutaneous and renal glomerular vasculopathy is a TMA of unknown etiology in dogs, characterized by ulceration of the distal extremities and acute renal injury.[10] Although first recognized in Greyhounds in the Southeastern United States of America, the disease has been recognized in multiple breeds in North America and Europe. Fibrinoid necrosis of glomerular arterioles with less frequent involvement of intralobular and arcuate arteries is accompanied by thrombosis. Fibrinoid necrosis of small dermal blood vessels underlies areas of cutaneous ulceration. Dermatohistologic features include atrophic sebaceous glands and hair follicles surrounded by variable numbers of neutrophils and foamy macrophages that obscure the follicular dermal epithelial interface.

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CHAPTER 94

Treatment of Hemostatic Defects

ROBERT GOGGS and ALEX M. LYNCH

Treatment of Hemorrhagic Disorders
- Transfusion for Platelet Disorders
- Transfusion for Coagulopathy and VWD
- Drug Therapy
  - Vitamin K Therapy
  - Desmopressin
  - Antifibrinolytic Drugs
- Topical Agents

Treatment of Thrombotic Disorders
- Clinical Thrombotic Disorders
- Antiplatelet drugs
  - Aspirin
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- Anticoagulants
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  - Unfractionated Heparin
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  - Direct Oral Anticoagulants
- Therapeutic Thrombolysis

Acronyms and Abbreviations

ACS, acute coronary syndromes; aPTT, activated partial thromboplastin time; AT, antithrombin; ATE, arterial thromboembolism; AXA, anti-Xa activity; BMBT, buccal mucosal bleeding time; COX, cyclooxygenase; CRASH-2, Clinical Randomization of an Antifibrinolytic in Significant Haemorrhage 2; CRI, constant rate infusion; CURATIVE, Consensus on the Rational Use of Antithrombotics in Veterinary Critical Care; DDAVP, desamino-8-D-arginine vasopressin (desmopressin); DIC, disseminated intravascular coagulation; DMSO, dimethyl sulfoxide; EACA, ε-aminocaproic acid; FP, frozen plasma; FFP, fresh frozen plasma; FWB, fresh whole blood; IM, intramuscular; IMHA, immune-mediated hemolytic anemia; IMT, immune-mediated thrombocytopenia; IV, intravenous; LMWH, low-molecular-weight heparin; PAR, protease-activated receptor; PC, platelet concentrate; PCI, percutaneous coronary intervention; PO, per os; PRP, platelet-rich plasma; PT, prothrombin time; PTE, pulmonary thromboembolism; SC, subcutaneous; tPA, tissue plasminogen activator; TXA, tranexamic acid; TXA_2, thromboxane A_2; UFH, unfractionated heparin; VTE, venous thromboembolism; VWD, von Willebrand disease; VWF, von Willebrand factor.

This section provides a management overview of hemorrhagic syndromes, and more in-depth review of antithrombotic agents, i.e., antiplatelet, anticoagulants, and thrombolytic drugs. Although knowledge gaps remain, the American College of Veterinary Emergency and Critical Care recently published consensus guidelines on the rational use of antithrombotics in veterinary medicine.[47]

TREATMENT OF HEMORRHAGIC DISORDERS

Transfusion should be considered for any patient with a documented bleeding disorder with signs of active bleeding or before invasive procedures. Blood component therapy (Table 94.1 and Section VIII) is usually the best means to provide cells or proteins to support hemostasis while minimizing risks of antigen exposure or volume overload. Since transfusion may transiently correct underlying deficiencies, baseline samples (EDTA and citrate blood) for laboratory assays should be collected before transfusion. At minimum, platelet count and coagulation screening tests such as the prothrombin time (PT) and activated partial thromboplastin time (aPTT) should be evaluated, with ancillary testing as needed to diagnose and manage complex disorders (see Chapter 88 and Section VI).[63,18] Global hemostatic tests may help monitor response,[48] but cessation

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TABLE 94.1 Blood products for hemorrhagic disorders.

Products	Dosage guidelines	Indications
Platelet-rich plasma[a]	6–10 mL/kg	Thrombocytopenia, platelet dysfunction
DMSO cryopreserved platelets[b]	1 unit/10 kg	Thrombocytopenia, platelet dysfunction
Lyophilized platelets[c]	3×10^6 particles/kg 1 × 8 mL vial/5 kg	Thrombocytopenia, platelet dysfunction
Fresh frozen plasma	6–12 mL/kg	Coagulation factor and VWF deficiencies
Cryoprecipitate[d]	1 unit/10 kg	Fibrinogen, factor VIII, factor XIII, and VWF deficiencies
Cryo-poor plasma	6–12 mL/kg	Deficiencies of factors II, VII, IX, X, and XI
Fresh whole blood	12–20 mL/kg	Replacement of platelets and all factors

[a]Platelet-rich plasma containing at least 200×10^6 platelets/mL.
[b]100 mL unit containing at least 5×10^{10} platelets total, minimum concentration 5×10^8 platelets/mL.
[c]Lyophilized powder, reconstituted with 8 mL water for injection, final concentration 1.5×10^9 particles/mL.
[d]1 unit cryoprecipitate prepared from 200 mL fresh frozen plasma.

of hemorrhage, cardiovascular stabilization, and a stable hematocrit remain the most important clinical endpoints.[28]

Transfusion for Platelet Disorders

Thrombocytopenia is the most common primary hemostatic defect, but acquired and hereditary platelet dysfunction may also cause hemorrhage (see Chapter 86). Management of the underlying disorder is the most important intervention for control of bleeding for thrombocytopenic patients. Severe, life-threatening hemorrhage may require transfusion, although transfused cells are often rapidly removed from circulation, thus precluding normalization of platelet count. Acquired platelet dysfunction typically causes mild to moderate bleeding that resolves after correction of the underlying disorder. By contrast, patients with hereditary platelet dysfunction typically require transfusion for treatment or prophylaxis. In human medicine, platelet counts above 10 000/μL are considered safe in the absence of bleeding, while counts below 100 000/μL associated with severe bleeding or central nervous system hemorrhage prompt intervention.[1]

Options for platelet transfusion include fresh whole blood (FWB), platelet-rich plasma (PRP), platelet concentrates (PC), cryopreserved platelets, and lyophilized platelet products (see Chapter 86).[21] FWB is often used in clinical practice as the source of platelet transfusion. Platelets cryopreserved at –30°C in dimethyl sulfoxide (DMSO) have longer shelf-life than fresh platelets (6 months vs 5 days),[65] but are less effective.[21,50] A second-generation, trehalose-stabilized, lyophilized canine platelet product is commercially available, and under evaluation in clinical trials. Other novel platelet products are also in development.[83]

Transfusion for Coagulopathy and VWD

Plasma product transfusion is the mainstay for management of severe coagulopathy and von Willebrand disease (VWD). The choice of product depends on the underlying disorder (see Chapters 88 and 100). Transfusion of components rather than FWB is preferred to reduce the risk of volume overload and antigen exposure. A shift toward whole blood transfusion for major trauma in people has resulted from landmark trials indicating survival advantage for red blood cells, plasma, and platelets transfused in a 1:1:1 ratio.[58] However, comparable indications and product availability are uncommon in veterinary medicine.

In single-factor defects such as hemophilia A or B, components optimize use of resources. Components for hemophilia A (FVIII deficiency) include fresh frozen plasma (FFP) or cryoprecipitate, whereas hemophilia B (FIX deficiency) is appropriately managed with FFP or cryo-poor plasma (Table 94.1). Simultaneous deficiency of many factors occurs in patients with severe liver disease, vitamin K deficiency, hemorrhagic disseminated intravascular coagulation (DIC), and following massive transfusion. These patients are typically best managed with FFP particularly if the nature of the underlying disorder is uncertain. The various products have distinct dosing guides (Table 94.1).

Drug Therapy

Vitamin K Therapy

Vitamin K deficiency is a common acquired coagulopathy, often arising due to anticoagulant rodenticide toxicity or hepatobiliary disease (see Chapter 89). Patients with severe hemorrhage should be transfused with FWB or components to immediately replace active factors. Pretransfusion samples for coagulation testing should be drawn to identify characteristic coagulopathy (e.g., prolonged PT and aPTT), whereas samples for toxicologic testing may identify specific products.Vitamin K replacement should be given as vitamin K_1 (phytomenadione or phytonadione), rather than vitamin K_3 (menadione). Oral administration is appropriate in the absence of gastrointestinal signs or malabsorptive disorders and may provide better bioavailability than subcutaneous (SC) injection. The SC route is preferred for parenteral

vitamin K_1, because intravenous (IV) administration may cause anaphylaxis, and intramuscular (IM) injection risks hematoma formation. Vitamin K_1 should be given at an initial dose of 5 mg/kg SC using a small-gauge needle, followed by a maintenance dosage of 2.5 mg/kg per os (PO) every 12 hours. New factor synthesis to correct coagulopathy occurs over a lag period of 12–24 hours from initiation of therapy. Prolonged vitamin K_1 therapy (4–6 weeks) may be required for long-acting rodenticide toxicities. A recheck PT to gauge response can be performed 48 hours after cessation of therapy. If the PT is prolonged, vitamin K_1 therapy should be continued for 1–2 weeks, with recheck after 48 hours. Gastrointestinal decontamination within 6 hours of known rodenticide ingestion may prevent the need for vitamin K_1 therapy, provided PT is normal at presentation (to identify prior exposure) and PT remains normal 48 hours later with no signs of hemorrhage.[96]

Desmopressin

Desmopressin (desamino-8-D-arginine vasopressin, DDAVP) is a synthetic analog of arginine vasopressin that produces antidiuresis without vasoactive side-effects. In people, DDAVP effectively induces (via V2 receptors) von Willebrand factor (VWF) release from endothelial stores.[80] Dogs demonstrate relatively low qualitative and quantitative VWF release in response to DDAVP, but the drug shortened in vivo bleeding time and improved surgical hemostasis in dogs with type 1 VWD and platelet dysfunction.[22] In addition to VWF release, DDAVP enhances platelet function via VWF-independent mechanisms including microparticle formation,[61] and enhanced procoagulant expression.[27] Desmopressin is most often used in human medicine to treat mild to moderate VWD, hemophilia A, and platelet dysfunction.[80] In dogs, DDAVP is used as adjunctive treatment of type 1 VWD, and for surgical prophylaxis (administered 30 minutes before surgery). Intravenous DDAVP reversed aspirin-induced buccal mucosal bleeding time (BMBT) prolongation in dogs.[35] Injectable or intranasal DDAVP is administered at 1–4 mcg/kg SC or IV.[69] Onset of action is approximately 30 minutes, duration is usually 2 hours, with diminished effect of repeated doses due to depletion of VWF stores. Response varies among individuals; thus, blood products should be available to control bleeding.

Antifibrinolytic Drugs

The lysine analogs tranexamic acid (TXA) and ε-aminocaproic acid (EACA) (Table 94.2) suppress fibrin degradation by reversibly blocking the lysine-binding sites for fibrin on plasminogen. In people, the potency of TXA is approximately 8–10 times that of EACA.[111] In 2010, the Clinical Randomization of an Antifibrinolytic in Significant Haemorrhage 2 (CRASH-2) human trauma trial showed reduction in mortality with early administration of TXA as a loading dose of 1 gram over 10 minutes, followed by 1 gram infused over 8 hours.[107] However, TXA administration more than 3 hours after injury appeared to increase the risk of fatal hemorrhage.[101] TXA is also used to reduce surgical blood loss.[94] Although efficacy data are limited, TXA has been used in dogs for clinical bleeding secondary to neoplasia, immune-mediated thrombocytopenia (IMT), trauma, surgery, anticoagulant intoxication, and liver failure.[66] Dogs may be relatively hyperfibrinolytic, suggesting higher dosages may be needed to inhibit fibrinolysis in dogs versus humans.[44] A recent study in healthy dogs demonstrated safety of TXA after a 10 mg/kg IV bolus followed by 10 mg/kg/h constant rate infusion (CRI) over 3 hours. Vomiting associated with initial doses of TXA of 15–20 mg/kg was noted.[67] In another canine study, TXA induced vomiting in a dose-dependent manner and a single IV dose of 50 mg/kg produced a prolonged duration of antifibrinolytic activity.[64] The use of EACA has been described in Greyhounds to prevent delayed postoperative bleeding,[72] and another study of limb amputation or gonadectomy demonstrated significantly decreased incidence of bleeding in EACA-treated greyhounds.[81]

Administration of antifibrinolytic agents to patients with prothrombotic disease is controversial.[52] Antifibrinolytic agents are generally contraindicated for secondary hyperfibrinolysis associated with DIC.[123] Experimental administration of TXA to cats caused adverse effects, including seizures and myocardial injury, thus warranting cautious use in this species.[98,62]

Topical Agents

Topical agents, classified as hemostats, sealants, and adhesives, are used for direct application to control bleeding and close tissue surfaces at sites of surgical or traumatic injury (Table 94.3).[116–118] Hemostats provide a

TABLE 94.2 Drug therapy for hemorrhagic disorders.

Drugs	Dosage guidelines	Indications
Vitamin K_1	2 mg/kg/day PO[a] or SC	Vitamin-K-dependent coagulopathy
Desmopressin	1 μg/kg SC[b]	Type 1 vWD, platelet dysfunction
ε-aminocaproic acid (EACA)	100 mg/kg IV or PO q6h	Perioperative hemorrhage, trauma, spontaneous hemoperitoneum
Tranexamic acid (TXA)	10 mg/kg IV q3–4h Or initial loading dose of 10 mg/kg IV then 30–50 mg/kg PO q3–4h	Perioperative hemorrhage, trauma, spontaneous hemoperitoneum

TABLE 94.3 Topical hemostatic agents.

Category	Products (tradename, source)
Hemostats: mechanical	Porcine gelatin (Gelfoam, Pharmacia; Surgifoam, Ethicon/J&J) Bovine collagen (Avitene, Davol/Bard; Helistat, Integra; Instat, Ethicon/J&J) Oxidized regenerated cellulose (Surgicel, Ethicon/J&J) Polysaccharide spheres (Arista, Medafor; HemoStase, CryoLife; Vitasure; Orthovita)
Hemostats: active	Bovine thrombin (Thrombin-JMI, King/Pfizer) Pooled human thrombin (Evithrom, Ethicon/J&J) Recombinant human (Recothrom, ZymoGenetics/BMS)
Hemostats: flowable	Human thrombin with bovine gelatin (Floseal, Baxter) Porcine gelatin ± thrombin (Surgiflo, Ethicon/J&J)
Hemostats: fibrin sealant	Pooled human plasma (Tisseel, Baxter; Evicel, Ethicon/J&J) Human plasma/bovine collagen/bovine thrombin (Vitagel, Orthovita) Pooled human plasma/equine collagen (Tachosil, Baxter)
Synthetic sealants	Polyethylene glycol (PEG) polymers (Coseal, Baxter) PEG and trilysine amine (DuraSeal, Covidien) PEG and human serum albumin (ProGel, NeoMend) Bovine serum albumin/10% glutaraldehyde (BioGlue, Cryolife) Octyl cyanoacrylate and butyl lactoyl cyanoacrylate (Omnex, Ethicon/J&J)
Adhesives	Octyl cyanoacrylate (Dermabond, Ethicon/J&J; Derma+Flex, Chemence) Octyl cyanoacrylate (LiquiBand Flex, AMS; Octylseal, Medline; SurgiSeal Adhezion) Butyl cyanoacrylate/polyester mesh (Indermil, Covidien; Histoacryl, TissueSeal) Pooled human plasma (Artiss, Baxter)

Source: Modified from Spotnitz and Burks.[118]

mechanical barrier and matrix for fibrin formation and include products derived from porcine gelatin, bovine collagen, cellulose, and polysaccharide spheres. Thrombin combined with hemostats enhances their activity, and thrombin is used with plasma-derived fibrinogen as "fibrin sealants." Synthetic products include cyanoacrylate adhesives for superficial skin closures and polyethylene glycol sealants for durotomy and abdominal procedures. A two-component adhesive consisting of bovine serum albumin and glutaraldehyde is also approved for cardiac and vascular surgery. The potential side-effects of the topical agents include allergic reactions, sensitization to heterologous proteins, embolization, wound infection, and delayed healing.

TREATMENT OF THROMBOTIC DISORDERS

Clinical Thrombotic Disorders

The Consensus on the Rational Use of Antithrombotics in Veterinary Critical Care (CURATIVE) guidelines assist in management of thrombotic disorders by categorizing patients as high-risk or low/moderate-risk,[33] accounting for the underlying disease. Dogs with immune-mediated hemolytic anemia (IMHA) or protein-losing nephropathy, cats with cardiomyopathy, and dogs or cats with more than a single thrombotic risk factor are categorized as high-risk. The low/moderate-risk category comprises dogs or cats with a single or transient risk factor. Formalized human thrombosis risk scores, such as the PADUA index, have not yet been developed for animals.[46]

In diseases associated with venous thromboembolism (VTE), fibrin-rich and platelet-poor thrombi typically form under low-shear conditions.[2] By contrast, arterial thromboembolism (ATE) is characterized by platelet-rich thrombi that form under high shear. This pathophysiologic difference leads to use of anticoagulants for VTE,[134] and antiplatelet agents for ATE.[112] However, platelets support fibrin formation,[54] with potential benefit for antiplatelet drugs in VTE,[17,110] and anticoagulants may be used for human acute coronary syndromes (ACS).[25] Many hypercoagulable states in small animals result in VTE and ATE, providing rationale for combined use of anticoagulants and antiplatelet drugs (Table 94.4).

Antiplatelet Drugs

Aspirin

Aspirin inhibits platelet aggregation by irreversibly acetylating platelet cyclooxygenase (COX), thereby preventing formation of thromboxane A_2 (TXA_2). Aspirin also inhibits endothelial cell COX activity, resulting in decreased prostacyclin synthesis and loss of anti-aggregating and vasodilating properties. Endothelial cell COX inhibition is reversible (via de novo synthesis), therefore aspirin exerts a net antithrombotic effect.

Multiple studies in various canine models demonstrate effective prevention of ATE by aspirin administration more than 2 days before thrombogenic insult. Aspirin therapy is effective in endarterectomy and angioplasty models,[20,42,84] and after therapeutic fibrinolysis in

TABLE 94.4 Antithrombotic Agents.

Drug	Drug class	Drug type	Dose
Aspirin	Oral antiplatelet agent	Cyclooxygenase inhibitor	Dogs: 1–2 mg/kg PO q24h Cats: Not recommended
Clopidogrel	Oral antiplatelet agent	ADP receptor antagonist	Dogs: 1.1–3 mg/kg PO q24h Cats: 18.75 mg PO q24h
UFH	Parenteral anticoagulant	Antithrombin potentiator FIIa, FXa inhibitor	Dogs: IV 100 U/kg bolus, then 20–37.5 U/kg/h CRI SC 150–300 U/kg q6h Cats: SC 250 U/kg q6h
Dalteparin	Parenteral anticoagulant	Antithrombin potentiator FXa inhibitor	Dogs: 100–175 U/kg SC q8h Cats: 75 U/kg SC q6h
Enoxaparin	Parenteral anticoagulant	Antithrombin potentiator FXa inhibitor	Dogs: 0.8 mg/kg SC q6h Cats: 0.75–1 mg/kg SC q6–12h
Fondaparinux	Parenteral anticoagulant	Antithrombin potentiator FXa inhibitor	Dogs: No data Cats: 0.06–0.20 mg/kg SC q12h
Rivaroxaban	Oral anticoagulant	Direct FXa inhibitor	Dogs: 1–2 mg/kg PO q24h Cats: 0.5–1 mg/kg PO q24h
Apixaban	Oral anticoagulant	Direct FXa inhibitor	Dogs: No data Cats: 0.2 mg/kg PO q24h

dogs.[102] Graft-model studies also suggest aspirin is efficacious in dogs.[125,19,9,41] Dosages as low as 0.5 mg/kg/day and as high as 15 mg/kg/day are reportedly safe and effective. Oral aspirin, given for 2–3 days prior to insult, may be effective for ATE prevention in dogs. No evidence base supports a defined dosage; however, the authors suggest 1–2 mg/kg/day.

Experimental studies yield conflicting effects of aspirin on feline platelet function, potentially due to varying dosages, outcome measures, and test methodologies.[100,10,23] In at-risk cats, ATE recurs in 20–28% cases treated with aspirin.[104,115] A randomized controlled trial of aspirin for secondary prevention of feline ATE found a 75% incidence of recurrence within 12 months. This trial demonstrated inferiority of aspirin to clopidogrel for feline ATE thromboprophylaxis;[56] thus, CURATIVE guidelines recommend against aspirin as a sole antithrombotic in cats at risk for ATE.

Clopidogrel

Clopidogrel is a thienopyridine that irreversibly inhibits platelet $P2Y_{12}$ ADP receptors. Clopidogrel must undergo hepatic biotransformation to its active metabolites. Inhibition of $P2Y_{12}$ impairs degranulation and aggregation, thus reducing thrombus stability under flow conditions. In people, clopidogrel's maximal effects develop after 3–5 days of daily administration, while activity disappears by 5–7 days after discontinuation. Clinical trials demonstrate that clopidogrel significantly reduces the risk of stroke, myocardial infarction, and vascular death compared to aspirin. Clopidogrel is used with aspirin as dual antiplatelet therapy for stable angina, ACS, and stroke prevention.[68]

Clopidogrel is an effective antiplatelet agent in canine studies,[136,59,128,127,13,12] particularly against provoked ATE. Dosages and efficacy vary, likely due to differences in model systems and thrombotic stimuli. A dose of 4 mg/kg PO q24h after a 10 mg/kg loading dose provides protection against provoked ATE in dogs.[12] The CURATIVE guidelines recommend clopidogrel 1.1–3 mg/kg PO q24h for ATE prevention in dogs.[47]

Multiple studies in cats suggest clopidogrel is effective for ATE thromboprophylaxis.[55,121,34,53] Dosages of 18.75 mg PO q24h are typically used, and a well-conducted randomized clinical trial of feline ATE demonstrated that clopidogrel is a more effective antithrombotic agent than aspirin.[56]

Other Antiplatelet Agents

Other antiplatelet agents target a wide range of cell-surface receptors.[45] Prasugrel, ticagrelor, and cangrelor target the $P2Y_{12}$ receptor.[130] Prasugrel is a potent, orally administered thienopyridine with more consistent biotransformation than clopidogrel. Ticagrelor is an active, orally administered, reversible $P2Y_{12}$ inverse agonist. Both prasugrel and ticagrelor are used for management of ACS. Cangrelor is a modified ATP derivative with high-affinity, reversible $P2Y_{12}$ inhibition immediately active upon intravenous CRI.[4] Cangrelor has a short half-life and is used for myocardial infarction or emergency percutaneous coronary intervention (PCI).

Abciximab, tirofiban, and eptifibatide are potent, reversible inhibitors of the platelet fibrinogen receptor (GPIIb/IIIa) given by IV infusion for PCI. There are no defined indications and no clinical studies have evaluated these drugs in veterinary practice. Experimental

studies suggest efficacy in dogs,[124,60] and abciximab demonstrated efficacy in a feline model of arterial injury.[16] Eptifibatide was associated with fatal cardiotoxicity in cats.[15]

Vorapaxar is an inhibitor of protease-activated receptors (PARs) that act as tethered ligand receptors for thrombin. Vorapaxar reduces the risk of cardiovascular death, myocardial infarction, and stroke in patients with stable atherosclerosis receiving standard therapy with aspirin and/or clopidogrel. Vorapaxar increases risk of bleeding and is contraindicated in patients with previous cerebrovascular events.[24] Lack of homology between human and animal platelet PARs may limit use of vorapaxar in veterinary medicine.

Anticoagulants

Warfarin

Warfarin (Coumadin), an oral vitamin K antagonist, was the principal anticoagulant in human medicine for decades. Warfarin inhibits vitamin K epoxide reductase, thus, preventing the cyclic conversion of vitamin K necessary for activation of the vitamin-K-dependent clotting factors.[133] Concomitant inhibition of vitamin-K-dependent anticoagulants, protein C and protein S, leads to a temporary prothrombotic tendency. An alternate anticoagulant (e.g., unfractionated heparin, UFH) is typically given for the first 4–5 days of warfarin therapy to prevent thrombosis.

Efficacy of warfarin has been described in dogs,[88,5,132] albeit with variation among studies.[32,71,95] The use of warfarin in cats is less well-defined.[113,114] Warfarin's therapeutic index is narrow, increasing the risk of excessive anticoagulation and life-threatening hemorrhage. Close monitoring is required through measurement of the PT, preferably with reporting of PT^{INR}.[109] The PT^{INR} minimizes the influence of assay variability to standardize anticoagulant intensity. Warfarin's anticoagulant effect is influenced by many factors,[30] including medications, diet, and protein binding.[93,11] The CURATIVE guidelines do not support the routine use of warfarin for dogs and cats.[11] If warfarin is administered, close monitoring to achieve a target PT^{INR} of 2–3 or a prolongation of 1.5–2.0 times the baseline PT is recommended.[109]

Unfractionated Heparin

UFH is a common, inexpensive anticoagulant that acts indirectly by greatly enhancing endogenous antithrombin (AT) activity.[133] UFH is a mixture of variably sized glycosaminoglycan molecules. UFH inhibits multiple serine protease factors, although its anticoagulant action is primarily due to inhibition of FIIa and FXa[133,37] Thrombin inhibition occurs via ternary complex formation between long-chained UFH and AT, and anticoagulant effects also result from loss of thrombin's activation of other procoagulant factors. UFH is administered parenterally (IV or SC);[91,36,106] neither oral nor inhalational administration is effective.[40,79] The CURATIVE guidelines for dogs suggest an initial IV bolus of 100 U/kg followed by a CRI of 480–900 U/kg/day (i.e., 20–37.5 U/kg/h). Alternatively, UFH may be administered at 150–300 U/kg every 6 hours SC in dogs or 250 U/kg every 6 hours in cats.[11]

Efficacy of UFH has been described in dogs,[91,36,106,51,6,40,79] with relatively few reports in cats.[3] Pharmacokinetics of UFH are unpredictable, due in part to heterogeneity of the drug preparation, variable protein and cell binding exacerbated by inflammatory states, and endogenous AT.[51] Individualized monitoring with dose adjustment is therefore advisable.[109] The dose of UFH can be adjusted to attain target factor Xa inhibitory activity (anti-Xa activity, AXA) within a range of 0.35–0.7 U/kg. This dosing target is extrapolated from human guidelines and supported by an experimental canine venous thrombosis model.[91] A clinical study of canine IMHA reported improved survival for individually adjusted UFH (based on AXA), compared to fixed doses.[51] Several point-of-care coagulation tests have been investigated as rapid alternatives to AXA for UFH monitoring.[6,82] Preliminary studies describe aPTT and RapidTEG® (i.e., thromboelastography with a proprietary activator) monitoring,[109] with a suggested target aPTT prolongation between 1.2 and 2.0 times baseline.[6]

Low-Molecular-Weight Heparin

Low-molecular-weight heparins (LMWHs) are produced by UFH depolymerization and consist of relatively short, uniform glycosaminoglycan chains that retain the ability to potentiate AT.[133] Few LMWH molecules have sufficient length to form the ternary complex required for thrombin inhibition, thus most anticoagulant effects result from FXa inhibition.[133,37] In people, LMWHs have predictable pharmacokinetic profiles and rarely require monitoring, except for renal insufficiency or body weight extremes.[57] Unfortunately, canine and feline LMWH pharmacokinetic profiles are less predictable.

Dalteparin and enoxaparin are the most commonly used LMWH in dogs and cats. The CURATIVE guidelines suggest initial dalteparin dosage in dogs of 100–175 units/kg SC,[11] given q.8 hours to attain human-defined target AXA (0.5–1.0 U/mL).[85,14,76] Coagulation screening tests (i.e., PT, aPTT) are not suitable for dalteparin monitoring.[86] Adverse effects associated with dalteparin in dogs are typically minor (e.g., local bruising from SC injections). One canine study reported fewer bleeding complications with dalteparin (100 U/kg SC twice daily) than UFH (100 units/kg IV then 900 units/kg/day).[106] Dalteparin dosing appears more problematic in cats.[86,105] A dose range of 75–150 units/kg SC with frequent dosing to attain AXA targets has been described.[3] The CURATIVE initiative recommends a starting dose of 75 units/kg SC every 6 hours,[11] with insufficient evidence for routine AXA monitoring.[109]

The CURATIVE guidelines recommend a starting canine enoxaparin dose of 0.8 mg/kg SC q.6 hours.[91,75,11] A breed variation in pharmacokinetics may exist, since this dose failed to attain AXA targets in healthy Beagles.[99] Clotting time tests (e.g., PT, aPTT) are insensitive to target enoxaparin concentrations; however, there is insufficient evidence to recommend routine AXA monitoring.[109] Few

reports describe enoxaparin usage in cats.[3,126,87] CURATIVE guidelines recommend a dosage of 0.75–1 mg/kg SC q.6–12 hours, with more frequent dosing to limit interindividual variation in AXA.[11]

Fondaparinux is a synthetic pentasaccharide FXa inhibitor that augments AT's anti-FXa activity, similar to LMWH. No clinical studies have investigated its usage in dogs; however, a feline study reported doses of 0.06 or 0.20 mg/kg SC q.12 hours achieved human therapeutic AXA targets without complications.[43]

Direct Oral Anticoagulants

Direct oral anticoagulants (DOACs) are replacing vitamin K antagonists and parenteral anticoagulants in human medicine due to predictable pharmacokinetic profiles and ease of administration. The DOACs inhibit the activity of single factors, targeting FXa or FIIa through direct competitive inhibition, with no reliance on endogenous AT.[29] Dabigatran is a direct thrombin inhibitor whose use in dogs and cats has not yet been investigated.[133] The DOACs rivaroxaban and apixaban inhibit FXa and prevent its interaction with FVa to form the prothrombinase complex (see Chapter 87).[92]

Rivaroxaban appears well-tolerated in healthy and sick dogs.[90,135,29] A dose of approximately 2 mg/kg twice daily was associated with a sustained anticoagulant effect in healthy dogs;[29] however, this dosage may be cost prohibitive in larger dogs. Consequently, lower doses and once daily dosing appear commonplace in clinical practice.[90,135] The CURATIVE guidelines recommend a dose of 1–2 mg/kg/day PO for rivaroxaban in dogs, but optimal dosing is unknown.[11] The pharmacokinetics and pharmacodynamics of rivaroxaban in healthy cats administered a fixed dose of either 2.5 mg or 5 mg per cat have been described.[38] Dose-dependent increases in rivaroxaban AXA were found, without adverse effects. Based on this data, the CURATIVE guidelines suggest 0.5–1 mg/kg/day in cats.[11] A single study has reported the pharmacokinetics and pharmacodynamics of apixaban in healthy cats. A single daily dose (0.2 mg/kg) effectively inhibited FXa without adverse effect. The bioavailability of rivaroxaban improves in people when taken with food. Although a pilot study failed to identify an effect of feeding on rivaroxaban AXA in healthy dogs,[78] further study is needed to confirm these findings.

The importance of rivaroxaban monitoring in animals is currently ill-defined, since indications for people are limited to emergency surgery or renal insufficiency.[7] The rivaroxaban assay AXA is appropriate for pharmacokinetic studies and long-term therapeutic monitoring; however, point-of-care assays are preferable for rapid dose adjustments. Prothrombin time may be a useful surrogate for rivaroxaban AXA in people and in dogs,[103,77] although variable reagent sensitivity may influence diagnostic utility.[122]

Therapeutic Thrombolysis

Supraphysiologic doses of agents that activate plasminogen to accelerate plasmin generation will induce thrombus dissolution. Agents may be infused systemically or delivered directly at the thrombus site (catheter-directed lysis).[39] Thrombolytic drugs include recombinant tissue plasminogen activator (tPA) products alteplase, reteplase, and tenecteplase. These thrombolytic drugs have distinct pharmacokinetics, activities, and fibrin specificities.[131] The tPA products activate fibrin-bound plasminogen more efficiently than circulating plasminogen. However, all tPA products are associated with risk of hemorrhage due to systemic fibrinolysis.[120] Alteplase has the shortest half-life,[97] followed by reteplase and then tenecteplase. Thrombolysis is generally safest and most effective when performed early, using a fibrin-specific drug with a short half-life such as alteplase.[74] The potential benefits and risks of administration depend on the location, size, and duration of thrombosis and the clinical, cardiovascular, and neurological status of the patient.

Veterinary clinical thrombolysis was first reported for feline ATE,[89,129] where it was associated with high rates of hemorrhage and reperfusion injury. Thrombolysis for feline ATE is not currently recommended,[73] although recent data suggest some potential benefit.[49] Alteplase was used in a dog with ATE,[26] and tPA used in experimentally induced canine pulmonary thromboembolism (PTE) .[119] Alteplase has been used for device-associated thrombosis in canine cardiology and dialysis patients.[31,70] Human studies suggest that low-dose tPA may be effective and safe for PTE.[108] A tPA protocol for PTE involves administration of half maximum dose (0.2–0.5 mg/kg IV), over the first 30–60 minutes followed by reassessment. No additional drug is given to hemodynamically stable patients, with the remaining half-dose administered after suboptimal response. This approach generally reduces dose-related bleeding complications. Reported tPA protocols in small animals include 0.2 mg/kg locally infused,[8] a locally infused CRI of 0.03 mg/kg/h (maximum 1 mg/kg),[31] and systemic administration of 0.2 mg/kg IV bolus, then 0.7 mg/kg IV over 30 minutes, then 0.5 mg/kg IV over 60 minutes (total 1.4 mg/kg).[5] Ultimately, clinical trials are needed to optimize patient selection and dosage regimens.

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CHAPTER 95

Avian Hemostasis

KAREN E. RUSSELL and J. JILL HEATLEY

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Coagulation Disorders
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    Vitamin K Antagonists
    Anticoagulant Rodenticides
  Infectious Disease and Disseminated Intravascular Coagulation (DIC)
  Hepatopathies
  Unknown Mechanisms

Acronyms and Abbreviations

ACT, activated clotting time; ADP, adenosine diphosphate; APTT, activated partial thromboplastin time; CI, confidence interval; DIC, disseminated intravascular coagulation; PT, prothrombin time; TCT, thrombin clotting time.

INTRODUCTION

Hemostasis is essential in the control of bleeding and is the first step in wound healing (see Chapter 87). It is achieved through complex, highly controlled interactions between the vasculature, blood cells, and proteins, all of which carefully balance procoagulant and anticoagulant forces. Hemostasis culminates in clot formation through the action of thrombin, which converts soluble fibrinogen to insoluble fibrin. These enzymatic reactions occur rapidly and almost immediately following vessel injury. Fibrinolysis completes the process of endothelial repair through slow dissolution of the clot. Imbalance of these complex processes may result in hemorrhage or thrombosis.

PHYSIOLOGY OF AVIAN HEMOSTASIS

Many of the enzymatic reactions of coagulation leading to thrombin generation are conserved in avian species; however, there are important differences from mammals (see Figure 95.1). Moreover, thrombocytes, the avian equivalent of mammalian platelets, are unique.

Thrombocytes

Thrombocytes are small, round to oval to slightly spindled, nucleated, bone-marrow-derived cells that represent the second most numerous cell type in avian peripheral blood after erythrocytes. They measure approximately 5 μm in width by 8–10 μm in length.

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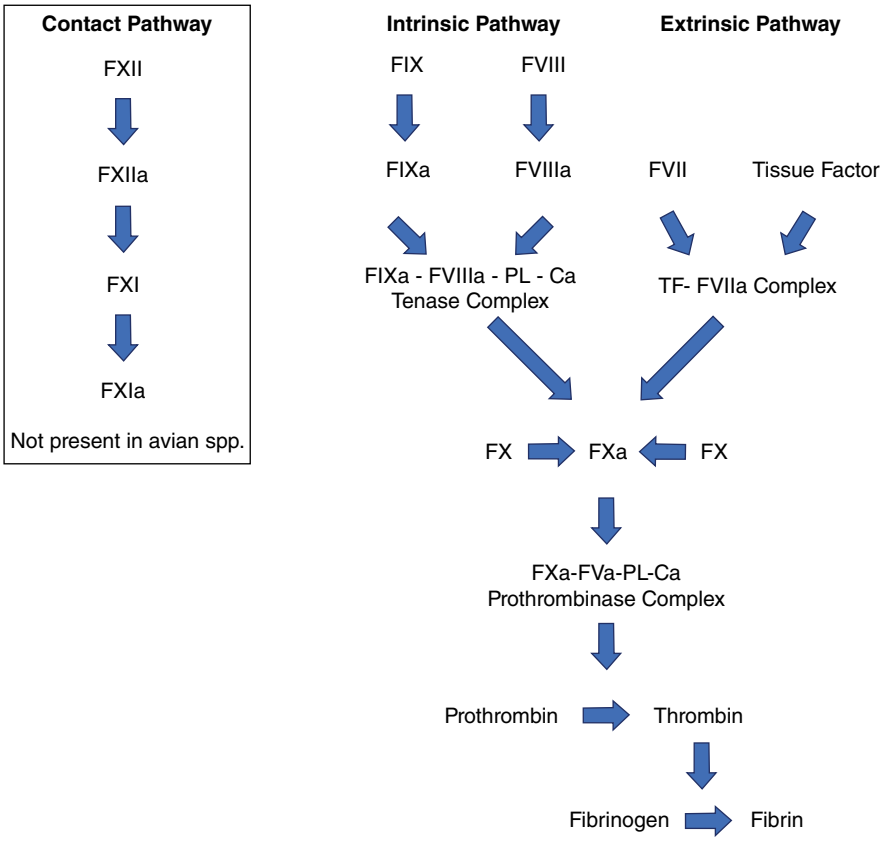


FIGURE 95.1 Diagram of Avian Intrinsic and Extrinsic Coagulation Factor Pathways. (**Source:** Modified from Thompson et al.[72])

A distinct mononuclear cell in marrow is thought to be the progenitor cell for avian thrombocytes. Stages of thrombopoiesis include thromboblasts, immature thrombocytes (early, mid, and late stages), and mature thrombocytes.[7]

Similar to mammalian platelets, the primary function of thrombocytes is hemostasis. Thrombocytes also play an important role in innate immunity because of their phagocytic ability to engulf foreign material.[28] Chicken thrombocytes constitutively express transcripts for several toll-like receptors, and have surface expression of major histocompatibility complex II and costimulatory molecules CD40, CD80, and CD86, suggesting a role in adaptive immunity.[20] Avian thrombocytes contain dense granules, mitochondria, and have microtubules and vacuoles that connect to a canalicular system or open to the surface.[6,20,28,63]

Thrombocytes possess much of the same molecular machinery and, with few exceptions, respond to the same primary agonists that activate mammalian platelets.[63] Thrombocytes are activated by collagen and thrombin, but contain little ADP and do not respond strongly to this platelet agonist.[3,64,66] Thrombocytes can adhere and spread on collagen, albeit less efficiently and more slowly than platelets. Thrombocytes have substantially lower levels of the glycoprotein IIb–IIIa receptor and do not appear to form shear-resistant aggregates.[64] Instead, thrombocytes form a single layer over a larger surface area, and the process takes longer to expand and cover the injured area.[56,64] Comparative genomics reveals that avian hemostasis involves fewer genes compared to mammals.[56] Avian thrombocytes have less than 50% of the sequence for the GPIbα, a glycoprotein that induces shape change in mammalian platelets upon engagement with its ligand, von Willebrand factor.[57] Incomplete GPIbα may therefore reduce thrombocyte aggregate strength and thrombin production, resulting in delayed fibrin clot formation.[56]

Coagulation Pathways

Avian species have many of the coagulation proteins found in mammals. These proteins are primarily synthesized in the liver, and like mammals, birds require vitamin K for post-translational modification of factors II, VII, IX, and X, and proteins C and S.[4,6,10,22,50,72]

While the tissue factor, or "extrinsic" pathway, is conserved in birds, activation of the intrinsic pathway differs from mammals (see Figure 95.1).[72] Birds lack prekallikrein and other contact pathway serine proteases, factors XII and XI, thus coagulation is not initiated by contact with negatively charged surfaces.[14]

Rather, the intrinsic pathway is initiated by activation of factor IX, which is accomplished by the tissue factor–FVIIa complex.[6,50] The tenase complex of FIXa–FVIIIa–calcium–phospholipid then assembles to amplify factor X activation, which in turn drives the reactions of the prothrombinase complex to form a burst of thrombin.

Avian coagulation inhibitory pathways are poorly defined. Tissue factor pathway inhibitor has not been confirmed to exist in avian species.[6,10] However, cDNAs with sequence identity for several anticoagulant factors, including protein C, have been found in chickens, and antithrombin has been cloned in various avian species including the chicken and ostrich.[4,6,10,22,23] Ostrich thrombin and antithrombin appear closely related to the corresponding proteins of other species based on physicochemical and kinetic properties.[23] Antithrombin has been quantitated for the adult chicken.[2]

DIAGNOSTIC TESTING

Common assays used to assess hemostasis in mammals are often problematic in avian species due to nucleated thrombocytes and the lack of optimized or species-specific coagulation reagents.

Thrombocyte Counts

The thrombocyte concentration of most avian species ranges from 20,000 to 30,000 per cubic millimeter of whole blood, representing approximately 10–15 thrombocytes per 1000 erythrocytes.[7] Automated thrombocyte counts are not possible due to the nucleated character of the cell. A hemocytometer method for counting thrombocytes has been described, but is rarely performed.[7] Avian thrombocytes are prone to clumping; large clumps on blood smears make accurate estimated thrombocyte counts challenging. The presence of approximately 2–5 thrombocytes over several erythrocyte monolayers at 1000× magnification is an indication of adequate thrombocyte numbers. Fewer than one to two thrombocytes over several monolayers suggests thrombocytopenia (in the absence of clumping), whereas greater than five thrombocytes over several monolayers suggests thrombocytosis.[7] Functional studies of thrombocyte aggregation have been described, but are rarely performed for diagnostic purposes.[27,48,78]

Coagulation Assays

Numerous studies describe coagulation testing in birds, with reported values for various assays and methods summarized in Table 95.1. The sample required for many coagulation assays is citrate anticoagulated plasma, collected at a ratio of one part 3.2% or 3.8% sodium citrate to nine parts whole blood. Venipuncture should be atraumatic, and the plasma should be free of hemolysis, which may interfere with colorimetric endpoint assays. Whole blood should be centrifuged to separate plasma within 30 minutes or less of collection. Frozen storage of plasma is described; however, freeze–thaw cycles may affect results. To the authors' knowledge, no commercial sources of avian-derived and avian-optimized coagulation reagents are available at this time.

A comprehensive study in Japanese quail described 20 coagulation and fibrinolysis assays.[4] While many assays were primarily applicable for research settings, the more readily available tests in this study, and others, are described in the following text.

Activated Clotting Time (ACT)

The ACT measures the time for clot formation in fresh whole blood in the presence of a contact pathway activator and is designed to evaluate the intrinsic and common pathway factors. Although birds lack key contact factors, the use of tubes containing diatomaceous earth has been described for avian ACT.[79]

Prothrombin Time (PT)

Prothrombin time (one-stage prothrombin time) assesses the tissue factor (extrinsic) and common pathways and is a functional test of factors V, VII, and X, prothrombin, and fibrinogen. The test methodology is based on the addition of a calcium and tissue factor/phospholipid reagent, referred to as thromboplastin, to warmed citrated plasma. The time for clot formation represents the PT. Avian PT varies widely depending on the source of thromboplastin (typically a brain extract). Clotting times are relatively prolonged and may not yield an endpoint using heterologous (mammalian) thromboplastins, thus avian-sourced thromboplastins are preferred.[4,22,71,79] Although the body temperature of most avian species is higher than 37 °C, incubation of the assay at 37 °C versus higher temperatures does not appear to affect results.[70]

Activated Partial Thromboplastin Time (APTT)

The APTT, similar to ACT, evaluates the intrinsic and common pathway factors: factors XII, XI, IX, VIII, X, and V, prothrombin, and fibrinogen. This assay measures the time of clot formation after the addition of calcium to citrated plasma preincubated with a negatively charged contact activator and phospholipid reagent. Because birds lack factors XII and XI, APTT endpoints are variable and often markedly prolonged compared to those of mammals.

Thrombin Time (TT, TCT)

The thrombin clotting time (TCT) measures the time for clot formation following the addition of a calcium and thrombin reagent to citrated plasma. The TCT is

TABLE 95.1 Summary of coagulation test results reported for avian species.

Common name	Scientific name	Prothrombin time (seconds)	Russell's viper venom time (seconds)	Activated partial thromboplastin time (seconds)	Thrombin time (seconds)	Bleeding time (min)	Whole blood clotting time (minutes)		Reference(s)
							20–22 °C	42 °C	
Domestic chicken	*Gallus domesticus*	72 (49–132) H		91 (66–296) H	29 (23–42.5) H				Guddorf et al.[31]
	A	(20–30)						26–69 (8–180) min	Bigland[5]
	Cockerel, pullet capon	13.2 (9–16.5)						306 (75–1021) sec	Juarez-Estrada et al.[35]
	Pullet							552 (<60–2340) sec	Juarez-Estrada et al.[35]
	Capon							270 (<60–450) sec	Juarez-Estrada et al.[35]
	2–24 weeks	13.02 (10.39–15.64)^	16.64 (12.08–21.21)^						Timms[74]
	Leghorn (2 weeks)	(16.8–25.4)				Comb: 4.47 ± 0.422 Wattle: 2.5 ± 0.151			Juarez-Estrada et al.[35]
Domestic turkey	*Meleagris gallopavo*	39 (36–70) H		112 (83–148) H	14.4 (12.4–17.5) H				Guddorf et al.[31]
		12.40 ± 0.11 (11.0–13.8)^	12.6 ± 0.27 (9–16.1)^				163–428 (15–720) *J*	188–557 (36–840) *J*	Timms[74], Bigland[5]
Macaw	*Ara spp.*	99 (62–141) H		111 (64–>500) H	13.4 (10.3–17.6) H				Guddorf et al.[31]
African grey parrot	*Psittacus erithacus*	69 (46–86) H		299 (72–>500) H	11.9 (10.2–13.2) H				Guddorf et al.[31]
Amazon parrots	*Amazona spp. A*	136.9 ± 171.6 H		229.5 ± 168.5 H 303 ± 171.6 H					Strindberg et al.[67]
Umbrella cockatoo	*Cacatua alba*	(10–15.8) C							Morrisey et al.[42]
Common buzzard	*Buteo buteo*	161 (95–206) H		256 (142–>500) H	16.7 (14.8–23.1) H				Guddorf et al.[31]
Kestrel	*Falco tinnunculus*	87 (53–112) H		326 (135–>500) H	10.9 (10–11.7) H				Guddorf et al.[31]
Kite	*Milvus migrans*	18.2 (13.5–24.4) A	13.2 (8–19.5)						Tahira et al.[70]
Vulture	*Neophron percnopterus*	16.2 (14.5–18.0) A	14.3 (11–17)						Tahira et al.[70]
American kestrel	*Falco sparverius*	12.4 (8.3–16.5)* C	18.53 (13.46–23.61)*						Hindmarch et al.[33]
Eastern screech owl	*Megascops asio*	9.3 (13–5.7)* C	19.02 (12.99–25.06)*						Hindmarch et al.[33]
Pigeon	*Columba livia A*	81 (61–154) H 25.0 (17.2–34.3) AS	14.6 (9.2–21)	290 (145–>500) H	13.9 (12.9–16) H		113 (50–220)	144 (25–540)	Guddorf et al.[31], Tahira et al.[70], Bigland[5]
Common wood pigeon	*Columba palumbus*	93 (60–109) H		142 (80–157) H	18.1 (14.9–25.4) H				Guddorf et al.[31]

Duck	*A*					123 (55–360)	47 (15–105)	Bigland[5]
Japanese quail	*Coturnix coturnix A*	12.7 (10.8–16.2) C				99 (5–360)	32 (3–90)	Bigland[5], Webster et al.[79]
Northern bobwhite quail	*Colinus virginianus A*	~15 C	~40 C					Rattner (2010)[53]
American flamingo	*Phoenicopterus ruber A*	68 ± 105 H		247.0 ± 94.6 H 325.1 ± 196.8 H				Strindberg et al.[67]
Helmeted guineafowl	*Numida meleagris A*	74.3 ± 6.5 H		241.1 ± 109.7 H 68.7 ± 111.4 H				Strindberg et al.[67]
Humboldt penguin	*Spheniscus humboldti A*	180.9 ± 48 H		98.14 ± 131.9 H 301.5 ± 93.1 H				Strindberg et al.[67]
Scarlet ibis	*Eudocimus ruber A*	142.6 ± 41 H		211.6 ± 206.7 H 279 ± 194.7 H				Strindberg et al.[67]

Legend: C = Chicken brain reagents; AS = Same avian species brain reagent; H = Human reagents; Median (min.–max.); *90% confidence interval (CI); ^95% CI; *A* = Adult; *J* = Juvenile

prolonged in cases of fibrinogen deficiency, dysfibrinogenemia, or in the presence of inhibitors such as heparin anticoagulants or fibrin degradation products.

Fibrinogen

The heat precipitation test can be performed on EDTA plasma to get an estimate of fibrinogen concentration. Clauss fibrinogen is a modified TCT that provides functional and quantitative determinations of fibrinogen concentration. In this assay, clotting times are inversely related to fibrinogen content and values are transformed to fibrinogen concentration (mg/dL or gm/L) by comparison with a calibrated fibrinogen standard.

Thromboelastography (TEG)

Thromboelastography is a viscoelastic assay that monitors the kinetics of clot formation in whole blood. The results represent a "global" assessment of platelet function, coagulation, fibrin clot strength, and fibrinolysis. Recent studies describe TEG analyses in different avian species (see Table 95.2).[36,67] A TEG study in Hispaniolan Amazon parrots revealed relatively slow clot formation times and a reduced clot strength compared to dogs or cats.[36] The use of a contact pathway activator (kaolin) and 37 °C reaction times may not represent optimized conditions for birds. Of note, a tissue factor activator yielded values more similar to feline and canine samples.[36,67]

COAGULATION DISORDERS

Vitamin-K-Deficiency States

Vitamin K's cofactor activity is required for carboxylation of glutamate residues on the vitamin-K-dependent factors. Without this modification, phospholipid binding via calcium bridging fails, preventing assembly of active coagulation complexes (see Chapter 87). Vitamin-K-dependent coagulopathies are reported in a variety of avian species due to dietary deficiencies, vitamin K antagonists, and anticoagulant rodenticides.[6,33,44,45,55,79]

Dietary-Related Coagulopathies

Early reports of hemorrhage in chicks fed low-fat diets were ultimately attributed to lack of an antihaemorrhagic factor that was found to be a fat-soluble vitamin designated "K" for German "koagulation." [9,32] Dietary vitamin K deficiencies in most avian species, except for poultry, are now relatively uncommon. In the hatchling chicken, very low levels of plasma vitamin K (approximately 2%) are not uncommon and relate to low levels in the maternal diet, lack of intestinal synthesis, extent of coprophagy, or the presence of sulfur drugs and other feed additives in the maternal diet.

A coagulopathy associated with high dietary vitamin E has been described in pink-backed pelicans (*Pelecanus rufescens*). Three of six affected birds succumbed with necropsy findings of extensive hemorrhage at subcutaneous,

TABLE 95.2 Summary of thromboelastography parameter results for select avian species.

Common name	Scientific name	Time to clot formation, R (min)	Clot formation time, K (min)	α-angle (°)	Maximum clot strength amplitude (mm)	References
Chicken	*Gallus domesticus*	[0.7–12]	[0.8–3.2]	67.6–78.3*	52.2–72.7*	Roy et al.[62]
Chicken	*Gallus domesticus*	5.3 ± 2.6 (3.9–6.7)	1 ± 0.4 (0.8–1.2)	77.3 ± 5.7 (74.3–80.4)	65.4 ± 4.4 (63.1–67.7)	Strindberg et al.[67]
Amazona spp.	*Amazona spp.*	4.3 ± 2.2 (1.9–6.6)	4.3 ± 2.5 (1.6–7)	55.4 ± 15.1 (39.6–71.3)	44.2 ± 6.6 (37.3–51.5)	Strindberg et al.[67]
Hispaniolan amazon	*Amazona ventralis*	2.6–15	4.3–20.8	12.7–47.9	26.3–46.2	Keller
American flamingo	*Phoenicopterus ruber*	5.6 ± 3.4 (3.5–7.7)	3.1 ± 2.2 (1.7–4.4)	58 ± 15.1 (48.9–67.2)	51.5 ± 4.3 (48.9–54)	Strindberg et al.[67]
Helmeted guineafowl	*Numida meleagris*	7.4 ± 5.3 (4.1–10.8)	2 ± 1.8 (0.8–3.2)	67.4 ± 15.6 (57.5–77.4)	66.7 ± 6.9 (62.3–71.1)	Strindberg et al.[67]
Humboldt penguin	*Spheniscus humboldti*	8.9 ± 5.8 (4.5–13.4)	3.7 ± 2.5 (1–5.7)	51.4 ± 19.6 (36.3–66.4)	49.9 ± 8.2 (43.6–56.2)	Strindberg et al.[67]
Scarlet ibis	*Eudocimus ruber*	3 ± 1.1 (2.4–3.7)	1.2 ± 0.4 (1–1.5)	72.7 ± 4.4 (70–75.3)	61 ± 5.9 (57.4–64.5)	Strindberg et al.[67]

Legend: data formats:	[Range]	Mean ± SD (95% CI)	Reference range*
Thromboelastographic parameters:	Parameter	Definition	Units
	R	Time from test start to initial fibrin formation; detected on tracing as first deviation from baseline	minutes (min)
	K	Time from initial fibrin formation to deviation of 20 mm from baseline	minutes (min)
	Alpha	Angle defined on tracing from baseline at R to K	degrees
	MA	Maximum width of tracing	millimeters (mm)

intramuscular, and multiorgan sites. Three survivors had hemorrhage and prolonged clotting times that responded to vitamin K treatment. The mechanism is unknown, but vitamin-K-responsive coagulopathies have been reported in humans and other species after chronic ingestion of high levels of vitamin E.[8,46,50,52] In vitro studies have shown the quinone metabolites of alpha-tocopherol may competitively inhibit vitamin K.[17,47,52,76]

Vitamin K Antagonists

In chickens, sulfonamide antibiotics (sulfaquinoxaline) and p-aminophenylarsonic acid (roxarsone) can act as vitamin K antagonists and have caused increased bleeding tendencies and prolongation of clotting.[21,30,69] These compounds have been used as growth promoters and coccidiostats; the latter compound, an organic arsenic, is no longer available.[19]

Anticoagulant Rodenticides

Anticoagulant rodenticides inhibit vitamin K_1-2,3-epoxide reductase, which in turn impairs vitamin K metabolism. Second-generation rodenticides include brodifacoum, bromadiolone, chlorophacinone, difenacoum, difethialone, and diphacinone. These longer-lasting rodenticides have high lipid solubility and enterohepatic circulation, increased hepatic accumulation, a greater affinity for vitamin K epoxide reductase, and the ability to inhibit the vitamin K epoxide cycle at various steps. This results in a longer acting and increased potency rodenticide that persists in the environment, thereby increasing risk of toxicity to nontarget species. Furthermore, a single high-level exposure can be lethal and multiple, sublethal exposures have the potential to be fatal.

Anticoagulant rodenticides pose a risk to nontarget avian species, most commonly birds of prey (raptors) and scavengers, but other species can be affected. Exposure can occur through ingestion of bait, or if the bird preys upon animals that have ingested bait. The toxicity of various rodenticides is dose-dependent, but differences in species sensitivities also occur. In general, raptors are more sensitive to pesticides and certain rodenticides than other bird species.[53–55]

Infectious Disease and Disseminated Intravascular Coagulation (DIC)

As in mammalian species, DIC triggered by vasculitis or bacterial infections (especially Gram-negative organisms) has been reported in avian species.[6,51] Acute infection with *Escherichia coli* in chickens can cause fibrin deposition in various tissues, thrombocytopenia, and elevations in bleeding times, clotting times, and PT.[6,80] *E. coli* lipopolysaccharide administration shortens TCT and decreases fibrinogen in broiler chickens.[49] Evidence for DIC and thrombocytopenia was found in chickens infected with *Salmonella gallinarum* and *Salmonella pullorum*.[37,80] Birds infected with *Erysipelothrix rhusiopathiae* have prolonged PT and APTT, and fibrin thrombi are found on histological sections of liver, kidney, and lung.[65] *Streptococcus zooepidemicus* is a recognized cause of vascular lesions in layer chickens, and hyperfibrinogenemia, prolonged PT, and TEG parameters of hypercoagulability have been described in chickens experimentally infected with this organism.[62]

Viral diseases may result in coagulopathies due to direct effects of the viral infection, secondary to the development of DIC, or immunosuppression and subsequent bacterial or fungal infections leading to DIC or hepatic injury. Varying signs of hemorrhage (superficial or internal), petechiation or ecchymosis, vasculitis, hepatic necrosis or hepatopathy, and combinations of these findings are often associated with avian viral infections. While hemorrhage provides evidence of hemostatic compromise, the underlying pathophysiology is often unknown.

Studies of chickens infected with highly pathogenic avian influenza A virus have revealed severe hemorrhage in multiple organs and fibrin thrombi in the lung.[43] Experimentally, highly pathogenic strains of influenza caused significantly decreased thrombocyte counts, prolonged PT, and high levels of tissue factor expression compared to control chickens infected with an avirulent strain. These findings support a consumptive coagulopathy as the underlying cause of hemorrhage.[43]

Infectious bursal disease is a highly contagious viral disease in chickens that causes marked hemorrhage, especially in skeletal muscles and the bursa of Fabricius. In addition to hemorrhage, studies comparing experimentally infected birds show thrombocytopenia, prolonged PT and APTT, and prolonged whole blood recalcification times.[82] Histological findings in the bursa are compatible with DIC.[82]

Pathologic findings associated with Eastern encephalitis virus infection in a flock of emus included petechial and ecchymotic hemorrhages in the viscera, liver, and rarely the kidney, hepatic necrosis, necrotizing vasculitis in the spleen and intestinal lamina propria, and hemoabdomen.[75,77]

Subcutaneous and/or multifocal hemorrhages, hepatic necrosis, profuse or prolonged bleeding, and hematuria have been reported in various psittacines and other avian species infected with polyomavirus.[24,34,38,39,57]

Psittacines infected with reovirus have hemorrhage under the skin, in the heart, and focal or disseminated hepatic necrosis.[26,58] Reoviral infections are also associated with hepatitis, immunosuppression, and secondary infections with other viruses and bacterial and fungal agents.

Infection with paramyxovirus-1, the causative agent for Newcastle disease, has been associated with multifocal petechiation and hemorrhages affecting periorbital tissues, thymus, tonsil, or proventriculus.[18,68] Gastrointestinal hemorrhage was also described in lories from which an unclassified paramyxovirus was isolated.[40,59]

Avian herpesviruses are host-specific, widespread, and can cause signs of hemorrhagic tracheitis, intestinal petechiation or ecchymosis, and hepatic necrosis.[25,29,60]

Pathological findings in psittacines infected with herpesvirus (Pacheco's disease) include an enlarged liver with areas of hemorrhage, intestinal hemorrhage, and hemoabdomen.[60]

Clinical findings in psittacines infected with adenovirus include cloacal hemorrhage, hepatitis, or enlarged, friable liver with whitish surface mottling and subcapsular hemorrhage.[29] Adenovirus in poultry or water fowl can be associated with hepatitis, hemorrhage, or hemorrhagic enteritis.[25]

Hepatopathies

Severe hepatic disease, of any etiology, can impair factor synthesis and result in hemorrhage. Aflatoxins and other mycotoxins in moldy feed contaminated with *Aspergillus* or *Fusarium* species are especially a problem in poultry, but other avian species can be at risk.[13,41] Aflatoxins are difuranocoumarin derivatives metabolized primarily in the liver. Aflatoxins have toxic and carcinogenic properties with effects of teratogenesis, immunosuppression, and reduced protein synthesis. High doses cause hepatocellular necrosis with subsequent coagulopathy. Ochratoxin A, another Aspergillus mycotoxin, is nephrotoxic and hepatoxic in poultry and has also been associated with coagulopathies; factors VII and V and fibrinogen are found to be especially sensitive to this mycotoxin.[15] T-2 toxin produced by *Fusarium spp*. primarily affects factor VII, prothrombin, and fibrinogen.[16]

Fatty liver hemorrhagic syndrome (FLHS) is a complex metabolic disorder of laying hens influenced by nutritional, environmental, genetic, and metabolic factors. Necropsy in affected birds reveals excessive abdominal fat and hepatic hemorrhages. Birds with enhanced susceptibility to FHLS have different plasma phospholipid composition that may influence factor activities and increase risk of hemorrhage.[73,81]

Unknown Mechanisms

Conure hemorrhagic syndrome has been recognized in several species of conures, but the etiology remains unknown.[51,61] Erythremic myelosis was initially suspected due to the presence of increased erythroid precursors in the liver; however, some birds appear to recover, making hematopoietic neoplasm unlikely.[61] Hepatopathy associated with poor-quality diet may play a role. Some birds respond to calcium treatment; however, hypocalcemia sufficient to cause coagulopathy is unlikely.[51]

Polysulfated glycosaminoglycans (PSGAGs), used to manage degenerative joint disease, have been implicated as a cause of fatal hemorrhage in birds. A case report of four different avian species and anecdotal reports of seven additional species from the American Association of Zoo Veterinarians LISTSERV describe hemorrhage from intramuscular injections of PSGAGs.[1] Necropsy findings described in the case report included hemorrhage into the left pectoral muscle and from injection sites. PSGAGs are synthetic heparinoids, suggesting that an anticoagulant action may cause bleeding. Transient prolongation of APTT, PT, and ACT in dogs and APTT and PT in cats has been reported following intramuscular injection of PSGAG; however, fatalities have not been attributed to PSGAGs in these species.[11,12] Coagulation studies of PSGAGs in birds are warranted to clarify the cause of hemorrhagic complications.

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SECTION VIII
Transfusion Medicine

CHAPTER 96

Erythrocyte Antigens and Blood Groups

MARIE-CLAUDE BLAIS and MARIA CECILIA T. PENEDO

Red Blood Cell Antigens and Blood Groups in the Dog
- Historical Background
- Nomenclature
- Dog Erythrocyte Antigen System
  - DEA 1 (A System)
  - DEA 3 (B System)
  - DEA 4 (C System)
  - DEA 5 (D System)
  - DEA 6 (F System)
  - DEA 7 (Tr System)
  - DEA 8
- Novel Blood Groups and Other Specificities
- Japanese Blood Group Systems
- Dal Blood Group
- Kai 1 and Kai 2 Blood Types

Red Blood Cell Antigens and Blood Groups in the Cat
- Historical Background
- Cat AB Blood Group System
  - Genetics
  - Prevalence of the Antigens
  - Molecular Characterization of the Antigens
  - Molecular Genetics
  - Transfusion Reactions
  - Neonatal Isoerythrolysis
- The Mik Red Blood Cell Antigen
- Naturally Occurring Alloantibodies Outside of the AB System

Red Blood Cell Antigens and Blood Groups in Livestock Species
- Historical Background
- Methods of Blood Group Detection
- Hemolytic and Agglutination Tests
- Blood Group Systems
  - Cattle
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  - Pig
  - Llama and Alpaca
- Blood Transfusion
- Neonatal Isoerythrolysis in Horses
- Neonatal Isoerythrolysis Disease in Mule Foals

Acronyms and Abbreviations

CMAH, cytidine monophospho-*N*-acetylneuraminic acid hydroxylase; DLH, domestic longhair; DSH, domestic shorthair; EA, erythrocyte antigen; FUT1, fucosyl-transferase 1; FUT2, fucosyl-transferase 2; NeuAc, N-acetylneuraminic acid; [NeuAc]2GD3, NeuAc-NeuAc-galactose-glucose-ceramide; NeuGc, N-glycolylneuraminic acid; [NeuGc]2GD3, NeuGc-NeuGc-galactose-glucose-ceramide; NI, neonatal isoerythrolysis; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SNPs, single nucleotide polymorphisms.

Inherited cell surface antigens located on the RBC membrane are referred to as blood group antigens. Their detection is primarily based on polyclonal or monoclonal antibody serology. The antigens can vary in immunogenicity and, therefore, in clinical importance. In veterinary medicine, the clinical significance of blood group antigens is usually in the areas of transfusion reactions and neonatal isoerythrolysis (NI). Blood group antigens are genetic markers and, in the past, were used for parentage testing. Though not yet proven

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in veterinary medicine, blood group antigens may play a role in immune-mediated hemolytic anemia and may serve as markers of disease. In this chapter, our current understanding of blood group nomenclature, serology, biochemistry, inheritance, incidence, and transfusion significance in the dog, cat, cattle, horse, sheep, goat, pig, llama, and alpaca is reviewed.

RED BLOOD CELL ANTIGENS AND BLOOD GROUPS IN THE DOG

Historical Background

Canine blood groups are among the first to be described in a species other than humans and were originally recognized by von Dungern and Hirszfeld in 1910, who defined four different blood groups based on immune isoagglutinins.[110] Animal experiments aimed to advance human medicine through multiple blood transfusions and organ/bone marrow transplantation led to the investigation of further canine blood groups. By a series of systematic experimental blood transfusions between dogs, Swisher and coworkers at the University of Rochester, NY, studied in vivo mechanisms of RBC destruction and characterized the in vivo and in vitro behavior of the alloantibody systems.[107,108]

Nomenclature

The first blood groups identified were named by letters from A to G in the order of their discovery.[107] Two categories of A cells were recognized: a strongly reactive group (A) and a distinct series of less strongly reacting cells designated as A′ that could only be detected by the Coombs antiglobulin test; they were eventually designated A1 and A2, respectively.[24,107] Subsequent studies led to the discovery of several other blood groups including He and Tr.[17,108] Anti-E and anti-G typing sera were lost, so no future comparisons could be made.[12,26,108]

In 1973 and 1976, two international workshops led to the standardization of canine blood group systems, as defined by their isoimmune sera.[119,120] The first workshop designated the terminology canine erythrocyte antigen (CEA) followed by a number to indicate the blood group antigen.[120] To avoid confusion with the carcinoembryonic antigen (CEA) system, the nomenclature was later modified to dog erythrocyte antigen (DEA). The second workshop specified that the number immediately after DEA designated a locus, whereas alleles within a locus would be identified by adding a number after a (.), e.g., DEA 1.1 and DEA 1.2. The previous edition of this chapter includes an extensive review of the dog blood group systems nomenclature, which is useful in understanding past and present literature on the subject.[4]

More recently, DEA 1 antigen analysis by flow cytometry using a monoclonal antibody revealed that the expression of DEA 1 is a continuum of reactions ranging from negative to strongly positive. Thus, all the alleles of the DEA 1 system have a similar epitope recognized by the monoclonal antibody.[1] Consequently, the historical nomenclature DEA 1.1, 1.2, 1.3, and DEA-1.1-negative should be substituted by DEA-1-positive and DEA-1-negative. An updated standardized international nomenclature system is required, which should include novel blood groups such as Dal and Kai 1/Kai 2.[14,35,49,71] Typing sera are commercially available in the United States, however, only for DEA 1, DEA 4, DEA 5, and DEA 7. There is limited accessibility to DEA 3, 5 and Dal reagents and a complete lack of accessibility to DEA 6 and 8 reagents. Other blood group factors and systems have been described,[64,84] but the lack of commercially available typing sera does not diminish their potential clinical significance in transfusion medicine.

Dog Erythrocyte Antigen System

DEA 1 (A System)

Originally designated by the letter A, the DEA 1 system is clinically the most important blood group system in dogs, mostly because of its well-documented immunogenicity, but also due to its overall prevalence, e.g., an almost equivalent proportion of DEA-1-positive and DEA-1-negative individuals.[46,92,119,120] Until recently, the DEA 1 system was named according to its subtypes: DEA 1.1, DEA 1.2, DEA 1.3, and DEA-1-negative. Historical accounts of these subtypes and their properties can be found in the publications of Swisher, Young, Symons, and Bell.[118,122] As stated in the earlier text under the "Nomenclature" section, recent findings by Acierno et al. suggested that this nomenclature should be replaced by DEA-1-positive and DEA-1-negative.[1]

The production and characterization of a murine monoclonal anti-DEA 1.1 antibody (IgM) in 1992 facilitated the clinical accessibility to DEA 1.1 blood typing thereafter.[2] Currently, two murine monoclonal anti-DEA 1.1 antibodies are utilized in clinical typing kits and in research settings.[1,16,69,70,72,92] Attempts to produce specific anti-DEA 1.2 and anti-DEA 1.3 antibodies have been unsuccessful without performing RBC absorption procedures.[107,110]

Analysis of the DEA 1 antigen by immunochromatography and flow cytometry suggested that the variation in the DEA 1 system is quantitative rather than qualitative. All the alleles within the DEA 1 system have a similar epitope that is recognized by a monoclonal antibody to varying degrees based on the surface expression of the DEA 1 antigen. Dogs that had been typed DEA 1.2 would actually be DEA-1-positive dogs with low antigenic expression, rather than having a morphologically different epitope.[1] However, a subsequent study by Polak et al. demonstrated no direct correlation between the historical DEA 1.2 and the strength of DEA 1 reactions.[88] The same group also studied the mode of inheritance in the DEA 1 system and confirmed an autosomal dominant Mendelian mode of inheritance. They determined that there were ≥4 alleles, which demonstrated differing degrees of antigenic expression based on a normalized blood PCV (20%): DEA-1-negative, DEA-1-positive weak (1+), intermediate (2+), and strong (3+ and 4+).[88]

Prevalence Several prevalence studies have been carried out in various countries with an overall prevalence of DEA-1-positive ranging from 33% to 65% in dogs of various breeds.[22,39,46,58,75,88]

Molecular Identity The biochemical and molecular characterization of the DEA 1 system requires further investigation, so that the data in the literature may be clarified. For example, western blotting experiments using the anti-DEA 1.1 monoclonal antibody characterized by Andrews et al. in 1992 identified two membrane proteins of 50 and 200 kDa.[2] Immunoprecipitation techniques using a polyclonal antisera to DEA 1.2 isolated a protein with a molecular mass of 85 kDa; however, this study lacks comparison to DEA-1.1-positive and DEA-1.1-negative dogs.[28] By contrast, using a second monoclonal anti-DEA 1 antibody, Polak et al. were unable to repeat these findings and did not detect the presence of any polypeptide bands in western blot experiments. They suggested that the anti-DEA 1 antibody may be specific for conformational or combinatorial epitopes lost during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).[88]

Transfusion Reactions From a practical standpoint, the DEA 1 system is the most important blood group system in the dog. Fortunately, naturally occurring anti-DEA 1 antibodies have not been documented and pregnancy has never been reported to cause sensitization in dogs;[15,126,127] however, the immunogenicity of DEA 1 remains well-documented.[46,128] Given this, first time acute hemolytic transfusion reactions do not occur in the dog; however, subsequent incompatible transfusions may result in severe hemolytic transfusion reactions (see Chapter 104).

Conflicting data have also been published regarding the immunogenicity of the historical DEA 1.2 (reported as A2), notably that DEA 1.2 (A2) dogs sensitized with DEA 1.1 (A1) RBCs can produced antibodies which react to DEA 1.1 (A1) RBCs.[107] Similarly, Hale reported in 1995 that the administration of DEA 1.1 RBCs to a sensitized DEA 1.2 dog results in an immediate hemolytic transfusion reaction.[57] Given the recent investigations into the DEA 1 system with monoclonal antibodies, modern transfusion studies are required to clarify the clinical significance of the DEA-1-positive in terms of antigenic strength. For instance, the effect of transfusing weakly DEA-1-positive blood to a DEA-1-negative dog or of transfusing strongly DEA-1-positive blood to a weak DEA-1-positive dog remains unknown.

DEA 3 (B System)

This is currently understood to be a one factor, two phenotypes system with the antigen DEA 3 dominant over a null phenotype. In the general canine population in the United States, the proportion of DEA-3-positive dogs is relatively low with a reported frequency varying from 6–11%.[17,57,58,105–107] Two studies in Japan have shown that some Japanese breeds such as Akita, Shikoku and Shiba Inu have more DEA-3-positive individuals. By contrast, non-Japanese breeds were predominantly DEA-3-negative.[32,33] Of 122 Labrador Retrievers tested in Australia, all were DEA-3-negative.[111] In a study from 2010 in retired racing Greyhounds in Ohio (n = 113 dogs), 25% were DEA-3-positive, which is similar to previous findings in the breed.[57,63] Naturally occurring anti-DEA 3 antibody has been reported in 2–20% of DEA-3-negative dogs.[57,107] The experimental administration of DEA-3-positive RBCs to a previously sensitized dog resulted in sequestration and destruction of the transfused RBCs within 5 days.[107] Given the rate of RBC loss, this degree of delayed post-transfusion reaction is most likely significant only if the recipient's ability to produce RBCs is compromised.[57]

Accessibility to typing sera for DEA 3 remains limited, threatening the ongoing ability to perform DEA 3 blood typing.

DEA 4 (C System)

This is a one factor, two phenotypes system with the antigen DEA 4 dominant over a null phenotype. DEA 4 is a high-frequency antigen with a prevalence of over 97% in the majority of tested populations.[17,34,46,57,58,63,69,107] Naturally occurring antibodies to DEA 4 have not been documented in the few DEA-4-negative individuals identified. Experimental studies have shown that DEA-4-negative dogs can produce antibodies to this antigen when exposed by transfusion, although sensitized dogs do not necessarily show RBC loss or hemolysis when subsequently transfused with DEA-4-positive RBCs.[108] Attempts to induce NI with DEA 4 (reported as C) incompatibility were unsuccessful.[126] However, a clinical report of an acute hemolytic reaction has been documented in a DEA-4-negative Shih Tzu previously sensitized by multiple transfusions.[77] The clinical significance of the DEA 4 is related to its high prevalence within the population, thus placing DEA-4-negative dogs at high risk of transfusion incompatibility when they require multiple blood transfusions. Immunoprecipitation techniques using polyclonal antisera have demonstrated that DEA 4 is a 32–40-kDa-sized protein present in a single band.[28] Blood typing for DEA 4 is performed with polyclonal canine antibodies available in a lyophilized formulation, which generate easily interpretable strong agglutination reactions (Animal Blood Resources International, Stockbridge, MI, USA).

DEA 5 (D System)

This is a one factor, two phenotypes system with the antigen DEA 5 dominant over a null phenotype. The prevalence of DEA 5 is usually less than 25% in the general population, although there are breed and geographic variations.[32,34,57,58,63,105–107,119] Naturally occurring anti-DEA 5 antibody has been reported in up to 10% of DEA-5-negative dogs. However, the titers expressed are generally weak (<1:16).[24] Swisher et al. reported sequestration and RBCs loss in less than 3 days in previously sensitized DEA-5-negative dogs transfused with DEA-5-positive blood.[108] Experimental attempts to induce NI with DEA 5 incompatibility were unsuccessful.[129] An in-house agglutination test card for DEA 5 was recently commercialized

(DMS Laboratories, Flemington, NJ, USA), which will facilitate clinical investigation of blood incompatibilities. The molecular structure of the DEA 5 has not been investigated.

DEA 6 (F System)

This is a one factor, two phenotypes system with the antigen DEA 6 dominant over a null phenotype. DEA 6 was initially suspected to be a high-frequency antigen with a prevalence greater than 98%.[106,107] However, subsequent studies have demonstrated a prevalence between 60% and 74%, suggesting significant breed and geographic variation of DEA 6 expression.[32,105,119] Naturally occurring anti-DEA 6 antibodies have not been documented. Following experimental sensitization of one DEA-6-negative dog, a moderate rate of RBCs destruction without clinical sign was observed.[107] Typing sera for DEA 6 no longer exist and the last prevalence study was published in 1986.[32]

DEA 7 (Tr System)

The DEA 7 system, previously referred to as Tr, was initially defined by a naturally occurring alloantibody and by an identically reacting antiserum produced by isoimmunization.[17] Factor O is an allele in the DEA 7 (Tr) system, resulting in the DEA 7 (Tr) system being a two factors, three phenotypes (Tr, O, and null) system with the order of dominance being Tr > O > null.[27] Unlike the other identified canine blood groups, DEA 7 antigen is not an integral RBC membrane antigen, rather it is believed to be produced elsewhere in the body, secreted into the plasma, and adsorbed onto the RBC surface.[20] The prevalence of DEA 7 varies significantly from one study to another, with reported prevalence of 8–82%, which is believed to be due to geographical and breed variations.[17,58,63,98,119] The existence and clinical significance of natural alloantibodies in DEA-7-negative dogs are controversial, but have been reported in 0–50% of DEA-7-negative dogs.[46,50,98,99] When present, their titers are rather low (rarely above 1:8).[57,97,98] Previously sensitized DEA-7-negative dogs transfused with DEA-7-positive RBCs show sequestration and loss of RBCs within 72 hours, without clinical evidence of hemolysis.[57] In this regard, the clinical importance of DEA 7 in transfusion reactions appears to be similar to that of DEA 3 and DEA 5. Immunoprecipitation experiments using polyclonal antisera to DEA 7 isolated three proteins of molecular mass 53, 58, and 66 kDa.[28]

DEA 8

DEA 8, originally designated as He, was discovered following systematic sensitization of dogs, and the antigen was present in 17–40% of dogs tested in the 1960s–1970s.[106,119] The clinical significance of DEA 8 has never been investigated. Similar to DEA 6, typing sera for DEA 8 no longer exist and the last prevalence study was published in 1976.[119]

Novel Blood Groups and Other Specificities

Japanese Blood Group Systems

As previously mentioned, two typing reagents (referred to as anti-D1 and anti-E) were produced in Japan and later found to be identical to the anti-DEA 3 antibody.[30] However, in contrast with the DEA 3 system, the Japanese D system describes two antigens (D1 and D2, codominant genes) and three phenotypes (D1, D2, and D1D2), based on agglutination testing with two rabbit heteroimmune antisera (anti-D1 and anti-D2). Although the D blood group system has been extensively studied in over 3000 Japanese dogs, the D2 antigen has not yet been identified outside of Japan. The incidence of D1 (i.e., DEA 3) is high in purebred dogs indigenous to Japan such as the Akita, Shiba Inu, Kishu, Shikoku, and in native Japanese Mongrel and mixed breeds, while most Western and European breeds are negative.[30,32,33] Ejima et al. observed severe acute hemolytic transfusion reactions following repeated transfusions of D2 type blood to a D1 type patient, or conversely of D1 type blood into a D2 type dog, indicating the clinical importance of this system in transfusion medicine.[33]

In Japan, a lectin extracted from the seeds of *Clerodendrum trichotomum* has demonstrated the ability to agglutinate RBCs in a small subpopulation of dogs (designated type C) at titers up to 128, while demonstrating no reaction for the majority of dogs (type c), which was later confirmed to be an autosomal dominant trait.[65] This system, designated as "C," has been compared to the DEA system and has been found to be different.[30,65]

Dal Blood Group

In 2007, a blood incompatibility between a previously transfused anemic Dalmatian patient and 55 non-Dalmatian blood donors led to the identification of a newly recognized blood type. The patient was found to be compatible to 4/25 Dalmatians, and the RBC antigen was therefore named Dal.[14] Although Dal was initially thought to be a high-frequency antigen, a survey of 1130 dogs from North America confirmed that it should actually be classified as a blood group system where Dal-positive is dominantly inherited over Dal-negative.[49] Goulet et al. identified several breeds with Dal-negative dogs, in addition to the previously reported Dalmatians (15/128; 11.7%), most notably Doberman Pinschers (183/432; 42.4%), Shih Tzus (12/21; 57.1%), and also sporadically in mixed-breed dogs (3/122; 2.5%), Lhasa Apsos (1/6), and Bichon Frises (1/3).[49]

At this time, no naturally occurring anti-Dal alloantibodies have been detected.[14,35,48] However, the strong immunogenicity of Dal has been documented following the sensitization of three Dal-negative research Beagles (published data on two of these dogs), with the detection of anti-Dal alloantibodies as early as 4 days post-transfusion and evidence of accelerated removal of Dal-positive RBCs using a gel column

technology.[48] In addition to the rapid production of anti-Dal alloantibodies, particularly high maximum agglutination titers were documented (i.e., as high as 2048) after a period of 1–2 months. Anti-Dal alloantibodies, which were confirmed to be IgG, remained detectable for as long as 2 years post-transfusion, thus providing evidence that they are long-lasting.[14,48]

Considering the fact that all non-Doberman Pinschers blood donors tested to date are Dal-positive, Dal-negative dogs are at high risk of transfusion incompatibility if they are to receive multiple blood transfusions. Therefore, Dal blood typing, in addition to standard DEA 1 typing, is recommended in Dalmatians, Doberman Pinschers, and Shih Tzus, especially in previously transfused dogs.[49] The Dal immunization study performed at the University of Montreal allowed for a considerable amount of anti-Dal polyclonal reagents stable through freezing to be banked for further clinical and research use.[48]

Kai 1 and Kai 2 Blood Types

Following the production of monoclonal antibodies by mouse hybridoma technique, two additional canine blood types (Kai 1 and Kai 2; Kai meaning "dog" in Korean) have recently been identified.[71] To date, Kai 1 and Kai 2 antigens have not been shown to coexist in any of the dogs tested (n = 203 dogs from South Korea; 42% Kai-1-positive, 37% Kai-2-positive), but both antigens may be absent (20% Kai-negative).[71] By contrast, the majority of dogs tested in North America (94%; n = 503) were Kai-1-positive, with barely 1% of dogs Kai-2-positive and 5% Kai-negative, with no dogs expressing both RBC antigens.[35] A comparative study confirmed that Kai 1 and Kai 2 are unrelated to DEA 1, 3, 4, and 7 and Dal.[35] No naturally occurring anti-Kai alloantibodies have been documented, but presumed anti-Kai alloantibodies were documented in four dogs 21 days post-transfusion (Kai-1-negative and Kai-2-negative dogs receiving mismatched Kai-positive blood).[71] Although the antigenic nature of Kai 1 and Kai 2 is likely, their clinical relevance is currently unknown.

Several additional specificities have been reported and proposed to represent new blood group systems.[26,30,105,111,119] As multiple blood transfusion recipients become more prevalent, novel blood groups are likely to be identified, as demonstrated by the discovery of the Dal blood type.

RED BLOOD CELL ANTIGENS AND BLOOD GROUPS IN THE CAT

Historical Background

The current nomenclature of blood groups A and B was first used by Eyquem et al. in 1962.[36] In addition to stating that these antigens are related to the ones previously described by Holmes in 1950, Eyquem also observed that 20% of cats possessed natural isoagglutinins.[36] In 1981, Auer and Bell used two naturally occurring antibodies, anti-A and anti-B, to further characterize the cat blood group system which they designated as AB, and first reported the rare AB phenotype where both antigens are present on the RBC.[7] They were also the first to describe immediate experimental and naturally occurring transfusion reactions in previously unsensitized cats of known incompatible blood type.[9]

Cat AB Blood Group System

The AB blood group system characterized by Auer and Bell is the predominant blood group system in cats.[7] Three phenotypes occur: type A, type B, and type AB. A null phenotype does not occur. The types are defined by naturally occurring alloantibody against the antigen they lack, which can be produced in kittens as young as 6–8 weeks of age.[19] Approximately one-third of type A cats have alloantibodies in their plasma capable of weak macroscopic agglutination of type-B RBC, which are weak anti-B hemagglutinins of the IgM class and weak hemolysins consisting of equal amounts of IgG and IgM. All type B cats have high-titered anti-A hemagglutinins and hemolysins mainly of the IgM class with lesser amounts of IgG.[19,123] The presence of naturally occurring alloantibodies, in particular of strong anti-A alloantibodies, is responsible for transfusion reactions and NI in blood type incompatible cats. Type AB cats do not possess alloantibodies against either A or B antigens.

Genetics

Systematic pedigree analysis of Abyssinian and Birman cat families confirmed that the blood group antigens A and B are inherited as simple autosomal Mendelian traits with A being dominant to B.[12,45] In spite of extensive breeding experiments and pedigree analysis, the mode of inheritance of the AB phenotype remains unclear due to apparently different inheritance patterns in different breeds.[55] The *ab* allele, causing blood type AB, was found to be separately inherited, being (co)dominant to *b* and recessive to the *A* allele.[55,68] Thus, all type B cats are homozygous for the *b* allele (genotype *b/b*), type A cats can be either homozygous (genotype *A/A*) or heterozygous (genotype *A/b* or *A/ab*), and the rare type AB cats can either be homozygous (genotype *ab/ab*) or heterozygous (genotype *ab/b*).

Prevalence of the Antigens

The percentage distribution of types A and B in domestic shorthair (DSH) and domestic longhair (DLH) cats can vary markedly with geographic location worldwide, ranging from 62–100% type A cats and 0–36% type B cats. Type B domestic cats are uncommon in some countries such as the United States and Switzerland; however, in other countries such as Australia, Greece, and Turkey, their prevalence can reach up to 36% of the nonpedigree feline population.[6,7,47,61,73,80] Similarly, the variation per breed is significant. For instance, the incidence

of type B in the United States ranges from 0% up to 60% among different purebred cats, with the highest prevalence found in Exotic and British Shorthair, Cornish Rex, and in Devon Rex.[45,47,56] To this day, type B has not been documented in Siamese cats worldwide.[40,45,94,118] By contrast, Turkish Van and Angora cats are reported to have a prevalence of type B cats of 60% and 46.4%, respectively.[5] Type AB is exceedingly rare and only found in breeds in which type B is detected.[55] In a survey of cats in the United States and Canada, 13 of 9239 cats (0.14%) were type AB.[55] Similarly, only seven of 1895 cats (0.4%) were type AB in a survey conducted in Australia.[7] Type AB has, however, been reported more frequently in certain random bred populations, based on surveys done in Israel (14.5%), Japan (9.7%), Portugal (6.3%), and England (8.5%).[31,78,94,112] Compared to all other cat breeds, Ragdolls are commonly type AB with reported frequency in Italy ranging of 18–24%.[89,96]

Molecular Characterization of the Antigens

The A and B blood types of cats are caused by differences in the neuraminic acid residues present on a ceramide dihexose backbone on the RBC membrane.[3,41] Type A RBCs have mainly N-glycolylneuraminic acid (NeuGc) and a small amount of N-acetylneuraminic acid (NeuAc). More precisely, type A RBCs have NeuGc-NeuGc-galactose-glucose-ceramide ([NeuGc]2GD3) as the major disialoganglioside, which is predominantly detected by feline anti-A antisera.[3] Type A RBCs also have NeuAc-NeuGc-GD3, NeuGc-NeuAc-GD3, and a very minor amount of [NeuAc]2GD3. Homozygous and heterozygous type A cats have some differences in their ganglioside profiles, which can be used to determine the genotype of type A cats.[54]

NeuAc is the determinant of the B antigen. Type B RBCs have [NeuAc]2GD3 as the only form of the disialoganglioside and do not express any detectable NeuGc.[3] Agglutination testing with the lectin from *Triticum vulgaris* (wheat), which binds sialoglycoproteins containing NeuAc, demonstrates selective agglutination of type B RBCs.[21] Type AB cats have both NeuAc and NeuGc present on the RBCs in similar quantities, each at approximately 50% normal expression levels.[54] Therefore, type AB RBCs are agglutinated by both anti-A and anti-B feline antisera, and by the lectin of *T. vulgaris* (see Chapter 107). Using a murine monoclonal anti-A antibody in agglutination testing, ganglioside thin-layer chromatography immunostaining, and flow cytometry, studies have suggested that there is more than one biochemical phenotype present among the AB type cats.[52,53]

Molecular Genetics

Cytidine monophospho-*N*-acetylneuraminic acid hydroxylase (CMAH) is the enzyme that catalyzes the conversion of NeuAc (B antigen) to NeuGc (A antigen).[79] Recent studies have identified several variants in the *CMAH* gene believed to disrupt the enzyme's function, so that it can no longer convert NeuAc to NeuGc.[13,42,43,85,112] The *b* allele was associated with several variants, including single nucleotide polymorphisms (SNPs) upstream of the *CMAH* start codon, a deletion in the 5′-untranslated region of exon 1, and four non-synonymous variants within the open reading frame.[13,112] The discovery of polymorphisms in *CMAH* ascribed to type B led to the development of commercial genotyping tests based on PCR and sequencing blood to identify the *AA*, *Ab*, and *bb* genotypes. However, despite high agreement reported, the assays have the significant limitation (particularly in pretransfusion settings) of not being able to differentiate between type A and type AB cats.[13] In addition, many examples of phenotype–genotype discordances for cats with type B and AB blood have been reported.[42,43,85] More recently, a study in Ragdoll cats identified a novel variant (c.364C>T) in *CMAH* exon 4 highly associated with blood type AB in the breed.[42] In addition, the feline *CMAH* coding regions were further examined in 70 cats, which allowed identification of 13 additional variants.[68] Analysis of three single nucleotide variants along with blood typing of an additional 351 typed cats, for which the phenotype was confirmed by additional typing assays (including backtyping), showed complete phenotype–genotype concordance. The authors concluded that three *CMAH* variants, i.e., c.179G>T (seen in type B in Turkish breed), c.268T>A (seen in type B cats of most breeds), and c.1322delT (seen in type B in some Ragdolls and DSH), are the main reasons for the defective NeuGc synthesis causing blood type B in domestic purebred and nonpedigreed cats.[68] These variants, combined with the variant identified for type AB in Ragdolls (c.364C>T), represent a promising molecular diagnostic scheme to genotype cats for the AB blood group system and differentiate type A, type B, and type AB.[42,68] It is likely that additional genetic variants will be identified in specific breeds and geographical regions.

Transfusion Reactions

The presence of naturally occurring alloantibodies is responsible for transfusion reactions in previously unsensitized cats (see Chapter 104). Of utmost clinical significance is the presence of highly potent anti-A agglutinins and hemolysins in virtually all type B cats, which can result in severe acute hemolytic transfusion reaction, and even death, if type A blood is administered to a type B cat.[8,9,124] Rapid intravascular destruction of the type A RBCs occurs within minutes to hours and is complement- and IgM-mediated. Transfusion of type B blood to type A cats also proved ineffective and is associated with extravascular hemolytic reactions, involving IgG and IgM without marked complement activity, and a half-life of transfused type B RBCs of about 2 days.[8,44]

Neonatal Isoerythrolysis

The strong anti-A naturally occurring alloantibodies present in type B cat can also be responsible for NI in type A or type AB kittens born to a type B queen.[55] The colostrum of type B queens contains high concentrations of anti-A antibodies, and colostral antibodies are

detectable in the serum of newborns as early as 4 hours after birth.[19] Clinical signs in affected kittens can be variable, ranging from unapparent to severe hemolytic anemia with hemoglobinuria, icterus, and death. NI is believed to be a major cause of the "fading kitten syndrome."[45]

The Mik Red Blood Cell Antigen

Based on the presence of a naturally occurring alloantibody in three blood donor cats, the Mik blood group system was described in 2007.[121] Incompatible crossmatch reactions with plasma from a transfusion-naïve blood donor cat (named Mike) resulted in a detailed survey of blood compatibilities within Penn Animal Blood Bank blood donors. Upon further investigation of 65 type A donors, a total of three unrelated cats (Mik-negative) were found incompatible with the same cats (Mik-positive), yet compatible to each other, indicating that they lacked the same RBC antigen (Mik antigen) and had natural anti-Mik alloantibodies. Three type B and one type AB cat also appeared to express the Mik antigen. The clinical relevance of anti-Mik alloantibodies was documented after an acute hemolytic transfusion reaction following inadvertent transfusion of Mik-positive blood to a thereafter confirmed Mik-negative renal transplant recipient upon its first blood transfusion.[121] Unfortunately, anti-Mik antibodies are currently not available, preventing future investigations.

Naturally Occurring Alloantibodies Outside of the AB system

There is some evidence for the presence of other naturally occurring alloantibodies outside the AB blood group system, like anti-Mik, but their clinical importance at the time of a first transfusion has not been defined.[51,74,122] Major crossmatch incompatibilities outside of the AB system in transfusion-naïve cats have been documented in 23 of 154 cases (14.9%) in one study and 10 of 52 (19%) in another. [74,109] However, the crossmatch screening of 112 cats in the United Kingdom failed to detect any non-AB incompatibilities.[112] In a prospective controlled study, 48 client-owned transfusion-naïve anemic cats were randomized to receive either type-compatible and crossmatched-compatible packed RBC transfusions or non-crossmatched blood-type-compatible pRBCs. Their findings did not detect any difference in the increase in PCV after transfusion or in the incidence of transfusion reactions between crossmatched and uncrossmatched groups; however, crossmatched-incompatible cats were not included (i.e., transfused) for obvious ethical reasons.[109]

Other RBC antigens outside of the AB blood group system can also be suspected based on the prevalence of non-AB RBC incompatibilities in previously transfused cats, which has been reported to be approximately 25–27% (see Chapter 99).[60,74,122]

RED BLOOD CELL ANTIGENS AND BLOOD GROUPS IN LIVESTOCK SPECIES

Historical Background

The identification of blood group antigens and analysis of their patterns of genetic inheritance in livestock species were the subject of intense investigation for about 50 years since the late 1940s. In contrast to humans and cats, naturally occurring antibodies are seldom found in the sera of cattle, horse, sheep, goat, pig, llama, and alpacas. Production of isoimmune and heteroimmune antisera by immunization and the use of RBC absorption procedures to isolate monospecific antibodies (blood typing reagents) were critical to uncover the genetic diversity of blood groups in these species. The molecular nature and function of the RBC antigens in farm animals remain largely unknown.

This section describes the blood group systems (or loci) of six livestock species: cow, horse, sheep, goat, pig, and llama (and the related alpaca). Although blood group systems were historically named as a single letter, we use here the more recent nomenclature convention in which the two letters "EA" for erythrocyte antigen precede the traditional system designation. The literature in blood groups of livestock species is extensive and cannot be fully represented in this section. Excellent reviews that offer historical accounts and details of the complex serological intricacies of blood group loci in the major livestock species are published by Stormont,[101] Bell,[12] Di Stasio,[29] Hines,[59] Juneja and Vogeli,[66] and Sandberg & Cothran.[91] The major application of blood type variation in farm animals was in the area of animal identification and parentage verification, because of the high level of individual discrimination that could be achieved and their classic Mendelian modes of inheritance. The advent of DNA genotyping methods and technologies in the late 1990s led to a replacement of methodology for this application in farm animals.[18,116,117] From a clinical perspective, knowledge of blood groups is significant only in horses and mules because of the risk of NI posed by maternal–fetal incompatibility for certain factors.

Methods of Blood Group Detection

Hemolytic and agglutination techniques are used to assay blood group antigens. The choice of procedure is largely determined by RBC characteristics of each species. Blood grouping assays for cattle, sheep, goat, llama, and alpaca are hemolytic tests because the RBCs of these species are not prone to agglutination. Horse and pig blood group antigens are assayed by saline agglutination or by hemolytic tests. Some pig factors require an antiglobulin test (Coombs test) or addition of dextran to facilitate agglutination and improve scoring of reactions.

Blood typing reagents (monospecific antisera) for livestock species are not commercially available. They have been traditionally prepared in laboratories that provided

blood typing services. Comparison tests sponsored by the International Society for Animal Genetics for several decades provided a forum for standardization of blood factor specificities and nomenclature for each species. Blood typing comparison tests are no longer held, and production of blood typing reagents has virtually ceased worldwide.

Hemolytic and Agglutination Tests

Hemolytic tests are typically set up in round-bottom, 96-well microtiter plates. A standard procedure consists of combining, in order, 50 µL of blood typing reagent with 25 µL of a 2–2.5% saline suspension of washed, packed RBCs and 25 µL of undiluted rabbit complement. The plate is then shaken, and reactions are read twice to record degree of hemolysis: once 30–45 minutes after set-up and a second time 2–3 hours later. A concave mirror is used for visual determination of hemolysis, which is graded as 0 (negative, no visible hemolysis, RBCs pelleted at the bottom), 1 (partial hemolysis), 2 (intermediate), 3 (strong, almost complete), and 4 (complete hemolysis, no visible RBCs). A complement control containing saline instead of reagent is run in parallel with the test reactions.

Rabbit serum is the source of complement for hemolytic tests, but because rabbits lack the heterophile Forssman antigen, their sera can have Forssman antibodies in high titers. Cattle and camelids are Forssman-negative and thus hemolytic tests in these species are not affected by Forssman antibodies. For the Forssman-positive horse, sheep, goat, and pig, the rabbit serum needs to be absorbed with RBCs from these species prior to use as complement.[103] Two serial absorptions, carried out at 4 °C, for 15–20 minutes each and a 1:2 volume ratio of washed, packed RBCs to rabbit serum are usually sufficient to remove heterophile antibodies without affecting complement function.

Agglutination tests are set up in the same way as hemolytic tests, but without complement. Reactions proceed at room temperature for 2–3 hours after which time the plates are shaken briefly to resuspend cells and degrees of agglutination are recorded 5–10 minutes later as 0 (no agglutination), 1 (partial clumping), 2 (intermediate clumping), and 3 (total clumping of red cells). A microscope helps distinguish between negative and weak positive reactions.

Antiglobulin tests are used with incomplete antibodies that require the addition of rabbit antiglobulin serum to produce agglutination. This procedure is used to detect some blood group factors in pigs. Addition of dextran to a saline agglutination reaction at a final concentration of 1.5% has been used with some incomplete antibodies in pigs as an alternative to the antiglobulin test.

Blood Group Systems

Cattle

The blood groups of cattle were the first to be studied in detail and provided a model for investigation in other species. The early studies in the 1940s demonstrated the value of isoimmunization and heteroimmunization for development of antibodies for blood group factors and established the procedures for preparation of monospecific antibodies through absorption and for assaying blood groups with hemolytic tests.[37,38]

Cattle blood groups remain the primary example of extreme genetic variation of RBC antigens, as evidenced by more than 70 blood group factors internationally recognized and by the extensive allelic series known in the complex *EAB* and *EAC* systems. An alphabetical nomenclature designated blood group factors (e.g., A, L, S, Z) with numerical subscripts added to describe serologically related subtypes (e.g., A_1, A_2, Y_1, Y_2). The use of symbols ′ and ″ (e.g., A′, B′, A″, G″) indicated second and third rounds of use of alphabet letters, respectively. One of the most important contributions of the early cattle blood group research was the demonstration that multiple factors segregated together in specific combinations (phenogroups) that defined allelic variants.[100,102] Evidence of infrequent recombination within *EAB* and *EAC* systems suggests that these complex systems may involve linked genes,[59] but the genomic basis of cattle blood group systems remains unknown. The *EAJ* system is not an intrinsic component of the RBC membrane and anti-J is the only antibody that occurs naturally. The J factor is a tissue and serum antigen that attaches to RBCs as serum concentrations become sufficiently high. The attached J substance has been shown to be a glycolipid, while the serum form occurs as both glycolipid and glycoprotein.[113] The J factor is serologically related to human A, sheep R, and pig A factors. Cattle blood group loci were mapped to chromosomes by linkage analysis.[67] Hines[59] provides a good review of the status of cattle blood group systems.

Horse

The first 16 blood group factors in the horse were identified through isoimmunization and heteroimmunization followed by agglutination and hemolysis testing.[104] Research worldwide led to the characterization of 34 factors distributed in seven systems as summarized in Table 96.1. By convention, factors within a system are assigned lower case letters and blood group alleles (phenogroups) are designated by the capital letter for the system, followed by lower case letters for the factor or factors detected. Five blood group loci were mapped to chromosomes by linkage analysis.[86]

The horse is the only livestock species for which blood group incompatibilities are clinically relevant. Blood group antibodies that cause transfusion reactions or NI can be found in the sera of horses either "naturally" or as a result of a blood group incompatible pregnancy. The majority of NI cases are explained by blood group incompatibilities between stallion and mare at either *EAA* or *EAQ*.[10] Naturally occurring anti-Aa (in any of its allelic combinations) and anti-Ac are occasionally found in the sera of Aa-negative horses, usually as agglutinins. Anti-Aa in mares may also be present as a hemolysin, particularly if produced by a blood group incompatible pregnancy. Where possible,

TABLE 96.1 Horse blood group loci, chromosome assignment (Chr), factors and alleles (phenogroups) defined.

Locus	Chr[a]	Factors	Recognized alleles[b]
EAA	20	a b c d e f g	A^a A^{adf} A^{adg} A^{abdf} A^{abdg} A^b A^{bc} A^{bce} A^c A^{ce} *Ae* A^-
EAC	Unknown	a	C^a C^-
EAD	14	a b c d e f g h i k l m n o p q r	D^{adl} D^{adlnr} D^{adlr} D^{bcmq} D^{cefgmq} $D^{cegimnq}$ D^{cfgkm} D^{cfmqr} D^{cgm} D^{cgmp} D^{cgmq} D^{cgmqr} D^{cgmr} D^{deklr} D^{deloq} D^{delq} D^{dfklr} D^{dghmp} D^{dghmq} D^{dghmqr} D^{dkl} D^{dlnq} D^{dlnqr} D^{dlqr} D^q (D^-)
EAK	2	a	K^a K^-
EAP	Unknown	a b c d	P^a P^{ac} P^{acd} P^{ad} P^b P^{bd} P^d P^-
EAQ	8	a b c	Q^{abc} Q^{ac} Q^a Q^b Q^c Q^-
EAU	24	a	U^a U^-

[a] From Penedo et al.[86]
[b] The absence of detectable factors is designated by the system letter followed by a dash (–).

hemolytic screening for this antibody (and others) prior to foaling can provide an effective test to identify mares whose foals are at risk for NI. In *EAQ* sensitization, the mare is negative for factor Qa (for most breeds this means negative for Q^{abc}), while the stallion has this blood group. The detectable antibody response for Qa has only been reported as a lysin and would be missed if antibody screening is based solely on agglutination testing. Occasionally, presence of antibody can be detected only very late in pregnancy. Antibodies for blood group factors in *EAC*, *EAD*, *EAK*, *EAP*, and *EAU* pose little risk as they do not occur naturally and have only rarely been implicated in NI cases, possibly as a result of blood-incompatible pregnancies.

Sheep and Goat

Eight blood group systems were described in sheep: *EAA*, *EAB*, *EAC*, *EAD*, *EAM*, *EAR*, *EAF30*, and *EAF41*.[83] The nomenclature of systems and factors followed that of the horse and pig. Although many antigen specificities were identified, only 22 blood group factors were internationally recognized in sheep. Hemolytic tests are the method of choice for sheep blood group factors with exception of the D system whose antigens are detected by agglutination tests. The sheep *EAB*, *EAC*, and *EAR* systems are homologous to cattle *EAB*, *EAC*, and *EAJ*. *EAR* locus R and O antigens, where EAR^R is dominant to EAR^O, are soluble substances found in the serum and saliva that attach to the RBC membrane and whose expression is under the control of the suppressor gene *I*. Sheep with genotype *i*/*i* do not express R or O antigens in RBCs, serum, or saliva. The *EAC* locus is closely linked to the *amino acid transport* gene. This association is of interest because RBCs with defective transport are never Cb-negative. The *EAM* locus is associated with RBC potassium transport such that low-potassium cells are always Mb-positive and high-potassium cells are always Mb-negative. It has been postulated that Mb inhibits active potassium transport into cells.[115] Several sheep blood group systems were mapped by linkage analysis. Di Stasio[29] provides a good review of sheep blood group systems.

In goats, six genetic systems were identified and named *EAA*, *EAB*, *EAC*, *EAE*, *EAF*, and *EAR*.[82] Several goat blood group factors cross-react with sheep blood typing reagents. The *EAA* system has a single specificity A1 homologous to sheep EAAa. In the *EAB* system, among 14 specificities identified, four are homologous to sheep factors Bb, Be, Bd, and Bi. The *EAC* system has a single specificity related to sheep EACa. The *EAE* system contains two factors E6 and E18 and the EAF system is defined by a single specificity F19. The *EAR* system is defined by a single specificity R related to sheep R.

Pig

Development and expansion of pig blood groups resulted in large part from work carried out in Denmark, Germany, Poland, and Russia using alloantibodies that behaved as agglutinins and a few as hemolysins. The 16 genetic systems internationally recognized are summarized in Table 96.2.[66] Some of these systems, e.g., *EAE* and *EAM*, approach the *EAB* and *EAC* systems of cattle in complexity and allelic diversity. Blood group loci were mapped to pig chromosomes by linkage analysis.[90] The nomenclature of pig blood group factors followed that of the horse, except for the *EAA* system.

The *EAA* system is related to cattle *EAJ*, human A, and sheep *EAR* systems. Pig EAA-A and EAA-O factors are soluble substances in the serum and saliva of A-positive and O-positive animals, respectively, that attach to the RBC membrane a few weeks after birth. The genetic inheritance of A and O factors is similar to sheep *EAR* system. EAA^A is dominant to EAA^O and their expression on RBCs is controlled by the suppressor gene, *EAS*.

The EAA^A allele codes for uridine diphosphate *N*-acetylgalactosamine transferase that adds a terminal fucose residue to the H substance.[76] The recessive EAA^O allele corresponds to a large deletion that removes part of intron 7 and exon 8 of the gene and results in a non-functional enzyme product.[81,125] The *EAH* and *EAS* systems correspond to fucosyl-transferase 1 (*FUT1*) and fucosyl-transferase 2 (*FUT2*) that are involved in the production of the H substance and are related to human *H* (*FUT1*) and *secretor* (*FUT2*) genes.[25,76] Antigens of the

TABLE 96.2 Sixteen loci of pig blood groups, chromosome assignment (Chr), test method, factors, and alleles (phenogroups) defined.

Locus	Chr[a]	Test method[b]	Blood factors	Number of alleles	Alleles[c]
EAA	1	a, h	A O	2	*A*, *O*
EAB	Unknown	a	a b	2	*a*, *b*
EAC	7[d]	h	A	2	–, *a*
EAD	12	a	a b	2	*a*, *b*
EAE	9	a	a b c e f g h i j k l m n o p r s t	17	*aeglns*, *bdgkmps*, *defhkmnps*, *deghkmnps*, etc.
EAF	8	a	a b c d	4	*ac*, *ad*, *bc*, *bd*
EAG	15	a	a b	2	*a*, *b*
EAH	6	h	a b c d e	7	–, *a*, *b*, *ab*, *bd*, *cd*, *be*
EAI	18	c	a b	2	*a*, *b*
EAJ	7	c	a b	3	–, *a*, *b*
EAK	9	h	a b c d e f g	6	–, *acf*, *acef*, *ade*, *adeg*, *bf*
EAL	4	c, d	a b c d f g h i j k l m	6	*adhi*, *bcgi*, *bdfi*, *agim*, *adhjk*, *adhjl*
EAM	11	c, h	a b c d e f g h i j k m	20	–, *ab*, *ade*, *aem*, *b*, *bd*, *bcdi*, *cd*, etc.
EAN	9	c	a b c	3	*a*, *b*, *bc*
EAO	6	d	a b	2	*a*, *b*
EAP	Unknown	c	A	2	–, *a*

[a] From Rohrer et al.[90]
[b] a: Saline agglutination; c: Coombs test; d: dextran test; h: hemolytic test.
[c] Dash (–) represents absence of detectable factors (null allele, recessive).
[d] Assignment based on linkage of C and J systems.

EAN system also occur as soluble substances in the serum and milk, but are not secreted into the saliva.

Llama and Alpaca

Little is known about blood group variation in the domestic South American camelids, llama and alpaca. From isoimmune and heteroimmune sera developed for these animals, six blood group factors were identified and given alphabetical designations in order of discovery: A, B, C, D, E, and F.[87] Factors A and B are inherited as codominant alleles and assigned to blood group system *EAA*. Factors C, D, E, and F were assigned to four separate systems, as they appeared to be transmitted independently from each other and from the *EAA* system. Blood groups in llamas and alpacas are detected by hemolytic tests.

Blood Transfusion

Clinically, knowledge of blood groups in cattle, sheep, goat, pig, and llama/alpaca has not affected conventional veterinary practice in transfusion. Large animals are not commonly transfused, and blood group matching is not practical because of the high variability between individuals and limited availability of donors. Blood transfusions in large animals are indicated for certain acute, life-threatening conditions and for plasma transfusion when failure of passive transfer occurs. Single, unmatched whole blood transfusions are generally safe and well-tolerated. A gross crossmatch, with hemolytic and/or agglutination tests according to species, is sufficient when repeated transfusions are required. Matching involves testing of the recipient's serum for antibodies against the RBCs of potential donors (major) and of the donors' sera for antibodies against the recipient's RBCs (minor) (see Chapter 107).

Pigs are often used as an animal model for organ or hematopoietic transplantation. Blood group compatibility is an important component of experimental protocols because of problems associated with immune-mediated tissue rejection or the need to provide transfusion support to patients.[95] In transplant experiments, mismatched transfusions for the *EAA* system result in adverse reactions that include disseminated intravascular coagulation, bleeding, and progressive hypotension[62,93] rather than typical hemolytic reactions seen in humans. Development of an efficient PCR-based DNA assay[23] allows accurate determination of EAA genotype for selection and matching of pigs for allotransplantation, and selection of EAA-O pigs in xenotransplantation experiments.

Horses merit special consideration because mares given a whole blood transfusion can be sensitized to blood group factors that may subsequently lead to NI problems for foals (see section in the following text). Selection of suitable donors that are negative for highly antigenic factors such as EAAa, and without antibodies in the serum, is recommended although currently not practical because very few laboratories can perform the serological test for blood group specificities. In the United States, horse blood typing service for veterinarians is

available through the Clinical Laboratory Services of the School of Veterinary Medicine, University of California, Davis, California. Mares that received a blood transfusion should always be screened for potential risk of NI prior to foaling.

A plasma transfusion in horses may fulfill the clinical requirements to restore fluid loss and replace necessary proteins when the RBC component is not compromised. Potential donors can be selected in a hematology laboratory by screening their sera against a RBC panel from 10 to 20 horses to identify those without antibodies against RBCs. Plasma can be collected and stored frozen to administer as needed.

Neonatal Isoerythrolysis in Horses

NI, or jaundice foal syndrome, is an acute hemolytic disease of newborn foals caused by immunologically mediated RBC destruction resulting from maternal–fetal blood group incompatibility. Affected foals are born healthy, but within 2–5 days develop signs of jaundice, lethargy, elevated pulse and respiration rates, and clinical evidence of anemia. NI foals are usually from the second or later pregnancies of a mare, but, on rare occasions, first foals can be affected. The antibodies that sensitize the RBCs of the foal are passively acquired from the dam's colostrum. Recovery may be spontaneous, or the disease may progress to severe anemia and death.

The most common antibodies involved in NI are anti-Aa and anti-Qa, although others are infrequently found. Not all mares negative for EAAa or EAQa factors become sensitized, although they may have produced Aa-positive or Qa-positive foals. One source of sensitization is whole blood transfusion, but this accounts for only a small number of cases. Mares negative for both Aa and Ca factors may be protected from sensitization to Aa by naturally produced anti-Ca antibodies.[11] At present, no simple hypothesis explains why blood group sensitization occurs in only a few percent of mares at risk.

A serum sample taken about 1–3 weeks before a pregnant mare is due to foal can be screened for evidence of blood group incompatibility. If test results are positive for hemolytic activity, it is strongly advised to withhold the foal from its dam's colostrum for 36–48 hours before putting it back to its dam's milk. An alternative colostrum and milk source must be provided to the foal during that period. Appropriate management for a severely anemic NI foal may be a RBC transfusion to alleviate the anemia and prevent death (see chapter 37).

Neonatal Isoerythrolysis in Mule Foals

Mule breeders are keenly aware that mares bred to jacks (male donkeys) can become sensitized to blood group factors of asses.[114] Monitoring of a pregnant mare's serum for antibodies against the RBCs of the jack to which she is bred is a prudent precaution to identify mule neonates at risk for NI.

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CHAPTER 97

Granulocyte and Platelet Antigens

JENNIFER S. THOMAS

Granulocyte Antigens
- Laboratory Techniques to Identify Neutrophil Antigens and Antigen–Antibody Interactions
- Clinical Disorders Associated with Neutrophil Antigens

Platelet Antigens
- Laboratory Techniques to Identify Platelet Antigens and Antigen–Antibody Interactions
- Clinical Disorders Associated with Platelet Antigens

Acronyms and Abbreviations

CD, cluster differentiation; CTL2, choline transporter-like protein 2; EDTA, ethylenediaminetetraacetic acid; GP, glycoprotein; HLA, human leukocyte antigen; HNA, human neutrophil antigen; HPA, human platelet antigen; IMN, immune-mediated neutropenia; IMT, immune-mediated thrombocytopenia; LFA-1, leukocyte function antigen-1; PCR, polymerase chain reaction; TRALI, transfusion-related acute lung injury.

Cell surface antigens on granulocytes and platelets play a role in recognition of self. Antigens vary in their ability to stimulate an immune response and, therefore, in their association with immunologic disorders. Exposure to an antigen during pregnancy, after transfusion or following transplantation, causes immune response if given to individuals that lack the antigen.[58] Immune-mediated destruction of platelets and neutrophils is implicated in a variety of disorders.[58] Much of published research has focused on the role of platelet and granulocyte antigens in immunologic disorders; however, antigen identification is also used as a marker of disease and antigens may play a role in a variety of disorders including infections, neoplasia, and cardiovascular disease.[63] In veterinary medicine, platelet or granulocyte antigens are identified to characterize myeloproliferative disorders, and expression of certain antigens is used to determine cellular activation or function.[64]

Immune responses targeting granulocyte or platelet antigens have been implicated in the pathogenesis of primary (autoimmune or idiopathic) and secondary immune-mediated neutropenia (IMN) or immune-mediated thrombocytopenia (IMT), neonatal alloimmune neutropenia or thrombocytopenia, and transfusion reactions.[10,39,40,47] Binding of antibodies to cell antigens may cause dysfunction of the target cells.[8,27] Immunologic disorders resembling those listed in the earlier text have been identified in veterinary species; however, the pathogenesis of these conditions is better characterized in people because transfusion therapy and neonatal cytopenias are more common.[47] Most of the information provided in this chapter is based upon current understanding of the role of platelet and granulocyte antigens in people. Whenever possible, comparisons between humans and veterinary species are presented.

Platelet or granulocyte antigens can be specific to one cell type, shared by other blood cells, or widely distributed on systemic cells. Antigens are generally classified based upon their biochemical nature into glycolipids, glycoproteins (GPs), or proteins.[40] The roles of some of these antigens in normal cellular function are known. The molecular structures of many antigens have been characterized in people, allowing development of molecular techniques to detect alterations in the nucleic acid structure of the genes encoding the antigens.

Antigens with variable structures that are present in some, but not all, individuals in a species are termed alloantigens. Alloantigens are the products of polymorphic genes. Most granulocyte and platelet alloantigens

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have been linked to single-nucleotide polymorphisms that cause a change in a single amino acid on a protein structure on the cell membrane.[58] The prevalence of the different alloantigens in people varies significantly between ethnic groups.[8,40] It is likely that similar differences exist in the expression of alloantigens among different breeds and species of animals.[47]

Alloantibodies are antibodies directed against alloantigens. Alloantibodies form when an individual receives a blood product that contains an alloantigen not present on self-cells or when there is incompatibility between fetal and maternal blood cells. Isoantibodies are antibodies that target a nonpolymorphic antigen found on the cells of the vast majority of normal individuals in a species. Isoantibodies usually form when an individual who has an inherited deficiency of an antigen is transfused with blood products from a normal individual.[47]

Antibodies that target self-antigens are termed autoantibodies. The production of autoantibodies may be idiopathic or may be secondary to an underlying disorder.[10,53,66] Autoantibody production sometimes follows drug administration. In some cases, the drug or its metabolite must be present for antibody binding to occur. In other cases, the drug induces an antibody that persists after drug administration ceases.[3]

GRANULOCYTE ANTIGENS

Granulocytes include neutrophils, eosinophils, and basophils. Neutrophils are present in highest concentrations in the blood and are most commonly implicated in immunologic disorders.[47] In people, immunologic disorders are associated with antigens in the human leukocyte antigen (HLA) system or the human neutrophil antigen (HNA) system.[17,55,58]

HLA antigens are widespread in distribution and found on many different cell types. HNA antigens are predominantly expressed on neutrophils (Table 97.1), but some are found on other cell types.[17] Because neutrophil antigens exhibit polymorphism, there are a variety of alloantigens. Currently 11 antigens have been characterized and are divided into five systems (HNA-1–HNA-5).[17,58] HNA-1 antigens are found on FcγRIIIb (cluster differentiation (CD)16b), a low-affinity receptor for the Fc portion of immunoglobulins. The HNA-1 system contains four identified alloantigens (HNA-1a, HNA-1b, HNA-1c, and HNA-1d).[17,58] The HNA-2 antigen is found on a 56–64-kDa GP (CD177) that is involved in adhesion to endothelial cells and neutrophil transmigration through the endothelium into the extravascular space.[17] The HNA-3 antigens are found on the choline transporter-like protein 2 (CTL2), a 68–72-kDa transmembrane protein. The HNA-3 system contains two alloantigens, HNA-3a and HNA-3b.[17,58] The HNA-4 antigens are located on the CD11b/α_M-subunit of the CD11b/CD18 complex (Mac-1, CR3), a β_2-integrin that plays a role in adhesion to endothelial cells, transmigration, phagocytosis, and cell-mediated toxicity.[17] Two alloantigens (HNA-4a and HNA-4b) have been identified.[17,58] The HNA-5 antigen system contains two antigens, HLA-5a and HLA-5b. It is found on the α_L-subunit (CD11a) of the CD11a/CD18 complex (leukocyte function antigen-1 [LFA-1]), a β_2-integrin involved in adhesion to endothelial cells.[8,17,55,58]

Some of the GPs in the HNA systems have been identified on neutrophils of domestic mammals using monoclonal antibodies. The relationship between the antigens recognized by these antibodies and the antigens associated with immunologic disorders is not currently known.[47] Antibodies to CD11a and CD11b bind to bovine neutrophils.[22,52] Antibodies to CD11a recognize antigens on neutrophils from dogs, horses, sheep, goats, and rabbits.[6,29,31,46,57] Antibodies to CD11b bind to neutrophils from cats, dogs, sheep, goats, and pigs.[6,46] Anti-CD16 antibodies bind to canine neutrophils.[31,57]

TABLE 97.1 Human neutrophil antigens and associated disorders.

Antigen location	CD designation	Antigen system	Clinical conditions in people associated with antibody–antigen interactions
FcγRIIIb	CD16b	HNA-1	Neonatal alloimmune neutropenia, primary immune-mediated neutropenia, TRALI*, drug-induced neutropenia
56-64-kDa GP	CD177	HNA-2	Primary immune-mediated neutropenia, drug-dependent neutropenia, TRALI
Choline transporter-like protein 2	Undefined	HNA-3	Neonatal alloimmune neutropenia, TRALI, febrile transfusion reaction
MAC-1; CR3	CD11b	HNA-4	Neonatal alloimmune neutropenia, primary immune-mediated neutropenia
LFA-1	CD11a	HNA-5	Unknown

*TRALI = transfusion-related acute lung injury.

Laboratory Techniques to Identify Neutrophil Antigens and Antigen–Antibody Interactions

Identification of neutrophil antigens or antineutrophil antibodies has traditionally relied on agglutination or immunofluorescence assays.[54,55] Monoclonal-antibody capture assays have been developed to detect antibodies to specific human neutrophil antigens.[54] Genotyping assays using PCR techniques are replacing agglutination or immunofluorescence assays in people for immunophenotyping neutrophils,[55] and several commercial kits are available for HNA-1, HNA-3, HNA-4, and HNA-5 antigens.[58] Similar assays are not readily available for veterinary species.

Flow cytometry is used to phenotype neutrophils in people and animals,[46,52,54,55,64] but is limited by availability of monoclonal antibodies that specifically target antigens of interest. Flow cytometry has been used to identify antineutrophil antibodies in animals, but specific target antigens have not been identified and the diagnostic usefulness has been questioned.[61,64]

Clinical Disorders Associated with Neutrophil Antigens

In people, clinical conditions associated with alloantibody interactions include neonatal immune neutropenia, transfusion-related acute lung injury (TRALI), alloimmune neutropenia after bone marrow transplantation, refractoriness to granulocyte transfusions, and febrile transfusion reactions.[8,10,17,55] Febrile transfusion reactions occur when the recipient has alloantibodies directed against antigens present on the donor leukocytes. TRALI occurs when alloantibodies to HLA antigens or neutrophil specific antigens cause neutrophils to become entrapped in the pulmonary vasculature.[55] Binding of alloantibodies to neutrophil antigens causes functional defects such as impaired adhesion and altered respiratory burst.[8,45]

Disorders associated with alloantibody production are not commonly recognized in veterinary species. Neonatal neutropenia rarely occurs in pigs and foals when the dam was previously exposed to paternal antigens on the fetal neutrophils and produces alloantibodies that are passed to the fetus in utero or to the neonate following ingestion of colostrum.[12,33] Febrile reactions in dogs following transfusion of platelet concentrates have been associated with leukocyte contamination.[1]

In people, autoantibody production is implicated in the pathogenesis of primary IMN, secondary IMN, drug-dependent neutropenia, and autoimmune neutropenia after bone marrow transplantation.[2,8,17] The cause of the autoantibody production is unknown in primary IMN. Studies in people have implicated antibodies targeting FcγRIIIb in many patients.[2,10] Secondary IMN is often multifactorial and is associated with systemic immune-mediated disorders (e.g., systemic lupus erythematosus), immunologic disorders, infectious diseases, malignant neoplasia, transplantation, and drug therapy.[2,10] The antigenic target in secondary IMN is often unknown, but antibodies targeting FcγRIIIb antigens have often been identified.[2] Antibodies bind to antigens on neutrophils and lead to premature neutrophil removal by macrophages. In some cases, autoantibodies target antigens on hematopoietic precursor cells in the bone marrow resulting in myeloid hypoplasia. Patients may also have defective neutrophil phagocytosis as well as concurrent thrombocytopenia or hemolytic anemia. Diagnosis often relies on identification of anti-neutrophil antibodies using agglutination, antigen-specific ELISA, immunofluorescence, or flow cytometric assays.[2,10,23,61]

IMN is infrequently reported in veterinary species.[7,14] Antibodies generally target antigens on circulating neutrophils, but bone-marrow-directed immune response causing white cell aplasia has been reported.[62] IMN is suspected when an animal has unexplained neutropenia that is responsive to immunosuppressive drugs. Identification of antineutrophil antibodies is supportive, but diagnostic assays required to detect these antibodies have limited availability and diagnosis is often made by exclusion.[14] Antineutrophil antibodies have been identified in dogs with IMN, but specific target antigens have not been identified.[59,61] Drug-associated neutropenia due to immune responses is infrequently reported. Antineutrophil antibodies have been identified in neutropenic dogs receiving cephalosporin.[4]

Antibodies to neutrophil isoantigens have been identified in human neonates born to mothers who had neutrophils deficient in FcγRIIIb.[19] Although not reported, a similar disorder is possible in dogs or cattle with leukocyte adhesion deficiency syndrome, a deficiency of membrane CD11/CD18 complex.[47]

PLATELET ANTIGENS

In people, platelet membrane surface antigens that play a role in immunologic disorders include those in the HLA system, ABO blood type antigens, and the human platelet antigen (HPA) systems (see Table 97.2).[11,20] HPA antigens are found predominantly on platelets, though some are expressed on other blood cells.[40] There are currently 35 identified HPA antigens[63] that are located on six platelet membrane GPs: $GP\alpha_{IIb}$ (IIb, CD41), $GP\beta_3$ (IIIa, CD61), GPIa (α2, CD49b), $GPIb_{\alpha}$ (CD42b), $GPIb_{\beta}$ (CD42c), and CD109.[11,20,39,40,63] The majority of HPA antigens are found on $GP\alpha_{IIb}$ or $GP\beta_3$.[11,20] Twelve of the antigens are located in six biallelic systems (HPA-1, HPA-2, HPA-3, HPA-4, HPA-5, HPA-15). Most result from a single-amino-acid substitution resulting from a single-nucleotide change. The frequency of the different alloantigens varies in different ethnic populations.[39,40] A total of 28 systems have been identified.[20]

$GP\alpha_{IIb}\beta_3$ is an integrin that is critical for platelet aggregation. Following activation, $GP\alpha_{IIb}\beta_3$ undergoes a conformation change that allows binding to adhesive proteins such as fibrinogen. Glanzmann's thrombasthenia, an inherited or acquired functional or physical deficiency of $GP\alpha_{IIb}\beta_3$, causes a bleeding disorder in people, dogs, and horses.[21] GPIa forms a complex with GPIIa(β_1) to act as a collagen receptor to participate in adhesion

TABLE 97.2 Human platelet antigens and associated disorders.

Antigen location site	CD designation	Human antigen systems	Clinical conditions associated in people with antibody–antigen interactions
$GP\beta_3$ (IIIa)	CD61	HPA-1, HPA-4, HPA-6, HPA-7, HPA-8, HPA-10, HPA-11, HPA-14, HPA-16, HPA-17, HPA-19, HPA-21, HPA-23, HPA-26	Neonatal alloimmune thrombocytopenia; post-transfusion purpura; primary IMT*
$GPIb_\alpha$	CD42b	HPA-2	Primary IMT
$GP\alpha_{IIb}$ (IIb)	CD41	HPA-3, HPA-9, HPA-20, HPA-22, HPA-24, HPA-27, HPA-28	Neonatal alloimmune thrombocytopenia; post-transfusion purpura; primary IMT
GPIa	CD49b	HPA-5, HPA-13, HPA-18, HPA-25	Neonatal alloimmune thrombocytopenia; post-transfusion purpura; primary IMT
$GPIb_\beta$	CD42c	HPA-12	Primary IMT
CD109	CD109	HPA-15	Neonatal alloimmune thrombocytopenia; post-transfusion purpura; refractoriness to transfusion

* IMT = immune-mediated thrombocytopenia.
Sources: Curtis and McFarland[11]; Hayashi and Hirayama.[20]

and signaling for activation of $GP\alpha_{IIb}\beta_3$.[20] $GPIb_\alpha$ and $GPIb_\beta$ covalently bind and associate with GPIX and GPV to form a complex that plays an important role in platelet adhesion to the vessel wall. A functional or physical deficiency of GPIb/GPIX/GPV, termed Bernard-Soulier syndrome, is found in people and is associated with thrombocytopenia, giant platelets, and functional defects.[20] The role of CD109 is not clear. It is detected on activated platelets and may be involved in cell binding to substrates and intercellular communication.[20]

Major platelet GPs appear to be conserved between the species, suggesting systems similar to those in people are likely in domestic mammals. Canine $GPIb_\alpha$ was cloned and is 82% identical with the human sequence in the carboxyl terminus.[24] The nucleotide sequence of canine $GP\beta_3$ is 92% homologous with the human sequence.[34] The amino acid sequence of canine $GP\alpha_{IIb}$ has 83% identity with the human peptide.[9] Monoclonal antibodies are used to identify platelet antigens in a number of species. The association between antigens recognized by the monoclonal antibodies and disease is not known. Monoclonal antibodies to CD41 ($GP\alpha_{IIb}$), CD42a (GPIX), and CD61 ($GP\beta_3$) bind to canine and equine platelets.[35,46] Monoclonal antibodies to CD41/CD61 ($GP\alpha_{IIb}\beta_3$) bind to bovine, porcine, and equine platelets.[35,41,49] Monoclonal antibodies to CD61a and CD62P (P-selectin) bind to bovine platelets.[52] Monoclonal antibodies to GPIb react with feline platelets.[56]

The number of different alloantigens associated with the major platelet antigens and their role in immunologic disorders in veterinary species is unknown.[5,9,34,37] In dogs, sequencing of the genes that encode $GP\beta_3$, $GP\alpha_{IIb}$, and $GPIb_\beta$ identified four single-nucleotide polymorphisms that resulted in amino acid substitutions in the gene encoding GPIIb and the identification of five protein isoforms. No association between breed and the development of thrombocytopenia was identified.[9] In a limited number of horses, sequencing of the genes that encode $GP\beta_3$, $GP\alpha_{IIb}$, GPIa, and $GPIb_\alpha$ identified several nucleotide polymorphisms that resulted in amino acid substitutions.[5]

Laboratory Techniques to Identify Platelet Antigens and Antigen–Antibody Interactions

Platelets are commonly typed in people for purposes of platelet transfusion and as part of a diagnostic workup for suspected immune platelet disorders. Genotyping using a variety of molecular techniques has become standard practice in human medicine to identify platelet antigens and high throughput assays are available.[11,16,58] Advantages include independence from availability of antibodies for typing, capacity to test for large numbers of antigens, and ability to extract DNA from leukocytes or from cell-free material plasma for fetal testing. Serologic phenotyping is still offered, but is more labor-intensive and limited by the availability of reliable antibodies.[16,39,58] Flow cytometry is used in human and veterinary medicine for serologic phenotyping.[16,64]

No single test has proven sufficient to detect antiplatelet antibodies in people.[51] A range of techniques including immunofluorescence, flow cytometry, ELISAs, and immunoradiometric methods have been used to detect antiplatelet antibodies, but testing in veterinary species is not commonly performed.[25,27,30,47,48]

Clinical Disorders Associated with Platelet Antigens

Clinical disorders related to platelet alloantigens are usually associated with the ability of antigens to induce an immune response; however, polymorphisms in the platelet receptors that occur with the different alloantigens may also alter function.[39] Studies suggest that some HPA alloantigens are associated with increased platelet

reactivity and increased risk for coronary heart disease and stroke, metastasis of certain neoplasms, and response to some infections.[63]

The binding of autoantibodies and alloantibodies to platelet membrane GPs often causes platelet destruction and thrombocytopenia. These antibodies may also cause platelet inhibition and increased risk for bleeding or platelet activation and increased risk for thrombotic disorders. Functional defects may be present independent of any associated thrombocytopenia.[13] One study suggested that platelets from dogs with IMT have impaired platelet function due to circulating antibodies.[28] Platelet aggregation was inhibited in mule foals with neonatal alloimmune thrombocytopenia, possibly due to alloantibody competition with collagen-binding sites.[43]

In people, disorders associated with alloantibody production include fetal/neonatal alloimmune thrombocytopenia, post-transfusion purpura, and refractoriness to platelet transfusions.[20,40,44] Neonatal alloimmune thrombocytopenia occurs when a mother produces alloantibodies against a paternal alloantigen expressed on fetal platelets. The majority of cases are associated with an alloantibody against fetal $GP\beta_3$ (HPA-1a). Alloantibodies against fetal GPIa (HPA-5a), CD109 (HPA-15b), or $GP\alpha_{IIb}$ (HPA-3a) are less commonly implicated.[44] Neonatal alloimmune thrombocytopenia has been described in pigs, horses, and mules.[18,42,43] The specific target alloantigen was not identified.

Post-transfusion purpura is a rare condition that occurs 5–12 days after an individual receives a blood transfusion. It is most common in HPA-1a-negative women who have been sensitized by prior transfusion or pregnancy. Other platelet-specific antigens or HLA antigens have been less frequently implicated. The immune response causes the destruction of the individual's own platelets as well as the transfused platelets.[40] Post-transfusion purpura has been described in dogs and pigs; however, an association with a specific alloantigen has not been identified.[32,60]

Platelet refractoriness occurs in people who receive multiple platelet transfusions. These individuals produce alloantibodies that result in shortened survival of transfused platelets. Anti-HLA antibodies are most commonly implicated; however, anti-HPA antibodies are found in a minority of individuals.[40] Platelet refractoriness has been experimentally reproduced in dogs.[50]

IMT occurs when antibodies bind to platelet antigens and cause premature destruction of platelets by the mononuclear phagocytic system or lysis in a complement-dependent manner.[26,66] Studies also implicate direct T-cell-mediated cytotoxicity and impaired megakaryopoiesis and thrombopoiesis in the pathogenesis of thrombocytopenia.[53] In people, target antigens are most commonly located on $GP\alpha_{IIb}\beta_3$.[66] Other targets include specific regions on GPIb/GPIX, GPIV, and $GP\alpha_2\beta_1$.[26] Antibodies to multiple antigens are found in many patients.[53]

Cryptic epitopes, particularly involving $GP\alpha_{IIb}\beta_3$, have been implicated in both initiation and perpetuation of thrombocytopenia in patients with IMT.[66] These epitopes are normally hidden, but may become exposed and initiate an immune reaction following an insult such as an infection. Exposure of cryptic antigens is also involved in EDTA-dependent thrombocytopenia in people. EDTA binds calcium, causing a conformational change in $GP\alpha_{IIb}\beta_3$ and exposing normally hidden epitopes which bind to circulating antibodies. EDTA-dependent pseudothrombocytopenia has been rarely reported in dogs, horses, and pigs. The underlying mechanism in these veterinary species has yet to be determined.[65]

IMT is commonly diagnosed in dogs and sporadically identified in other veterinary species (see Chapter 80).[15,48] In primary IMT, the immune response is directed against autoantigens and an underlying disease is not identified. In secondary IMT, the immune response is associated with an underlying disorder (e.g., systemic immune-mediated disease, infection, neoplasia). The antibodies can be directed against either autoantigens, foreign antigens absorbed to platelet membranes, or altered platelet antigens. Platelet-surface-associated antibodies (PSAIg) have been demonstrated in dogs, cats, and horses with IMT.[15,25,27,30,38,48] In most cases, the target antigens are not identified. $GP\alpha_{IIb}\beta_3$ and GPIb were identified as target antigens in some dogs with a clinical diagnosis of primary IMT.[30]

Isoantibodies have been detected in people with an inherited deficiency of platelet membrane antigens. People with a deficiency of $GP\alpha_{IIb}\beta_3$ produce isoantibodies following transfusion of platelets from normal individuals.[36] The isoantibodies appear to target epitopes on β_3 or intact $\alpha_{IIb}\beta_3$, leading to inhibited function and rapid removal of transfused platelets. Although not reported, similar responses could occur in animals with inherited membrane GP deficiencies.[47]

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CHAPTER 98

Principles of Canine and Feline Blood Collection, Processing, and Storage

ANTHONY C. G. ABRAMS-OGG and SHAUNA L. BLOIS

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Acronyms and Abbreviations

ACD, acid-citrate-dextrose; APS, anticoagulant–preservative solution; AS, additive solution; CPDA-1, citrate-phosphate-dextrose-adenine-1; DPG, 2,3-diphosphoglycerate; EBV, estimated blood volume; FeLV, feline leukemia virus; FFP, fresh frozen plasma; FP, frozen plasma; FWB, fresh whole blood; Hgb, hemoglobin; MAP, mean arterial pressure; PAGGGM, phosphate-adenine-glucose-guanosine-gluconate-mannitol; PCV, packed cell volume; pRBCs, packed red blood cells; PC, platelet concentrate; PRP, platelet-rich plasma; RBC, red blood cell; RT, room temperature; SAGM, saline-adenine-glucose-mannitol; WB, whole blood; WBC, white blood cell.

BLOOD BANKING—INTRODUCTION

The advent of large-scale veterinary blood banks has made the practice of high-quality transfusion medicine possible by increasing availability of blood components. Strict adherence to blood collection, processing, and storage guidelines is essential to providing safe and effective transfusions. This chapter details the currently accepted blend of human protocol and veterinary modifications where necessary for the collection, processing, and storage of canine and feline blood components.

DONOR CONSIDERATIONS

Canine

Veterinary blood banks currently use resident dogs, client-owned dogs, or a combination of the two to meet supply demands. Because human blood packs are used for collection, a donation has historically been one unit of blood (450 ± 45 mL), which was the human standard. In some countries, including the USA, some 450 mL packs are no longer available, necessitating the use of packs provided for a collection of 500 ± 50 mL.

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Based on an estimated blood volume (EBV) of 85 mL/kg body weight[42] and a recommended collection volume of 15–22% EBV,[1,18] the maximum donation volume is approximately 19 mL/kg. In a study evaluating changes in systolic blood pressure in 19 Greyhounds (27–41 kg) after donating 450 mL blood, the mean systolic pressure dropped from 145 to 134 mmHg, not a clinically relevant decrease.[18] Recommended minimal donor body weight varies among veterinary blood banks from 24 to 27 kg, with dogs of this size able to donate a 450–500 mL unit as often as every 3–4 weeks on a short-term basis.[1,66] Donor blood-type considerations are discussed in Chapters 96, 97, 99–101, and 104.

For the safety of both donor and recipient, the health of a dog is evaluated prior to enrollment as a donor and then annually through a complete medical history, physical examination, and laboratory evaluation including a hemogram, serum biochemistry profile, and infectious disease screening. In addition, a brief history, physical examination, and measurement of PCV or hemoglobin (Hgb) concentration are performed prior to each donation; donor PCV or Hgb concentration should be at least 40% or 13 g/dL, respectively. Donors should be current on vaccinations and maintained on regionally appropriate parasite prophylaxis. Most other drugs are unacceptable for use during the donation period because of unknown effects on blood quality and potential for adverse reactions in the recipient.

Minimal and optimal screening for infectious diseases are detailed in the American College of Veterinary Internal Medicine Consensus Statement[86] and elsewhere.[21] Briefly and considering endemic diseases, blood donor candidates should be negative for infectious diseases including *Anaplasma spp*., *Babesia canis* and *Babesia gibsoni*, *Bartonella henselae* and *Bartonella vinsonii*, *Ehrlichia canis*, and *Mycoplasma haemocanis*. Screening for other *Babesia* and *Bartonella spp*.; *Brucella canis*; other *Ehrlichia spp*.; *Hepatozoon spp*.; *Leishmania donovani*; *Mycoplasma haematoparvum*; *Neorickettsia risticii*; *Rickettsia felis*; and *Trypanosoma cruzi* should be considered depending on region. Exclusion from a blood donor program does not imply that the animal should be treated for the disease. Iron supplementation in canine blood donors is not a common practice,[1,41] although frequent blood donation (e.g., 13% EBV every 2 months, or more frequently) and long-term donation can result in iron deficiency.[27,29,95] Donation intervals should be considered in light of these findings.

Prior transfusion is an exclusion criterion for dogs to become blood donors. Following a transfusion, dogs may develop antibodies against foreign RBC antigens, resulting in an increased risk of incompatibility reactions to their plasma. Previous pregnancy had long been considered an exclusion criterion in the event that a dog negative for a specific RBC antigen became sensitized by her pups positive for that antigen. This risk is minimal considering zonal placentation of dogs, and there is little evidence to support this exclusion.[7]

Feline

Because cats have typically been sedated/anesthetized for blood collection, most blood banks have relied on resident or fostered cats. The EBV of cats varies with the reference, ranging from 40 to 60 mL/kg in the anesthetized cat,[33] to as high as 67 mL/kg.[80] Donation volume should be calculated based on lean body weight; recommendations for maximum donation range from 11 to 15 mL/kg, and standard volumes collected (including anticoagulant) range from 45 to 70 mL.[1,34,38,39] Because of sedation and blood volume collected, hypotension during donation is a major consideration, especially given the emerging concept that cumulative episodes of subclinical acute kidney injury contribute to chronic kidney disease.[19] Further details regarding sedation and hypotension are provided in the following text in the "Preparation and Collection of Whole Blood" section. Supplemental iron can be given, but is not a common practice.[1,41] Assuming a similar mean concentration of 0.5 mg iron/mL blood in cats as in humans, a 60 mL donation represents a loss of 30 mg of iron. This may be replaced with 150 mg oral ferrous sulfate, typically divided into several doses in between donations and adjusted based on predonation PCV.

Initial and predonation health assessment and health maintenance are similar to canine donors.[63] Because cats may have subclinical heart disease, thorough cardiac auscultation should be performed prior to each donation and periodic screening by echocardiography and pro-brain natriuretic peptide should be considered. Donor PCV or Hgb concentration should be at least 30% or 10 g/dL, respectively. Feline donors should be kept indoors and be given parasite prophylaxis to minimize the risk of infectious disease transmission and maintain optimal health. Similar to dogs, minimal and optimal infectious disease screening recommendations are outlined in published guidelines.[50,63,86] Briefly, cats should be negative for *Anaplasma phagocytophilum*, *Bartonella spp*., feline leukemia virus (FeLV), feline immunodeficiency virus, and hemotropic *Mycoplasma spp*. Due to the possibility of regressive FeLV infection being transmitted via blood transfusion,[59] updated guidelines recommend FeLV provirus PCR testing in addition to, or in lieu of, antigen testing.[50,59,63,86] Screening for *Cytauxzoon felis* and rickettsial diseases may be considered based on exposure risk. As with dogs, exclusion from a blood donor program does not imply that the cat should be treated for the disease. In addition, one must consider the potential ramifications of false positive results for feline retroviral infections, especially unwarranted euthanasia.

BLOOD COLLECTION PRINCIPLES

Blood is usually collected as whole blood (WB) and then separated into components by centrifugation. Alternatively, single components may be collected by apheresis (see Chapter 101). For optimal quality, WB should be collected into commercially available, sterile, airtight systems that allow for subsequent processing and storage of components without exposure to the environment. These systems, known as closed systems, prevent exposure of blood components to air except when the component unit is entered with an

administration set for transfusion. Conversely, an open system has one or more additional sites of entry and possible bacterial contamination, such as occurs when preparing for blood collection into syringes or bags into which anticoagulant is added after manufacture. Components prepared from blood collected into an open system are not intended for storage. If an open collection system is used, the American Association of Blood Banks and European Directorate for the Quality of Medicines advise that RBC components (stored at 1–6 °C) should be used within 24 hours and platelet components (stored at 20–24 °C) should be used within 4 hours.[26,30] The shelf-life of plasma from an open system is unaffected if it is frozen within 8 hours.[26,30] In veterinary medicine, current standard canine and feline donations use closed and open systems, respectively, but the feline components are stored similarly to the canine ones. Detailed illustrated methods of blood collection and processing have been previously described and will be summarized here.[1,2,43,49,72,92]

BLOOD COLLECTION SUPPLIES

Canine

Commercial 450–500 mL collection bags manufactured for human blood banking are used for canine donations. These contain an anticoagulant–preservative solution (APS) and a sterile collection line with a 16-gauge thin-walled needle attached to the bag. The bags are available in a variety of configurations consisting of a primary collection bag with attached satellite bags (0–4). These satellite bags, some of which may contain RBC nutrient additive solutions (AS), are used for the separation of components as discussed later. After centrifugation and removal of plasma from the main collection bag, the nutrient AS is added to the packed red blood cells (pRBCs). Various AS are composed of saline, adenine, and dextrose ± mannitol ± citrate–citric acid, and are intended to extend the shelf-life of stored pRBCs (see Storage – "Red Blood Cell Components" section and Chapter 99).

Collection bags are available in a variety of other sizes (150 mL, 250 mL, and 350 mL) and contain the appropriate amount of APS and AS. Another option for collection of <450–500 mL WB is to transfer the unwanted portion of the APS from the primary bag of a 450–500 mL pack into an empty satellite bag, which is later discarded. This allows collection of proportionately smaller units in a closed system without excessive concentration of anticoagulant; the desired ratio of APS to blood is 1.4:10.[30]

Feline

The closed collection systems used in dogs have been used in cats, although some find the large needle unwieldy.[1] A closed collection system using a 21-gauge needle and citrate-phosphate-dextrose-adenine-1 (CPDA-1) designed specifically for feline blood donation has recently become commercially available (TEC 724 Kit, Futurlab Srl, Limena, Padova, Italy).[22] A feline closed collection system has also been prepared by sterilely docking a collection needle to a 75 mL pediatric transfer pack (Pedi-Pak, Genesis BPS, Hackensack, NJ) containing APS using a commercial sterile tubing welder (Terumo BCT or Genesis BPS).[81] However, most blood collection systems currently in use for cats are open using a 19- or 21-gauge butterfly needle attached to a syringe or collection bag. These systems include: (1) collection into syringes for prompt transfusion of WB; (2) commercial 100 mL bags and collection tubing with attached butterfly needle for gravity collection; (3) attached three-way stopcock, 60 mL syringe, and butterfly needle for syringe collection and subsequent transfer to bag (Animal Blood Resources International, Stockbridge, MI); and (4) collection into syringes (preferably 35 or 60 mL) and then careful injection (to avoid hemolysis) into a transfer pack, either 75 mL (Pedi-Pak) or 150 mL (Fenwal). If an open system for cats is used, care must be taken to maintain sterility where possible, especially if the blood or blood components are to be stored.[22] Periodic culture of stored bags is recommended. The APS used most often are CPDA-1 at an APS:blood ratio of 1:7 (e.g., 7.5 mL CPDA-1 + 52.5 mL blood = final volume 60 mL) or acid-citrate-dextrose (ACD)-A at an APS:blood ratio of 1:6.4–6.5 (e.g., 7 mL ACD-A + 45 mL blood = final volume 52 mL; 8 mL ACD-A + 52 mL blood = final volume 60 mL). As with dogs, pRBCs may be prepared and an AS added.

PREPARATION AND COLLECTION OF WHOLE BLOOD

Canine

A full unit contains 450–500 mL of WB, but 10% variation (405–495 mL, 450–550 mL, respectively) is acceptable considering the volume of anticoagulant in the collection bag. The volume of collected blood is estimated by weight. The specific gravity of whole blood at 37 °C is 1.053, so in an ideal 450 mL unit the blood weighs 474 g and the acceptable range is 426–521 g, while in an ideal 500 mL unit the blood weighs 527 with a range of 474–579 g.[30,85] The minimum acceptable underdraw into a 450 mL or 500 mL bag for blood to be used to prepare pRBCs is 300 mL (316 g) or 333 mL (351 g), respectively, in which case the plasma is discarded as it contains excessive anticoagulant. Weight during collection may be determined by placing the bag on a scale or by using a blood collection mixer scale which automatically stops the collection at the desired weight (many brands are available).

Fasting prior to donation is not necessary. Postprandial lipemia is not problematic in most blood components and indeed is beneficial for preparing platelet-rich components.[5,70] The main exception is when pRBCs are resuspended in plasma rather than an AS, where lipemia in the plasma will cause hemolysis.[5] Most donors habituate to the procedure and do not require sedation. If sedation is required, butorphanol, 0.2–0.4 mg/kg IM, is

recommended. The jugular vein is the only recommended site for routine blood donation. The authors' choice of position for the donor is lateral recumbency on a table, but sternal recumbency or sitting on a table or floor may be better in some circumstances. The venipuncture site is clipped and surgically prepared.

Blood may be drawn into collection bags aided only by gravity and donor blood pressure, in which case the bag should be continuously and gently rocked by an assistant during collection, which typically takes 5–15 minutes (mean 6 minutes in one study[16]). Alternatively, light suction may be used, shortening collection time to 3–10 minutes (mean 4 minutes[16]). The blood collection bag is hung from a hook inside a clear cylinder with a flat lid (Animal Blood Resources International) such that the blood will be forced to pass through the APS. The collection line with attached needle is brought through a notch between the cylinder and lid, and a tube clamp is placed on the line near the needle. A vacuum source is connected via tubing to an inlet in the chamber. The cylinder is placed on a scale, the scale is tared to zero, and the suction is adjusted to −127 to −178 mmHg (−5 to −7 inches Hg).[43] With one hand, the phlebotomist puts gentle pressure on the jugular vein below the prepared skin site, and the needle held in the other hand is inserted through the prepared site into the donor's jugular vein in either direction. When the tubing clamp is removed, blood flows into the collection line to the bag as the scale measures the grams of blood collected. When the desired amount has been obtained, pressure is released from the jugular below the venipuncture site, the line is clamped, and the needle is removed from the vein as pressure is applied over the site.

During blood collection, the well-being of the donor should be continuously monitored. Mucous membrane color, pulse rate and strength, respiratory rate, and donor attitude are easily evaluated by the phlebotomist and assistant. Mild tachycardia is common. Monitoring of blood pressure and other parameters may be considered.[16,52,54] Any indication of donor compromise is cause for discontinuation of the collection.

Once the needle is removed from the donor, the blood in the tubing is stripped into the bag using a tube stripper, and the bag is gently rocked to ensuring adequate mixing. The bag is then manually compressed to refill the tubing, which is sealed (using hand sealer clips or a thermal sealer) at several points to provide small segments containing WB for quality and compatibility testing.

Feline

As noted in the earlier text, sedation/anesthesia is frequently used to enable blood collection from cats. Oral sedation with gabapentin and/or trazodone prior to transport of fostered donors to the clinic is frequently used at the authors' institution to decrease anxiety associated with transport and facilitate initial handling and further sedation. Reported drug protocols for collection include: ketamine and midazolam (ketamine 5–10 mg/kg + midazolam 0.2 mg/kg IM, or ketamine 2 mg/kg + midazolam 0.1 mg/kg IV), with or without butorphanol (0.2 mg/kg); tiletamine and zolazepam (2.5 mg/kg of each, IM); dexmedetomidine plus butorphanol (dexmedetomidine 10 μg/kg + butorphanol 0.2 mg/kg, IM); alfaxalone plus butorphanol (alfaxalone 2 mg/kg + butorphanol 0.2 mg/kg, IM); or sevoflurane by facemask.[1,2,4,22,24,32,38,39,45,49,51,71,79,84] Optimal sedation/anesthesia protocols will vary between institutions and individual donors. One study comparing alfaxalone plus butorphanol versus dexmedetomidine plus butorphanol revealed similar sedation, ease of blood collection, and recovery scores between the two protocols.[71] A recent study described only minor movement or anxiety-related concerns when using unsedated client-owned cats.[24] However, it is notable that 3 cats in the unsedated group experienced cardiovascular or respiratory distress, while none of the sedated cats exhibited these events.[24] Vascular access ports have also been evaluated for donation to avoid anesthesia.[4,57]

The cat is positioned in lateral (preferred by authors due to steadiness) or sternal recumbency, and the jugular venipuncture site surgically prepared. If a syringe or vacuum chamber is used, only gentle suction (−76 mmHg [−3 inches Hg]) is applied to avoid collapsing the vein or causing hemolysis. Blood collection is typically completed within 3–5 minutes. Careful monitoring, including blood pressure assessment, of the cat is essential because hypotension may be encountered despite all precautions. Studies have reported transient hypotension after collection of 50–55 mL blood in cats sedated with varying protocols. Cats did not show clinical signs related to hypotension in these studies.[4,39,45] Intravenous fluid therapy to treat hypotension was necessary in 84% of blood donors anesthetized with sevoflurane and 42% anesthetized with a combination of ketamine, butorphanol, and midazolam in one study; there was no significant difference in blood pressure between the groups.[45] In that same study, postprocedure hyperthermia and a longer return to normal behavior were noted in donors given ketamine, butorphanol, and midazolam.[45] Signs of hypotension or possibly vasovagal reactions were seen in another study of donation of unsedated cats.[24] Collection via vascular access ports also resulted in suspected vasovagal reactions in two lightly sedated cats from one study,[4] while in another study, 10 mL/kg blood collection from vascular access reports from awake cats did not result in clinical signs of hypotension.[57] A recent study showed that while donor cats sedated with alfaxalone plus butorphanol had a significantly decreased blood pressure following blood collection, no cats experienced hypotension.[32] In contrast to the abovementioned study, one study reported mild increases in oscillometric tail blood pressure readings in donor cats sedated with tiletamine and zolazepam, presumed due to, in part, the increase in sympathetic tone caused by these agents.[79] In the authors' experience, cats donating less than 11 mL/kg are less likely to have hypotension-related problems. Some blood banks routinely give donors intravenous and/or subcutaneous

saline at a total dose of one to three times the volume of blood drawn, while others only treat the donors if hypotension or signs of hypotension occur.

LEUKOREDUCTION

Leukoreduction (LR), or leukodepletion, is the process of removing residual WBC from transfusion components aimed at reducing transfusion-induced inflammatory responses.[10,11,13,55] Leukoreduction is usually performed prior to storage by passing blood through a leukoreduction filter at room temperature (RT), aided by gravity. Blood is typically refrigerated for approximately 4 hours prior to filtration to enhance leukoreduction,[10,11] but this may not be required with some newer filters and manufacturers should be consulted in this regard. Filters retain WBC using either selective pore sizes or adsorption membranes. Most filters also exclude platelets; a platelet-sparing filter is available.[75] The process removes >99.9% of WBC from the blood.[11] Subsequently, blood can be stored as whole blood or separated into components.

Prestorage leukoreduction has been shown to be effective in markedly reducing WBC counts in canine blood, and RBC viability is preserved following filtration.[11,55] In addition to removing WBC, leukoreduction is effective in attenuating the accumulation of bioactive substances in canine packed red cells.[17,31,35,58,74,94]

Leukoreduction has been shown to decrease some transfusion reactions in people, such as febrile nonhemolytic transfusion reactions and complications related to alloimmunization, and improve outcome in some patient subpopulations.[10] In veterinary medicine, leukoreduction has mainly been investigated in healthy dogs. Healthy dogs showed an inflammatory response following transfusion of autologous non-LR pRBCs, while LR pRBCs did not induce this same effect in one study.[55] However, leukoreduction did not mitigate post-transfusion inflammation in another study.[13] A prospective, blinded study of 23 clinically ill dogs receiving pRBC transfusions showed that most markers of inflammation were similar after transfusion of LR versus non-LR pRBCs and no changes in outcome were observed between the groups.[10] Clinical benefits of leukoreduction in veterinary medicine are currently unknown. Drawbacks of leukoreduction include increased processing time for blood components, cost of the filters, and loss of a small volume of blood component in the filter.

IRRADIATION, ULTRAVIOLET TREATMENT, AND PATHOGEN INACTIVATION OF BLOOD COMPONENTS

Irradiation, ultraviolet illumination, and photochemical treatment of blood components may be used to decrease alloimmunization, graft-versus-host disease, and pathogen contamination. These treatments of canine blood have been reported in research trials, but are not used clinically at this time.[60,67,94]

SEPARATION OF WHOLE BLOOD INTO COMPONENTS

A flowchart for the separation of fresh whole blood (FWB, less than 8 hours old) by centrifugation is presented in Figure 98.1. Separation requires a large-capacity, floor-model, swing-bucket, temperature-controlled centrifuge. If a centrifuge is not available, some components may be prepared by gravity separation.[1,48]

Canine Packed Red Blood Cells and Plasma

Standard separation of canine FWB into pRBCs and fresh plasma is begun by centrifugation of the blood bags at 5000 × *g* for 5 minutes at 4 °C. The centrifugation time includes acceleration time, but not deceleration time. To transfer the plasma to a satellite bag, the primary bag is placed in a plasma extractor, and the seal in the tubing of the primary bag is broken. The plasma extractor is slowly engaged and allowed to gently squeeze the primary bag, which expresses the plasma into the satellite bag. Plasma may be transferred entirely to one satellite bag or divided between two or three bags. Expression of plasma is stopped by clamping the tube. If the pRBCs are to be stored in CPDA-1 without an AS, the pRBCs must have a PCV ≤ 80%. This requires leaving ≥50 mL of plasma with the pRBCs, which may be accomplished by stopping the expression of plasma when the buffy coat/plasma interface is about 2 cm from the top of the bag. Once the plasma has been removed to one or more satellite bags, the AS is added to the pRBCs. The seal in the tubing to the AS bag is broken, and the solution is transferred into the primary bag by gravity and gently mixed. If desired, half of the pRBCs can then be transferred to an additional empty satellite bag if available to prepare half-units. The tubing attached to each component bag is then sealed.

Each bag of component is weighed, and the weight of the component calculated by subtracting the weight of the bag. Volumes of plasma and pRBCs at RT are then estimated by dividing by 1.026 and 1.056, respectively[85] (these values are for humans, and specific gravity varies with temperature, PCV, and protein concentration, but effects on volume estimates are clinically negligible). Each component bag is labeled as to component, blood type, donor, volume, and dates of collection and expiration.

Canine Platelet-Rich Plasma and Platelet Concentrate

Fresh whole blood can also be separated into platelet-rich plasma (PRP) and pRBCs. The FWB may be allowed to rest for 1 hour after collection to minimize platelet activation, and the bag should be gently massaged

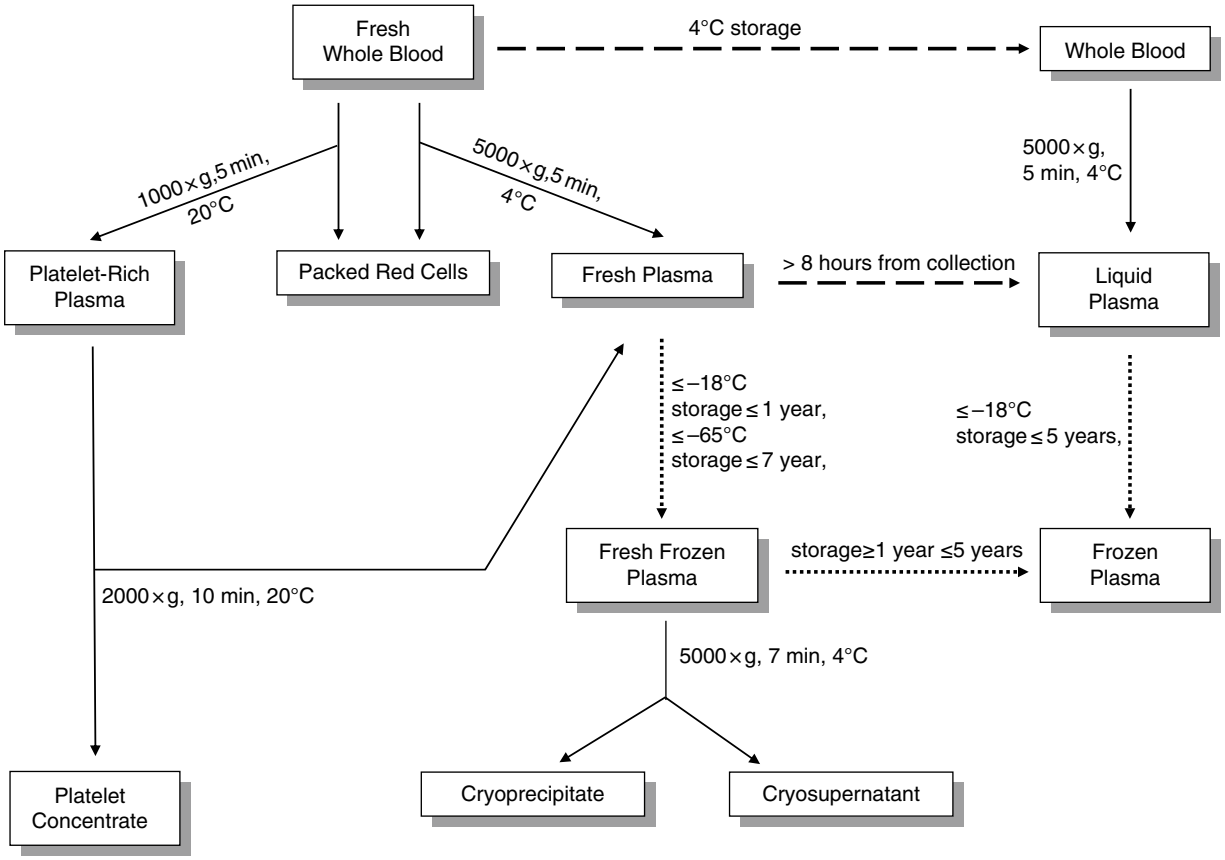


FIGURE 98.1 Flowchart for separation of canine fresh whole blood into components. Further preparation of the various plasma components is discussed in Chapter 100. (**Source:** Abrams-Ogg[1])

before centrifugation to resuspend platelets.[44] Centrifuge temperature should be set at 20–24 °C. Centrifugation to make PRP uses lighter gravitational forces than for standard separation of pRBCs and plasma, and isolates the platelets in the plasma above the buffy coat. When the PRP is expressed into the satellite bag, expression should be stopped when the RBC–plasma interface is 1 cm from the top of the bag to minimize leukocyte and RBC content of PRP while not sacrificing platelet yield.[14] One unit of FWB (450 ± 45 mL; 500 ± 50 mL) yields one unit of PRP.

Percent platelet yield (platelet count in PRP/platelet count in FWB × 100) and PRP platelet count vary with the donor, donor platelet count, blood volume being centrifuged, technician, centrifuge, and centrifugation protocol. In a study comparing protocols to make PRP from small volumes of canine blood, protocols with shorter centrifugation times and higher gravitational forces had better yields than protocols with longer times and lower forces.[15] Most current blood bank protocols use about 1000–1600 × *g* for 4–6 minutes or 2000 × *g* to 2500 × *g* for 2.5–3 minutes.[3,37,70] The brake on the centrifuge should be turned to a low setting or turned off. In the protocol used by the authors (1000 × *g* for 4 minutes), average yield from 450 mL FWB is about 80% (range 35–97%), resulting in a mean of 6×10^{10} platelets/unit (range 3×10^{10} to 10×10^{10} platelets/unit).[3] Donors with high normal platelet counts are preferred.[70] A second centrifugation of the FWB can be used to increase yield with Greyhounds which have low normal platelet counts (W. J. Dodds, personal communication).

Platelet concentrate (PC) may be prepared by centrifugation (2000 × *g* for 10 minutes) of PRP to pellet the platelets. The platelet-poor plasma (PPP) is expressed, leaving 35–70 mL of plasma and the platelet pellet behind in the satellite bag. The resulting PC is left undisturbed for 60–90 minutes to promote disaggregation. Gentle manual kneading and agitation are then used to disperse macroscopic leukocyte–platelet aggregates and resuspend the platelets. Adding prostaglandin E1 to the PRP prior to centrifugation reduces this aggregation and facilitates resuspension without reducing platelet function.[73] The PPP may be used as fresh plasma or fresh frozen plasma (FFP). One unit of PRP yields one unit of PC. Units of PC can be pooled via transfer tubing. The pooled units are centrifuged at 570 × *g* for 15 minutes and PPP is expressed, leaving behind 8–10 mL per unit pooled. Once pooled, the concentrate needs to rest for at least 15 minutes at room temperature (60 minutes preferred), prior to resuspension (K. J. Wardrop, personal communication).

The buffy coat method is an alternate method for producing PC and is used more than the PRP method for humans.[37,64] In a canine study, WB was centrifuged at 3000 × *g* for 11 minutes to push platelets into the buffy coat.[37] All but 30 mL of the PPP was transferred to a

satellite bag, and the remaining plasma, buffy coat, and 20 mL of the upper portion of RBCs transferred to another satellite bag and centrifuged at 1000 × *g* for 5 minutes to separate the platelets from WBCs. Platelet yield was similar to the PRP method, but the buffy coat method resulted in more WBC and RBC contamination. In human blood banks, buffy-coat-derived PC are typically pooled and leukoreduced prior to storage.

Feline Blood Components

Feline FWB is placed in one bag, and the tubing between this bag and an empty satellite bag is obstructed by folding and securing with a rubber band or a hand sealer clamp. Due to the relatively small volume of blood being separated, the collection/satellite bags may be bound (via rubber band or tape) to a 500 mL bag of saline to prevent the bags from collapsing during centrifugation. The blood bags are centrifuged at 3000 × *g* for 10 minutes at 4 °C, and separation is continued as for dogs. The pRBCs may be stored without addition of AS (in which case 10 mL 0.9% sodium chloride is added just prior to administration to the patient), or 10 mL AS may be added to pRBCs prior to storage.

Feline PRP is infrequently prepared due to a limited clinical need, as well as technical challenges; FWB is more likely to be administered to thrombocytopenic or thrombocytopathic cats with severe bleeding. However, preparation of feline PRP has been described, with the transfused platelets exhibiting in vivo efficacy.[15,20]

STORAGE

Red Blood Cell Components

Units of WB and pRBCs are stored at 1–6 °C with lower in the range preferred especially with longer storage times.[6] A refrigerator with an alarm to indicate unsafe temperatures is ideal. Alternatives include a regularly checked thermometer placed in a container of fluid the volume of a small unit, or use of HemoTemp II blood bag thermometer labels (Biosynergy, Elk Village, IL).

Red blood cell viability and function decrease during storage as a result of physical, morphologic, metabolic, and immunologic changes collectively known as the storage lesion, with potential to negatively impact the transfusion recipient.[61] Major cellular changes during storage include depletion of ATP and 2,3-diphosphoglycerate (DPG) and alteration of nitric oxide activity.[61,87,89,91] These changes can impact RBC oxygen delivery, although the significance of 2,3-DPG depletion is questionable, as it is restored following transfusion and does not play a primary role in feline hemoglobin–oxygen modification.[61]

The RBC morphological changes observed early in storage can be reversible following transfusion. Over time, however, changes become irreversible and RBC membranes begin to shed microparticles. Decreased RBC deformability leads to diminished circulation within smaller vessels and RBC adhesion to endothelium, impairing oxygen delivery.[34,61] The accumulating microparticles express negatively charged proteins such as phosphatidylserine and have procoagulant and pro-inflammatory properties.[35,53,76] Progressive hemolysis during storage releases free hemoglobin, heme, and iron into the supernatant.[9,28]

Extracellular changes include accumulation of ammonia, sodium, chloride, and lactate, while pH and glucose concentrations decline; potassium changes are variable over time and between species.[22,23,34,47,90] Some proinflammatory eicosanoids and cytokines have been documented to accumulate in stored blood components.[8,17,23,58,69,94]

The storage lesion impacts RBC shelf-life, which is defined as the number of days after collection, assuming proper closed system collection and storage, at which 75% RBC viability is maintained.[26,30] Viability is measured as 24-hour post-transfusion survival of radiolabeled or biotinylated RBCs.[88] Red cell AS provide substrates for RBC metabolism and help delay onset of storage lesion.[9,47,87,89]

Canine WB in CPDA-1 is by convention stored for up to 35 days, supported by one viability study.[12] Canine pRBCs, based on viability studies, have a maximum storage time of 20 days in CPDA-1,[68] whereas canine pRBCs stored in AS-1 (Adsol) and AS-3 (Nutricel) may be stored for 35 days.[87,89] Similarly, in a study using in vitro tests, including percent hemolysis, of an AS consisting of saline-adenine-glucose-mannitol (SAGM), 35-day storage was acceptable.[28] Maximum storage of pRBCs in AS-5 (Optisol) is also 35 days by convention. As noted in the earlier text, leukoreduction can diminish the accumulation of pro-inflammatory cytokines and other substances, and is thought to reduce development of some storage lesions.[17,25,69,94] One study reported that viability was maintained at 35 days in leukoreduced pRBCs stored in Adsol,[11] and another study suggested a maximum storage of 42 days with SAGM based on in vitro tests.[25] However, using percent hemolysis and other in vitro tests, similar rates of storage lesion development were observed with the AS phosphate-adenine-glucose-guanosine-gluconate-mannitol (PAGGGM), SAGM, Adsol, and Optisol, and a maximum storage of 21 days was suggested.[47]

Feline WB in CPDA-1 is by convention also stored up to 35 days, which is supported by one viability study, and several studies using in vitro; tests including osmotic fragility and degree of hemolysis.[12,22,51,77,78] Feline pRBCs in an AS are also by convention stored for up to 35 days. However, a study of feline pRBCs stored in SAGM recommended a maximum storage of 28 days based on in vitro hemolysis.[9] Other studies of feline pRBC documented similar changes in pRBCs stored in Optisol for 25 days and Nutricel for up to 35 days.[4,34] Clinical implications of the storage lesion and shelf-life are discussed in Chapter 99.

Cryopreservation is approved for longer-term storage of human pRBCs and is feasible for canine and feline pRBCs.[65] Another option to prolong shelf-life is the use of blood substitutes including polymerized hemoglobin, perfluorocarbons, and hemoglobin–albumin clusters under various stages of development.[93]

Plasma and Platelet Components

All plasma components should be stored at −18 °C or lower in a cardboard plasma box which protects the bag from breakage (see Chapter 100). A freezer with an alarm to indicate unsafe temperatures is ideal. If a regular household freezer is used, exposure of the FFP to repeated freeze/thaw cycles will result in loss of the more labile plasma coagulation factors.

Platelet-rich plasma and PCs are usually stored at room temperature in plastic bags under continuous gentle agitation (see Chapter 101).[83] The composition of the plastic bag, high surface area:volume ratio, and agitation facilitate gas exchange and sustain aerobic metabolism, and thereby platelet viability. Platelet additive solutions may potentially be used to prolong storage time.[36]

QUALITY CONTROL

Properly collected blood is anticipated to have a low level of bacterial contamination.[56] Nonetheless, transfusion-associated sepsis is always a risk, and units of WB and pRBCs should be visually inspected during storage and prior to administration. The presence of murky, purple, brown, or red supernatant RBC mass that appears purple and has visible clots or differences in color between the unit and the tubing segments gives reason to suspect bacterial contamination.[38,46,86] Pretransfusion hemolysis is also always a risk.[62] Visual inspection to identify in vitro hemolysis is not an accurate method in determining the precise level of hemolysis, and free hemoglobin measurement can be considered to ensure that red cell units have <1% hemolysis.[28,40]

Plasma components should be inspected when issued for transfusion and discarded if cracks or leaks are noted. Inadvertent thawing during storage may reduce levels of factor VIII and von Willebrand factor, and allow for bacterial proliferation. In the absence of a freezer alarm or chart recorder, the following techniques may be used to detect inadvertent thawing and refreezing of a unit after its original freezing: (1) Store the plasma box initially lying flat in the freezer. After complete freezing, shift the box to sit on one end. Air bubbles present in the bag will move from the front edge to the upper edge of the bag if thawing occurs, and the unit will also be thicker on the lower edge. (2) Place a rubber band around the unit before freezing, so that it makes an indentation in the bag. After complete freezing, cut the rubber band. If thawing occurs, the indentation left by the rubber band will be less obvious or vanish.

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CHAPTER 99

Red Blood Cell Transfusion in the Dog and Cat

MARY BETH CALLAN

Indications
- Transfusion Threshold
- Whole Blood Versus Packed RBCs
- Fresh Versus Stored RBCs

Pretransfusion Compatibility Testing
Xenotransfusion in Cats
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- Warming of Blood
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Patient Monitoring

Acronyms and Abbreviations

AABB, American Association of Blood Banks; ADCAS, anemic dog clinical assessment score; FDA, Food and Drug Administration; FFP, fresh frozen plasma; FWB, fresh whole blood; Hct, hematocrit; Hgb, hemoglobin; [Hgb], hemoglobin concentration; NTBI, non-transferrin-bound iron; pRBCs, packed red blood cells; SWB, stored whole blood; WB, whole blood.

The term RBC *transfusion* includes administration of packed red blood cells (pRBCs), fresh whole blood (FWB), or stored whole blood (SWB). With the establishment of more commercial animal blood banks, the administration of pRBCs rather than whole blood (WB) has become more commonplace for treatment of anemia in both dogs and cats. Blood component therapy addresses a patient's specific transfusion needs while maximizing the use of a blood unit and decreasing potential adverse events associated with WB transfusion.

INDICATIONS

Red blood cell transfusions are indicated in the treatment of anemia caused by hemorrhage, hemolysis, or ineffective erythropoiesis. Because oxygen is poorly soluble in plasma, nearly all oxygen contained in blood is carried by hemoglobin (Hgb). Therefore, RBC transfusions increase the oxygen-carrying capacity of the anemic patient and thereby treat or prevent inadequate delivery of oxygen to tissues, with consequent tissue hypoxia. Hemorrhage (acute or chronic) was the major cause of anemia in 64–68% of dogs receiving RBC transfusions,[12,23] while ineffective erythropoiesis was the most common indication (47–75%) for RBC transfusions in cats.[24,34,40]

Transfusion Threshold

The decision to administer a RBC transfusion is usually based on a measurement of the patient's PCV, hematocrit (Hct), or Hgb concentration ([Hgb]) and, more importantly, on clinical evaluation of the patient. A "transfusion trigger" or threshold PCV, below which a RBC transfusion is administered, has not been clearly defined in human or veterinary medicine. A landmark multicenter, randomized, controlled clinical trial, the Transfusion Requirements in Critical Care (TRICC), found 30-day mortality was similar with "restrictive" and "liberal" RBC transfusion strategies in which critically ill human patients received blood when the [Hgb] fell below 7 g/dL to maintain the [Hgb] at 7–9 g/dL (restrictive group) or to maintain the [Hgb] at 10–12 g/dL (liberal group).[13] However, the mortality rate during hospitalization was significantly lower in the restrictive group, suggesting that a restrictive strategy of RBC transfusion is at least as effective as and possibly superior to a liberal transfusion strategy in critically ill patients.[13] Based on evaluation of results of 31 randomized clinical trials evaluating

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restrictive and liberal RBC transfusion thresholds, the American Association of Blood Banks (AABB) made the following recommendations in 2016: (1) a restrictive threshold of 7 g/dL is recommended for hospitalized adults who are hemodynamically stable, including critically ill patients; and (2) a restrictive threshold of 8 g/dL is recommended for patients undergoing orthopedic surgery or cardiac surgery or those with pre-existing cardiac disease.[3]

While there are no clinical trials evaluating RBC transfusion thresholds in anemic dogs and cats, it is clear that measurement of Hct, [Hgb], or PCV alone is an inadequate determinant of the threshold for RBC transfusion because many additional factors (e.g., cardiac output and oxygen consumption) are involved in adequacy of tissue oxygenation. In a prospective study of 24 client-owned euvolemic anemic dogs receiving pRBC transfusions, an "anemic dog clinical assessment score" (ADCAS) based on five clinical variables (mucosa color, heart rate, pulse quality, respiratory rate, and mentation/exercise tolerance; clinically normal dog, score = 0, severely affected dog, maximum score = 12) was developed to provide an objective means to include clinical examination findings in the transfusion decision-making process.[19] There was a significant decrease (improvement) in all ADCAS variables after receiving pRBCs, with a total score ≤3/12 post-transfusion (compared to ≥5/12 pretransfusion) in all dogs that were deemed to not need additional pRBCs.[19] Although the ADCAS has not been validated for reproducibility and reliability, results suggest that the higher the score, the more likely a RBC transfusion would be clinically beneficial.[19]

Whole Blood Versus Packed RBCs

The majority of canine and feline blood collected at commercial blood banks and veterinary teaching hospitals is processed into pRBCs and fresh frozen plasma (FFP) (see Chapter 98). Administration of pRBCs is appropriate in the management of anemia resulting from any cause, but WB transfusion may be considered in certain situations. Fresh whole blood contains functional platelets, coagulation factors, and plasma proteins in addition to RBCs. Potential indications for FWB include anemia and a combined hemostatic disorder; anemia and thrombocytopenia or thrombopathia resulting in uncontrolled or life-threatening bleeding; and possibly massive transfusion. Stored whole blood contains plasma proteins and RBCs, but not functional platelets or coagulation factors. A possible indication for SWB is anemia and hypoproteinemia (e.g., chronic gastrointestinal bleeding), although administration of pRBCs and, if there is a clinical need to increase the patient's oncotic pressure, a synthetic colloid, plasma, or lyophilized canine albumin (Animal Blood Resources International, Stockbridge, MI) would be appropriate. In anemic patients with underlying cardiac disease, pRBCs would be preferable to WB in an effort to avoid circulatory overload.

Fresh Versus Stored RBCs

Although storage of blood components allows for a readily available supply of RBCs for transfusion, it has been well documented that during storage RBCs undergo a number of physical and chemical changes, collectively referred to as the "RBC storage lesion," which reduce RBC function and viability after transfusion (see Chapter 98). By the United States Food and Drug Administration (FDA) criteria, human pRBCs have a maximum refrigerated shelf-life of 42 days. Results of retrospective and observational studies in humans have suggested that transfusions of "older" pRBCs are associated with increased morbidity and mortality.[10,35] However, five randomized controlled trials comparing transfusion of "fresher" (generally stored for 10 days or less) and "standard-issue" pRBCs (storage duration ranging from 2 to 5 weeks) did not find any clinically significant outcome differences.[6,8,14,20,33] It is important to note, however, that none of the randomized controlled trials evaluated pRBCs in their last week of storage. A parallel-group, randomized trial comparing the effects of transfusing healthy volunteers with 1 autologous pRBC unit after 1, 2, 3, 4, 5, or 6 weeks of refrigerated storage found increased extravascular hemolysis, serum ferritin, and serum non-transferrin-bound iron (NTBI) in volunteers transfused pRBCs stored for 6 weeks compared with transfusion after 1–5 weeks of storage.[29] Furthermore, a higher prevalence of infection has been reported in human patients that received pRBCs stored 35 days or more compared to 21 days or less.[10] Due to concern about the potential for increased morbidity and mortality associated with transfusion of pRBCs stored 35–42 days in critically ill patients, there has been a recommendation to reduce the FDA's maximal allowable storage period to 35 days, as has already been done by the National Institutes of Health.[29]

Similar to the situation in humans, the maximal duration of RBC storage for animals that could adversely affect patient outcomes is uncertain. In using the FDA requirement for human pRBC storage of a minimum of 75% post-transfusion recovery (i.e., at least 75% of transfused RBCs should be in the circulation 24 hours later) at the end of the storage period, studies of canine pRBCs stored in additive solutions (Adsol or Nutricel) suggest that 35-day storage is acceptable, while 42-day storage is too long.[37,38]

As is evident from RBC transfusions in humans, acceptable post-transfusion recovery does not equate with clinical safety or effectiveness. In a prospective crossover study of 20 healthy research dogs randomized to two autologous pRBC transfusion events, one after 7 days (fresh) and one after 28 days (old) of storage or vice versa, a proinflammatory cytokine response, exemplified by monocyte chemoattractant protein-1, was observed after only old pRBC transfusions and was accompanied by increased neutrophil counts and decreased platelet counts compared to the fresh pRBC and saline control groups.[2] In another study of autologous RBC transfusions administered to 10 healthy dogs after either 3 or 35 days of storage, only the older pRBC transfusions resulted in evidence of intravascular hemolysis with concomitant marked increases in circulating free iron and cell-free Hgb.[41] In an experimental canine model with the extreme conditions of *Staphylococcus*

aureus-induced pneumonia with sepsis and exchange transfusions with 42-day-stored (vs 7-day-stored) allogeneic pRBCs, administration of older pRBCs increased circulating cell-free Hgb and NTBI, as well as mortality.[32] However, in the absence of established infection, exchange transfusion with neither 7-day-stored nor 42-day-stored pRBCs resulted in mortality.[36]

Retrospective studies evaluating the effect of duration of pRBC storage on morbidity and mortality in dogs after transfusion have not identified a clear association between older pRBC transfusions and increased mortality.[12,23] In a study of 3095 dogs that received 5412 PRBC units with a median storage duration of 23 days (range, 0–40 days), longer duration of pRBC storage was associated with development of new or progressive coagulation failure and thromboembolic disease.[12] In a study of 210 dogs that received 321 pRBC units with a median storage duration of 28 days (range, 0–46 days), age of stored pRBCs was associated with increased risk of transfusion-associated hemolysis.[23]

There is not a consensus of opinion among veterinary blood banks regarding an appropriate maximal storage time for canine (or feline) pRBCs. However, in light of the poor post-transfusion recovery of canine pRBCs stored for 42 days, a maximal allowable storage time of 42 days appears too long. Furthermore, the laboratory evidence of post-transfusion intravascular hemolysis and inflammation documented in healthy dogs receiving autologous pRBCs stored for 35 days and 28 days, respectively, raises the question of whether even 28–35-day storage is too long for canine pRBCs.[2,41] The maximal allowable storage time for canine pRBCs will likely remain a controversial point until there are carefully designed clinical trials in appropriate patient populations.

PRETRANSFUSION COMPATIBILITY TESTING

Blood typing and crossmatching (see Chapters 96 and 107) are essential to providing RBC transfusions that are safe and efficacious. However, the need for a blood crossmatch prior to a *first* blood transfusion in both dogs and cats is controversial. Although naturally occurring RBC alloantibodies have been identified in dogs (see Chapter 96), they are generally not considered of clinical significance, since neither acute nor delayed hemolytic transfusion reactions have been documented in transfusion-naïve dogs receiving RBC transfusions. In a retrospective study of 149 transfusion-naïve dogs, 25 dogs (17%) were found to be incompatible to at least one donor on a tube major crossmatch, and the mean change in Hct was higher in dogs that received crossmatch-compatible RBCs (12.5 ± 8.6%) compared to those for whom a crossmatch was not performed (9 ± 4.3%), though acute adverse reactions were not observed in either group.[26] By contrast, in a prospective study of 80 transfusion-naïve dogs, no RBC incompatibilities were noted on pretransfusion testing performed using both a gel column crossmatch and an immunochromatographic strip crossmatch kit (both antiglobulin-enhanced).[11] Based on the conflicting data available and questionable clinical significance, performing a blood crossmatch prior to a dog's *first* transfusion could be considered optional.

As for dogs, there is not a uniform opinion on the need for a pretransfusion crossmatch for a transfusion-naïve cat receiving AB-matched RBCs. A retrospective study investigating the influence of crossmatch on post-transfusion PCV found that administration of type-specific, crossmatch-compatible pRBCs resulted in a significantly greater mean increase in the post-transfusion PCV when compared to administration of typed, non-crossmatched pRBCs, though pretransfusion crossmatches were performed mostly for previously transfused cats.[40] In a subsequent prospective study comparing change in PCV and incidence of adverse events post-transfusion in transfusion-naïve cats with and without a crossmatch performed, major crossmatch incompatibilities were noted in seven of 24 cats (29%).[34] There was no difference in change in PCV or adverse events when comparing cats that received type- and crossmatch-compatible pRBCs and cats that received non-crossmatched, type-compatible pRBCs, leading to the conclusion that a major crossmatch does not improve efficacy of or decrease adverse events associated with administration of AB type-matched RBCs to transfusion-naïve cats.[34] In a retrospective study of 300 cats receiving RBC transfusions, a pretransfusion crossmatch was performed for 154 transfusion-naïve cats, with 23 cats (14.9%) having major crossmatch incompatibilities.[24] Administration of crossmatch-compatible RBCs was not associated with a greater increase in PCV compared to type-specific, non-crossmatched RBCs.[24] Although the clinical relevance of major crossmatch incompatibilities detected in transfusion-naïve cats is unknown, previous documentation of anti-*Mik* RBC alloantibodies causing acute hemolytic transfusion reactions in transfusion-naïve cats suggests erring on the side of caution.[39]

XENOTRANSFUSION IN CATS

Xenotransfusion, the transfusion of blood from another species, can be viewed as a potentially life-saving option when species-compatible blood is not available and the patient is in imminent danger of dying without additional oxygen-carrying support. Five studies from the 1960s reported on the experimental administration of canine blood to 61 cats, with no evidence of severe adverse events after the first transfusion but anaphylaxis, frequently fatal, upon repeat transfusion with canine blood after 4–6 days.[1] A recent case report described two cats that received canine blood on an emergency basis, both of which stabilized after the transfusion and survived to hospital discharge.[7] Intravascular hemolysis was noted 1 day post-transfusion, and canine RBCs were no longer detectable 4 days after the xenotransfusion.[7] A pretransfusion plasma sample available from one cat did not show an agglutination reaction with canine RBCs;

however, strong (3+ to 4+) agglutination reactions were noted using post-transfusion plasma samples from both cats and random canine RBCs.[7] Despite the apparent transient improvement in the clinical condition of cats that have received canine RBCs, such xenotransfusions should only be considered in a dire situation when compatible feline RBCs are not immediately available.

ADMINISTRATION

Careful attention to administration is essential to prevent damage of the blood product and harm to the patient. Most canine and feline pRBCs purchased from commercial veterinary blood banks are stored with an additive solution, such as Adsol, and can be administered directly, while pRBC products without an additive solution should be diluted with physiologic saline (e.g., 100 mL of saline to 250 mL canine pRBCs) to decrease the viscosity of the donor blood. Concurrent administration of any drugs or fluids other than physiologic saline through the same catheter should be avoided.

Warming of Blood

In the routine administration of RBC products to normovolemic anemic patients, refrigerated blood components do not require warming prior to transfusion. A study comparing the temperature changes in chilled canine pRBCs in an administration line after passing through a dry heat fluid/blood warming device *versus* no warming device at rates ranging from 10 to 500 mL/hour found that, without the heating device, the pRBCs reached an ambient temperature (20–21 °C) at all fluid rates tested within 1 minute.[4] With the warming device (set at 46 °C), canine pRBCs achieved a maximal outlet temperature of 35.8 °C at a flow rate of 100 mL/hour; RBC integrity was not evaluated.[4] In studies evaluating the effect of warming on hemolysis of human pRBCs, there was no difference between warming devices calibrated to 37.5 °C and 41.5 °C,[28] and warming devices with temperatures up to 46 °C did not appear to increase hemolysis.[27] It is uncertain whether this upper temperature limit would be applicable to the warming of canine and feline pRBCs. Indications for the use of blood warmers in human medicine include rapid infusion of blood components (e.g., in trauma or surgery settings) and transfusions to neonates in order to prevent exacerbation or development of hypothermia, the consequences of which may include cardiac arrhythmias and coagulopathies.[9]

Blood Filters and Infusion Devices

Red blood cell transfusions must be administered through a filter designed to remove clots and particles potentially harmful to the patient. Standard blood infusion sets have in-line filters with a pore size ranging from 170 µm to 260 µm that trap large cells, cellular debris, and coagulated proteins. A neonatal syringe (30–60 mL) set with a 150 µm filter (Charter Medical, Winston-Salem, NC) is useful for administration of whole blood or PRBCs, as well as plasma products, to small dogs and cats. A pediatric microaggregate blood filter with an 18 µm pore size and a priming space of 0.7 mL (Hemo-Nate syringe filter, Utah Medical Products, Midvale, Utah) can be used to administer small volumes of blood (<50 mL WB or <20 mL pRBCs); however, administering larger volumes could result in hemolysis due to a progressive decrease in pore size as more blood is filtered. With both the neonatal syringe set and the Hemo-Nate syringe filter, the blood is filtered as it is removed from the blood storage bag, the filter is discarded, and the syringe with extension tubing is then placed on a syringe pump for administration.[9]

In most instances, the blood flow rate through an administration set by gravity alone is adequate to meet a patient's needs. Some electromechanical infusion devices that deliver crystalloid solutions at a controlled rate have been approved by the manufacturer for administration of blood to human patients. In a study comparing three different techniques (volumetric peristaltic pump, syringe pump with a postsyringe 18 µm filter, and gravity flow with a standard in-line 170–260 µm filter) for administration of autologous whole blood (washed RBCs labeled with biotin and reconstituted with plasma) to healthy dogs, there was a significant difference in the number of dogs with detectable labeled RBCs 1 day post-transfusion depending on the technique: biotin-labeled RBCs administered via gravity were detected in all eight dogs, but in only four of eight dogs and one of seven dogs when administered via a volumetric and syringe pump, respectively.[25] After 24 hours, there was no significant difference in the circulating half-life of RBCs administered via volumetric pump compared to gravity flow.[25] It is important to recognize that the syringe pump method of delivery was not evaluated without a postsyringe 18 µm filter, so results might be different for prefiltered whole blood or pRBCs placed on a syringe pump, as recommended by the manufacturer of the Hemo-Nate filter and as done for human neonatal transfusions.[9] In a similar study performed in healthy cats, fresh autologous whole blood was administered via gravity and a pump delivery system using a 20 mL syringe and an 18 µm filter, with no difference in short- or long-term RBC survival noted between groups during a 6-week period.[15] The reason for the striking contrast in results for syringe pump delivery system in the canine and feline studies is unclear. If similar to the situation with blood from humans, there are many factors besides pump mechanics that can influence infusion-pump-mediated hemolysis, including flow rate (low flow rates increase hemolysis secondary to increased RBC exposure to mechanical shear force) and viscosity of the blood (higher hematocrit in pRBC units without additive solution has been associated with increased

hemolysis).[17] If considering the use of an infusion pump designed for crystalloid solutions to administer RBCs, the manufacturer should be consulted first, and ideally the system should be tested to confirm the pump will not damage canine and feline blood components before their use.

Route, Volume, and Rate of Transfusion

The intravenous route of administration is recommended for all RBC transfusions; however, intraosseous administration is an excellent alternative if venous access cannot be obtained. Central veins allow for the use of large-bore catheters and thereby more rapid transfusion in the case of hemorrhagic shock. However, peripheral veins may be preferred in animals with an increased bleeding tendency.

The volume of blood to be administered depends on the degree of anemia, the patient's clinical status, and the size of the animal. Similar to the transfusion threshold, a "target PCV," or the desired PCV post-transfusion, has not been clearly defined and is likely to vary depending on the patient's overall condition. It is typically not necessary to transfuse RBCs with the aim of restoring a normal PCV. In a retrospective study evaluating 4 formulae to predict post-transfusion PCV in 31 anemic dogs administered pRBCs, the most accurate formula was (donor PCV × pRBC volume infused in mL)/(90 mL × body weight in kg).[31] In a similar retrospective study evaluating five formulae in anemic cats administered WB, the best performing formula for predicting the increase in PCV was (WB volume infused in mL)/(2 × body weight in kg), but no single formula was highly accurate.[30] As an alternative to calculating the volume of donor blood required to reach a target PCV, one may administer an average volume of 10–15 mL/kg of pRBCs or 20 mL/kg of WB to a normovolemic dog and, if deemed necessary based on clinical evaluation of the patient post-transfusion, follow with additional blood. For practical purposes, most anemic cats will initially receive one unit of WB (typically 40–50 mL) or one unit of pRBCs (typically 20–25 mL).

The rate of blood administration depends on the patient's overall condition. In normovolemic anemic patients, the maximum rate of transfusion is 10–20 mL/kg/hour to avoid circulatory overload; hypovolemic anemic patients may tolerate replacement of their blood volume as quickly as the blood can be infused. In animals with advanced cardiac disease, the infusion rate should not exceed 2–4 mL/kg/hour; pRBCs are preferable to WB in such cases where the transfusion volume tolerated by the patient may be a limiting factor in providing sufficient oxygen-carrying support. In all cases, including those in which the donor and recipient have been blood typed and/or crossmatched, the initial rate of transfusion should be slow while observing for any immediate adverse reactions. In general, a blood transfusion should be complete within 4 hours to ensure administration of functional blood components and to prevent growth of bacteria in the event of contamination.[9]

Massive Transfusion

Massive transfusion represents a special situation with regard to volume and rate of blood transfusion, as well as blood components administered. In veterinary medicine, the following definitions of massive transfusion have been used: transfusion of a volume of whole blood or blood components that is greater than the patient's estimated blood volume within a 24-hour period, replacement of half the patient's estimated blood volume in 3 hours, or the replacement of 150% of the patient's blood volume irrespective of time.[18,21,22] Massive transfusion protocols have been established in human medicine to treat intravascular volume deficits, preserve oxygen-carrying capacity, and prevent dilutional coagulopathy, with blood products administered in a balanced ratio that is closely approximated to reconstituted whole blood. In a randomized clinical trial of patients with severe trauma transfused plasma, platelets, and pRBCs in ratios of 1:1:1 or 1:1:2, there was no difference in 24-hour and 30-day mortality, though exsanguination was significantly decreased in the 1:1:1 group.[16] In the case of massive transfusion during cardiac surgery, administration of plasma to pRBCs in a ratio ≥1:1 was associated with lower mortality and less organ dysfunction than with lower ratios.[5]

Guidelines for massive transfusion in dogs and cats have not been established. In a retrospective study of dogs receiving massive transfusion for a variety of causes, including intra-abdominal neoplasia and trauma, the mean volumes of pRBCs and FFP administered were 66.5 mL/kg and 22.2 mL/kg, respectively, resulting in a mean plasma to RBC ratio of 1:3, with four of 15 (27%) dogs surviving to discharge.[18] Massive transfusions (with unspecified plasma to pRBC ratios) have been reported for dogs following trauma, with seven of 10 dogs (70%) surviving.[22] In a study of 83 dogs with hemoperitoneum (90% of which were associated with splenic hemorrhage, most commonly due to neoplasia) and undergoing surgery, four dogs met the criteria for massive transfusion, with only one dog (25%) surviving.[21]

PATIENT MONITORING

Early recognition of transfusion reactions (see Chapter 104) requires careful evaluation of patient's attitude, vital signs, and perfusion (i.e., capillary refill time and pulse quality) before, during, and after a RBC transfusion. Measurement of PCV and total protein pretransfusion and post-transfusion (at a minimum, immediately and at 24 hours) and evaluation of the plasma and urine for the presence of Hgb are recommended. Any unexpected change in attitude, vital signs, or laboratory parameters

may be indicative of an adverse reaction, in which case the RBC transfusion should be stopped immediately, the intravenous line left open with physiologic saline, and other supportive care provided as necessary.

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CHAPTER 100

Transfusion of Plasma Products

MARJORY B. BROOKS

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  Albumin and Immunoglobulins
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Acronyms and Abbreviations

CPD, citrate-phosphate-dextrose; DIC, disseminated intravascular coagulation; FFP, fresh frozen plasma; FP, frozen plasma; IMHA, immune-mediated hemolytic anemia; ITP, immune-mediated thrombocytopenia; IVIG, intravenous immunoglobulin; RBC, red blood cell; rhFVIIa, recombinant human factor VIIa; TRALI, transfusion-related acute lung injury; TRIM, transfusion-related immunomodulation; VWD, von Willebrand disease; VWF, von Willebrand factor.

PLASMA CONSTITUENTS

Plasma contains a myriad of proteins that maintain homeostasis within the body and respond to environmental challenges. The major therapeutic constituents of plasma include hemostatic proteins, albumin, and immunoglobulins. The term "plasma component" is reserved for products prepared by differential centrifugation using blood banking techniques, while "plasma derivative" refers to products prepared by fractionation in manufacturing facilities.[1,3,8] The advent of commercial, referral, and academic veterinary blood banks facilitates routine transfusion of animal plasma components in clinical veterinary practice. By contrast, sources of plasma constituents are relatively limited. Currently available commercial constituents include canine albumin (Animal Blood Resources International) and equine immunoglobulin preparations (Sera, Incorporated).

Hemostatic Proteins

Plasma contains all the procoagulant factors required for fibrin clot formation, adhesive proteins, potent anticoagulants, and fibrinolysins. The transformation of soluble plasma fibrinogen to insoluble, polymerized fibrin occurs through the action of serine protease coagulation factors (factors II, VII, IX, X, XI, and XII), cofactors (factors V and VIII), and a transglutaminase (factor XIII). Platelet adhesion under conditions of high shear requires the large multimeric glycoprotein, von Willebrand factor (VWF). The major anticoagulant proteins in plasma are antithrombin, protein C, and protein

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TABLE 100.1 Half-lives in circulation of hemostatic proteins.

Adhesive proteins	Half-life (hours)
Fibrinogen	120
Fibronectin	24
Von Willebrand factor	12
Anticoagulant proteins	
Antithrombin	72
Protein C	6
Protein S	60
Fibrinolytic pathway proteins	
Antiplasmin	60
Plasminogen	60
Plasminogen activator inhibitor-1	2
Procoagulant factors	
Factor II	100
Factor V	25
Factor VII	5
Factor VIII	10
Factor IX	20
Factor X	65
Factor XI	65
Factor XII	60
Factor XIII	150

S. Plasma also contains the fibrinolytic enzyme plasminogen and its primary regulatory proteins, antiplasmin and plasminogen activator inhibitor-1. Fibrinogen is the highest concentration hemostatic protein, circulating in plasma at approximately 300 mg/dL. The plasma half-lives of hemostatic proteins vary widely (Table 100.1), thereby influencing appropriate transfusion intervals for different disease syndromes.[13,15]

Albumin and Immunoglobulins

Albumin is the major oncotic protein in plasma and a carrier protein for lipophilic compounds. The immunoglobulin fraction of plasma consists of IgG (up to 85%), IgA, and IgM. The plasma albumin concentration of most species ranges from approximately 2 g/dL to 4 g/dL, with immunoglobulins generally present at equivalent or slightly lower concentration. Derivative concentrates, rather than plasma components, are used in human medicine to supply therapeutic levels of albumin and globulins.[4,7]

PLASMA COMPONENTS

Centrifugation of whole blood sediments the cellular elements from the supernatant plasma. Separation within 8 hours of blood collection optimizes retention of the quantitative and functional properties of all plasma proteins.[1,3] The supernatant plasma can be transfused immediately as fresh plasma, but it is usually frozen and stored as fresh frozen plasma (FFP) for subsequent transfusion, or further processed to yield cryoprecipitate and cryosupernatant (Figure 100.1). The general procedures for preparation of animal components in the United States follow human blood banking guidelines, e.g., collection of whole blood in citrate-phosphate-dextrose (CPD) anticoagulants, initial quick-freeze of separated plasma, and storage temperatures at or below −18 °C.[3,14]

Fresh Frozen Plasma

Fresh frozen plasma refers to plasma separated from whole blood within 8 hours of collection and quick-frozen within 1 hour of separation. It contains essentially all the hemostatic proteins, albumin, and globulin as the plasma from which it was prepared. The term *FP24* is used in human blood banking for plasma separated and frozen within 24 hours from collection. Compared to FFP, this product has diminished activity of labile factors, but is approved as a source of coagulation factors for patients with moderate, combined factor deficiencies.[1,15] Similarly, canine plasma processed up to 24 hours from collection demonstrated retention of factor activity.[20] Additional studies of canine, equine, and feline FFP demonstrated stability of factor content under a variety of storage conditions.[10,21,23,24]

Frozen Plasma

Frozen plasma (FP) refers to FFP units stored longer than 1 year or thawed FFP units stored under refrigeration for more than 24 hours before transfusion. Hemostatic proteins retain variable activity in FP, and the product does not supply replacement levels of the most labile coagulation factors, factors V and VIII. FP is a source of albumin and globulins.

Cryoprecipitate

Cryoprecipitate is prepared by slowly thawing FFP at temperatures between 1 and 6 °C, followed by centrifugation to sediment the large, cold-insoluble proteins.[1,15] The resultant cryoprecipitate fraction is then refrozen within 1 hour of separation for storage times of up to 1 year. Cryoprecipitate contains fibrinogen, the VWF–factor VIII complex, factor XIII, and fibronectin. "Units" of cryoprecipitate may be defined based on the volume of starting FFP, or fibrinogen or factor content. The process of cryoprecipitation yields approximately 50% factor activity, in one-tenth volume, of the starting FFP. The factor content in cryoprecipitate is also related to that of donor plasma.[6] Volume reduction is the major advantage of cryoprecipitate transfusion; therapeutic levels of factors are attained within minutes over the course of a single bolus infusion. Cryoprecipitates are most often used for dogs as sources of VWF and factor VIII.

Cryosupernatant

Cryosupernatant is the plasma component remaining from production of cryoprecipitate.[1,15] This product is deficient in fibrinogen, VWF–factor VIII, and other cold-insoluble proteins, but retains proteins such as albumin and most hemostatic proteins and immunoglobulins.[5]

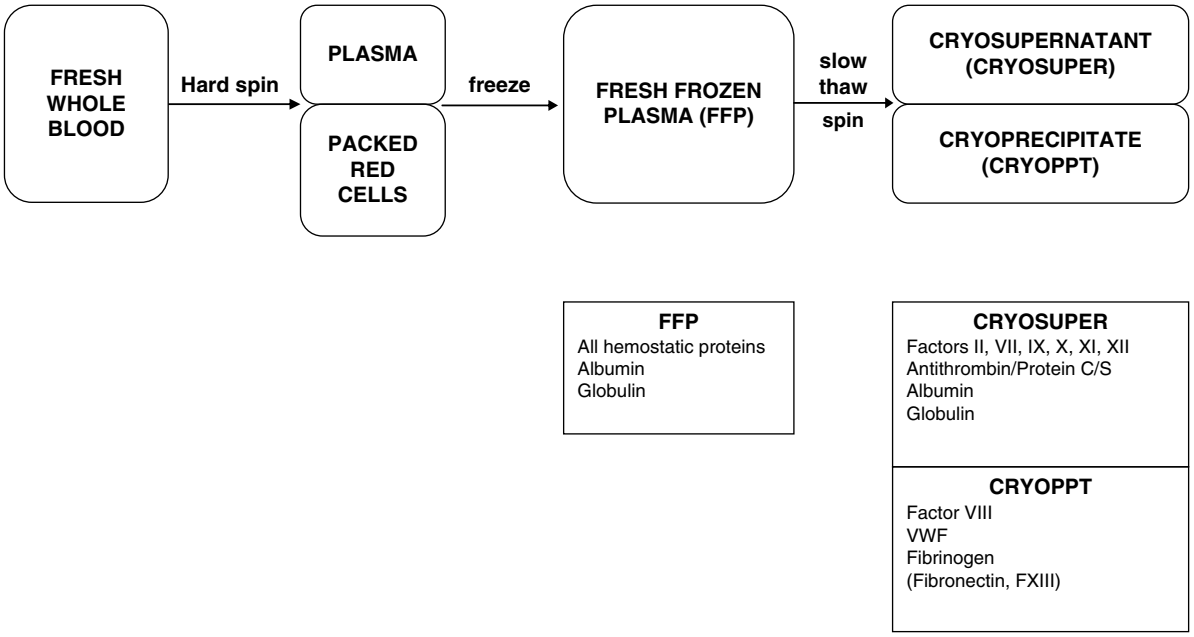


FIGURE 100.1 Flow chart of plasma component processing and composition.

Cryosupernatants are often used as a source of the vitamin-K-dependent coagulation factors (factors II, VII, IX, and X) and are stable for this purpose for at least 1 year of storage.

PLASMA DERIVATIVES AND RECOMBINANT PROTEINS

Human apheresis plasma provides most of the source material for commercial manufacture of pharmaceutical protein derivatives.[8] Human albumin, immunoglobulin, and factor concentrates (factor VIII and VWF) are among the most commonly used derivatives in medical practice. The first production methods developed in the 1940s and 1950s (Cohn's fractionation) separated constituent proteins based on differential solubility under conditions of varying temperature, pH, and ethanol concentration. Subsequent advances include the use of chromatography and immunopurification processing techniques. The final protein concentrates are supplied as solutions or lyophilized powders with long (2–3 year) shelf-lives at room temperature. The manufacturing process of human protein concentrates incorporates donor and plasma screening and highly effective pathogen inactivation procedures.

In addition to high-purity factor VIII and IX concentrates, recombinant human factor VIII and IX products are approved to prevent and control hemorrhage in hemophilic patients. Although recombinant factors carry no risk of blood-borne disease transmission, they do not prevent development of inhibitory alloantibodies, a severe complication of replacement therapy. Recombinant human factor VIIa (rhFVIIa) was developed as a bypassing agent to treat hemophiliacs and other patients with acquired coagulation inhibitors.[12] At supraphysiologic levels, rhFVIIa generates thrombin by directly activating factor X on the surface of procoagulant platelets.

INDICATIONS FOR PLASMA THERAPY

Plasma components are the mainstay for treatment of hemorrhagic disorders caused by hemostatic protein deficiencies. Plasma components are also transfused to animals with the goal of providing albumin and immunoglobulins due to a lack of allogeneic derivative protein concentrates.[13] The indication and therapeutic target for each transfusion should be clearly established in order to select the most specific replacement product (Table 100.2). This approach maximizes the use of scarce plasma resources and helps minimize recipient exposure to foreign antigens.

Hemorrhagic Disorders

Hereditary Bleeding Disorders

Plasma components are indicated to control active hemorrhage and for preoperative prophylaxis for animals with hereditary coagulation factor deficiencies and von Willebrand disease (VWD) (see Chapters 83 and 90). In many cases, appropriate component therapy will eliminate the need for RBC transfusion with its inherent risks of sensitization and adverse reaction to RBC antigens.

Von Willebrand disease, among the most common hereditary bleeding disorders of dogs, horses, and people, is often managed with plasma component therapy. Patients with severe forms of VWD invariably require replacement therapy to safely undergo invasive

TABLE 100.2 Plasma components and plasma derivatives.

Product	Dosage guidelines	Indications
Fresh frozen plasma	6–12 mL/kg q8–12 h	Inherited and acquired coagulation factor deficiencies, von Willebrand disease, hypoproteinemia, hypoglobulinemia
Cryoprecipitate[a]	1 unit/10 kg q4–12 h	Hemophilia A, von Willebrand disease, hypofibrinogenemia
Cryosupernatant	6–12 mL/kg q8–12 h	Hemophilia B, hereditary deficiencies of factors II, VII, X, XI, vitamin K deficiency, hypoproteinemia, hypoglobulinemia
Frozen plasma	12–20 mL/kg q12–24 h	Hypoproteinemia, hypoglobulinemia
Human albumin solution[b]	0.5–1.0 g/kg	Hypoproteinemia
Human intravenous immunoglobulin[b]	0.5–1.0 g/kg	Refractory immune-mediated disorders
Recombinant human FVIIa[b]	35–120 μg/kg	Factor bypassing agent, surgical hemostasis

[a]One unit cryoprecipitate prepared from 200 mL fresh frozen plasma.
[b]Administration of human protein products may induce severe adverse reactions in animals.

procedures and are subject to spontaneous mucosal bleeds that may persist without transfusion. Transfusion requirements are generally related to residual VWF protein levels; however, each patient's risk of hemorrhage should include consideration of prior history of abnormal bleeding, presence of concurrent disorders, and the nature and site of surgery or trauma. The highest potency VWF plasma component is cryoprecipitate; however, FFP can be transfused as a source of VWF if cryoprecipitate is unavailable.

Hemophilia occurs in many different breeds and species. Hemophilia A (factor VIII deficiency) is much more common than hemophilia B (factor IX deficiency). Both are X-linked recessive traits, and, therefore, signs of a bleeding disorder are seen predominantly in males. Heritable coagulopathies caused by deficiencies of fibrinogen and factors II, VII, IX, X, and XI are less common than hemophilia, but have been identified in animals. Transfusion requirements vary among hereditary factor deficiencies. Clinical expression of a bleeding tendency depends on the physiologic role of the deficient factor and the severity of factor deficiency. For example, hemophilia A and B often manifest as clinically severe coagulopathies, whereas most patients with factor VII deficiency have no signs of a bleeding tendency (see Chapter 90). Hemophilia A and fibrinogen deficiency or dysfunction (dysfibrinogenemia) respond to cryoprecipitate or FFP, if cryoprecipitate is unavailable. All other hereditary factor deficiencies can be treated with FFP or cryosupernatant.

Acquired Factor Deficiencies

Acquired coagulopathies are caused by simultaneous deficiencies of many factors rather than specific deficiency of a single factor (see Chapters 89, 92, and 94). Acquired coagulopathies are more common than hereditary disorders and often develop in patients with vitamin K deficiency, liver disease, disseminated intravascular coagulation (DIC), and massive blood loss treated with large-volume fluid replacement.

Transfusion is required for vitamin-K-dependent coagulopathies if fulminant hemorrhage develops in critical sites, e.g., respiratory tract or central nervous system, since vitamin K replacement therapy corrects coagulopathy only after a period of 12–24 hours. Cryosupernatant is the most cost-effective plasma component to supply the vitamin-K-dependent factors (factors II, VII, IX, X),[5] but FFP is an acceptable alternative (Table 100.2).

While FFP provides all coagulation factors and fibrinogen, it also contains plasminogen, anticoagulant proteins, protease inhibitors, and albumin. Volume overload, however, can limit the efficacy of FFP for ongoing replacement of these proteins.[1,2,13,15] The combined use of FFP with platelet and packed RBC transfusion is considered effective for short-term replacement of hemostatic proteins in patients with massive blood loss.[1,15] New, freeze-dried FFP products have also shown clinical benefit in early human trauma studies.[17] A clinical benefit of FFP or cryoprecipitate has not been shown across treatment trials of DIC in humans.[2,15] Therefore, use of component therapy is generally restricted to DIC patients with signs of hemorrhage caused by severe fibrinogen and factor depletion.

Nonhemorrhagic Disorders

Hypoproteinemia

Hypoproteinemia develops in numerous disease conditions including vasculitis, protein-losing disorders, hepatic failure, and peritonitis. In medical practice, albumin concentrates are used to restore the oncotic properties and carrier functions of albumin, and veterinary studies have described the use of human albumin in sick dogs and cats.[7,18,19] Antigenic differences among species, however, render human albumin immunogenic, and severe adverse reactions were described in a study of healthy dogs receiving pharmaceutical human albumin solutions.[9] Off-label use of human albumin solutions for veterinary patients should be carefully considered and proceed only after obtaining owners' informed consent. The plasma components FFP and cryosupernatant are potential sources for albumin replacement therapy. However, high-volume transfusions (20–25 mL/kg) of these components are predicted for calculated increments of 0.5 g/dL plasma albumin in recipients.

Immune Disorders

Human intravenous immunoglobulin (IVIG) concentrates were initially developed for treating immunodeficiencies; however, these products are now used to modulate the immune response for diverse human disease syndromes including immune cytopenias, myasthenia, rheumatic disease, and immune-mediated dermatopathies.[4] The clinical use of human IVIG in animals includes management of refractory immune-mediated disorders, including immune-mediated hemolytic anemia (IMHA), immune-mediated thrombocytopenia (ITP), pemphigus foliaceus, and cutaneous drug eruptions.[16] The immunoglobulin content of plasma components does not supply supraphysiologic levels of immunoglobulins for immunosuppression; however, FFP (and cryosupernatants) can serve as sources of immunoglobulin replacement therapy for neonates with failure of passive transfer, and potentially to augment response to infectious agents.

GUIDELINES FOR PLASMA ADMINISTRATION

Sources of Plasma Products

The options for obtaining veterinary blood products now include regional and national veterinary blood banks, and local academic centers and specialty clinics. Guidelines for screening canine and feline donors have been recently updated;[22] however, nationwide standards do not exist for unit definition, potency, or processing techniques to prepare plasma components. Information on quality assurance, quality control, and specific dosage and handling recommendations must be obtained from each supplier.

Plasma Product Administration

Plasma Components

Plasma components are prepared in sterile plastic bags and then stored and shipped frozen in individual boxes.[1,13] Plasma bags are brittle and must be handled carefully to prevent breakage. All products should be stored at −20 °C or lower and then warmed to 37 °C in a water bath or incubator just before transfusion. Unopened bags can be refrozen without significant loss of factor activity when kept at 37 °C for up to 1 hour,[24] but any product remaining in opened bags should be discarded if not transfused within 3–4 hours.

Intravenous transfusion of plasma components is the preferred route of administration; however, intraosseous transfusion may be indicated in emergency situations when attempts at vascular access have failed. Catheter sites for transfusion should be aseptically prepared and the catheters reserved for plasma component administration (or flushed with sterile saline before and after plasma transfusion). Plasma components should be infused through blood filters and should not be mixed with other fluids, drugs, or additives to prevent contamination, precipitation, or clot formation.[1]

In the author's experience, routine pretreatment with corticosteroids or antihistamines is not required before plasma component transfusion. Plasma products should be infused at a slow initial rate of 0.1 mL/kg/minute for 15–20 minutes and the recipient closely monitored for signs of transfusion reaction. Maintenance of this rate is advisable for patients at risk for volume overload (e.g., puppies, small dogs, cats, cardiac patients). Transfusion rates of 0.2–0.3 mL/kg/minute (to total transfusion time of 1.5–2 hours) are generally well tolerated for more rapid factor replacement and for larger dogs. Low-volume cryoprecipitate can be transfused as a slow intravenous bolus over a period of 10–20 minutes.

The dose and frequency of transfusion vary for different products and different disease conditions (Table 100.2). In general, FFP, FP, and cryosupernatant are transfused at 10–12 mL/kg, with an interval of 6–8 hours between doses to prevent volume overload. Cryoprecipitates are supplied in arbitrary "units" defined by each blood bank and should be transfused, therefore, according to the suppliers' recommended dosages. The best strategy for rapidly restoring hemostasis in patients with severe factor deficiencies is to initiate high-dose and short-interval component transfusion. The high end of dosage range is also appropriate for preoperative prophylaxis. The dose can be decreased and transfusion interval increased after hemostasis is restored. While coagulation assays and point-of-care monitors aid in assessing patient response (Figure 100.2), clinical assessment and confirmation of rising or stable hematocrit are important parameters to guide subsequent transfusion.[3] Patients with severe factor deficiencies usually require more than a single preoperative transfusion to undergo surgery or to control hemorrhage severe enough to cause blood loss anemia. A second (postoperative) transfusion should be given routinely, with access to additional components as needed. Transfusion over a maximum period of 2–3 days is usually sufficient to support hemostasis and postoperative wound healing in patients with even the most severe factor deficiencies.

Plasma Derivatives and Recombinant Proteins

Administration of human albumin, immunoglobulin, and rhFVIIa has been reported in dogs; however, treatment trials adequately powered to judge clinical efficacy have not yet been performed. In retrospective reviews,[18,19] the use of human albumin was described in canine and feline patients, with disparity among studies in the number of observed clinical complications. Moreover, a study of albumin infusion to healthy dogs described immune-mediated reactions to the heterologous protein.[9] Human IVIG has been used in the dosage range of approximately 0.5–1.5 g/kg for refractory IMHA and ITP in dogs.[16] Cytopenias generally resolved in more than two-thirds of the dogs treated with a single IVIG dose. The hemostatic effects of rhFVIIa have been demonstrated in research dogs

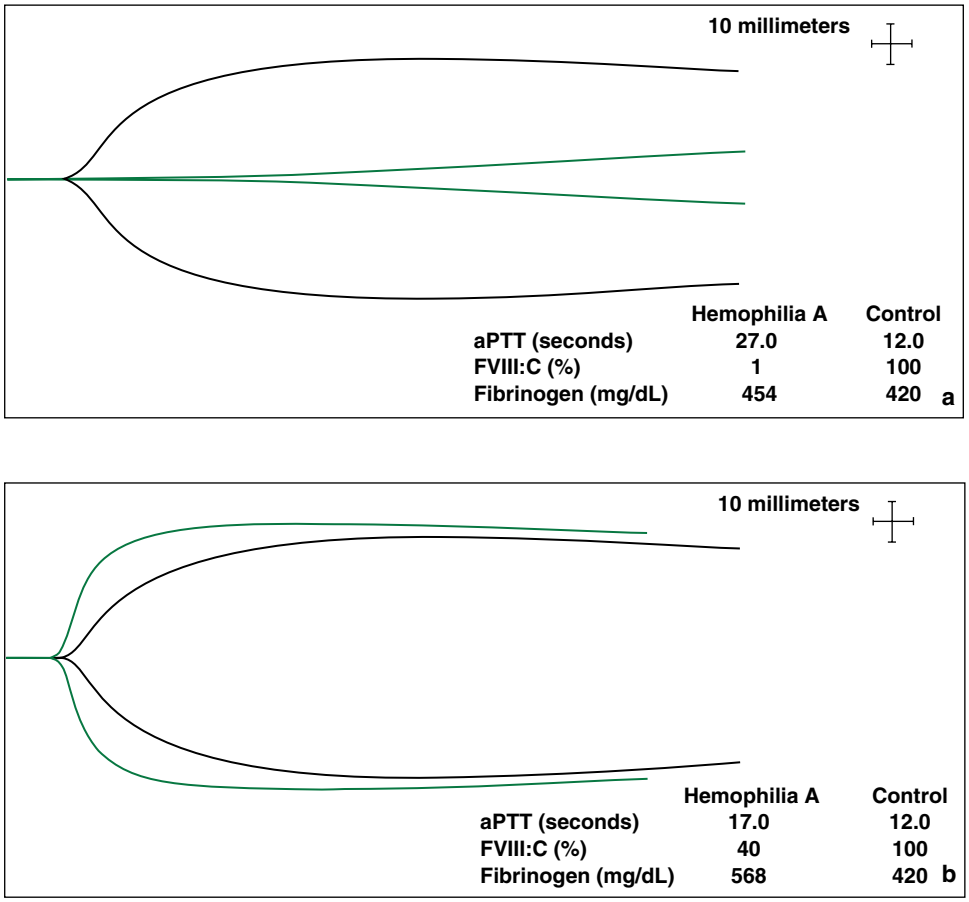


FIGURE 100.2 Response to cryoprecipitate in a dog with severe hemophilia A. Cryoprecipitate was transfused to a hemophiliac dog preoperatively and postoperatively for cutaneous mass excision. Citrate-anticoagulated blood samples were collected before transfusion and 45 minutes after postoperative transfusion to monitor activated partial thromboplastin time (aPTT), factor VIII coagulant activity (FVIII:C), fibrinogen, and thromboelastography (TEG) using recalcified blood and no trigger reagent. In each panel, the hemophiliac dog's TEG tracing (green line) is superimposed over a normal dog tracing (black line). Inset tables display aPTT, FVIII:C, and fibrinogen values. (a) Pretreatment TEG depicts minimal fibrin formation in the absence of factor VIII; (b) Post-treatment TEG depicts a marked increase in the rate and tensile strength of the fibrin clot formed in the presence of hemostatic levels of factor VIII.

with hereditary factor deficiencies (hemophilia A, hemophilia B, and factor VII deficiency), at dosages ranging from 5 to 30 μg/kg.[12,25] Pending additional veterinary studies, any potential benefit of human plasma-derived or recombinant concentrates, must be weighed against their high cost and cross-species immunogenicity.

ADVERSE REACTIONS TO PLASMA PRODUCTS

All plasma recipients should be observed during transfusion, with minimum monitoring of temperature, pulse, and respiration before and after transfusion. Based on the indication for plasma therapy, the appropriate samples to monitor coagulation status, hematocrit, or protein concentration should be collected before and after transfusion to gauge recipient response. Plasma product administration should be stopped immediately, and the product saved for analysis if the recipient displays any signs of discomfort or change in clinical status (see Chapter 104).

Immune-Mediated Reactions

Plasma components and concentrates are acellular but contain potentially immunogenic proteins and other compounds (e.g., anticoagulants) capable of eliciting immune reactions. Acute allergic reactions characterized by urticaria, erythema, and facial edema are among the most common reactions and may occur during a first transfusion, or in patients previously transfused many times. Signs typically manifest within the first 30–60 minutes of infusion. The antigens or factors responsible for these reactions have not been defined, but most patients respond quickly if the transfusion is discontinued and they are given antihistamines and/or short-acting steroids. Cats have clinically significant, naturally occurring alloantibodies to foreign RBC antigens. Therefore, cats should be transfused only with type-matched plasma products. A minor crossmatch (recipient RBCs plus donor plasma component) can be performed in any species to

detect hemolysins and agglutinins before transfusion (see Chapter 107). The off-label use of pharmaceutical agents containing human proteins or any cross-species plasma transfusion carries an inherent risk of potentially fatal acute and/or delayed immune reactions.

The acronyms TRALI (transfusion-related acute lung injury) and TRIM (transfusion-related immunomodulation) refer to deleterious antibody and/or cytokine-mediated plasma component reactions.[11] TRALI is primarily attributed to alloantibodies in donor plasma-activating recipient granulocytes, which results in severe pulmonary edema. Biolipids and soluble CD40 ligand shed from platelets and lymphocytes may also contribute to neutrophil activation. TRIM is an immunosuppressive syndrome caused by donor leukocytes or leukocyte-derived intermediaries inducing an increased susceptibility to cancer recurrence or bacterial infection in recipients.

Nonimmune Reactions

Nonimmune reactions to plasma products are caused by improper collection, storage, or administration procedures, or by disease transmission from infected donors.[1,11] Volume overload is a common nonimmune reaction caused by too rapid rate or too high volume transfusion. Clinical signs of this reaction include emesis, vocalization, and dyspnea. Signs of respiratory distress may also develop secondary to transfusion of unfiltered plasma products containing fibrin clot fragments or precipitates formed by plasma contact with calcium- or glucose-containing solutions. Although RBC and platelet products are more frequently implicated in disease transmission, appropriate donor screening and careful attention to aseptic procedures for catheter placement are important to minimize risk of infection.[22] Blood banking recommendations to minimize plasma component contamination include avoidance of transfusion times longer than 4 hours and prohibiting introduction of any drugs or solutions into the plasma product.

The demand for animal plasma components and derivatives is likely to intensify as veterinarians provide increasingly complex medical and surgical services. The challenge remains for clinicians and researchers to develop mechanisms for evaluating the risks and benefits of plasma transfusion and protocols for optimizing plasma usage and clinical outcomes.

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CHAPTER 101

Platelet and Granulocyte Transfusion

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Platelet Transfusion
Assessment of Platelets for Transfusion
Whole Blood-Derived and Apheresis Platelets
Storage of Platelet-Rich Blood and Components
  Chilled Platelets
  Pathogen Inactivation
  Cryopreserved Platelets
  Lyophilized Platelets and Platelet Substitutes
Transfusion of Platelet
  Fresh Whole Blood
  Platelet-Rich Plasma and Platelet Concentrate
  Platelet-Poor Blood Components
Platelet Transfusion Reactions
Granulocyte Transfusion

Acronyms and Abbreviations

AABB, American Association of Blood Banks; ABRI, Animal Blood Resources International; DMSO, dimethyl sulfoxide; EDQM, European Directorate for the Quality of Medicines; FWB, fresh whole blood; G-CSF, granulocyte colony-stimulating factor; GC, granulocyte concentrate; PC, platelet concentrate; PPR, percent platelet recovery; PRP, platelet-rich plasma; RT, room temperature; WB, whole blood.

PLATELET TRANSFUSION

Platelet transfusion is indicated for the control of bleeding due to thrombocytopenia or thrombopathia. It may be *therapeutic*, given to stop active bleeding, or *prophylactic*, given to prevent spontaneous or excessive bleeding. In humans, platelet transfusions were initially given primarily to patients with leukemia and other neoplasms receiving intensive cytotoxic treatments, which promoted prophylactic transfusion against spontaneous bleeding. More recently, therapeutic platelet transfusion has been employed with increasing frequency in trauma and other non-neoplastic disorders.[29] Platelet transfusion has not been as widely used in veterinary medicine, in part because of short shelf-life of traditional components and short post-transfusion platelet circulation time. Early reports concerned experimental bone marrow transplantation, but, with improved blood banking, the use of more aggressive anticancer therapy, and the increased willingness to treat complex hematologic disorders, there is increasing use of platelet-rich blood and components, including fresh whole blood (FWB), platelet-rich plasma (PRP), and platelet concentrate (PC).[2,8,14,30,35] The latter may be prepared by centrifugation of FWB (see Chapter 98) or by plateletpheresis, the selective removal of platelets from the donor's blood with return of RBCs and plasma to the donor.[9]

ASSESSMENT OF PLATELETS FOR TRANSFUSION

During platelet collection and storage, deleterious physical and metabolic changes known as the *storage lesion* occur, which are greater than that expected with senescence alone. The storage lesion affects both platelet quantity and quality. Methods for the collection and storage of platelets to improve quantity and quality are being continuously evaluated, with results compared on the basis of in vitro and in vivo tests of PRP and PC. The pH of the component is an important aspect of the storage lesion, and standards for human PCs produced by centrifugation specify a pH > 6.2 (American

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Association of Blood Banks [AABB]) or >6.4 (European Directorate for the Quality of Medicines [EDQM]) in all units at the end of the allowable storage period.[17,20]

The number of transfused platelets is important for hemostatic effect. Platelet *quantity* in the component is typically determined using standard methods of platelet counting for hemograms, but hematology analyzers are not specifically calibrated for this purpose and results will vary with the method (see Chapter 137). Platelet *yield* from centrifugation is calculated by dividing the number of platelets in the final component by the number of platelets in the initial blood or component. Platelet *survival during storage* is calculated by dividing the number of platelets remaining at the end of the storage period by the number present at the beginning. Standards specify that human PCs produced by centrifugation have $\geq 5.5 \times 10^{10}$ (AABB) or $\geq 6 \times 10^{10}$ (EDQM) platelets per unit.[17,20] Plateletpheresis should result in $\geq 3 \times 10^{11}$ (AABB) or $\geq 2 \times 10^{11}$ (EDQM) platelets in ≥90% of units.[17,20]

Platelet *quality* in the blood component refers to both post-transfusion hemostatic effect (in vivo function) and circulation time (in vivo viability). Platelet viability is most important for prophylactic transfusion. Platelet viability is determined by radiolabeling or biotinylating platelets and measuring a decrease in labeled platelets over time[5, 43-49] In the clinical setting, platelet viability is determined by measuring the incremental increase in platelet counts following transfusion. One-hour and 24-hour platelet increments are standard measurements; 10-minute increments have also been used.[15,37]

For assessing platelet function, many of the in vitro tests discussed in Chapter 79 have been used in a somewhat unsuccessful effort to predict post-transfusion function and viability.[1,4,50] The poor predictive value occurs at least in part because the metabolic changes in stored platelets may be reversible after transfusion.[41] The most useful tests, in addition to pH, are lactate concentration, response to hypotonic stress, flow cytometry of activation markers, and evaluation of platelet morphology.[1,10,50] The latter includes shape change in response to agonists, morphology scoring using light or electron microscopy, and evaluation of swirling in the blood bag with the unaided eye. Of these, only swirling is practical as a routine test in most clinics; the others are used primarily in a research capacity. Given the limited data on clinical platelet transfusion in dogs and cats, the following in vitro tests are recommended for routine use: (1) measurement of platelet count in FWB, PRP and PC (2) assessment of swirling in PRP and PC; and (3) measurement of pH and lactate in PRP and PC after storage.

The best proof of platelet function is control of bleeding in experimental or naturally occurring thrombocytopenia or thrombopathia.[27] Improved hemostasis may be documented by subjective observation of the bleeding patient and comparison of pretransfusion and post-transfusion bleeding times or other point-of-care tests of platelet function (see Chapters 79 and 88). In addition to the quality of the PRP or PC, improved hemostasis is dependent on recipient factors affecting platelet function and viability.

WHOLE BLOOD-DERIVED AND APHERESIS PLATELETS

Preparation of PRP and PC from FWB is described in Chapter 98. Leukoreduction during collection with a platelet-sparing filter reduces the storage lesion in human and canine (author unpublished observations) PCs.[38] All PCs should be labeled with donor blood type, as these components invariably contain RBCs.

The main benefit of apheresis PC over whole blood-derived PC or PRP is a greater total platelet yield per donor. Plateletpheresis has been performed successfully in dogs (10–28 kg body weight) using both automated continuous and discontinuous flow centrifugation.[8,9] Blood is typically collected through a 14–16-gauge jugular venous catheter, and platelet-poor blood is returned via a peripheral venous catheter. Platelet concentrate volume and platelet yield from plateletpheresis have ranged from 120 to 322 mL and 1.26×10^{11} to 4.5×10^{11} platelets/PC, respectively.[8,9] Collection time is approximately 1–2 hours, depending on desired platelet yield.[8,9] Platelet recovery (viability 1–2 hours post-transfusion) is approximately 80%, and transfused apheresis platelets have a mean half-life of 3.5–3.8 days and life span of 7–9 days.[8,9]

The main adverse effects of plateletpheresis on the canine donor are thrombocytopenia and hypocalcemia. Thrombocytopenia is usually mild, with most counts remaining above 100,000/μL and returning to baseline by 4–6 days. Ionized calcium levels drop during plateletpheresis because of citrate toxicosis during reinfusion of platelet-poor plasma and RBCs to the donor. Clinical signs of hypocalcemia were noted in three of 14 dogs in one study and were generally mild, but occurred despite intravenous calcium supplementation.[8,9] No long-term side effects have been noted.

STORAGE OF PLATELET-RICH BLOOD AND COMPONENTS

Veterinary clinics with blood-component-producing capabilities usually prepare PRP and PC on an as-needed basis, but PRP and PC may be stored for 5–7 days at room temperature (RT, 20–24 °C) with continuous or intermittent agitation; FWB is standardly stored up to 8 hours at RT, although an overnight holding period is feasible.[53] Due to the increased risk for bacterial proliferation at RT storage, especially in leukoreduced units, there has been re-evaluation of refrigerated storage of platelets as well as studies of pathogen inactivation. Cryopreservation and lyophilization are also long-term storage options for platelets that reduce bacterial proliferation.

Chilled Platelets

Prior to 1969, human platelets were refrigerated at 4 °C, similarly to RBCs.[34] Evidence of reduced viability after 8–24 hours of chilling then prompted the current standard of preparation and storage at RT.[3,7,8,18,47,50] However,

during the first 24 hours of refrigeration, platelets actually had normal-to-increased in vitro aggregation responses and demonstrated immediate in vivo hemostatic efficacy in humans with thrombocytopenia and thrombocytopathia.[7,18,51] Nonetheless, after 24 hours of refrigeration, in vitro aggregation, in vivo viability, and hemostatic efficacy rapidly diminished.[7,18,51] Various mechanisms contributing to reduced in vivo viability have now been elucidated, and there is a resurgence in the use of chilled platelets for therapeutic transfusion and investigations into techniques to improve quality.[23,40,54] There are no reports on the function or viability of chilled canine PRP or PC, and, given the effects of chilling on survival of both murine and human platelets, refrigerated storage of canine platelet-rich PRP and PC is not recommended. However, in vitro aggregation was normal in FWB during the first 6 hours of refrigeration, and remained at approximately 50–75% of baseline over 3 weeks; so, if FWB has been refrigerated immediately after collection, it is reasonable to assume that platelet function in the blood would be acceptable if transfused the same day.[36]

Pathogen Inactivation

Pathogen inactivation uses photochemical and/or ultraviolet illumination to damage pathogen nucleic acids.[54] This is effective against many pathogens, but also reduces platelet quality.[54] Pathogen inactivation is being used increasingly in human blood banks for RT storage, but in dogs has only been used experimentally.[49]

Cryopreserved Platelets

Cryopreservation is approved for human PC and has been used to store canine PC for up to 2 years.[4,5,8,24,54] Platelets are cryopreserved using a cryoprotectant solution in approved plastic bags at a freezing rate of 1–3 °C per minute. In general, cryopreserved human platelets have reduced function and viability compared to RT platelets. For dogs, platelets cryopreserved in 6% dimethyl sulfoxide (DMSO) also have impaired in vitro function based on marked reduction of aggregation, although cryopreserved platelets can be activated in vitro as demonstrated by P-selectin expression.[4,5,8,24] Compared to fresh PC, cryopreserved canine platelets also have had reduced in vivo recovery (approximately 50%) and survival (half-life, approximately 2 days), but had hemostatic efficacy in irradiated dogs.[5,8] These results may reflect the poor predictive value of the in vitro tests and/or the clinical benefit of transient platelet support. Canine platelets cryopreserved in polyethylene glycol–DMSO were also effective in controlling bleeding in Basset Hounds and Otterhounds with thrombopathia (W. J. Dodds, personal communication).[39] Commercial availability of cryopreserved canine PCs (Animal Blood Resources International, ABRI) provides a source of platelets for transfusion for clinics that do not own blood component centrifuges. In one retrospective study, thrombocytopenic dogs transfused with this component tolerated the transfusion well and platelet count significantly increased 24 hours following transfusion. Clinical bleeding scores were not significantly improved following transfusion compared to a control group, but the control group was comprised of dogs that did not receive a platelet transfusion and therefore disease severity could have differed between groups.[35]

Lyophilized Platelets and Platelet Substitutes

Lyophilization has also been used for long-term storage of human and canine platelets. The latter are commercially available as StablePlate RX™ (BodeVet, Rockville, MD). A randomized trial of this component in thrombocytopenic dogs compared outcomes after transfusion of fresh PC versus lyophilized PC.[14] There was no difference in bleeding scores of dogs treated with the different components.[14] Both groups had similar rates of acute transfusion reactions, which were mild.[14] Efficacy of the different platelet components could not be compared in the study due to a low number of dogs.[14]

Several synthetic platelet substitutes are under development as are methods for deriving platelets from cultured progenitor cells.[19]

TRANSFUSION OF PLATELETS

Platelet transfusion is most useful in the management of bleeding in patients with thrombocytopenia due to decreased platelet production, where transfused platelet life span is normal. It is clinically most rewarding when prompt marrow recovery is anticipated, e.g., following anticancer chemotherapy. Platelet transfusion may also be beneficial in dilutional thrombocytopenia associated with massive transfusion. Platelet transfusion is less beneficial in disseminated intravascular coagulation, but may be given if the patient is bleeding. Platelet transfusion is least beneficial in immune-mediated thrombocytopenia because transfused platelets may be rapidly destroyed; however, platelet transfusion has been useful in some cases and should be considered when there is critical bleeding.[8,11,14,35] Platelet transfusion is beneficial in most cases of bleeding due to thrombopathia.

Platelet transfusion triggers and doses have been reviewed.[2,15,25,28,29] Prophylactic transfusion, although controversial, is a common practice in human medicine, with transfusion triggers against spontaneous bleeding of 10,000/μL in the absence of other risk factors for bleeding, and 20,000/μL with other risk factors, and periprocedural triggers of 50,000–100,000/μL. Prophylactic transfusions increase the use of platelet components. Most transfusions given to dogs and cats are therapeutic. The latter approach requires close patient monitoring and readily available FWB, PRP or PC.

Platelet dose is also controversial.[2,15,25,28,29] A strategy of higher-dose, less-frequent transfusions may be used, versus one of lower-dose, more-frequent transfusions. Platelet survival is increased at higher platelet counts, supporting the first strategy, which is particularly applicable if transfusions are given prophylactically against

spontaneous bleeding on an outpatient basis. However, this may increase the total number of units used. Economics and resources often dictate a minimal transfusion dose in animals, which are usually hospitalized and transfused therapeutically, promoting the latter strategy. It is often not necessary to raise the platelet count by large increments—critical spontaneous bleeding can usually be prevented by transfusions to keep the platelet count above 10,000–15,000/μL.[2]

As with RBC and plasma components (see Chapters 99 and 100), platelet transfusions should be given using standard blood administration sets with 170 μm filters. Transfusion sets should be free of latex, which can bind platelets. Most infusion pumps may be used without damaging platelets, but the manufacturer should be consulted.

The success of a platelet transfusion is judged by control of bleeding and by comparing expected and measured platelet increments.[2,15,27] If the transfusion was beneficial, active bleeding will be reduced. If bleeding is well-controlled, existing petechiae and ecchymoses will fade over 12 hours. The expected 1-hour post-transfusion platelet count is calculated as:

Expected 1-hour platelet count = platelet count pre-transfusion + expected 1-hour platelet increment, where expected 1-hour platelet increment = unit platelet count × unit volume × percent platelet recovery (PPR)/estimated blood volume.

The PPR corrects for a fixed loss of transfused platelets due to splenic sequestration and other factors, and may be based on post-transfusion counts (increments) of labeled or unlabeled platelets in normal subjects or on platelet increments in clinical patients in previous studies.[15,25,28,47] If the measured platelet count is much below the expected count, there is accelerated destruction, consumption, increased sequestration, or the quality of the transfused blood or component was suboptimal. If there is minimal platelet production, but no accelerated platelet loss or sequestration, the recipient's platelet count should drop by approximately 33% each day following transfusion.[3] When interpreting the success of a platelet transfusion, it should be noted that platelet counts at low values are imprecise and hematology analyzer calibrations have not been optimized to count platelets altered by various storage methods.

Fresh Whole Blood

Often FWB is the only platelet-rich blood or component available to veterinary clinics. It also has maximum hemostatic properties. In a human trial, 1 unit FWB was equivalent to 10 FWB-derived PCs after open heart surgery, at least partially because the most functional platelets remain in the RBC fraction after centrifugation.[32,33] However, as is consistent with the observation that marked bleeding is required to lower platelet counts, large-volume transfusions are required to substantially raise them. As a rule of thumb, a FWB transfusion of 10 mL/kg will raise the recipient's platelet count by a maximum of 10,000/μL and will stop critical bleeding if there is no ongoing platelet loss.[2,3] The transfusion is given over 1 hour unless a slower rate is indicated.

Platelet-Rich Plasma and Platelet Concentrate

The recommended initial dose for FWB-derived PRP or PC is 1 unit per 10 kg, although resources may dictate a smaller dose. If the units contain on average 6×10^{10} platelets, platelet dose is 6×10^{9} platelets/kg, and the 1-hour platelet increment should be approximately 35,000/μL based on 51% PPR.[3,48] Using an approximately 50% fixed platelet loss may be extreme given that other studies suggest approximately 20% loss.[5,8] However, in the authors' experience, platelet increments are even lower than that predicted with approximately 50% loss, perhaps reflecting decreased platelet survival in the presence of thrombocytopenia.[25,27] Assuming canine apheresis PC contains on average 2.4×10^{11} in 200 mL, a minimum initial dose would be 1 apheresis PC/40 kg. Apheresis PCs are clearly superior for high-dose platelet support and transfusion to large dogs. Cryopreserved apheresis PCs (ABRI) are available as half-units of a minimum of 0.5×10^{11} platelets (typically $0.6–0.8 \times 10^{11}$ platelets) in 100 mL (600,000–800,000 platelets/mL), with a recommended dose of 1 PC/10 kg and an expected 1–2 hour increment of 20,000/μL, i.e., about 50% of that expected with FWB-derived PC.[24] Fresh or cryopreserved PCs are transfused over 1 hour and PRP at 10 mL/kg/hour, unless a slower rate is indicated.

For lyophilized platelet transfusion, StablePlate RX™ is provided as a 1.5×10^{10} particles/50 mL vial, which when reconstituted contains 1.5×10^{9} particles/mL. The recommended dose is 3×10^{9} particles/kg (one vial/5 kg), administered by slow bolus intravenous infusion. This dose is approximately 50% of the FWB-derived and cryopreserved PC dose, and, using the abovementioned formula, predicted 1-hour platelet increment would be at most approximately 15,000/μL.

Platelet-Poor Blood Components

If FWB, PRP, or PC are not available, fresh frozen plasma (10 mL/kg) or cryoprecipitate (1 unit/10 kg) could be considered for therapeutic transfusion. These components contain hemostatically functional platelet microparticles, and have been beneficial in treating thrombocytopenic bleeding.[22] Benefit may also be via augmentation of other aspects of hemostasis. Stored WB or packed RBCs may also reduce thrombocytopenic bleeding in anemic patients, as bleeding time is longer at lower hematocrits.[52] This is likely a benefit in dogs with immune-mediated thrombocytopenia. In addition, platelets retain some function in stored WB.[36]

PLATELET TRANSFUSION REACTIONS

Transfusion reactions are discussed in Chapter 104. Febrile reactions are the most common reaction to PC; frequency is reduced by prestorage leukoreduction.[38] Alloimmunization to platelet antigens occurs readily in immunocompetent dogs receiving multiple transfusions, resulting in platelet transfusion refractoriness, a failure to achieve an expected increment in platelet count in response to a platelet transfusion.[42–49] Two currently

accepted methods for minimizing the development of platelet alloimmunization include leukoreduction and ultraviolet irradiation.[43-45,49] The combination of centrifuge- and filter-leukoreduction was shown to be superior to either procedure alone for prevention of platelet alloimmunization in dogs.[50] Dogs being treated with cyclosporine are less likely to develop alloimmunization.[45]

Transfusion-associated sepsis is a particular concern with RT-stored PRP or PC. The rate of contamination in human PC without pathogen reduction is one in 2000–5000 units.[31] The rate for canine PC is not known, and measurement of this rate is affected by the comparatively low number of units produced. Visual inspection of a contaminated unit may reveal gaseous distention of the bag and loss of swirling, but contaminated units may have a normal appearance. Veterinary blood banks should closely monitor RT-stored platelet components microbiologically.

GRANULOCYTE TRANSFUSION

Granulocyte concentrate (GC) transfusion produces a transient increase in neutrophil numbers to a critical level that may permit survival in otherwise fatal sepsis.[21] While promoted in the 1970s and early 1980s in human medicine, usage of GC then declined because of expense, febrile reactions, controversial benefit, and newer antibiotics. The availability of granulocyte colony-stimulating factor (G-CSF) paradoxically led to a resurgence of GC transfusion.[13] Granulocyte concentrate is usually prepared by continuous-flow centrifugation leukapheresis, with pre-administration of G-CSF (with or without corticosteroids) to the donor to increase neutrophil yield. Donors should be blood-type-compatible, and irradiation of GC (25–50 Gy) is recommended to minimize transfusion-associated graft-versus-host disease.[20]

Therapeutic GC transfusions in dogs have been used primarily in experimental models of myelosuppression and neonatal sepsis.[12,16] They have been rarely used in clinical veterinary medicine. In addition to leukapheresis, centrifugation of FWB, with or without colloid-facilitated sedimentation, may be used to isolate canine and feline buffy coats; sedimentation alone may also be used in the cat.[6,42] Granulocytes are stored at RT without agitation for 24 hours.[20] The initial dose is 1×10^9 granulocytes/kg in a volume of 15 mL/kg once to twice daily.

Granulocytes may also be used diagnostically. Infusion of radiolabeled autologous granulocytes and scintigraphy may be used to detect inflammation in animals with nonspecific clinical signs.[26]

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CHAPTER 102

Blood Transfusion in Large Animals

MARGARET C. MUDGE

Indications for Blood Product Transfusions
- Whole Blood
- Packed Red Blood Cells
- Plasma
- Hemoglobin-Based Oxygen Carriers

Blood Donor Selection
Blood Typing and Crossmatching
Collection Techniques
Administration and Adverse Reactions
Ruminants and Camelids
- Indications for Transfusion
- Donor Selection
- Collection Technique
- Administration and Adverse Reactions

Acronyms and Abbreviations

CPDA-1, citrate-phosphate-dextrose with adenine; FFP, fresh frozen plasma; FPT, failure of transfer of passive immunity; HBOC, hemoglobin-based oxygen carrier; NI, neonatal isoerythrolysis; pRBC, packed red blood cell; TP, total protein; USDA, United States Department of Agriculture; WB, whole blood.

Blood product transfusion is an integral part of equine practice, both in referral institutions and in ambulatory practice. Blood products may be administered for conditions ranging from life-threatening acute hemorrhage to failure of transfer of passive immunity (FPT). The available equine blood products and indications for transfusion will be discussed in this chapter. Practical considerations for donor selection and collection and administration of blood will also be provided. A brief discussion of whole blood (WB) and blood product transfusion in ruminants and camelids is presented at the end of the chapter.

INDICATIONS FOR BLOOD PRODUCT TRANSFUSIONS

Whole Blood

Whole blood transfusions are most often indicated for horses that have suffered acute blood loss from trauma, surgery, or other conditions such as splenic rupture or uterine artery hemorrhage. In cases of blood loss, the transfusion serves to restore blood volume as well as oxygen-carrying capacity. While there are no set variables that serve as "transfusion triggers," a combination of physical examination and clinicopathologic parameters can be used to guide the decision to transfuse. It is important to remember that the PCV may initially remain normal in cases of acute hemorrhage due to splenic contraction. Some fluid redistribution occurs almost immediately after blood loss, but it may take hours to days for complete restoration of blood volume, further complicating the assessment of the patient with acute blood loss. Serial monitoring of PCV and total protein (TP) as the horse is rehydrated with intravenous fluids will give an indication of the extent of blood loss. Suspicion of large-volume blood loss, combined with tachycardia, tachypnea, pale mucous membranes, lethargy, and decreasing TP, may lead to the decision to transfuse.[18] Blood transfusion is likely needed during an acute bleeding episode when the PCV drops below 20%, although in acute severe cases, transfusion may be needed before there is a significant drop in PCV.

Estimation of blood loss at surgery can be used to guide the decision to transfuse, with greater than 30% blood loss generally requiring transfusion.[13] Anesthetized horses may have very stable heart rate

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and PCV despite massive blood loss; pale mucous membranes with prolonged capillary refill time, decreasing TP, hypotension, and hypoxemia are better indicators of blood loss.[47]

Oxygenation status can help to determine the need for blood transfusion in cases of both acute hemorrhage and chronic anemia. A rise in blood lactate concentration despite volume replacement with crystalloid or colloid fluids may indicate continued tissue hypoxia and a need for blood transfusion.[15,23] The oxygen extraction ratio is also a useful measure:

$$ER_{O_2} \approx \frac{S_{aO_2} - S_{vO_2}}{S_{aO_2}}, \quad (102.1)$$

where ERo_2 is the oxygen extraction ratio, S_ao_2 the arterial oxygen saturation, and S_vo_2 the mixed venous oxygen saturation. A ratio greater than 40–50% in the context of blood loss may indicate a need for blood transfusion.[24]

Transfusion should be considered a temporary measure to restore oxygen-carrying capacity, relying on the horse's erythropoietic response, control of hemorrhage, or resolution of underlying disease to provide long-term resolution.

Packed Red Blood Cells

Packed RBCs (pRBCs) are indicated for normovolemic anemia, such as neonatal isoerythrolysis (NI), erythropoietic failure, and chronic blood loss. In cases of chronic or hemolytic anemia, markers of tissue oxygenation, such as lactate and oxygen extraction, are still useful. PCV is a better "transfusion trigger" for chronic anemia compared to acute hemorrhage, with transfusions suggested for horses with evidence of tissue hypoxia and a PCV less than 10–12%. Transfusions may be given at a higher PCV for horses with concurrent conditions (e.g., respiratory disease, anesthesia, sepsis) or risk of further blood loss. When pRBCs are not available, WB may be used for the same indications, although attention should be paid to the total volume given so that volume overload is avoided.

Plasma

Plasma transfusion is indicated for the treatment of clotting factor deficiency, hypoalbuminemia, decreased colloid oncotic pressure, and FPT. Fresh and fresh frozen plasma (FFP) contain immunoglobulins, coagulation factors (fibrinogen and factors II, VII, IX, X, XI, and XII), and cofactors (factors V and VIII), as well as the anticoagulant proteins antithrombin, protein C, and protein S. Plasma has also been used for treatment of disseminated intravascular coagulation in horses.[45]

Colloid support is generally recommended in patients with a total protein less than 4 g/dL or serum albumin concentration less than 2 g/dL. Other indications for colloid support are colloid oncotic pressure less than 14 mmHg, clinical signs such as ventral edema, and conditions which increase microvascular permeability, such as sepsis. When plasma is not necessary for clotting factor replacement, a synthetic colloid such as hydroxyethyl starch (hetastarch) is preferred for volume expansion and more effective oncotic support. In healthy horses, hetastarch and plasma infusion increased colloid oncotic pressure similarly, with an increase of approximately 2 mmHg after a 10 mL/kg IV infusion.[29]

Failure of transfer of passive immunity in neonatal foals more than 12 hours of age is best treated by plasma transfusion, as colostrum absorption is greatly diminished after 12 hours.[19] An IgG concentration less than 200 mg/dL is considered complete FPT, and IgG between 400 and 800 mg/dL is considered partial FPT. Although plasma transfusion is not always needed for foals with partial FPT, it is recommended for foals that have preexisting infection or exposure to pathogens. Commercially available fresh frozen hyperimmune plasma is most commonly used for treatment of neonatal foals (Table 102.1). Equine FFP is a USDA-licensed product, and most products have a minimum guarantee for IgG concentration and a 2–3-year shelf-life when frozen. Although commercially available hyperimmune plasma has very high IgG concentrations (1500–2500 mg/dL), plasma from local donor horses may provide better protection against specific local pathogens.

There are multiple hyperimmune plasma products with bacterial- or viral-specific antibodies. There is some evidence for the efficacy of *Escherichia coli* (J5) and *Salmonella typhimurium* hyperimmune plasma for the treatment of equine endotoxemia; however, there are also reports which dispute the efficacy of such products.[10,33,38,39] *Rhodococcus equi* hyperimmune plasma has been shown to decrease the severity of pneumonia in foals experimentally infected with *R. equi*, although it did not reduce the incidence of naturally acquired *R. equi* pneumonia.[6,14,35] Other plasma products available for specific disease treatment include plasma from donors vaccinated against *Clostridium botulinum*, Rotavirus, and West Nile virus antibody.

Hemoglobin-Based Oxygen Carriers

A hemoglobin-based oxygen carrier (HBOC) solution, Oxyglobin, has been used in ponies with experimentally induced normovolemic anemia.[3] This product improved

TABLE 102.1 Commercial sources of fresh frozen equine plasma.

Veterinary Immunogenics (Cumbria, UK)	www.veterinaryimmunogenics.com
Lake Immunogenics, Inc. (Ontario, NY)	www.lakeimmunogenics.com
Mg Biologics (Ames, IA)	www.mgbiologics.com
EquiPlas (Templeton, CA)	www.plasvaccusa.com

hemodynamic and oxygen transport parameters; however, one pony did have an anaphylactic reaction. The use of an HBOC solution was also reported for treatment of a pony mare with chronic hemorrhage and a history of acute transfusion reactions.[27] Oxyglobin is no longer on the market, but there are newer generations of HBOCs in development.

BLOOD DONOR SELECTION

The ideal equine blood donor is a healthy, young gelding weighing at least 500 kg. Donor horses should be up-to-date on vaccinations, including rhinopneumonitis, tetanus, eastern and western encephalitis, rabies, and West Nile virus. Donors should be tested annually for equine infectious anemia. Because RBC antigens Aa and Qa are the most immunogenic, the ideal donor should lack the Aa and Qa alloantigens. There are breed-specific blood factor frequencies, so a donor of the same breed as the recipient may be preferable, especially when blood typing is not available. Horses that have received blood or plasma transfusions and mares that have had foals are not suitable as donors, as they have a higher risk of carrying RBC alloantibodies. Donkeys have a RBC antigen known as "donkey factor," which is not present in horses; therefore, donkeys or mules should not be used as donors for horses, as the transfused horses can develop anti-donkey factor antibodies.[28]

When a surgical procedure is planned in advance and there is a high risk of blood loss, preoperative autologous donation should be considered, as the horse would be its own ideal blood donor.[31] The half-life of fresh allogeneic transfused RBCs is approximately 34 days for crossmatch-compatible blood and less than 5 days for crossmatch-incompatible blood, and approximately 50 days for autologous transfused RBCs.[32,41] Intraoperative or posthemorrhage cell salvage is also an option for autotransfusion, and its use has been reported in a horse with postcastration hemorrhage.[42] Red blood cell recovery can be performed with specialized cell salvage equipment which washes and filters collected blood, but cell salvage can also be performed with simple anticoagulation and filtration.[20,43]

BLOOD TYPING AND CROSSMATCHING

In an emergency situation, an immediate blood transfusion may be given for the first time with a very minor risk of serious transfusion reaction. Horses can develop alloantibodies within 1 week of transfusion, so blood typing and crossmatching are recommended before a second transfusion is performed.[48] However, a second blood transfusion may be performed safely within 2–3 days of the first transfusion without a blood crossmatch.

Blood typing and alloantibody screening can be used to help find the most appropriate donor horse for the patient requiring transfusion (see Chapters 96 and 107). Unfortunately, since blood typing is time-consuming and laboratories performing blood typing are very limited, this is not often a practical method of donor selection (Table 102.2). Blood typing and antibody screening prior to initial transfusion are more important for horses for which subsequent blood transfusions are anticipated and for broodmares which may produce foals with NI if sensitized to other blood group factors.[48] A rapid agglutination method (Alvedia, Limonest, France) is available for detection of equine RBC antigen Ca, which may be more practical for limited pretransfusion testing.

TABLE 102.2 Equine blood typing laboratories.

University of California, Davis
Clinical Laboratory Services
Central Laboratory Receiving, VMTH Rm 1033
One Garrod Drive
University of California, Davis
Davis, CA 95616
Phone: 530-752-8684
www.vetmed.ucdavis.edu/hospital/support-services/lab-services

Rood and Riddle Veterinary Laboratory
2150 Georgetown Rd.
Lexington, KY 40511
Phone: 859-233-0331
www.roodandriddle.com/services/laboratory

A blood crossmatch is recommended prior to a transfusion, especially for any horse which may have previously been exposed to RBC antigens (see Chapters 96 and 107). Although preexisting alloantibodies are uncommon in horses, RBC survival time is decreased and febrile reactions are more common when crossmatch-incompatible blood is transfused.[41] Hemagglutination crossmatching is widely available and rapidly performed; however, it will not predict all transfusion reactions, namely, the hemolytic reactions. Rabbit complement can be added to the reaction mixture to detect hemolytic reactions.[2] The gel column crossmatch technique has been shown to have excellent accuracy for agglutination reactions, but not for hemolytic reactions.[21]

COLLECTION TECHNIQUES

Blood is collected from the jugular vein of the donor horse, either via direct needle cannulation or catheterization. When a large volume of blood is needed, a 10- or 12-gauge catheter is recommended, although a 14-gauge catheter is also sufficient. Blood flow may be improved by placing the catheter opposite the venous blood flow (catheter directed toward the head). A healthy horse can donate approximately 20% of its total blood volume every 30 days.[26] When 15% or greater blood volume is collected, volume replacement with approximately 10–20 mL/kg intravenous crystalloid fluids is recommended. The donor horse's heart rate, respiratory rate, and attitude should be monitored during the blood collection. Vital parameters should normalize within 1 hour of collection.

Plastic bags and vacuum-collection glass bottles are available for blood collection in sizes ranging from 450 mL to 4 L (Baxter Fenwal, Deerfield, IL; MWI Veterinary Supply, Meridian, ID; Animal Blood Resources International, Dixon, CA). The glass bottles are preferred by many due to the speed of collection; however, the glass inactivates platelets and causes some damage to RBCs.[30,36]

When blood is collected for immediate transfusion, anticoagulation with 3.2% sodium citrate is adequate. However, when blood is stored for later transfusion, optimal pH and support of RBC metabolism are necessary to sustain RBC viability (see Chapter 98). A post-transfusion viability study demonstrated a viability of 73% at 24 hours post-autologous transfusion, for blood stored for 28 days in citrate-phosphate-dextrose with adenine (CPDA-1).[32] Recommended storage conditions for equine blood are similar to those for canine and human blood (see Chapter 98).

Equine blood can be processed to provide plasma and pRBC components, as with other species. Due to the rapid sedimentation of equine RBCs, the RBC component can be administered without specialized processing; however, the pRBCs will still contain plasma components unless centrifugation and repeated washing are performed. Washing of RBCs is the preferred technique when a transfusion is given to an NI foal using the mare as a donor. When RBC washing or other processing is planned, blood should be collected into bags rather than bottles due to ease of centrifugation and sterile transfer.

Plasma processing can be performed by gravity sedimentation, centrifugation using a double-bag system, or plasmapheresis. Plasmapheresis is the preferred technique for commercial plasma production, as it is more rapid than WB collection and processing, and results in plasma with minimal RBCs and leukocytes.[11] Plasmapheresis of between 4 and 11 L can be performed every 30 days on donor horses.[25] Immunoglobulins are well-maintained for at least 1 year in FFP; however, coagulation factor activity may decrease after 2–4 months of storage.[17]

Plateletpheresis has been described in horses using commercially available apheresis equipment. Platelet concentrations in horses were lower using this technique compared to the concentrations reported in other species, possibly due to the human-specific calibration of the equipment.[40] Equine platelet concentrate can also be produced through processing of whole blood with multiple centrifugations. This technique was reported to produce platelet concentrate that meets the standards of the American Association of Blood Banks.[4]

ADMINISTRATION AND ADVERSE REACTIONS

The volume of blood to be transfused depends on estimated blood loss, estimated total blood volume, and donor PCV:

$$\begin{aligned}&\text{Blood transfusion volume (mL)}\\&= \text{Body weight (kg)}\\&\quad \times \text{Blood volume (mL/kg)}\\&\quad \times \left[\frac{(\text{Desired PCV} - \text{Actual PCV})}{\text{Donor PCV}}\right]\end{aligned} \qquad (102.2)$$

In cases of acute blood loss, PCV is often not useful for estimates of volume to be transfused, since it does not accurately reflect blood loss. Instead, estimates of blood loss and evaluation of clinical parameters are used to determine the volume of blood needed. Between 25% and 50% of the total blood lost should be replaced by transfusion, since much of the circulating volume will be replaced by fluid shifts. It is important to remember that up to 75% of RBCs lost into a body cavity (e.g., hemoperitoneum) are autotransfused back into circulation within 24–72 hours.[34] Therefore, lower percentages of blood volume replacement may be needed in cases of intracavitary hemorrhage. Blood and plasma products should be delivered with an in-line filter to remove small clots and fibrin.

Volumes of plasma for treatment of hypoproteinemia can be estimated by TP or albumin concentrations, although the use of plasma to normalize severe hypoproteinemia can be prohibitively expensive in the adult horse:

$$\begin{aligned}&\text{Plasma transfusion volume (mL)}\\&= \text{Body weight (kg)} \times 45\text{(mL/kg)}\\&\quad \times \left[\frac{(\text{Desired TP} - \text{Actual TP})}{\text{Donor TP}}\right]\end{aligned} \qquad (102.3)$$

Volumes of plasma given for treatment of hypoproteinemia or coagulopathy are often determined by clinical and clinicopathological response. The volume of plasma needed in a foal with FPT can be determined if the IgG concentrations of the foal and the plasma are known. A dose of 20 mL/kg of plasma (IgG approximately 1200 mg/dL) will generally raise the foal's IgG concentration by 200–300 mg/dL, and hyperimmune plasma (IgG > 2500 mg/dL) should increase the IgG by approximately 400 mg/dL. A larger volume of plasma may be needed to achieve a similar rise in IgG in clinically ill foals.[46]

In order to monitor for transfusion reactions, blood should be delivered at a rate of approximately 0.3 mL/kg over the first 10–20 minutes, while monitoring heart rate, body temperature, and respiratory rate. Horses should also be monitored for signs of muscle fasciculation, piloerection, and urticaria. Adverse reactions reported in horses receiving blood transfusions include urticaria, hemolysis, and acute anaphylactic reactions. The rate of adverse reaction to WB transfusion has been reported as 16%, with one of 44 horses (2%) having a fatal anaphylactic reaction.[18] The rate of transfusion reactions to commercial equine plasma was reported as 0% in adult horses and 8.7% in foals, with no fatal reactions.[16] If no signs of reaction are seen, the rate of administration can be increased to 5 mL/kg/hour for normovolemic horses and up to 20–40 mL/kg/hour for hypovolemic horses. If signs of anaphylaxis are present, epinephrine (0.01–0.02 mg/kg IV) should be administered immediately. More mild transfusion reactions, such as urticaria, fever, and tachypnea, may be treated with an anti-inflammatory (e.g., flunixin meglumine 1.1 mg/kg IV) or an antihistamine (e.g., tripelennamine or diphenhydramine 1.1 mg/kg IM).

Similar to the risks in other veterinary species, bacterial contamination of blood, transmission of blood-borne disease from donor to recipient, and hypocalcemia associated with citrate toxicity are all potential concerns related to transfusion in the equine patient (see Chapter 104). An additional concern in horses is the possible sensitization of a broodmare to blood group antigens, leading to the risk of NI in subsequent foals.[47] Although plasma transfusions are not commonly associated with serious adverse reactions, serum hepatitis has been reported in association with transfusions of commercial plasma.[1]

RUMINANTS AND CAMELIDS

Indications for Transfusion

The indications for WB and plasma transfusion in ruminants and camelids are similar to those for horses. Gastrointestinal parasites, especially *Haemonchus contortus*, and ectoparasites (e.g., *Haematopinus spp*. and *Linognathus spp*.) are causes of chronic blood loss anemia, and iron-deficiency anemia can affect neonatal calves.[37] The blood parasites *Anaplasma* and *Babesia* are both associated with the development of chronic anemia, although by different mechanisms. Infections in llamas and alpacas due to *Mycoplasma haemollama* result in a chronic anemia due to extravascular hemolysis, frequently leading to a PCV of less than 10%. In the case of acute hemorrhage or ongoing blood loss (e.g., abomasal ulcers), transfusion should be considered when the PCV falls below 20%. Ruminants with chronic blood loss may not require blood transfusion until the PCV is below 12%.

Failure of transfer of passive immunity is a common indication for plasma transfusion in neonatal calves and crias. While a TP less than 5 g/dL is suggestive of FPT in neonates, radial immunodiffusion can be performed to quantitatively determine IgG concentration.[5,44] Commercial FFP is available for ruminant and camelid transfusion (Animal Blood Resources International, Stockbridge, MI; Triple J Farms, Bellingham, WA), although fresh plasma or even WB can be effective for treatment of FPT. Hyperimmune serum products are available for subcutaneous and intramuscular dosing in ruminants, including products with antibodies against *E. coli*, *Pasteurella*, *Arcanobacterium pyogenes*, *S. typhimurium*, and *Mannheimia haemolytica* (Colorado Serum Company, Denver, CO). The use of Oxyglobin has been reported in a sheep with anemia due to gastrointestinal parasites and in two alpacas with hemolytic anemia, although Oxyglobin is not currently available.[8,12]

Donor Selection

The initial blood transfusion should generally be safe, regardless of the donor; however, a J-negative donor (cattle) is ideal (see Chapter 96).[9] Crossmatching in ruminants must include complement to test for hemolytic reactions, as these are more clinically important than agglutination reactions. Initial transfusions are generally safe to administer without a blood crossmatch. However, if more than 48–72 hours have elapsed since the initial blood transfusion, crossmatching is recommended. Blood donors should be free of disease, including bovine leukosis virus, anaplasmosis, and bovine viral diarrhea virus. If a donor animal is selected from the herd, it is especially important to check PCV and TP prior to blood collection, since the donor animal may also be affected by the same endoparasites or ectoparasites.

Collection Technique

Up to 20–25% of total blood volume can be removed from the donor animal, usually via needle cannulation or jugular catheterization. Total blood volume can be estimated at 80 mL/kg in cattle, and this estimate is also reasonable for goats and sheep, while blood volume of alpacas should be estimated at 100 mL/kg.[22] As with equine transfusions, blood can be collected into bottles or bags with citrate anticoagulant. Blood stored in CPDA-1 may retain adequate viability for 2–3 weeks.[7] Since bovine blood does not readily separate, a centrifuge is needed to process WB into plasma and pRBC components.

Administration and Adverse Reactions

Blood should initially be delivered slowly in order to monitor for transfusion reactions. Complement-mediated hemolysis is the primary transfusion reaction which occurs with ruminant blood type incompatibility. Volume overload should be avoided, and total volumes delivered should especially be considered in neonates and small ruminants.

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CHAPTER 103

Blood Transfusion in Exotic Species

ANNELIESE STRUNK and ANKE C. STÖHR

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Acronyms and Abbreviations

ACD, acid-citrate-dextrose; BV, blood volume; BW, body weight; CPD, citrate-phosphate-dextrose; CPDA-1, citrate-phosphate-dextrose-adenine; CRI, constant rate infusion; Hct, hematocrit; Hgb, hemoglobin; HBOC, hemoglobin-based oxygen carrier; IMHA, immune-mediated hemolytic anemia; IO, intraosseous; IV, intravenous; RCS, red cell survival; RHD, rabbit hemorrhagic disease; WB, whole blood.

Blood transfusions can be a lifesaving tool for all exotic species. While basic transfusion medicine concepts can be directly extrapolated from domestic species and applied to exotic species, there is a growing body of peer-reviewed literature and research about blood transfusions in exotic species.

ANEMIA IN EXOTIC SPECIES

Anemia can occur in all species to the point where transfusions may become necessary. Causes of anemia in exotic species are similar to domestic animals, and can include blood loss, hemolysis, and decreased production. Sequestration in the spleen may also lead to a low hematocrit (HCT) or PCV; e.g., in ferrets associated with isoflurane anesthesia.[28,29] Lymph (or cerebrospinal fluid) contamination occurs frequently during reptile venipuncture and accuracy of the PCV should be questioned and confirmed before making the diagnosis of anemia in reptiles.

INDICATIONS FOR TRANSFUSION

A transfusion should be considered in patients with a low PCV (less than 20% in birds and mammals and 10% in reptiles) or in patients experiencing hypovolemic shock from acute blood loss. However, the clinical presentation, chronicity of the anemia, underlying cause, risk of continued loss, and especially the ability of the patient to tolerate the stress of handling are very important considerations to take into account when deciding on this plan of action. Classification and identification of the underlying cause for anemia, as well as associated clinical signs, should be critically evaluated before electing blood product administration, especially in chronically sick animals. In those patients, treatment of the

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underlying disease and supportive care may be superior to the risks and stress associated with a blood transfusion.[8]

CROSSMATCHING

Crossmatching is recommended for first transfusions in all exotic species except for ferrets, who have no identifiable blood groups. Crossmatching should be performed prior to all subsequent transfusions.

See Chapter 107 for more details on crossmatching, and Table 103.1 for more information on donor selection. A full crossmatch (major and minor) may not be feasible when managing most exotic species, as they are often much smaller than domestic species. A modification for small exotics to minimize the total blood volume needed (0.3–1 mL) includes the use of plasma instead of serum for full crossmatching. Plasma samples are used commonly in exotics, as it maximizes the volume available for analysis compared to serum.[26] EDTA is the ideal anticoagulant for this testing and has been used successfully for detection of incompatibilities via crossmatching by the authors. However, heparin is also routinely used, especially in avian and some reptile species. An optimized protocol for crossmatching of sea turtle blood using this anticoagulant has been published recently.[13] There are variations in reactions to different anticoagulants among exotic species. Heparin is commonly used for many avian species, as EDTA has been noted to cause hemolysis or reactions in a number of species, including crowned cranes (*Balearica sp*.), jays, brushturkey, crows (*Corvus corone*), and magpies (*Pica pica*).[26] In reptiles, it has been found that EDTA can cause hemolysis in Hermann's tortoises (*Testudo hermanni*)[35] and other chelonian species. Heparin, however, has been noted to cause hemolysis in some snakes (ball pythons (*Python regius*)).[46]

If a full crossmatch is not possible, a simplified crossmatch, using a drop of whole blood with two drops of plasma on a slide and looking for agglutination, can be tried before blood administration.[24] It should be noted that this technique cannot predict hemolysis and that weaker antibody reactions are likely to be missed.

BLOOD COLLECTION AND STORAGE

As a rule, sedation or anesthesia is recommended for blood collection techniques in all exotic species (except for tail collection in reptiles) in order to minimize stress from handling as well as the possibility of excessive blood loss or complications from hematomas, vascular laceration, or hemopericardium.[23,48] Blood collection sites can vary with the species. See Section IX "Species-Specific Hematology" for further information on blood collection sites.

A safe volume to collect from healthy animals is 10% of their blood volume. Blood volume in birds is variable (between 4.8% body weight (BW) in ring-necked

TABLE 103.1 Donor selection criteria for exotic species.

Species	Ideal donor	Donor screening
Avian	Healthy animal of appropriate weight Same species or genus preferred Housed singly or in closed aviary	Yearly physical examination, CBC, and biochemical profiles Screened for polyoma, psittacine beak and feather disease, and chlamydiosis Major or simplified crossmatch
Ferret	Male, less than 3 years of age	Yearly examination, CBC, and biochemical profiles Annual heartworm testing (antibody and antigen) and heartworm prevention in endemic areas Vaccinated yearly for canine distemper and rabies Major or simplified crossmatch after first transfusion
Rabbit	Female, less than 3 years of age Related animals may reduce disease transmission	Yearly examination, CBC, and biochemical profile Negative testing for *Encephalitozoon cuniculi* Screened (and/or vaccinated) for viral hemorrhagic disease (rabbit hemorrhagic disease (RHD)) and myxomatosis (in endemic countries) Major or simplified crossmatch
Rodents	Male guinea pigs less than 3 years of age Female chinchillas less than 5 years of age Male rats less than 1 year of age	Yearly examination, CBC, and biochemical profile in larger species Major or simplified crossmatch
Hedgehogs and other mammalian species	Largest available individual of the same species with an age that is less than half the expected life span of the species	Yearly examination, CBC, and biochemical profile if species size allows Major or simplified crossmatch
Reptiles	Young, healthy animal of appropriate weight Same species or genus preferred Same collection, singly housed animal or no animal added to collection for at least 3 months	Yearly examination, CBC, and biochemical profile, PCV and total solids in small species Screen for hemoparasites, viral testing Major or simplified crossmatch

TABLE 103.2 Anticoagulants for blood transfusions/known storage times. If blood will be stored, it needs to be cooled appropriately and warmed to 25–30 °C before administration.

Anticoagulant	Proportion of anticoagulant to blood	Avian	Mammal	Reptile	Comments
ACD	1:9	X	X	X	American alligators (packed RBCs), at least 35 days at 4 °C[14]
CPD	1:9	X	X	X	
CPDA-1	1:9	X	X	X	Preferred for stored whole blood in domestic species Rabbits, 28–35 days at 4–6 °C[6] Ferrets, 7 days at 4 °C[41] Rats, maximum 24 hours at 4 °C[7] American alligators (packed RBCs), at least 35 days at 4 °C[14]
Sodium heparin (1000 IU/mL)	Avian: 1:40 (0.25 mL per 10 mL blood) Reptile: 1:20–1:10 (5–10 IU per 1 mL/blood)	X		X	Preferred for short-term storage in sea turtles (24 hours at 4–8 °C)[40] Heparin may cause hemolysis in some snakes[46]
Sodium citrate (3.2%)	1:9	X			Citrate binds calcium in blood (use caution in hypocalcemic animals or very small animals)

pheasants (*Phasianus colchicus*) and 20% BW in domestic pigeons (*Columba livia domestica*); average bird blood volume is considered to be 10% of BW. The acceptable safe volume to draw from a healthy bird is 1% BW.[26] In small mammals, there is some variation in blood volume among species: rats/mice (5.5–7% BW),[36] guinea pigs (7.5% BW),[32] rabbits (5–7% BW),[32] ferrets (5–6% BW).[49] In reptiles, blood volume is 5–8% BW.[55] The maximum volume to draw from a healthy reptile is 1% BW.[34] Table 103.2 includes anticoagulants that may be used for blood collection, and known storage times. However, fresh blood (administered within 4 hours after collection) remains the primary and preferred transfusion type in all exotics. There are currently no long-term storage options for avian samples.[17]

BLOOD ADMINISTRATION—GENERAL CONSIDERATIONS

Blood transfusions should be administered in a similar technique to dogs/cats using an intravenous (IV) or intraosseous (IO) catheter via constant rate infusion (CRI). In most cases, the transfusion volume is 10–20% of the recipient's BW. In small species, multiple donors may be needed to achieve an appropriate volume. Warm blood should be administered via pediatric syringe pump and a standard 18 micron blood filter in most species.[22,37,38]

In mammals and birds, it is advisable to start the transfusion slowly (0.25 mL/kg/hour) for 15 minutes and monitor attitude heart rate, respiratory rate/effort, temperature, and appearance of skin for erythema or swelling at 5-minute intervals during that time.[6] The rate can be increased to 10–20 mL/kg/hour if there are no signs of a reaction, and monitoring should be continued at regular intervals until the transfusion is complete. Due to anatomical and physiological differences in reptiles, specific considerations should be taken into account and can be found later in this chapter in the reptile section.

Faster delivery should be considered in cases of acute blood loss. The transfusion may be administered as a bolus using a butterfly catheter, but should be given over at least 5 minutes to prevent circulatory overload.[3,38]

AVIAN SPECIES

For hematology information in different avian species groups, please see Chapters 123–127.

General Considerations

Birds are resistant to the effects of acute blood loss and rapidly regenerate blood cells (the half-life of avian RBCs is short (28–48 days, mean 39.7 ± 3.3 days) compared with other species groups).[44] A comparison of response to acute blood loss in pigeons to IV crystalloids alone, homologous transfusions, and heterologous transfusions demonstrated the best response in the IV crystalloid group.[5] The LD50 of acute blood loss was noted to be 60% of blood volume in mallard ducks (*Anas platyrhynchos*).[25] In this study, regeneration of cells started within 24 hours. In Japanese quail (*Coturnix coturnix japonica*) that underwent a 30% decrease in blood volume, blood volume returned to normal within 1 hour without replacement of volume with crystalloids, and most hematologic values returned to normal within 72 hours.[16,45] Chickens (*Gallus gallus domesticus*) that had acute hemorrhagic shock induced with 50% reduction in blood volume had a notable rise in PCV and hemoglobin (Hgb) within 3 days, and a return to normal values within 7 days, regardless of the presence or absence of fluid resuscitation.[57] If a patient is clinically stable, and

there has been less than 25% of blood volume lost (and the source of hemorrhage is arrested), it may not be necessary to perform a transfusion and the patient is likely to recover with supportive care and time.

Birds that are weak and showing profound clinical signs from anemia would benefit from a whole blood transfusion. However, these birds are more at risk of death from the stress-related effects of handling needed for performing a transfusion, requiring clear communication with clients.

Donor Selection/Blood Groups/Compatibility

There are 28 blood group antigens identified in chickens, with more than 10 alleles for most antigens.[12] Blood groups in psittacines have not been identified,[20] but serologic incompatibility between species of parrots has been noted using crossmatching, existing both prior to initial transfusion and after a heterologous transfusion has occurred.[9,10] A study evaluating crossmatches between 14 different avian species showed crossmatch reactions of varying degrees in 66% of heterologous transfusions.[51] However, heterologous transfusions between birds found to have mild incompatibility via crossmatching did not result in any identifiable negative clinical effects.[9,10]

Autologous transfusions resulted in red cell survival (RCS) of 35 days in red-tailed hawks (*Buteo jamaicensis*), and almost 27 days in pigeons. RCS from homologous transfusions ranges from 6–11 days; RCS from heterologous transfusions ranges from 12 hours–3 days (with the longer time frame for transfusions within the same genus, and the shorter time frame for transfusions between more distantly related species (i.e., pigeon to raptor)), noting that the RCS decreases with subsequent transfusions from the same donor.[9,43]

Ideally, one should aim for donors of the same species. If not available, donors from the same genus are preferred to more distantly related species.[9,10] In an emergency, using blood from a donor from a different class (i.e., chickens to psittacine) can be considered as long as any crossmatch incompatibility is mild. The transfused RBCs will have a very short half-life, but the transfusion may be beneficial as a colloid. See Table 103.1 for more information on donor selection.

Transfusion Reactions

Information from crossmatching can aid in donor selection in birds who have already had multiple transfusions. There are no reports of a bird clinically reacting to a single transfusion, regardless of relation to donor. However, there have been reports of deaths in birds after multiple heterologous transfusions, with histological evidence of a hemolytic reaction.[3]

Incompatibilities may be noted on crossmatch prior to transfusion, or may develop after the first or subsequent transfusions. Despite mild incompatibilities, one heterologous transfusion between conures (different *Aratinga sp.*) and two subsequent heterologous transfusions in cockatiels still did not result in identifiable clinical reactions.[9,10] However, when some species (African greys (*Psittacus erithacus*) and umbrella cockatoos (*Cacatua alba*)) were crossmatched with cockatiels, the incompatibility (agglutination) was noted to be so severe that transfusions were not performed.[9] It is also important to keep in mind that most studies are being performed in healthy animals with limited volumes. Birds with severe clinical signs that require a transfusion may not tolerate crossmatch-incompatible heterologous transfusions.[9,18]

SMALL MAMMALS

General Considerations

For hematology information in different small mammal species groups, please see Chapters 114–118. Transfusion medicine in small mammals most directly correlates with domestic species, and principles/guidelines for fresh blood transfusions in dogs/cats should be followed (see Chapter 99 for details on RBC transfusions in dogs/cats).

Donor Selection/Blood Groups/Compatibility

See Table 103.1 for recommendations for donor selection. There are no blood groups in ferrets, and repeated transfusions from the multiple donors have not led to development of anti-RBC antibodies.[20,42] There is no known information about blood groups in rabbits and other small mammals. Crossmatching should be performed for all transfusions except for a ferret's initial transfusion (crossmatching for subsequent ferret transfusions is advised).

Transfusion Reactions

Acute transfusion reactions have not been reported in small mammal species, but following the same protocols for administration in dogs/cats (slow rate to start and regular monitoring) will allow detection of adverse reactions, should they occur.

REPTILES

General Considerations

Limited research has been done to assess the applicability of blood transfusion medicine in reptiles, but the principles and techniques established in other species should apply. However, species-specific modifications due to hematological variations should be considered (see Chapters 132–134). Blood product administration has been reported with various outcomes in different reptilian species including lizards,[31] chelonians,[15,33,54,56] snakes,[31,54] and crocodilians.[19]

Anemia is caused by similar mechanisms as in other species, but anemia of chronic disease appears to be most common. The physiological PCV of most reptiles ranges between 20 and 40%.[50] Therefore, a PCV < 20% may indicate anemia.

Donor Selection/Blood Groups/Compatibility

Blood donor criteria are presented in Table 103.1. During the last decade, an increasing amount of viruses has been identified in reptiles, especially snakes.[21,30,52,53] The possible transmission of viral diseases via blood should be critically assessed and discussed with the pet owner. Due to the high number of various reptilian species kept in private and zoological collection, an ideal donor from the same species and the same household is often not available. A heterologous blood transfusion may need to be considered and has been performed successfully.[31,54,56] Similar to findings in avian species, preliminary research data support the hypothesis that reptilian post-transfusion RCS can be much shorter after a heterologous blood transfusion in comparison to a homologous blood transfusion (Stöhr, personal communication).[10] Therefore, a donor from the same or a closely related species is preferred.

Blood typing is not established in reptiles, but genomic studies indicate that a blood group system exists in reptiles.[2] Animals that receive repeated blood transfusions may be more likely to experience adverse reactions including hemolysis. However, incompatibilities in crossmatching between individuals from the same as well as between different species have been described in sea turtles,[13] and have also been observed by the authors in other reptiles that didn't have a previous history of blood transfusion. Crossmatching is recommended to assess blood compatibility, but has not been largely studied in reptiles and may be challenging due to limited volume of blood available from small donors and recipients. See section on cross-matching for a more detailed explanation.

Blood Administration in Reptiles

Evidence-based research focusing on blood administration in reptiles is lacking. Based on extrapolation from other species, anecdotal case reports, one study, personal opinions, and the authors' experience, we recommend the following protocol:[8,15,31,37,47]

1. Rehydration of the recipient with crystalloid fluids and increasing animal's temperature to their preferred optimal temperature zone.
2. Sedation (if indicated and safe for the individual).
3. Gain venous access via placement of IV or IO catheter with an aseptic technique. A butterfly catheter can also be used for temporary venous access. A surgical cutdown procedure may be indicated for IV catheter placement in hypovolemic or small animals.
4. Administration of warm blood via pediatric syringe pump, extension set, and a blood filter after negative crossmatch. The use of blood filters has historically not been recommended in reptiles due to concerns of destruction of the large reptilian RBC during the procedure.[27] However, 18 µm filters did not appear to cause hemolysis in red blood cells from American alligators (*Alligator mississippiensis*),[37] have been used by the authors in other reptile species without adverse effect, and its benefit appears to overweight the risk. Caution may be indicated in species with known large RBCs like chelonians or tuataras (*Sphenodon punctatus*).[11] Initial transfusion rate should not exceed 5 mL/kg/hour and can be increased to 10–20 mL/kg/hour if no adverse reaction is seen after 30 minutes; a bolus may be given over 3–5 minutes to reptiles in shock. The total blood volume given should not be more than 20 mL/kg, but 40 mL/kg of blood have been given to a bearded dragon (*Pogona vitticeps*) within a few minutes without adverse reaction.[31]

Transfusion Reactions

No acute transfusion reactions have been documented in reptiles. However, there was suggestion of a potential immune reaction in a diamond python (*Morelia spilota spilota*) after multiple heterologous blood transfusions.[31] Monitoring for the development of acute and delayed transfusion reactions is recommended similar to other animal species.[4] Parameters such as body temperature and blood pressure cannot be applied in reptiles, but monitoring for tachycardia, tachypnea, restlessness, and edema may be useful. Assessing blood for hemolysis may be indicated if a hemolytic reaction is suspected.

OTHER SPECIES/TRANSFUSION ALTERNATIVES

Transfusion protocols are similar between species. Approaching transfusions in unique species should be similar to methodologies listed in the earlier text, noting variability in blood volume, RBC size, and response to anticoagulants.

Hemoglobin-based oxygen carrier (HBOC) products such as Oxyglobin® have been used in many species and are a good substitute for RBCs and are beneficial as colloid support. Unfortunately, this product is no longer available. If it becomes available in the future, the reader is directed to refer to existing literature on its use.[1,24,39,56]

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CHAPTER 104

Transfusion Reactions

NICOLE M. WEINSTEIN

Immune Complications
- Hemolytic Transfusion Reactions
  - Acute Hemolytic Transfusion Reactions
  - Delayed Hemolytic Transfusion Reactions
  - Xenotransfusion
- Nonhemolytic Transfusion Reactions
  - Febrile and Allergic Reactions
  - Uncommon Immune Reactions

Nonimmune Complications
- Infectious Disease Transmission
  - Transfusion-Associated Sepsis
  - Uncommon Nonimmune Complications

Prevention and Recognition of Transfusion Reactions
Evaluation of a Patient with a Suspected Transfusion Reaction

Acronyms and Abbreviations

AHTR, acute hemolytic transfusion reaction; DEA, dog erythrocyte antigen; DHTR, delayed hemolytic transfusion reaction; FFP, fresh frozen plasma; FNHTR, febrile nonhemolytic transfusion reaction; HPA, human platelet antigen; HTR, hemolytic transfusion reaction; PCV, packed cell volume; pRBCs, packed red blood cells; PTP, post-transfusion purpura; RBC, red blood cell; TA-GVHD, transfusion-associated graft-versus-host disease; TRALI, transfusion-related acute lung injury; VetALI, veterinary acute lung injury; WB, whole blood.

Blood is a complex biologic product which, like any tissue transplant, can result in as much harm as benefit to the recipient. The term transfusion reaction denotes any adverse event associated with transfusion of blood or a blood component. The reaction may occur during or within hours to weeks after administration of a blood product. Understanding how to lessen the risks of a transfusion reaction and recognizing the potential clinical and clinicopathologic signs of an adverse reaction should one occur are key to good transfusion medicine practice.

Transfusion reactions are most often characterized by their immunologic and nonimmunologic mechanisms. There have been several reviews in the veterinary literature on blood transfusions in cats, dogs, and horses, with the incidence and type of transfusion reactions varying between institutions and species, but ranging from 3–13%.[10,13,14,28,33-35,37] The present chapter deals primarily with reactions in dogs and cats. Additional information on reactions in dogs and cats can be found in consensus statements on reaction definitions, prevention, and diagnosis and treatment.[16,17,48] Further information on transfusion reactions in large animals is given in Chapters 37, 96, and 102. Reports of adverse reactions underscore the need for further education on their prevention and identification.

IMMUNE COMPLICATIONS

Hemolytic Transfusion Reactions

Hemolytic transfusion reactions (HTRs) jeopardize the health of an already compromised patient. An immune-mediated HTR is the result of naturally occurring or induced antibodies present in the recipient plasma, which can destroy donor RBCs immediately upon transfusion or within hours to weeks following transfusion (see Chapter 96). These reactions are classified as a type 2 hypersensitivity and are mediated by antibodies

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directed against antigens present on the surface of RBCs.[1] After reacting with the RBC surface antigen, IgG or IgM antibodies activate the complement system, culminating in the formation of a membrane attack complex which disrupts the lipid bilayer of the RBC membrane, causing intravascular hemolysis.[1] Also, antibody or complement fragments, primarily C3b or C4b, can adhere to the RBC surface (opsonization), enhancing RBC susceptibility to phagocytosis by leukocytes expressing receptors for these proteins.[1] Intravascular and/or extravascular hemolysis can result, depending on whether the leukocytes recognize the opsonized RBCs while in circulation or within the reticuloendothelial system. The severity of a HTR is influenced by the concentration of recipient alloantibody, the immunoglobulin type, whether the antibody is warm- or cold-reacting, the amount of blood transfused, and the condition of the recipient.

Acute Hemolytic Transfusion Reactions

Acute hemolytic transfusion reactions (AHTRs) present within 24 hours of an RBC transfusion.[15,54] Preformed antibodies in the recipient react with transfused donor RBCs. The recipient has previously formed an alloantibody against an antigen present on the donor's RBCs, which is not present on the recipient's RBCs. Cats are similar to humans, as both have naturally occurring alloantibodies which can result in a potentially fatal AHTR. Anti-A alloantibodies in type B cats are strong hemagglutinins and hemolysins, and are present in a high concentration[11,40] (see Chapter 96). Transfusion of type A blood to a type B cat can be fatal if not immediately recognized, with subsequent discontinuation of the transfusion and institution of supportive care.[14,22,23] Within seconds to a few minutes of receiving as little as 1 mL of type A blood, a type B cat may exhibit restlessness, vocalization, vomition, urination, salivation, and recumbency.[22,23] Bradycardia, cardiac arrhythmias, decreased pulse strength, pale mucous membranes, and prolonged capillary refill time are also expected in the initial phase.[23] If the reaction is recognized and the transfusion stopped, hemoglobinemia and hemoglobinuria will follow, as these reactions are IgM- and complement-mediated with obvious intravascular hemolysis.[22,23] Acute kidney injury and failure, which are seen in humans with blood type ABO transfusion reactions, have not been documented in type B cats receiving type A blood under experimental conditions.

As type B blood is not commonly stored and type B cats comprise a small percentage of cats (outside of a few breeds), inadvertent transfusion of type B blood to a type A cat is unlikely. Inaccurate blood typing results, however, could result in a type A or AB cat being mislabeled as type B with subsequent transfusion of type B blood.[47] Although type A cats generally have lower concentration of weak anti-B antibodies which are of both IgM and IgG classes, transfusion of type B blood can still cause both clinical and clinicopathologic features of a transfusion reaction including discomfort, listlessness, tachycardia, and tachypnea, as well as mild hemoglobinemia, hemoglobinuria, and bilirubinuria.[11,23,40]

A blood type AB cat could receive either type A or B packed red blood cells (pRBCs), but plasma or whole blood (WB) from a blood type A or B cat could increase the risk of a HTR reaction due to the presence of anti-B and anti-A alloantibodies, respectively. Given the generally weaker nature of anti-B alloantibodies, plasma or WB from a type A cat would be preferred. Type AB WB or plasma would be ideal, but unrealistic in most settings given the low frequency at which type AB cats are found.

The risk of hemolytic transfusion reactions in cats can be greatly minimized by AB blood typing both the donor and recipient. Crossmatching is also advised even in transfusion-naïve cats who are otherwise AB-compatible with the intended donor. A crossmatch test (see Chapter 107) can identify preformed alloantibodies against a blood group antigen, thus preventing an AHTR. Blood types and naturally occurring alloantibodies outside of the AB blood group system can result in incompatible crossmatches, even in transfusion-naïve cats (see Chapter 96).[27,29,32,44,59,66,67] *Mik*, a non-AB blood group in cats, can result in HTRs as well as incompatible crossmatch results.[67] A cat negative for the *Mik*-RBC antigen can experience an AHTR, similar to those described in type A cats receiving type B blood, after transfusion with *Mik*-positive RBCs.[67] Given evidence of alloantibody production in as soon as 2 days post RBC transfusion, earlier crossmatch surveillance prior to additional RBC transfusions in cats is advised.[32] An incompatible crossmatch test in the face of AB blood-type compatibility supports alloantibody formation and should not be ignored.

Some dogs may have naturally occurring RBC alloantibodies (see Chapter 96), though the clinical significance is less clear.[26,55,56] Generally, HTRs require prior sensitization through a previous blood transfusion. Sensitization is unlikely to develop secondary to pregnancy in dogs.[5] A dog erythrocyte antigen (DEA)-1-negative dog who has been sensitized by transfusion of DEA-1-positive blood will develop anti-DEA 1 antibodies. A second transfusion with DEA-1-positive blood will cause an AHTR. Such a reaction has been documented clinically: a DEA-1-negative recipient had been sensitized to the DEA 1 antigen through a previous transfusion, and upon inadvertently receiving DEA-1-positive blood 3 years later exhibited a 2°C rise in temperature within 1 hour of transfusion and developed hemoglobinemia and hemoglobinuria.[24] Following an AHTR plasma will be red-tinged due to hemoglobinemia (Figure 104.1); this is most often identified when a post-transfusion packed cell volume (PCV) is measured. An AHTR is not exclusive to the DEA 1 antigen; alloantibody production and HTRs have been documented for other RBC antigens in dogs, including known DEA, as well as RBC antigens without DEA designation.[4,12,45] When a compatible donor is not easily identified on a blood crossmatch, testing both siblings of the patient or potentially dogs of the same breed has been shown to be successful in identifying a compatible donor.[4,12]

Delayed Hemolytic Transfusion Reactions

Delayed hemolytic transfusion reactions (DHTRs) are defined as those occurring more than 24 hours

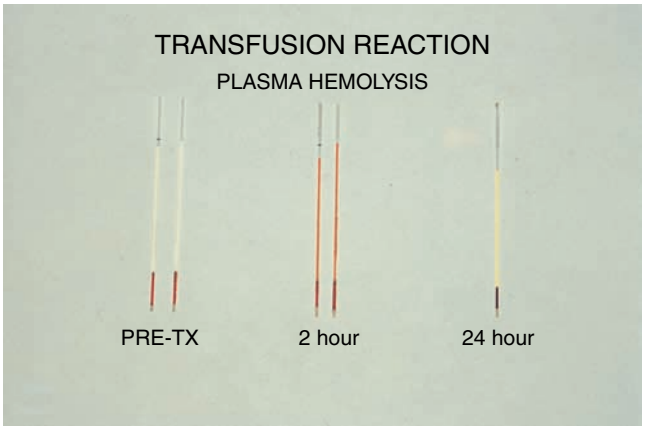


FIGURE 104.1 Serial packed cell volumes (PCVs) from a dog experiencing an acute hemolytic transfusion reaction. Note the transfusion of plasma from clear to hemolyzed to icteric over 24 hours. As a consequence of the transfusion reaction, the PCV fell following the transfusion. (**Source:** Courtesy of *Schalm's Veterinary Hematology*)

following a transfusion, but the time of onset can vary up to 48 hours post-transfusion depending on the reference.[15] DHTRs are often the result of an anamnestic response to a RBC antigen that the recipient lacks, with the previously produced reactive alloantibody weak or present in very low concentrations. Pretransfusion crossmatch testing may not identify the potential incompatibility if the test is insufficiently sensitive. Alternatively, DHTRs may be a result of primary alloimmunization to a RBC antigen, with hemolysis occurring weeks later after sufficient time for alloantibody production.[15]

A DHTR should be suspected if there is a more rapid decline than expected in PCV in the weeks following a blood transfusion, based on the patient's underlying disease. Additional clinicopathologic signs to support a diagnosis of DHTR include hyperbilirubinemia, bilirubinuria, or hemoglobinuria: fever can develop but often goes unnoticed. Confirmation of a DHTR requires documentation of development of antibodies by the recipient against the donor(s) RBCs.

Preventing HTRs requires knowledge of blood groups present within a species, presence of naturally occurring antibodies, prior transfusion history, and blood crossmatch testing. Regardless of the designation of acute versus delayed, the majority of HTRs can be prevented through proper blood typing and crossmatch testing.

Xenotransfusion

Xenotransfusion or the practice of transfusing blood of one species to another species is primarily described in a clinical setting with anemic cats receiving dog blood.[8,19,49] Although not universally accepted or recommended, xenotransfusion was utilized in these cases due to the lack of a suitable feline donor and the severity of the patient's disease necessitating RBC support (see Chapter 99). Xenotransfusion should not be considered a viable therapy for cats in need of ongoing RBC support as subsequent transfusions with canine blood will result in anaphylaxis and possible death.[8,19]

Nonhemolytic Transfusion Reactions

Febrile and Allergic Reactions

Febrile and allergic reactions are two of the most common types of transfusion reactions reported in veterinary medicine, comprising 60–90% of reported reactions.[10,29,34,35] Febrile nonhemolytic transfusion reactions (FNHTRs) are defined in human medicine as a rise in temperature of either 1.8°F and greater or 1°C or greater during or within 4 hours post-transfusion without an obvious other cause for the rise in temperature.[10,39,54] Additional clinical signs can include vomiting and tremors: the vomiting will often resolve without treatment, but a decreased transfusion rate may help to alleviate this symptom.[13,54]

Febrile nonhemolytic transfusion reactions are frequently attributed to recipient alloantibodies which react with histocompatibility leukocyte antigens or other antigens present on donor lymphocytes, granulocytes, or platelets.[6,54] Cytokines released from leukocytes and platelets within stored blood are also implicated as causes for FNHTRs and some allergic-type reactions.[39] Prestorage leukocyte reduction, as a means of minimizing cytokine production and release, has had variable demonstrated success in minimizing the most common of transfusion reactions in dogs.[7,9] In a recent study, the likelihood of FNHTRs did not decrease following transfusion of leukoreduced units to dogs.[7] The total number of transfused dogs and the total rate of reactions were low, when compared to the reported incidences of febrile and allergic reactions in the literature.[7] Although leukoreduction remains a still-examined subject in human transfusion medicine, prestorage leukoreduction has been repeatedly shown to decrease FNHTRs in people.[36,53] Additional investigations in veterinary transfusion medicine are likely needed to fully determine the potential benefit of prestorage leukoreduction on reaction rates and severities and weigh this against the added expense leukoreduction filters contribute. Admittedly, even in leukoreduced WB or pRBCs, platelet antigens would remain a potential source of reactivity, and this may be the source of the febrile reactions not otherwise controlled by leukoreduction. Age of stored RBC products does not obviously increase the risk of a febrile reaction in dogs, but transfusion-related hemolysis is more likely in transfused patients receiving older blood.[43] Given the known storage lesions RBCs can develop, both immunologic and nonimmunologic factors may be at work. Recommendations for management of FNHTRs include stopping the transfusion but maintaining the intravenous line open with saline while excluding an AHTR or transfusion-related sepsis due to bacterial contamination of the unit (Figure 104.2).

Allergic reactions are more often associated with transfusion of plasma products and are triggered by

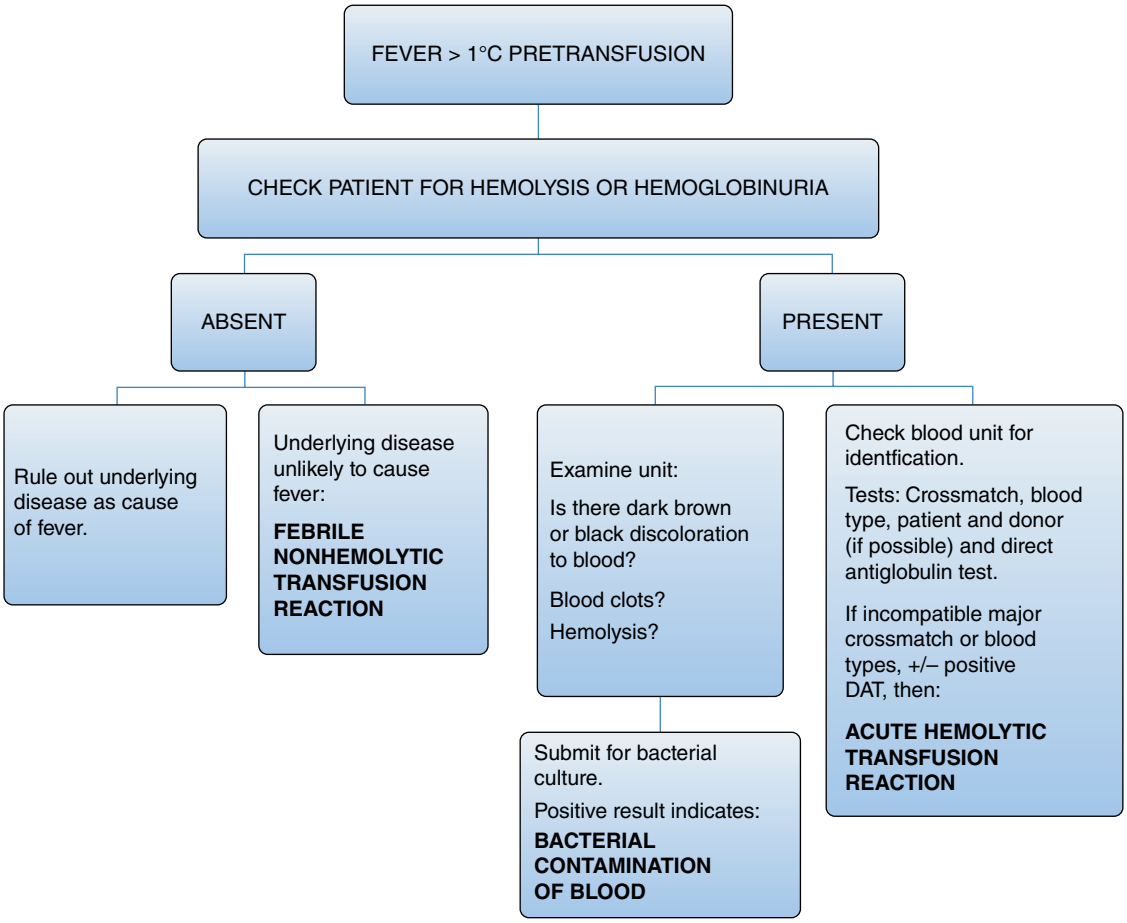


FIGURE 104.2 Algorithm for evaluation of a patient with a febrile transfusion reaction.

exposure to a substance, likely a protein, present in donor plasma to which the recipient has been sensitized.[54] Signs of an allergic reaction typically start within the first 15 minutes of a plasma transfusion, but can occur during or within a few hours of administration. Clinical signs include mild to sometimes dramatic urticaria, pruritis, and erythema (Figure 104.3). Additional signs can include vomiting, nausea, diarrhea, and/or abdominal pain.[15] Treatment of an allergic reaction to a plasma product includes stopping or, at least, decreasing the rate of transfusion, as well as administering an antihistamine such as diphenhydramine (1–2 mg/kg IM). Although administration of diphenhydramine to humans prior to leukoreduced platelet transfusions has not shown to decrease the frequency of allergic-type urticarial reactions, recent veterinary literature supported pretreatment with antihistamines decreasing the incidence of acute allergic reactions in dogs receiving blood transfusions.[10,36,63]



FIGURE 104.3 An urticarial reaction in a dog receiving plasma to treat rodenticide intoxication. Treatment with antihistamines and steroids induced resolution in 24 hours. (**Source:** Courtesy of *Schalm's Veterinary Hematology*)

Uncommon Immune Reactions

Post-transfusion purpura (PTP) is a relatively uncommon transfusion reaction in humans and has been described once in veterinary medicine.[54,65] Thrombocytopenia develops 1–2 weeks post-transfusion due to recipient-produced antiplatelet antibodies. These antibodies are most often directed against a platelet-specific antigen (e.g., in humans, human platelet antigen (HPA)-1a)

which the recipient lacks; platelets targeted for destruction are typically those of the donor, but targeting of recipient/patient platelets has also been documented.[54] PTP must be differentiated from worsening underlying disease or development of a concurrent disorder.

Anaphylaxis secondary to IgA deficiency has been reported in a single dog.[2] Anaphylaxis results from patient anti-IgA antibodies reacting to IgA antibodies in the donor plasma.[2] Clinical signs vary from facial swelling, urticaria, and erythematous skin to respiratory distress, vomiting, diarrhea, and shock.[1] An IgA-deficient patient who previously demonstrated a severe anaphylactic reaction would require future transfusions that are free of IgA.

Transfusion-related acute lung injury (TRALI) is characterized by dyspnea, hypoxia, pulmonary edema, and fluffy, bilateral infiltrates on thoracic radiographs which develop during or within 6 hours of a transfusion, most often secondary to a plasma and/or platelet transfusion. TRALI is well-documented in humans with an incidence approaching 25%.[52,61] It has recently been described as likely occurring in 2 dogs in a prospective observational study of nearly 55 transfused dogs, with the terminology veterinary acute lung injury (VetALI) used in place of TRALI.[60] Although there were several limitations to the study that the authors highlight, the study emphasizes that awareness of the syndrome, the shared features with other commonly seen complications in critically ill patients, and the related risk factors are paramount to correct identification, evaluation of prognosis, and treatment.[60] TRALI or VetALI clinically mimics acute respiratory distress syndrome, circulatory overload, other respiratory diseases, cardiac failure, and potential fever.[61] Transfusion-related acute lung injury may result from several mechanisms, including both antibody-mediated pathways and nonimmune causes, with neutrophil activation and subsequent leakage from capillaries (pulmonary microvasculature leakage) being the common final pathway; underlying inflammatory conditions can increase risk.[52,61] Treatment typically involves oxygen therapy and ventilation, when needed.[54]

Transfusion-associated graft-versus-host disease (TA-GVHD) has yet to be reported in veterinary medicine. It results from the proliferation of viable donor T lymphocytes, which go unrecognized as foreign by the recipient's immune system; these donor T lymphocytes recognize and subsequently attack the recipient (or host).[15] In humans, certain cellular immunodeficiencies predispose the transfusion recipient to this reaction; also, recipients of blood transfusions from close relatives place the recipient at greater risk for TA-GVHD.[15] Since leukoreduction is insufficient in preventing TA-GVHD, component irradiation is recommended in patient populations at increased risk of developing TA-GVHD (e.g., hematopoietic progenitor cell transplant recipients).[15] Clinical signs of TA-GVHD in man reportedly manifest within 2–50 days of transfusion and include a rash, diarrhea, liver dysfunction, and pancytopenia. Mortality is greater than 90%.[15]

NONIMMUNE COMPLICATIONS

Infectious Disease Transmission

Transmission of infectious agents to a blood transfusion recipient, though a relatively infrequent complication in both human and veterinary transfusion medicine, is not a true transfusion reaction, but is obviously an avoidable and potentially devastating complication of transfusion. Infectious diseases documented to have been transmitted via transfusion in veterinary medicine include babesiosis, leishmaniasis, bartonellosis, and diseases caused by hemic *Mycoplasma sp*. as well as viral diseases.[3,18,21,41,42,50,57,62] Although WB and pRBCs carry a greater risk of infectious disease transmission, plasma is not without risk; administration of commercially prepared plasma to horses resulted in inadvertent transmission of herpesvirus.[3] A recently updated consensus statement from the American College of Veterinary Internal Medicine includes guidelines for recommended testing for both canine and feline blood donors based on the literature and known blood-borne agents (see Chapter 98).[64] Documentation of a transfusion-acquired infection requires testing of pretransfusion recipient blood, in addition to testing the donor; unfortunately, pretransfusion recipient samples are not always available. Proper transfusion records will allow for easy identification of the suspected infected donor, removal of any remaining blood units from that donor, and appropriate treatment of the donor.

Transfusion-Associated Sepsis

Bacterial contamination of a blood unit can result in transfusion-associated sepsis. Clinical signs associated with transfusion of a contaminated blood unit include fever, vomiting, diarrhea, hypotension, and hemolysis.[15] Contamination can occur at the time of collection from inadequate preparation of the venipuncture site or contamination of materials used in collection. Once contamination of a unit occurs, bacterial numbers tend to increase with storage time, even when the unit is refrigerated, although this ultimately depends on the bacteria type(s). Storage at room temperature, which is necessary to preserve the post-transfusion survival of platelets in fresh WB and platelet-rich preparations, can also lead to bacterial proliferation in contaminated units. It has been documented that units of human pRBCs stored for longer than 21 days are more likely to contain proliferating bacteria.[25] Monitoring of refrigerated whole canine blood units at various timepoints for 42 days revealed infrequent positive culture results. On a quantitative (q)PCR assay, low amounts of bacterial DNA from at least one timepoint were identified in 53% of the units.[46] It was concluded that the risk of contamination of WB was low, and the common bacteria implicated by qPCR were unlikely to play a role in transfusion-associated sepsis.[46] Ultimately, the occurrence and severity of a reaction will depend on the bacterial species, the number of bacteria present, the blood product and related storage conditions, and the clinical condition of the recipient/patient.[30]

Reports of bacterial contamination of blood products and transfusion-associated sepsis are infrequent in veterinary medicine.[2,31,46] Contamination of feline blood units with *Serratia marcescens* was attributed to contaminated supplies used in donor collection. Vomiting was the most common clinical sign seen in cats receiving contaminated units, and four recipients died.[31] This report illustrates the importance of bacterial culture of materials other than the unit and demonstrates the steps necessary to identify the source of contamination. Contaminated WB or pRBC units are typically discolored (dark brown, purple, or black), and clots and air bubbles may also be present.[31,54] The discoloration associated with bacterial contamination indicates deoxygenation, hemolysis, and the formation of methemoglobin. Blood units with such an abnormal appearance should not be used, and an investigation to confirm bacterial contamination and identify the source of contamination should be pursued. Lastly, administration of a blood component unit should ideally take no longer than 4 hours to reduce the potential for bacterial proliferation. Once a unit is opened, even if refrigerated, it should be used within 24 hours. If bacterial contamination is suspected after the transfusion has been started, the transfusion should be stopped immediately and the unit submitted for a Gram stain and bacterial culture. Blood culture of the recipient may also be warranted.

Circulatory Overload Patients with chronic anemia and a compensatory expanded plasma volume or patients with compromised pulmonary and/or cardiac function may be at risk for circulatory overload.[54] Expected clinical signs include respiratory distress and, in some cases, congestive heart failure; signs may be seen during or soon after transfusion. Management of fluid overload with diuretics is advised. Slowing the transfusion rate and using pRBCs rather than WB in anemic patients can help to decrease the risk of circulatory overload.

Uncommon Nonimmune Complications

Citrate Toxicity Citrate is the anticoagulant used in most standard blood collection systems. Plasma and WB have the greatest concentrations of citrate when compared to pRBCs. Patients most at risk for citrate toxicity include those receiving large volumes of blood products, such as in cases of massive transfusion (see Chapter 99) and, potentially, patients with severe liver disease, since citrate is metabolized by the liver. Ionized hypocalcemia and hypomagnesemia result from chelation of these cations by citrate, and associated clinical signs include muscle tremors, vomiting, cardiac arrhythmias, ear twitching, hypotension and/or tetanic seizures.[2,34] Intravenous calcium can be administered to treat clinically significant transfusion-related hypocalcemia.

Nonimmune Hemolysis Unrelated to Bacterial Contamination Hemolysis unrelated to a blood group incompatibility or bacterial contamination may occur in vitro due to improper storage or handling of blood units, aged units, use of a hot water bath, inadvertent freezing, use of pumps not approved for administration of blood products, and concurrent administration of incompatible fluids, such as 5% dextrose or hypertonic saline through a shared intravenous line.[20,38,43,51,54,58]

PREVENTION AND RECOGNITION OF TRANSFUSION REACTIONS

Prior to blood product administration, baseline values of temperature, heart rate, and respiratory rate should be determined and pretransfusion PCV should be recorded (see Chapters 99–103). Pretransfusion WB and serum samples should be collected from the recipient, if not already submitted for other routine testing. These samples can be invaluable in cases of HTRs and in documentation of infectious disease transmission. Transfusion of any blood product is ideally started at a slower rate for the first 15 minutes while monitoring for signs of any adverse reactions. Clearly, in cases of severe, ongoing blood loss, rapid administration from the start may be necessary. Transfusion monitoring should be performed every 15 minutes for the first hour, with serial measurement of temperature, heart, and respiratory rates. If there is any concern about an AHTR, evaluation of plasma and/or urine for the presence of hemoglobin is indicated. Lack of an expected increase in PCV post-transfusion may also be suggestive of an AHTR.

EVALUATION OF A PATIENT WITH A SUSPECTED TRANSFUSION REACTION

Investigation of hemolytic and febrile reactions should include inspection of the patient's plasma for evidence of hemolysis or icterus; evaluation of the blood product unit administered (including unit labels) and of the administration set; blood culture ± cytology of the transfused product; comparison of patient bilirubin both pretransfusion and post-transfusion; retesting of patient and donor blood type, especially for reactions involving RBC-containing units; and post-transfusion crossmatch testing of recipient–donor compatibility. If a crossmatch test was performed pretransfusion, it should be repeated in the case of a HTR; if one was not performed, a crossmatch test should be done using both pretransfusion and post-transfusion plasma/serum, if available. In general, if a transfusion reaction is suspected, the transfusion should be stopped, but the intravenous line kept open with saline until the patient and blood product can be further evaluated.

Adequately screening potential blood donors, transfusing patients only when clinically indicated, selecting the most appropriate blood component, properly administering blood products, and carefully monitoring transfusion recipients will greatly diminish the risk of a transfusion reaction.

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CHAPTER 105

Cellular Therapy

STEVEN E. SUTER and STEVEN DOW

Hematopoietic Stem Cell Therapy
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Mesenchymal Stem Cell Therapy
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- Anti-Infective Therapy with MSC

Acronyms and Abbreviations

BM, bone marrow; DLA, dog leukocyte antigen; GVHD, graft-versus-host disease; GVT, graft-versus-tumor; HCT, hematopoietic cell transplantation; HDC, high-dose cytoxan; HLA, human leukocyte antigen; HPC, hematopoietic progenitor cell; IDO, indoleamine-dehydrogenase; IL, interleukin; iPSC, induced pluripotent stem cell; MHC, major histocompatibility complex; MSC, mesenchymal stem cell; PBMC, peripheral blood mononuclear cells; PGE2, prostaglandin E2; OA, osteoarthritis; rhG-CSF, recombinant human granulocyte colony-stimulating factor; TBI, total body irradiation; TBV, total blood volume; TGF-β, transforming growth factor-beta; VEGF, vascular endothelial growth factor.

HEMATOPOIETIC STEM CELL THERAPY

The hematopoietic system is an exquisitely controlled hierarchical tissue with self-renewing, multipotent stem cells residing mainly in the bone marrow that produces all the mature hematopoietic cells that circulate in the peripheral blood. Based on observations of survivors of the atomic bomb explosions toward the end of World War II, it became clear that bone marrow was the most radiosensitive tissue in the body and that marrow transplantation had the potential to rescue radiation victims from death due to complete marrow aplasia. Early unsuccessful attempts at transplantation led to the use of suitable animal preclinical models, to help elucidate the fundamental rules of marrow transplantation biology.

This chapter will briefly summarize important aspects of the preclinical murine/canine models of hematopoietic cell transplantation (HCT) and review the canine HCT literature as it relates to veterinary medicine. The chapter will also contain information on mesenchymal stem cells (MSC) derived from either bone marrow or adipose tissues, and used in either an autologous or allogeneic setting.

Preclinical Studies: Murine Models

Early research using inbred mice proved that the protection of lethally irradiated mice could be achieved by shielding either the spleen or one femur with lead during the radiation[43] or also by infusion of syngeneic spleen or bone marrow after irradiation.[9] For these

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"autologous" transplants (progenitor cells harvested from the patient), both "humoral" and "cellular" theories were advanced.[5,43] In 1956, three independent groups confirmed the cellular theory by showing repopulation through donor cells by following various blood genetic markers.[32,61,89] Subsequent studies in mice began to address important issues of marrow transplantation biology, including graft rejection, graft-versus-host disease (GVHD), and the importance of the major histocompatibility complex (MHC). Unfortunately, by 1970, a report covering 200 patients treated with HCT for hematologic malignancies, in which all recipients died of graft failure, GVHD, or disease relapse,[10] made it clear that a more suitable preclinical animal model of HCT needed to be identified.

Preclinical Studies: Canine Models

Dogs were deemed especially useful for understanding marrow transplantation biology for many reasons. They are the only mammalian species that share the following human qualities: they are random bred, have a large body size, have a long life span, exhibit wide genetic diversity, and represent a well-mixed gene pool.[63] In addition, dogs have short gestation times and large litter sizes which allowed studies of donor–recipient combinations that were matched or mismatched for dog leukocyte antigen (DLA or dog MHC antigens), which faithfully mimics human leukocyte antigen (HLA) donor–recipient pairs. Finally, the value of the canine model was heightened by early observations that dogs develop a variety of spontaneous malignant diseases, including hematologic malignancies, that had well-recognized counterparts in humans.

For these reasons, dogs have provided an enormous wealth of information relating to bone marrow biology and HCT that has been directly translated over the past six decades to the clinical setting for a wide variety of human disease. Data from dog studies of total body irradiation (TBI), irradiation and chemical myeloablation, engraftment and GVHD, progenitor cell dose requirements, and graft-versus-tumor effects, to name a few, have been essential to solve the clinical problems seen in human HCT, especially relating to "allogeneic" grafts (progenitor cells harvested from a donor).

Based on the use of random-bred dogs, mainly at the Fred Hutchinson Cancer Research Center (Seattle, WA) by a group led by Dr. Donnall E. Thomas, HCT quickly advanced from an investigational therapy in the early 1960s to a viable treatment for people that produced amazing clinical results in the 1970s.[82,85] In 1990, Dr. Thomas was awarded the Nobel Prize in Medicine for his pioneering work in marrow transplantation. An excellent overview of this work was published in 2007.[52]

Hematopoietic Progenitor Cells

Based on the early successful murine HCT experiments proving that "cellular" components were responsible for engraftment post myeloablation, it was clear that specific cells residing in the bone marrow were able to lead to complete hematologic reconstitution. However, although autologous HCTs in syngeneic mice led to successful engraftment, allogeneic HCTs between unrelated strains of mice were not successful, with most animals succumbing to overt GVHD and graft rejection. These same results were seen in early canine allogeneic HCT experiments and in humans transplanted with unconventional donors. In an effort to reduce the morbidity and mortality associated with allogeneic HCT, clinical investigators expanded their search for more refined sources of hematopoietic progenitor cells (HPC). During this time, the definition of a true HPC evolved: an inexhaustible, self-renewing cell that can give rise to the multilineage clonal expansion of all peripheral blood lineages.[86]

With the development of in vitro clonogenic assays of primitive progenitor activity, such as colony-forming units-spleen (CFU-S),[7] Dexter cultures for observing myeloerythroid differentiation,[25] Whitlock–Witte cultures for observing B-cell lineage differentiation,[96] and the thymus colony-forming cell (CFU-T) assay for testing T-lineage potential,[27] in conjunction with the development of fluorescence-activated cell sorting (FACS),[88] identification of populations of putative murine HPC became possible.

In mice, primitive HPC were found in a subpopulation of bone marrow cells expressing low levels of Thy1.1.[58] This population was further refined to include Thy1.1/Sca-1$^+$ cells, with further refinement to Thy1.1/Sca-1$^+$/Lin1^{-lo} cells. The smallest population of bone marrow cells in mice that can reconstitute lethally irradiated recipients, which represents approximately 0.05% of all bone marrow cells, includes Thy1.1/Sca-1$^+$/Lin1^{-lo}/c-kit$^+$ cells.[70]

The discovery of the surface glycophosphoprotein CD34 was the result of a strategy to develop antibodies that do not recognize mature blood or lymphoid cells, but do recognize small subsets of human and primate marrow cells.[16] CD34+ cells constitute approximately 1.5% bone marrow mononuclear cells in both species and early primate HCT experiments proved that CD34+ cells, but not CD34− cells, could reconstitute hematopoiesis in an autologous setting after myeloablative therapy.[8] CD34+ cells are phenotypically and functionally heterogeneous; therefore, other surface markers, including CD38, Thy-1, and Lin1^{-lo}, can isolate smaller, more primitive subsets of human/primate HPC. However, CD34+ cells remain the "cells of choice" for human HCT, since they are reasonably plentiful, easy to isolate, have a long history of being able to reconstitute the immune system, and can be easily cultured ex vivo. Subsequent research identified enough CD34+ cells in umbilical cord blood[48] to consider its use as a source for HCT and, importantly, identified small numbers of CD34+ cells in peripheral blood.[6,99]

Canine CD34 was cloned in 1996 and mouse anticanine CD34 monoclonal antibodies quickly became available.[56] Early studies in dogs using these antibodies essentially recapitulated human/primate findings. Canine CD34+ cells comprised 1–3% of total bone marrow cells, were 25–50-fold enriched for

granulocyte–macrophage colony-forming units when compared to unfractionated bone marrow cells, and were able to repopulate the bone marrow leading to complete hematologic recovery after myeloablative therapy.[57]

Hematopoietic Progenitor Cell Collection

The source of HPC in early human transplant studies was whole bone marrow collected via multiple bone marrow aspirations that entailed either general or spinal anesthesia, a brief hospital stay, and significant postprocedure discomfort. Whole bone marrow was also initially used in murine, primate, and canine studies. However, bone marrow harvests in people many times did not collect enough cells for HCT and repeating the procedure impacted quality of life and increased anxiety scores. Two important developments led to our ability to harvest enough CD34+ cells from the peripheral blood, instead of bone marrow, to consider HCT: (1) the cloning and large-scale production of recombinant hematopoietic cytokines, and (2) the development of sophisticated apheresis machines/systems to harvest specific populations of cells from the peripheral blood.

Dogs were instrumental in showing that both autologous[13] and allogeneic[73] HCT could be successfully performed using peripheral blood mononuclear cells (PBMCs), although approximately tenfold more PBMCs than marrow cells were necessary to ensure stable engraftment.[2] To induce expansion/peripheralization of bone marrow CD34+ cells, a variety of recombinant mobilizing agents, including canine granulocyte colony-stimulation factor (rcG-CSF),[18,67] canine stem cell factor (rcSCF),[18,67] and canine granulocyte–macrophage colony-stimulating factor (rcGM-CSF),[59] were cloned and introduced in the 1990s. More recently, the CXCR4 antagonist, AMD3100 (Mozobil, Genzyme), was shown to be an effective mobilizing agent in both humans and dogs.[12] To date, human and canine patients undergo mobilization or "G-priming" using recombinant human granulocyte colony-stimulating factor (rhG-CSF) (Neupogen, Amgen: rcG-CSF is not commercially available) or a combination of rhG-CSF and Mozobil in preparation for leukapheresis in both autologous and allogeneic settings.

The large-scale use of PBMC grafts in people could not occur until the development of techniques to collect large numbers of PBMCs and preclinical validation of PBMC grafting protocols. Although leukaphereses were reported in dogs as early as 1967,[73] a variety of harvesting techniques, including artery–jugular shunts and intermittent centrifugation techniques, finally led to the development of continuous flow techniques.[50] Many studies subsequently showed both autologous and allogeneic PBMC grafts reconstituted normal hematopoiesis in dogs after myeloablative therapy. With the introduction of recombinant hematopoietic cytokines and the commercial availability of anti-CD34 monoclonal antibodies, it became possible to isolate large numbers of CD34+ cells from the peripheral blood of dogs as the technology of apheresis advanced. In addition, with the development of highly automated microprocessor-controlled blood cell separators, the desired PBMC component could be retained with the remainder of cells returned to the circulation.[55] Today, most apheresis systems, such as the Optia (Terumo BCT, USA) and Amicus (Fresenius Kabi, USA), can isolate enough PBMCs to ensure postmyeloablation engraftment. In people, a single PBMC procedure yielded 3.7 times more CD34+ cells than a standard bone marrow harvest.[68] In addition, PBMC is generally preferred by human donors over traditional bone marrow (BM)donation.[4] Now >90% of all human autologous HCT protocols for the treatment of hematological malignancies include the use of mobilized PBMC grafts as a source of CD34+ progenitor cells because of higher cell yields, faster engraftment, decreased procedural risks, and shorter hospitalization compared to whole bone marrow grafts. In addition, the generation of canine apheresis data has made it clear that peripheral blood grafts can be harvested from both normal dogs and client-owned dogs with hematological malignancies in a clinical setting.[45,51,65,79]

DLA Typing

As stated previously, early allogeneic HCTs in people failed, with leukemia patients dying from either allograft rejection or recurrent disease. Early canine allogeneic HCT studies also failed, with most dogs dying for the same reasons. Based on these results, it was concluded that our understanding of the histocompatibility barriers between donors and recipients needed to be dramatically expanded. Dogs were the first random-bred species where the impact of donor–recipient matching on HCT outcome was demonstrated.

Beginning an intense period of experimental allogeneic HCT research, a serological canine histocompatibility typing system (DLA or dog leukocyte antigen) was described in 1968,[29] which showed that compatibility between littermate donors and recipients was essential in reducing the risks of both graft rejection and GVHD. Long-term engraftment using both related and unrelated recipients could also be achieved when matching was performed either serologically or with mixed leukocyte culture nonreactivity assays. Antigenic canine histocompatible polymorphisms, later named DLA, were later divided into class I and class II regions, whose identification could be ascertained using microsatellite polymorphisms within class I and II regions that were inherited according to Mendelian segregation (see Chapter 7).[90] With the publication and further characterization of the canine genome,[41] this research has moved rapidly forward, with DLA now a well-defined complex.

The use of the canine model of DLA-matched donor–recipient pairs and post-HCT immunosuppression directly led to physician's confidence when treating humans with aplastic anemia and hematologic malignancies with allogeneic HCTs.

Pre-HCT Conditioning

The lack of pre-HCT conditioning in early human HCTs was postulated to be the leading cause of patient death.

In an autologous setting, conditioning is used for tumor cytoreduction or, ideally, elimination, since an early study proved that TBI itself did not completely eliminate leukemia in human patients.[84] The other beneficial effect of conditioning is thought to be the creation of "space" in the bone marrow via cellular death, so donor HPCs can occupy favorable niches that enhance their ability to repopulate the bone marrow. However, since some animal studies have shown successful engraftment after autologous HCT without conditioning,[71] the question of the absolute necessity for pre-HCT conditioning in an autologous setting remains open. Regardless, early studies showed that dogs given high-dose cyclophosphamide (Cytoxan),[28] in place of lethal TBI, could be rescued from lethal immunosuppression using either autologous or DLA-matched allogeneic grafts, leading to successful engraftment and mixed donor–recipient chimerism. High-dose Cytoxan conditioning regimes were then quickly translated to human patients with aplastic anemia undergoing allogeneic HCTs. Other types of chemotherapy, including dimethyl myleran (DMM), or combinations of DMM and antithymocyte serum (ATS) or DMM, ATS, and procarbazine, were also found to be efficacious.

TBI conditioning regimes have also been extensively studied in dogs to help understand engraftment, toxicity, and the treatment of hematologic malignancies. All dogs treated with 4Gy TBI who did not receive an autologous marrow infusion died from complications of marrow failure, while those that did lived.[83] In addition, it was quickly found that both the total dose and the dose rate that TBI was administered were important parameters for acute toxicity. Finally, it was determined that the overall TBI dose tolerated by healthy dogs given autologous HCTs could be increased from 10 to 14 Gy by reducing the dose rate from 10 to 5 cGy/minute.[46] In general, acute toxicity was related to the TBI dose rate, while chronic toxicity was related to the total TBI dose.[23]

Dogs were also instrumental in determining if TBI-related toxicities in people could be decreased using fractionated TBI treatment schemes. When using total TBI doses of 12–21 Gy given in multiple fractions of 1.5–2 Gy separated by 3–6 hours (also with variable dose rates from 2 to 20 cGy/minute), acute toxicities were lower than seen in nonfractionated treatment schemes at the highest dose rate of 20 cGy/minute.[20]

Pre-HCT conditioning eliminates most, but not all, precursors and mature cells of the recipient immune system. In the allogeneic HCT setting, the immune system in the recipient is reconstituted by the graft and originates from the donor. Therefore, the primary clinical concerns in allogeneic HCTs are not only to prevent graft rejection by recipient cells that survive the conditioning regime, but also to prevent donor cells from causing severe immune-mediated injury, GVHD, in the recipient, while allowing immunologic reconstitution for control of pathogens. Therefore, pre-HCT conditioning in this setting is used to possibly "make space" as described previously, and also to allow the development of some non-life-threatening level of GVHD, since the resultant graft-versus-tumor (GVT) phenomenon is more effective than chemical or irradiation conditioning regimes at tumor cell killing.[94] The cause of acute and/or chronic GVHD, sensitization of donor T cells for disparate major and minor histocompatibility antigens of the recipients, was first described in dogs.[72] Initial studies showed that engraftment of DLA-identical marrow was consistently achieved with a single dose of 4.5 Gy TBI delivered at a dose rate of 70 cGy/minute or a single dose of 9 cGy delivered at a dose rate of 7 cGy/minute.[75] When administering DLA-nonidentical grafts, only higher TBI doses of 15–18 Gy, given in fractionated dosing schemes to prevent acute toxicities, led to sustained engraftment.[21] Importantly, during these studies it was found that DLA-incompatible donor buffy coat cells or lymphocytes could lead to sustained engraftment,[22] which suggested that peripheral blood mononuclear cell grafts may be an alternative to bone marrow grafts.

Further studies using DLA-identical allogeneic grafts in conjunction with 8 Gy TBI in dogs with lymphoma and leukemia proved the graft-versus-lymphoma effect by demonstrating prolonged disease-free survivals ($p < 0.005$) when compared to autologous grafts using the same conditioning regimes.[94]

This large body of canine pre-HCT conditioning research has translated into the routine use of high-dose cyclophosphamide/TBI and other chemotherapy-only protocols for treatment in people.

Graft-versus-Host Disease Prevention

The canine preclinical model of GVHD has produced extremely valuable strategies for GVHD prevention and treatment in people. In humans and dogs, GVHD prevention was initially difficult, in spite of the use of MHC-matched donor–recipient pairs. As mentioned previously, GVHD was first recognized in dogs and the cause, donor-activated T cells attacking host tissues, including gut, liver, skin, and the hematopoietic system, was elucidated. In unrelated DLA-nonidentical dogs, acute and rapidly fatal GVHD occurred within 2 weeks of HCT.[74] This could be delayed, with a prolongation of survival, using a variety of postgrafting immunosuppressive drugs, including methotrexate, cyclosporine, azathioprine, succinylacetone, tacrolimus, or a combination of these.[55] Based on these studies, post-HCT immunosuppressive regimes were successfully translated to human GVHD prophylaxis and to treat acute GVHD once it was established. It was also proven in dogs that postgrafting immunosuppression could be discontinued approximately 3–6 months after HCT. In this allogeneic model, the therapeutic benefit of GVT prompted the exploration of less-aggressive (thus, less-toxic) nonmyeloablative HCT protocols, such as 2 Gy TBI combined with post-HCT immunosuppression with mycophenolate mofetil and cyclosporine.[76] This "minitransplant" protocol was successfully translated to people who were not eligible for conventional myeloablative HCT.

Overall, based almost entirely on this very large body of canine preclinical research, human HCT has become routine in physician-based medicine. However, the awareness of HCT among veterinarians and the

potential beneficial impact of HCT in veterinary medicine, while growing, have been slower to expand.

HCT to Treat Nonhematologic Diseases in Veterinary Medicine

In a research setting, a positive response to HCT has been noted when treating a wide variety of inherited canine and feline monogenetic, recessive genetic diseases including X-linked severe combined immunodeficiency (X-linked SCID),[31,40] mucopolysaccharidoses I, VI, VII (MPS),[11,35,66] α-mannosidosis,[91] α-fucosidosis,[81] pyruvate kinase deficiency,[101] canine leukocyte adhesion deficiency (CLAD),[69] and cyclic neutropenia (Gray Collie syndrome),[100] although this is not a complete list. As such, these spontaneous diseases have provided extremely valuable genetic disease HCT models whose findings can been translated to the treatment of similar human diseases. Importantly, these models provide a necessary intermediate to optimize gene correction protocols (i.e., gene therapy) and assess the safety and efficacy of treatment before initiating human protocols.

HCT to Treat Canine Hematologic Malignancies–Autologous HCT

Attempts to cure client-owned dogs with leukemia and lymphoma, who were referred by local veterinarians, with both autologous and allogeneic HCT were pioneered by Thomas's group in Seattle. As early as 1979, it became clear that dogs with lymphoma who received a combination of chemotherapy-induced remissions and TBI could be long-term survivors after autologous HCT.[93] In this study, 4/17 dogs (23.5%) survived 1–2 years post HCT with no further therapy. Soon thereafter in 1986, 3/12 (25%) dogs with lymphoma became long-term disease-free survivors (334, 631, and 727 days) after chemotherapy-induced remission and TBI when given autologous peripheral blood mononuclear cells grafts collected before TBI.[1] Although these very early results were encouraging, further proof that client-owned dogs with hematologic malignancies could be successfully treated in a clinical setting with HCT was slow to develop.

Currently, autologous (and allogeneic) HCT is offered by four facilities (three private practices and one university hospital) in the United States to treat dogs with hematologic malignancies. The reader is referred to published studies for protocols and results of these HCT treatments.[30,92,98]

HCT to Treat Hematologic Malignancies–Allogeneic HCT

In 2006, a dog with T-cell lymphoma underwent an allogeneic HCT at a private veterinary clinic using a DLA-matched PBMC graft harvested using a COBE Spectra apheresis system.[53] A second case report,[77] in 2015, describes an allogeneic HCT used to treat a dog with a rare acute large granular lymphocytic leukemia of splenic origin.[87] Although a partial retraction relating to the diagnosis was submitted by the authors, the conclusion that allogeneic HCT may be a feasible treatment in dogs remained unchanged.[78]

To date, allogeneic HCTs have been performed by the aforementioned facilities to treat 24 dogs with hematologic and nonhematologic malignancies (Suter, personal communication). And as apheresis systems become more available to veterinarians and the literature supporting the benefits of autologous and allogeneic HCT over conventional chemotherapy expands, a greater number of dogs will be cured of what were once considered incurable hematologic malignancies.

MESENCHYMAL STEM CELL THERAPY

Mesenchymal stem cells as they are currently defined are multipotent stromal cells that can differentiate primarily into osteoblasts, chondrocytes, and adipocytes. In this section, we will confine our discussion to mesenchymal stem/stromal cells derived from either bone marrow or adipose tissues, and used in either an autologous or allogeneic setting. Nearly all stem cell studies currently described in veterinary medicine involve the use of allogeneic or autologous MSC. A number of recent reviews have been published describing the use of MSC to treat companion animals and horses.[17,24,33,38,42,54,97] True tissue-culture-derived MSC have a number of important properties, particularly immune modulatory properties, which make them attractive for treatment of conditions associated with pathologic inflammation, including immune-mediated diseases, osteoarthritis (OA), and bacterial infections.[14,44]

The other major categories of "stem cell therapy" in veterinary medicine, which will not be discussed further here, include stromal vascular fraction cells, which actually consist of a poorly defined mix of cells and debris isolated from enzymatic digestion of adipose tissues, as well as minimally manipulated bone marrow aspirate samples, and platelet-enriched cell products obtained from blood.

Biology of Mesenchymal Stem Cells

Mesenchymal stem cells derived from bone marrow or adipose or other stromal tissues should more appropriately be termed mesenchymal stromal cells, since the true stem cell properties of MSC are relatively limited.[33,38] These cells can be readily cultured as adherent, proliferating cells from bone marrow or enzyme-digested adipose tissues collected from young or old animals. Their proliferative capacity is relatively limited and after typically 10 or fewer passages MSC proliferation greatly slows and the cells become terminally differentiated as stromal cells. Thus, MSC are considered multipotent but not pluripotent, and can generally only generate new osteoblasts, adipocytes, and chondrocytes in vitro, though there are reports of generation of other cell types under very specialized cell culture conditions. Following in vivo injection, MSC rarely generate any new tissues, and typically persist for only several weeks at sites of injection before undergoing either apoptosis or immune destruction.

So why is MSC treatment being considered for so many different disease states? The common denominator appears to involve a reduction in inflammation achieved with this treatment.[26,36,47,49] Numerous studies have established that MSC are inherently immunomodulatory, via a variety of mechanisms. In culture, MSC spontaneously secrete immune modulatory factors, which vary from species to species. In mice, the major immune suppressive factors include reactive nitrogen intermediates, transforming growth factor-beta (TGF-β) and interleukin (IL)-10, galectin, and prostaglandin E2 (PGE2), while in humans the major factors include indoleamine-dehydrogenase (IDO), TGF-β, soluble IL-1R, vascular endothelial growth factor (VEGF), and PGE2.[49] Studies in dogs have identified TGF-b, PGE, and adenosine as major immune suppressive factors, while studies in cats also identify PGE and TGF-b as immune suppressive factors.[3,14,95] While MSC spontaneously secrete immune modulatory factors in vitro, their immune modulatory function can be further modulated and enhanced in vivo, particularly by inflammatory cytokines such as IFN-g and TNF-a, in a process referred to as MSC "licensing."[47] Thus, MSC respond to their inflammatory environment by modulating the local immune responses.

Use of MSC for Treatment of Orthopedic Conditions

The majority of studies using MSC in companion animals and horses have been for treatment of orthopedic disease, typically OA in dogs and tendon and cartilage injuries in horses.[19,33,34,37,39] In the case of tendon injuries, MSC are purported to stimulate healing by differentiating into tenocytes, though direct evidence for this is lacking in equine studies. For treatment of cartilage injuries, the primary goal is replacement of missing or dysfunctional chondrocytes with MSC-derived chondrocytes. Again, with the possible exception of rodent studies and a few studies in goat and rabbit models, evidence for direct cartilage replacement is scant. Beneficial effects in dogs with OA likewise are unlikely to result from replacement of damaged or missing cartilage. Instead, the primary effects of MSC injection for orthopedic injuries appear to be stimulation of endogenous healing processes (e.g., recruitment of host endogenous stem cells, stimulation of angiogenesis), along with suppression of local inflammatory processes, which can be quite profound. Thus, cellular therapy with MSC is best considered as a biological approach to alter the local injury environment to improve healing and reduce inflammation.

Immune Modulation with MSC

Perhaps the most pronounced activity of MSC in vivo is to reduce or modulate inflammatory processes, which is why MSC cellular therapy has increasingly been viewed as a new means of modulating intractable inflammatory diseases. For example, in animal models and in clinical trials in humans, autologous or allogeneic MSC have been evaluated following systemic administration for treatment of severe and unrelenting inflammatory diseases including graft-versus-host disease, inflammatory bowel disease, multiple sclerosis, rheumatoid arthritis, and scleroderma.[26,47]

In veterinary medicine, use of MSC has been evaluated for treatment of asthma and gingivostomatitis in cats, inflammatory bowel disease in dogs and cats, and anal fistulas in dogs.[3,39,42,62,64] Other studies have investigated the use of MSC to treat spinal cord injury, chronic keratitis sicca, and other degenerative or inflammatory conditions.[38,97,17] All of these diseases would be expected to respond to a treatment that suppresses inflammation and stimulates healing processes, regardless of whether there was any true replacement of lost cells or tissues. Dramatic responses have been observed in animals with chronic immune-mediated diseases that have failed to respond to months of conventional immune suppressive therapy (Dow, S., unpublished data).

Anti-Infective Therapy with MSC

An additional and until recently unappreciated property of MSC is their antimicrobial activity. For example, in vitro studies have shown that MSC from multiple different species, including dogs and horses, can exert direct bactericidal activity, mediated by secreted antimicrobial peptides.[44] Moreover, in vivo MSC have been shown to interact with the host innate immune cells to increase antibacterial activity, including stimulation of neutrophil phagocytosis and neutrophil extracellular trap (NET) formation, as well as enhancement of monocyte recruitment.[44,80] A number of studies in acute infection models have demonstrated the ability of MSC to help eradicate infection.[60]

More importantly, MSC can be used effectively to manage chronic bacterial infections, including infections associated with biofilm formation and with drug-resistant bacteria.[44] In these studies, MSC administered IV accumulate at the site of infection, where they significantly reduce the bacterial burden and stimulate wound healing. In a clinical trial in dogs with a variety of different chronic, drug-resistant bacterial infections, infusion of activated MSC several times was an effective means to resolve previously refractory infections.[44] Importantly, the treatments were done using allogeneic MSC, which were administered safely for multiple IV treatments without eliciting immune rejection reactions. This new application of cellular therapy offers considerable promise as an adjunctive therapy to prolonged antibiotic administration for management of chronic infections.

Cellular therapy using MSC is increasingly viewed as an effective tool for managing certain diseases that are difficult to manage with conventional therapy. Future developments may include the use of single sources for cell production and expansion in vitro (e.g., induced pluripotent stem cell (iPSC)-derived MSC), as well as more standardized methods of cell expansion and preparation for injection or infusion.[15]

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CHAPTER 106

Clinical Use of Hematopoietic Growth Factors

STEVEN E. SUTER

Erythropoietin (EPO)
  Background
  Use in Veterinary Medicine
Granulocyte Colony-Stimulating Factor (G-CSF)
  Background
  Use in Veterinary Medicine
Granulocyte–Macrophage Colony-Stimulating Factor (GM-CSF)
  Background
  Use in Veterinary Medicine
Thrombopoietin (TPO)
  Background
  Use in Veterinary Medicine
Interleukin-3
  Background
  Use in Veterinary Medicine
Stem Cell Factor
  Background
  Use in Veterinary Medicine
Conclusions

Acronyms and Abbreviations

AIDS, acquired immune deficiency syndrome; BMT, bone marrow transplantation; CKD, chronic kidney disease; CRF, chronic renal failure; FDA, Food and Drug Administration; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; HGFs, hematopoietic growth factors; IL, interleukin; M-CSF, macrophage colony-stimulating factor; PBPC, peripheral blood progenitor cell; rhEPO, recombinant human erythropoietin; SC, subcutaneously; SCF, stem cell factor; TBI, total body irradiation; TNF, tumor necrosis factor; TPO, thrombopoietin.

The hematopoietic system is an exquisitely controlled hierarchical tissue, with multipotent stem cells residing mainly in the bone marrow eventually becoming nonreplicative mature hematopoietic cells that circulate in the peripheral blood. The endogenous elaboration of hematopoietic growth factors (HGFs), stimulatory glycoproteins that affect the growth and differentiation of bone-marrow-derived cells from these stem cells, drives this process. Some of these HGFs exert their effects on multiple cell lineages, while others are cell-lineage-specific. The availability of these HGFs has become invaluable in human medicine, with marked clinical advancements in the setting of bone marrow transplantation (BMT) and chemotherapy-induced myelosuppression, as well as a variety of other disorders.

Several canine and feline HGFs have been cloned, although none are commercially available for clinical applications. The use of recombinant human HGFs is relatively commonplace in veterinary medicine, but still considered strictly an extra label application. The following brief review will discuss the most clinically relevant HGFs commonly used in veterinary medicine.

ERYTHROPOIETIN (EPO)

Background

EPO, a RBC lineage-specific hormone, is produced mainly by juxtatubular interstitial cells of the renal cortex, with a small amount being produced by the liver. EPO cooperates with various other HGFs (interleukin-3 [IL-3], stem cell factor [SCF], thrombopoietin [TPO]) in the development of colony-forming unit-erythroid cells in the bone marrow from a common myeloid precursor. EPO promotes erythroid progenitor cell survival through apoptosis evasion via upregulation of the antiapoptotic protein Bcl-xL[19] and by reducing the level of

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cell-cycle inhibitors. The end result is increased production of proerythroblasts and release of more RBCs into the circulation. It is now recognized that hypoxia, the most potent stimulus of EPO production, increases the activity of the primary EPO transcription factor, hypoxia-inducible factor-1α (HIF-1α).[45]

In 1989, EPO became the first HGF to become commercially available for clinical use in the United States. Recombinant human erythropoietin (rhEPO, epoetin alfa) (Epogen®, Amgen, Thousand Oaks, CA; Procrit®, Ortho Biotech, Bridgewater, NJ) is approved for the treatment of anemia associated with chronic renal failure (CRF), with zidovudine (Retrovir) therapy in patients infected with human immunodeficiency virus, and with chemotherapy for nonmyeloid malignancies, as well as for anemia in patients scheduled for elective, noncardiac, nonvascular surgery. In 2001, darbepoetin alfa (Aranesp®, Amgen), a hyperglycosylated molecule based on the protein backbone structure of human EPO, was approved by the Food and Drug Administration (FDA) to allow a new option for treatment of anemia of CRF. The increased level of glycosylation allows a longer duration (up to three times longer than EPO) of the drug in the circulation.[13] Subsequently, darbepoetin alfa was approved for the treatment of chemotherapy-induced anemia in patients with nonmyeloid malignancies.

Use in Veterinary Medicine

Similar to the human experience, EPO most likely plays an important role in the pathogenesis of anemia in companion animals. The suppressed rate of RBC formation caused by lack of renal production of EPO is a major problem in the treatment of patients with CRF.[36,41]

Fortuitously, the structure of EPO is well-conserved across species lines. When given to dogs and cats with CRF, rhEPO causes a dose-dependent increase in the hematocrit (Hct) and corrects anemia.[8] A transient, moderate reticulocytosis is initially seen and the bone marrow myeloid:erythroid ratio decreases, illustrating the increased erythropoietic response. Depending on the dose given and the initial Hct, correction of the Hct to low normal takes from 2 to 8 weeks. When RBC production is noted, it usually results in a 1% increase per day in the dog's or cat's Hct.[8]

Dosing schedules for EPO have not been standardized for veterinary patients. Both intravenous and subcutaneous routes of administration of rhEPO appear effective, although plasma concentrations persist longer after subcutaneous administration, which allows lower total doses to be given. Starting doses of 50–150 U/kg given subcutaneously three times per week are currently recommended for dogs (100 U/kg in cats) with weekly monitoring continued until a target Hct is achieved (30–40% in dogs and 25–30% in cats).[3,18,30,55] Thereafter, the dosing interval should be decreased to twice weekly. To maintain RBC counts and Hcts in the target range, most animals require 50–100 U/kg one to two times weekly, although the dosage required varies significantly among treated individuals. Therefore, periodic monitoring of the Hct is required for proper adjustment of the dose or dosing interval or both. Pharmacological activity in dogs has also been demonstrated using a mucoadhesive tablet containing 100 U/kg rhEPO.[56] Supplementation with oral ferrous sulfate (100–300 mg/day for dogs and 50–100 mg/day for cats) is highly recommended for all patients treated with rhEPO due to the high demand for iron associated with stimulated erythropoiesis, even if the pretreatment transferrin saturation is normal.[24]

Reported adverse reactions to EPO include transient cutaneous or mucocutaneous reactions with or without fever (rare and self-limiting upon cessation of rhEPO), hypertension, seizures, polycythemia, vomiting, discomfort at the site of injection, and, most significant clinically, the development of neutralizing antibodies and RBC aplasia. Approximately 20–30% of rhEPO-treated animals develop neutralizing antibodies against the foreign protein, leading to RBC aplasia.[8,18] Although anti-rhEPO antibody titers may decline after discontinuation of treatment and pretreatment levels of erythropoiesis can be attained, the severe anemia seen before treatment remains, since these levels are not sufficient to maintain adequate RBC numbers. Due to the possible long-term prevalence of anti-rhEPO antibodies in treated dogs and cats, administration of rhEPO is generally not recommended until the anemia of CRF is negatively impacting the animal's quality of life.

The clinical safety and efficacy of recombinant canine EPO (rcEPO) were investigated in dogs with CRF and rhEPO-induced RBC aplasia.[42,44] rcEPO stimulated RBC production in dogs with nonregenerative anemia secondary to CRF without causing the profound erythroid hypoplasia that can occur in rhEPO-treated dogs, although rcEPO was not as effective in restoring RBC production in dogs that had previously developed rhEPO-induced RBC aplasia. More recently, darbepoetin alfa, given weekly at a median starting dose of 0.5 μg/kg subcutaneously (SC), was shown to be an effective treatment for anemia secondary to chronic kidney disease (CKD) in dogs, although adverse events, including high blood pressure, seizures, vomiting, diarrhea, and pure red cell aplasia, were noted.[15]

Recombinant feline EPO (rfEPO) has also been produced in Chinese hamster ovary cells.[1,43] The biological activity of rfEPO was broadly equivalent to rhEPO with the median Hct and absolute reticulocyte count in cats with CRF treated with rfEPO increasing significantly during the first 3 weeks of treatment. Unfortunately, development of RBC aplasia during treatment with rfEPO was also seen, most likely due to the development of antibodies against a Chinese hamster ovary antigen that cross-reacts with feline EPO. Similar to dogs, darbepoetin alfa appears to have activity in approximately 50% of cats that receive a dosage of 1 μg/kg/week or higher.[6] Importantly, a common side-effect of epoetin administration in cats, pure red cell aplasia, appears to be less common with darbepoetin.

GRANULOCYTE COLONY-STIMULATING FACTOR (G-CSF)

Background

G-CSF, also called colony-stimulating factor 3 (CSF 3), is a 19.6-kDa glycoprotein that exists in two forms, 174 and 177 amino acid long molecules. The 174 amino form is the most abundant and the most active. G-CSF is produced by bone marrow stromal cells, monocytes/macrophages, and endothelial cells. Granulocyte colony-stimulating factor binds to primarily granulocyte progenitor cells in the bone marrow, which stimulates the proliferation and maturation of neutrophil precursors. At higher doses, G-CSF can act in a multilineage fashion, stimulating the differentiation of several other hematopoietic progenitors. Granulocyte colony-stimulating factor also promotes neutrophil migration across the vascular endothelium and primes neutrophils for cell killing. FDA-approved, commercially available human G-CSFs include: lenograstim (Granocyte, Chugai Pharma, USA), filgrastim (Neupogen®, Amgen), and PEGylated filgrastim (Neulasta®, Amgen).

Human G-CSF was cloned in 1986 and approved by the FDA in 1991 to protect cancer chemotherapy patients. In humans, G-CSF administration causes a dose-dependent rise in neutrophils, as well as a moderate increase in monocytes. G-CSF is now routinely given to human patients undergoing chemotherapy to help prevent episodes of severe neutropenia; G-CSF may also be beneficial in the treatment of established afebrile or febrile neutropenia by reducing the length, and thus expense, of hospitalization stays.[7]

Granulocyte colony-stimulating factor has also been used in humans for the treatment of cyclic neutropenia, an inherited disease characterized by recurrent episodes of neutropenia occurring every 20–24 days. Following rhG-CSF administration the mean neutrophil counts increase, and the severity of neutropenia associated with the cycles decreases. Granulocyte colony-stimulating factor does not completely eliminate the neutrophil cycling or the clinical signs associated with disease, suggesting a defect in receptor signaling, rather than a paucity of G-CSF production.

Another important setting in human medicine where G-CSF is routinely used is in association with BMT. The administration of G-CSF after high-dose chemotherapy and autologous BMT improves the rate of peripheral blood neutrophil recovery, reducing the morbidity and mortality associated with febrile neutropenic episodes. Granulocyte colony-stimulating factor also mobilizes hematopoietic CD34+ progenitors for collection prior to myeloablative therapy. After treatment with G-CSF for 5 days, high concentrations of CD34+ progenitor cells are found in the peripheral blood, which can be collected by leukapheresis and administered to a patient after high-dose chemotherapy or total body irradiation (TBI) in place of marrow. "G-priming" of donors is now standard practice in human transplant protocols. Other uses of G-CSF include treating patients with myelodysplasia and acquired immune deficiency syndrome (AIDS)-associated bone marrow aplasia.

Use in Veterinary Medicine

Canine G-CSF (rcG-CSF) has been cloned[57] and produced synthetically for research purposes, but is not commercially available. rcG-CSF transiently increases neutrophil and macrophage numbers in dogs and cats. At a dose of 5 μg/kg/day, mean neutrophil counts in healthy dogs increased to 26,330/μL with 24 hours of the first injection of rcG-CSF and reached a maximum of 72,125/μL by day 19.[38] A similar effect on neutrophil counts was noted in cats following administration of rcG-CSF at a dose of 5 μg/kg/day.[39]

Mirroring the human experience, rcG-CSF is able to reduce the duration and severity of chemotherapy-induced myelosuppression.[40,58] In both studies, the administration of serial daily doses of rcG-CSF beginning 1–5 days after chemotherapy infusion accelerated neutrophil recovery and also decreased the severity of neutropenia. Both studies suggest that rcG-CSF can increase the feasibility of using high-dose chemotherapy in dogs by mitigating the toxic effects of these drugs on bone marrow hematopoietic precursors.

Cyclic hematopoiesis has also been identified in Gray Collie dogs.[59] Dogs with this disease respond similarly to humans given rhG-CSF, although the development of neutralizing antibodies and subsequent prolonged neutropenia with chronic rhG-CSF usage occurs.[26] This problem was negated by giving rcG-CSF at a dose of 1–2.5 μg/kg SC every 12 hours.[33]

The use of G-CSF in the setting of TBI and BMT has also been reported in the veterinary literature. Granulocyte colony-stimulating factor leads to complete bone marrow reconstitution within 21 days after lethal TBI[49,50] and can also mobilize bone marrow CD34+ progenitor cells to the peripheral blood for peripheral blood stem cell transplantation.[11] Granulocyte colony-stimulating factor can also "prime" dogs before marrow harvesting in the setting of malignant lymphoma. rcG-CSF increased donor peripheral blood mononuclear cell numbers (containing CD34+ progenitor cells) for harvesting via leukapheresis with subsequent allogeneic BMT to an unrelated dog with stage V(b) T-cell multicentric lymphoma who received 8 Gy TBI.[29] The transplanted dog maintained a complete remission for at least 58 weeks after BMT. rcG-CSF has also been used to successfully "prime" dogs before bone marrow harvesting via bone marrow aspiration.[16] The reader is referred to a comprehensive review of the use of hematopoietic cytokines, including G-CSF, in the BMT setting,[28] as well as an original study describing the use of leukapheresis, both with and without rcG-CSF "priming," to harvest peripheral blood progenitor cells (PBPCs) from dogs.[27]

GRANULOCYTE–MACROPHAGE COLONY-STIMULATING FACTOR (GM-CSF)

Background

GM-CSF, also called colony-stimulating factor 2 (CSF 2), was first cloned in 1985. It is a variably glycosylated

(10–30 kDa) monomeric glycoprotein produced by fibroblasts, endothelial cells, monocytes, and T lymphocytes that has a variety of biological activities. These include prolonging the survival of hematopoietic progenitors and mature neutrophils, eosinophils, and macrophages, stimulating the proliferation of progenitor cells, and enhancing the functional capacity of the mature cells.

The FDA-approved pharmaceutical analogs of naturally occurring rhGM-CSF (sargramostim) are Leukine® (Partner Therapeutics, USA) and Prokine (Amgen). Both are approved for use in human patients more than 55 years of age with acute myelogenous leukemia following induction chemotherapy. Leukine is also approved for use in a number of hematopoietic stem cell transplant settings, including BMT failure or engraftment delay, myeloid reconstitution after autologous and allogeneic BMT, and mobilization and following transplantation of autologous PBPCs. The reported side-effects of rhGM-CSF in human patients include bone pain, fever, and flu-like symptoms.

Use in Veterinary Medicine

Recombinant canine GM-CSF (rcGM-CSF) has been cloned,[35] although it is not commercially available for clinical use. rcGM-CSF given to normal dogs caused a significant increase in circulating neutrophils and monocytes, whereas the effect on circulating eosinophils was more variable.[36] rhGM-CSF is active in dogs[32], inducing neutrophilia and eosinophilia, although it produced variable results in dogs with chemotherapy-induced myelosuppression.[50] This is most likely because rcGM-CSF is only 70% homologous with the human protein; therefore, higher doses of rhGM-CSF may be necessary to achieve the same effect as rcGM-CSF. rhGM-CSF also prevents recurrent neutropenia in Gray Collie dogs with cyclic hematopoiesis, although most dogs developed antibodies to the protein within 2–3 weeks of treatment and hematopoietic cycling persisted.[20]

Importantly, GM-CSF also has the ability to activate monocytes and macrophages, increasing their ability to recognize and destroy malignant cells.[17] DNA plasmid vectors containing the coding sequence for GM-CSF can deliver GM-CSF directly to tumors, which theoretically allows the local recruitment and activation of macrophages. rhGM-CSF cDNA was cloned into a xenogeneic expression vector and injected intramuscularly biweekly for a total of four vaccinations into three dogs diagnosed with oral malignant melanoma. In this same study, nine dogs were injected with both rhGM-CSF and human Tyrosinase.[2] The authors reported a median survival time of 148 days for dogs receiving rhGM-CSF alone and >402 days for dogs receiving both rhGM-CSF and human Tyrosinase. Another antimalignancy strategy is to remove the primary tumor or metastatic lesion, culture the tumor cells in vitro, and transfect them with a eukaryotic vector encoding GM-CSF or use GM-CSF-transfected cell lines established from primary tumors.[13,21] There are currently a wide variety of human clinical trials examining the safety and efficacy of both viral and nonviral vectors encoding GM-CSF for the treatment of many types of solid and hematopoietic malignancies.

THROMBOPOIETIN (TPO)

Background

TPO, a 35-kDa glycoprotein produced primarily in the liver by parenchymal cells and sinusoidal endothelial cells and in the kidney by proximal convoluted tubule cells, is an important regulator of thrombopoiesis via its role as a late-acting megakaryocyte maturation factor. It also stimulates CD34+ hematopoietic progenitor cells to undergo proliferation and differentiation into megakaryocytic colonies and synergizes with SCF and IL-3 to stimulate hematopoietic progenitor cell division and differentiation. The receptor for TPO is encoded by the proto-oncogene c-mpl. Eltrombopag (Promacta®, Novartis, USA or Revolade, GlaxoSmithKline, EU) is a small molecule agonist of the c-mpl (TPOR) receptor. Eltrombopag is FDA-approved to treat idiopathic thrombocytopenic purpura and cirrhosis due to hepatitis C in people. Neumega® (oprelvekin, Wyeth, USA) is a man-made, synthetic 19-kDa version of human IL-11 with FDA approval for use to prevent severe thrombocytopenia and reduce the need for platelet transfusions following myelosuppressive chemotherapy. Nplate® (romiplostim, a thrombopoietin analog, Amgen) is a commercially available peptibody protein with FDA approval for the treatment of thrombocytopenia in adults with chronic immune thrombocytopenia purpura.

In the setting of BMT, TPO can stimulate enhanced platelet recovery and improve the recovery of other hematopoietic lineages after BMT and act synergistically with G-CSF to accelerate neutrophil recovery in mice after radiation or chemotherapy. The reader is referred to two review articles for a more thorough discussion of TPO and the development of TPO mimetics.[54,5]

Use in Veterinary Medicine

Nplate (romiplostim) administration to five dogs with refractory immune thrombocytopenia (ITP, three primary and two secondary) increased platelet counts in all five dogs.[23] Four of these dogs attained remissions with no relapses occurring over a 3–10-month follow-up period. Feline, but not canine, TPO has been cloned.[31] However, there are no reports in the veterinary literature describing the clinical use of rfTPO, Neumega, or eltrombopag in companion animals.

INTERLEUKIN-3

Background

Interleukin-3 is a T-cell-derived, pluripotent 25-kDa glycoprotein that is required for the survival and proliferation of CD34+ hematopoietic progenitor cells. Interleukin-3

promotes the survival and expansion of these cells and also, to a lesser extent, produces the same effects on committed progenitors of the granulocyte–macrophage, erythroid, eosinophil, basophil, megakaryocyte, and mast cell lineages.[10] IL-3 also enhances myeloid cell functions. Clinically, IL-3 leads to multilineage expansion of myeloid cells, including platelets, neutrophils, and monocytes, when used alone or in combination with rhGM-CSF. Therefore, IL-3 may be useful to treat chemotherapy-induced myelosuppression, although signs of toxicity, including fever, fatigue, and headaches are common. The reader is referred to these review articles describing the role of IL-3 in hematopoietic cell development.[8,34]

Use in Veterinary Medicine

Canine IL-3, which was cloned in 2001,[22] has approximately 37% amino acid homology to the human protein. This may explain the lack of efficacy of the human protein (rhIL-3) in increasing neutrophil or macrophage numbers when administered to dogs. rhIL-3 failed to correct recurrent neutropenia in Gray Collie dogs with cyclic hematopoiesis, although it did stimulate eosinophilia.[20]

STEM CELL FACTOR

Background

SCF (also known as kit ligand [KL], or steel factor) is a 30-kDa glycoprotein produced by fibroblasts, endothelial cells, and bone marrow stromal cells, which exists as a transmembrane protein and a soluble protein. These different forms are the result of alternative splicing of the RNA transcript. The SCF receptor is encoded by the proto-oncogene c-kit that is expressed on hematopoietic progenitor cells, mast cells, and melanocytes.

Stem cell factor acts synergistically with many other HGFs to promote the production of lymphoid, myeloid, and erythroid lineages from hematopoietic progenitor cells.[53] Therefore, SCF has potential benefit in a number of clinical settings involving either hematopoietic precursor damage or defects and in the setting of BMT. Administration of rhSCF to humans results in significantly increased hematopoietic progenitor cells in the bone marrow and blood. SCF can enhance the proliferation of bone marrow cells derived from patients with aplastic anemia when used with other HGFs. Other uses include the administration of SCF after BMT or chemotherapy to enhance hematopoietic recovery.

Use in Veterinary Medicine

Canine SCF was cloned in 1992 and shares approximately 85% amino acid homology to the human protein.[47] Feline SCF, which has a high degree of homology at both the nucleic acid and protein levels to other species, has also been cloned.[12] The vast majority of veterinary literature describes the use of rcSCF in the settings of hematopoietic recovery after BMT and canine mast cell disease.[51,52] rcSCF supports the survival of hematopoietic progenitors in long-term marrow culture[37] and acts synergistically with rcG-CSF to decrease the time to hematopoietic recovery in dogs after TBI.[46] rcSCF, either alone or in combination with rcG-CSF, also significantly increases the level of PBPCs and stem cells.[11,48] rcSCF logically enhances IgE-mediated histamine and tumor necrosis factor (TNF)-alpha release in canine mast cells, since mature mast cells express c-kit.[4,25] rcCSF, alone or in combination with rcG-CSF, has also been used to treat Gray Collies with cyclic neutropenia.[9]

CONCLUSIONS

Hematopoietic growth factors have significant potential in the treatment of veterinary patients in a wide variety of settings. The greatest benefit associated with the use of HGFs is for those patients with profound blood cell dyscrasias secondary to chemotherapy administration or BMT. Mirroring the human experience, the use of HGFs in conjunction with the administration of chemotherapies that are toxic to the bone marrow may provide an arena to allow the safe exploration of increased anticancer dose intensity which could result in increased survival times while improving quality of life. In addition, as more reports of canine BMT are published, HGFs will most likely become an important part of these protocols in an effort to increase our ability to harvest hematopoietic progenitor CD34+ cells and aid in post-BMT hematologic recovery. Unfortunately, until recombinant canine and/or feline HGFs become affordable and commercially available products, a veterinarian's only option is to use recombinant human proteins which are quite often expensive, ineffective, and have the potential to lead to the development of debilitating neutralizing antibodies with chronic use.

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CHAPTER 107

Clinical Blood Typing and Crossmatching

K. JANE WARDROP

Blood Typing
- Laboratory Testing and Submission
- Blood Typing in the Dog
- Blood Typing in the Cat
- Clinical Blood-Typing Methods in the Dog and Cat
- Blood Typing in Large Animals

Crossmatching
- Indications and Limitations
- Crossmatching in Large Animals
- Standard Tube Crossmatching Procedure
- Commercial Crossmatching Kits

Acronyms and Abbreviations

ACD, acid-citrate-dextrose; DEA, dog erythrocyte antigen; EDTA, ethylenediaminetetraacetic acid; IAT, indirect antiglobulin test; NI, neonatal isoerythrolysis.

Blood typing and crossmatching are techniques used in the selection of blood components before transfusion. When blood typing is performed, antisera or other reagents are used to identify specific RBC antigens. In crossmatching, antibodies are detected by reacting donor sera and donor RBCs with recipient sera and recipient RBCs. The primary purpose of this pretransfusion testing is to ensure that the transfused RBCs have optimal survival when transfused and do not cause harm to the recipient.

Pretransfusion testing in humans generally involves a type and screen procedure. This consists of an initial typing for ABO and Rh(D) antigens and detection of patient antibody by mixing patient serum with commercially available reagent RBCs that contain known antigens. An indirect antiglobulin test (IAT) is used during this antibody screen. When no clinically significant antibodies are detected, an immediate spin crossmatch, in which serum is mixed with saline-suspended RBCs and centrifuged immediately, can be performed to ensure ABO compatibility. If clinically significant antibodies are found, RBCs that lack the corresponding antigens are used, and an IAT crossmatch is performed. The IAT is best for detecting IgG antibodies. Use of a low-ionic strength saline solution in the IAT further enhances its sensitivity. Techniques for antibody detection using tubes (hemagglutination), red cell adherence solid-phase systems, and column agglutination (gels) have been compared.[5]

Blood typing and crossmatching can also be used before transfusion in most domestic animals. Transfusions performed with untyped or uncrossmatched blood can sensitize recipients to future transfusions or can result in reactions that range from enhanced removal of the transfused RBCs to death of the patient. Pretransfusion testing in veterinary clinical practice is frequently less complex than that performed in the human field, largely because of a lack of appropriate reagents.

BLOOD TYPING

Laboratory Testing and Submission

Blood typing of some animal species can be performed in commercial veterinary laboratories. A partial listing of these laboratories is provided (regional veterinary

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diagnostic or clinical pathology laboratories may also offer these services and can be contacted):

1. Animal Blood Resources International (dogs, cats) (www.ABRINT.net).
2. University of California (Davis) Hematology Laboratory (horses) (www.vetmed.ucdavis.edu/vmth/lab_services/clinical_labs/Featured_tests/equine_blood_typing.cfm).

To perform blood typing, laboratories usually require the submission of either EDTA or acid-citrate-dextrose (ACD) whole blood. Serum also may be required for antibody testing.

Blood Typing in the Dog

Canine RBC antigens have been previously identified by use of polyclonal antibodies generated through canine alloimmunization. Dog erythrocyte antigen (DEA) 1, an antigen capable of producing acute hemolytic reactions in sensitized animals, can be identified with a monoclonal antibody.[1] DEA typing for dogs varies, depending on the laboratory used, with some laboratories only typing for DEA 1, and others typing for DEA 1, 4, 5, and 7. Other RBC antigens may be present; however, lack of available antisera prohibits or prevents their identification. This is true for both well-known antigens and newly discovered antigens. For example, DEA 6 and 8 are common antigens (DEA 6 is present in 99% of dogs; DEA 8 may be present in 40% of dogs) previously identified in dogs.[4,21] Lack of available antisera prevents their identification by most typing laboratories. The Dal antigen is a more recently discovered RBC antigen of dogs, with Dal-negative dogs found in Dalmatians (11.7%), Doberman Pinschers (42.4%), and Shih Tzus (57.1%).[2,9] Testing is available through the Section of Medical Genetics at the University of Pennsylvania School of Veterinary Medicine (PennGen, http://www.vet.upenn.edu/research/academic-departments/clinical-sciences-advanced-medicine/research-labs-centers/penngen), or at the University of Montreal Small Animal Blood Bank at chuv.umontreal.ca/banque-de-sang/. The newest documented blood types are Kai 1 and Kai 2, whose clinical relevance remains to be elucidated.[6]

Blood Typing in the Cat

The best characterized blood-group system in cats is the AB system. This system consists of three blood types, A, B, and AB. Type A is the most frequent blood type, occurring in more than 95% of domestic short-hair and long-hair cats in the United States. Type-B blood occurs in less than 5% of domestic short-hair and long-hair cats; however, the frequency of types A and B in cats varies with the breed of cat and the geographic location.[7,8] For example, as many as 45% of Exotic and British Shorthair cats and Cornish and Devon Rex cats have the B blood type. Type-AB cats are rare. Most laboratories type for both the A and B antigens in cats.

All type-B cats older than 3 months of age have high titers of anti-A antibodies. These antibodies act as strong hemolysins and hemagglutinins.[3] Type-B cats transfused with type-A RBCs have a significant hemolytic transfusion reaction, and type-A or type-AB kittens born to type-B queens are at risk of developing neonatal isoerythrolysis (NI). Cats that have type-A blood have weak anti-B alloantibodies that produce a delayed transfusion reaction characterized by premature removal of transfused cells. Type-AB cats have no anti-A or anti-B alloantibodies in their plasma.

The newest non-AB blood-group RBC antigen to be described is the Mik red cell antigen.[24] The absence of this antigen was found to be associated with naturally occurring anti-Mik alloantibodies, capable of eliciting a hemolytic transfusion reaction.

Clinical Blood-Typing Methods in the Dog and Cat

Typing of dogs and cats has been simplified for clinical use by production of commercial typing kits. Either typing cards, typing gels, or an immunochromatographic test can be used.

Typing cards (DMS Laboratories, Inc./RapidVet-H, www.rapidvet.com) contain monoclonal antibody that has been lyophilized onto the card wells. A small amount of diluent and patient EDTA whole blood is added, and the card is rocked for approximately 2 minutes. Agglutination indicates a positive response (Figure 107.1). For the dog, cards are available that type for DEA 1. Positive control wells and wells to detect autoagglutination are included with the canine DEA 1 typing cards to aid in reading the reactions. Cats that have types A, B, or AB can be identified using the feline blood-typing cards. The advantage of these tests lies in their speed and simplicity.

Typing gels are being redeveloped for the typing of DEA 1 in dogs (Alvedia Veterinary Diagnostic Company, https://www.alvedia.com) and A, B, and AB in cats (Alvedia, RapidVet-H). Gel tubes contain a monoclonal antibody (for typing determination) in a gel matrix. Antigen–antibody reactions cause agglutinates to form that then become trapped within the gel. Free RBCs pass though the gel and form a button at the bottom of the tube upon centrifugation.

In the immunochromatographic technique, RBCs mixed with a buffer are allowed to migrate on a membrane that contains monoclonal antibodies against either DEA 1 (Alvedia) or against feline types A and B (Alvedia, RapidVet). In a positive reaction, RBCs will agglutinate with the antibodies and form a visible line on the membrane (Figure 107.2). A study comparing this technique with the typing card and the standard tube agglutination typing technique in cats showed good concordance of typing results.[18]

Blood Typing in Large Animals

Blood typing for large animals is complex and best performed by a commercial veterinary laboratory. In the horse, over 30 different erythrocyte antigens from seven

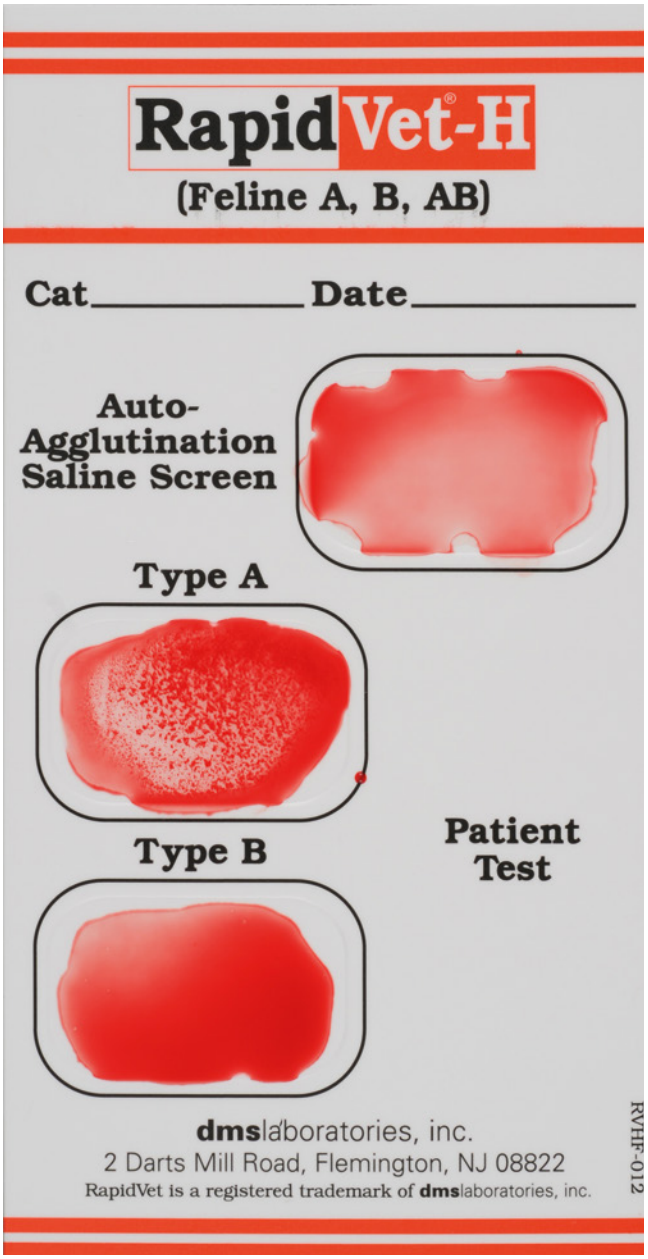


FIGURE 107.1 An example of a feline blood-typing card. The feline patient blood shows agglutination in the type A well and is type-A-positive. (Courtesy of dmslaboratories, inc.)



FIGURE 107.2 An example of a canine DEA 1 immunochromatographic blood-typing test. The presence of a red line by the "DEA 1" arrow indicates a positive reaction. The red line by the "C" arrow is a control line that indicates the test has been run successfully. (Courtesy of Alvedia Veterinary Diagnostic Company, France.)

internationally recognized blood groups have been recognized.[16] This means that identification of a blood donor with identical alloantigen and antibody profiles is difficult, if not impossible. Alloantigens Aa and Qa are the most immunogenic of the equine RBC antigens and are responsible for the majority of equine NI cases.[12] Mares that lack Aa and Qa are at risk of producing antibody to them upon exposure. Mares known to be at risk, either because of blood type or because of previous foaling of an affected foal, can have sera tested for antibody before foaling. In mules, a unique donkey RBC antigen known as the donkey factor has been associated with NI.[22]

Recently, an in-clinic test for the equine Ca antigen (Alvedia Quick Test BT Ca) was evaluated.[13] The test is performed using an immunochromatographic technique with an equine anti-Ca monoclonal antibody. In-clinic typing for detection of Ca and Aa antigens has also been described, using antisera from horses previously identified as having anti-Ca and anti-Aa antibodies.[16]

CROSSMATCHING

Indications and Limitations

Blood typing permits the identification of RBC antigens in a patient. It does not allow the detection of antibodies between a patient and a potential blood donor. In a clinical situation, this is generally determined by crossmatching. Even in animals that have been blood-typed, crossmatching should be performed to detect antibodies against RBCs.

Crossmatching does have its limitations. A compatible crossmatch does not necessarily indicate that the donor and recipient have the same blood type, merely that antibodies against RBCs cannot be detected. Also, the crossmatch technique may lack the appropriate sensitivity to detect some antibodies; thus, it is possible to have a compatible crossmatch and still have increased removal of the transfused cells. A standard crossmatch does not detect platelet or granulocyte antibodies. It cannot predict some types of transfusion reactions, such as urticarial reactions. Nevertheless, crossmatching can be a useful technique and should be performed before transfusion. Incompatibilities in transfusion-naïve dogs have been documented;[15] therefore, crossmatching should be considered before all transfusions. At minimum, crossmatching should be performed before those transfusions where the recipient has a history of previous blood exposure. Crossmatching should always be performed before feline transfusions, regardless of the previous transfusion history, because of the presence of naturally occurring antibody in the cat and the serious consequences of transfusing type-A blood into a blood type-B cat.

Crossmatching in Large Animals

Crossmatching in large animals generally requires both a saline-agglutinating technique and a technique that can detect hemolysis. Agglutination tests can be

performed by most veterinary practices; however, the hemolytic tests require the addition of exogenous complement, usually provided by rabbit or guinea pig serum.[20] This serum is diluted or adsorbed first with RBCs of the species to be tested, to remove the natural heterolysins and heteroagglutinins present in the rabbit or guinea pig serum. Consequently, detection of hemolytic antibodies is usually confined to the veterinary laboratory. In a study evaluating a standard tube crossmatch and a gel column crossmatch in horses, both the tube and gel procedures had excellent accuracy for agglutination, but the gel procedure had marginal accuracy for hemolysis.[13]

One crossmatching technique used in the identification of mare antibody in cases of suspected NI is the jaundiced foal agglutination test.[12] In this test, colostrum from the mare is reacted with RBCs from the foal. The colostrum can also be tested with potential donor blood. Serial dilutions (1:2–1:128) with saline of colostrum are made, and one drop of EDTA foal blood is added to 1 mL of the dilution. Blood is also added to a saline control tube that lacks colostrum. The tubes are centrifuged and judged for agglutination. Positive reactions at dilutions of 1:16 or greater are considered significant in horses.

Standard Tube Crossmatching Procedure

The tube crossmatching procedure described in Table 107.1 is a saline-agglutination crossmatch performed by veterinary laboratories. Some laboratories also use an IAT in this procedure, adding species-specific Coombs reagent after the initial RBC or sera reactions have been interpreted. Antisera-treated RBCs are washed in saline, the Coombs reagent is added in an amount and dilution determined by the manufacturer, and the mixture is incubated. The mixture is then centrifuged and the sample is checked for agglutination. A saline replacement technique is useful to detect rouleaux in species such as horses and cats, in which rouleaux formation is common and can be erroneously identified as agglutination in the crossmatch procedure. Steps in the saline replacement technique[3] are as follows:

1. Recentrifuge the serum or the RBC mixture when rouleaux formation is suspected.
2. Remove the serum.
3. Replace the serum with an equal volume of saline (two drops) and gently mix.
4. Centrifuge the saline or the RBC mixture at 1000 × *g* for 15 seconds.
5. Resuspend the saline or the RBC mixture and observe for agglutination.

Rouleaux disperse when suspended in saline, whereas true agglutination remains. Grading of reactions or antibody titration can also be performed.[14]

Gel column crossmatching methods, requiring gel column cards, low-ionic-strength saline, special centrifuges, and special incubators, have also been described.[2,24]

TABLE 107.1 Crossmatching procedure.

1. Obtain an anticoagulated (EDTA) and non-anticoagulated specimen of blood from both patient and donor. The EDTA tube will serve as the source of RBC (antigen), and the non-anticoagulated tube will serve as the source of serum (antibody). Plasma can be used, but it should be noted that EDTA can interfere with detection of some complement-dependent hemolytic antibodies.
2. Centrifuge and separate plasma or serum from RBC.
3. Wash RBC by adding saline or phosphate-buffered saline (PBS) to a small amount of packed RBC, mixing and centrifuging at 1000 × *g* for 1 minute. Decant the saline and repeat three times, filling the tubes with saline, mixing, centrifuging, and decanting.
4. After last wash, decant supernatant and resuspend cells with saline to give a 4% suspension of RBCs; 0.1 mL blood in 2.4 mL saline gives a 4% suspension.
5. Make the following mixtures by adding the indicated amount of the well-mixed RBC suspension and serum or plasma to 10–12 × 75 mm tubes:
   Major crossmatch: two drops patient sera/plasma, one drop donor 4% RBC.
   Minor crossmatch: two drops donor sera/plasma, one drop patient 4% RBC.
   Controls: two drops patient sera/plasma, one drop patient 4% RBC; a donor control of two drops donor sera/plasma with one drop donor 4% RBC can also be prepared.
6. Incubate tubes 15 minutes at 37 °C.

Note: To detect hemolysis in large animal species, add two drops of complement to the RBC/serum tubes and incubate at 37 °C for 90 minutes.

7. Centrifuge for 15 seconds (3400 rpm/1000 × *g*).
8. Read tubes:
   Macroscopic: Examine tubes first for hemolysis. Then rotate tubes gently and observe cells coming off the red cell "button" in the bottom of the tube. Rotation of the control tubes prior to or simultaneously with rotation of the other tubes allows for optimum comparison of reactions. In a compatible reaction, i.e., where there is no antigen–antibody reaction, the cells should float off freely, with no clumping/hemagglutination (compare to the control tubes). Rouleaux formation can be falsely interpreted as a reaction. If rouleaux formation is suspected, a saline replacement technique can be used.[17] Proceed to the microscopic examination in those tubes with weak or no obvious reactions.
   Microscopic: After tubes have been viewed macroscopically, place a drop of the cells/sera mixture on a slide, apply a coverslip, and examine microscopically. The RBC should normally appear as individual cells, with no clumping or rouleaux formation.

Commercial Crossmatching Kits

Crossmatching kits are available. The RapidVet®-H major and minor companion animal crossmatch test (DMS Laboratories, Flemington, NJ, http://www.rapidvet.com) uses gel tubes to detect incompatible crossmatches.[23] The Alvedia company (www.alvediavet.com) provides both gel tests and an immunochromatographic,



FIGURE 107.3 An example of a canine antiglobulin-enhanced immunochromatographic crossmatch test. The presence of the red line by the "XM" arrow indicates a positive (incompatible) reaction. The red line by the "C" arrow is a control line that indicates the test has been run successfully. (Courtesy of Alvedia Veterinary Diagnostic Company, France.)

antiglobulin-enhanced crossmatch test (Figure 107.3) to detect incompatible crossmatches in dogs, cats, and horses.[11] Care must be taken to follow the manufacturer's centrifugation recommendation when using the gels, as this can impact dispersion of cells through the gel. Kits from both companies can be stored at room temperature for at least a year. They can be ordered directly from the companies or from commercial veterinary laboratories. Microscopic or weak incompatible results or hemolysis seen in the standard tube crossmatching technique are unlikely to be picked up using the gel- or strip-based technique.[10] In addition, a recent study indicated that the tube and gel crossmatching techniques, but not an immunochromatography strip technique, were useful methods for the identification of DEA 7 incompatibilities caused by anti-DEA 7 antibodies.[19]

Blood typing and crossmatching are valuable techniques that must be considered before transfusion in all species. Although some testing should be performed in specialized veterinary laboratories, other tests, such as saline-agglutinating crossmatching and DEA 1 and feline AB blood typing, can be performed in most clinical veterinary practices and provide information that aids in the safe selection of blood components.

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SECTION IX
Species-Specific Hematology

Hct:	hematocrit
Hgb:	hemoglobin
MCV:	mean corpuscular volume
MCH:	mean corpuscular hemoglobin
MCHC:	mean corpuscular hemoglobin concentration
M:E ratio:	myeloid to erythroid ratio
N:C ratio:	nuclear to cytoplasmic ratio
nRBC:	nucleated red blood cell
PCV:	packed cell volume
RDW:	red cell distribution width
RI:	reference interval

CHAPTER 108

Hematology of Dogs

MAGGIE R. McCOURT and THERESA E. RIZZI

Erythrocytes
Leukocytes
  Partitioning Factors
Platelets
Reference Intervals
Bone Marrow
Hematologic Disease
  Changes in the Erythron
  Changes in the Leukon
  Infectious Agents

Acronyms and Abbreviations

ASVCP, American Society of Veterinary Clinical Pathologists; PARR, PCR for antigen receptor rearrangement (PARR).

ERYTHROCYTES

The canine erythrocyte is a biconcave disc, approximately 7 μm in diameter when measured microscopically on a blood smear and a MCV of 60–71 fL.[3] The biconcave shape is more pronounced in the dog than in other domestic animals, giving their RBCs a clearly visible central pallor when viewed in the monolayer of well-made blood films (Figure 108.1a). This prominent central pallor, which normally encompasses approximately the central one-third to one-half of the cell's diameter, makes spherocyte recognition possible in this species. Central pallor may be lost at the smear edges or in very thin smears, where sheer forces distort normal morphology (Figure 108.1b).

Canine erythrocytes normally display mild anisocytosis and poikilocytosis. Echinocyte formation may be an artifact related to smear preparation (i.e., crenation), but has also been reported in certain disease states. Clear, refractile areas (water artifacts) within RBCs may be seen if the blood smear is not adequately dried before staining. Stain precipitate may sometimes adhere to erythrocytes and must not be confused with parasites (Figure 108.1c).

When blood smears are stained with Romanowsky stains, such as Wright's stain, polychromatophilic (blue-staining) cells may be seen occasionally. In health, these comprise less than 1% of the cells (Figure 108.1d) and are recognized as reticulocytes when blood is stained with supravital stains, such as new methylene blue (Figure 108.1e). Rare nRBCs and Howell–Jolly bodies can be seen in blood smears from normal adult dogs with increased numbers found in normal neonates. Hgb crystals are square to rectangular structures which, depending on staining technique, are similar, slightly lighter, or darker than Hgb in mature RBC and often deform the RBC that contains them (Figure 108.1f). Low numbers (1–10 per slide) of Hgb crystals have been reported in blood smears of clinically normal dogs and dogs less than 3 months of age.[36] They have not been associated with abnormal Hgb molecules (see Chapter 24).

Reticulocytes are released from the canine marrow in a cyclic manner with a periodicity of approximately 14 days.[38] Aggregate reticulocytes, containing one or more large, distinct clumps of blue-staining material, are most common (Figure 108.1e). In dogs, polychromasia noted on Romanowsky-stained smears correlates well with reticulocytosis, although automated reticulocyte counts are a more accurate reflection of the number of early RBCs in circulation especially when polychromasia is not prominent[9] (see Chapters 23 and 137). Reticulocyte RI vary between laboratories based on the methodology and reference population used. Reticulocyte counts should be interpreted using the RI provided by the laboratory, made in accordance with

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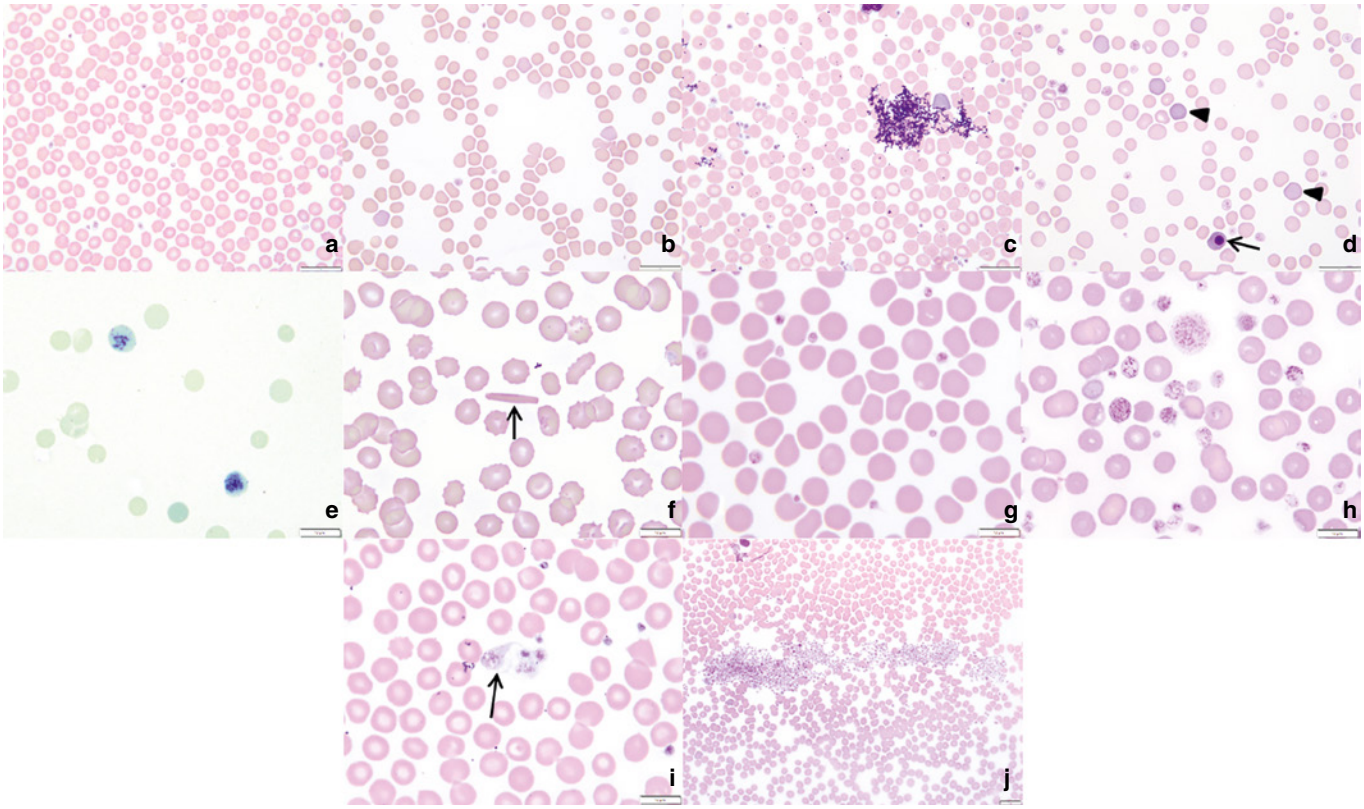


FIGURE 108.1 Morphology of canine erythrocytes and platelets. Wright–Giemsa stain. (a) RBCs with normal central pallor; (b) RBCs from the same blood film have lost their central pallor due to shear and spreading forces close to the feathered edge; (c) A large aggregate of stain precipitate is present, and precipitate is adhered to RBCs; (d) Regenerative anemia showing polychromatophils (arrowheads) and a nRBC (metarubricyte, arrow); (e) Two reticulocytes with large blue RNA aggregates (new methylene blue); (f) A single rectangular Hgb crystal is present (arrow); (g) Platelets with a uniform small size; (h) Reactive thrombocytosis with variably sized platelets including large and giant platelets; (i) Platelets with a long filamentous cytoplasmic projection (arrow); (j) A large platelet clump within the monolayer.

American Society of Veterinary Clinical Pathologists (ASVCP) reference interval guidelines. Neonatal animals have significantly higher reticulocyte counts, a manifestation of increased erythropoiesis needed for replacement in a rapidly expanding vascular volume. Reticulocyte counts of up to 10% can be seen in apparently healthy dogs during the first 2 months of life.[15,50] This number decreases to adult levels by approximately 5–6 months of age. Greyhounds have significantly increased reticulocyte counts immediately after racing; however, reticulocyte counts return to baseline 1–2 hours post-race.[27] Recently, an increased prevalence of reticulocytosis in the absence of anemia has been recognized in dogs. Reticulocytosis was defined as >2.5% reticulocytes or >110,000 reticulocytes/μL and was associated with several underlying diseases, joint supplements, and nonsteroidal anti-inflammatory medications; however, some dogs were apparently healthy and unmedicated.[16,42]

All three measurements of RBC mass, Hgb, Hct, and RBC count, are interrelated and, in the absence of marked alterations of RBC size and Hgb concentration, tend to parallel each other. Hct, the percentage of blood volume comprising erythrocytes, can be determined either by centrifugation PCV or calculated based on the MCV and RBC count. Most automated hematology analyzers calculate Hct, although manual PCV is commonly determined in practice. PCV should be used as a gold standard when artifactual, calculated Hct values are suspected (see Chapter 137).

The RBC mass of neonatal animals varies significantly from that of adults and changes as dogs age. At birth, Hgb, Hct, and the RBC count are near adult values, but decline rapidly over the first 2 months of life. Hct values reach a nadir in the high 20s to low 30s (%), which is clinically significant and should not be diagnosed as anemia.[46,50] After this, values start to increase, reaching adult levels by 6 months to 1 year of age.[24] Anemia of the elderly (defined as the general population >65 years of age) is well described in human medicine and a recent study showed that this may also occur in the canine population.[21] In one study, senior (8–11.9 years) and geriatric (>12 years) dogs had lower Hct than adult dogs (1–8 years), as well as lower MCV and serum iron (not total body stores) suggesting that as dogs age they develop some degree of iron-restricted erythropoiesis and inflammation.[44] Decreased erythroid mass in geriatric dogs is supported by another study that developed RIs from a large population of apparently healthy senior and geriatric dogs; however, subclinical disease was possible in this population.[2]

Some authors have found a sex effect with males having a slightly higher Hgb concentration than females; however, it is not clinically significant as all values are well within RIs for adult dogs. Pregnant females have significant changes during gestation. In one study, the Hct of pregnant dogs fell from a mean baseline of 53% to 32% at term.[6] Dogs in diestrus may have a 7–8% decrease in Hgb, Hct, and RBC counts compared to dogs in other phases of the estrus cycle, suggesting a possible component of hemodilution which may be related to the elevated progesterone concentrations present in both diestrus and during pregnancy.[57]

Numerous studies have shown that greyhound dogs have increased erythroid mass, with Hct up to 65% reported in clinically healthy animals.[49,58] These higher RBC indices become evident by 9–10 months of age.[49] Erythroid indices in greyhounds may increase further immediately after racing (PCV up to 68%), but return to baseline within 30 minutes post-race.[47] Dachshunds have also been reported to have higher RBC indices than the general dog population with mean PCVs of 52% (46–58%).[53] Akita dogs, with the high potassium RBC phenotype, have slightly lower Hgb, Hct, and RBC counts than their low potassium RBC phenotype counterparts.[10] A slightly higher RI for dogues de Bordeaux erythroid values as compared to the general dog population has been reported. Breed-specific RIs are warranted for these values.[33]

These RBC indices characterize the size and Hgb content of the RBC population and vary in some breeds. In the dog, fetal RBCs are larger than those of adults. The MCV of umbilical cord blood is 100–110 fL and in newborn pups is 95–100 fL. Rapid replacement of fetal erythrocytes after birth brings this value to adult levels by 2–3 months.[39,50] Some lines of toy and miniature poodles have an inherited macrocytosis with abnormal erythropoiesis.[7] These animals have RBC counts that are below standard canine RIs, but due to the large size of the RBCs (84.5–106.7 fL) and increased Hgb content, their Hct and Hgb are comparable to other breeds. Affected poodles may also have circulating nRBCs and a case of an apparently healthy poodle with a concurrent reticulocytosis was reported.[5,7] Greyhound dogs have also been reported to have a higher MCV and MCHC than other breeds.[58]

Japanese Akita and Shiba Inu breeds have microcytic RBCs (55–65 fL).[13,22] A subset of Akitas that display the high-potassium RBC phenotype have been shown to have increased MCV and lower MCHC than their low-potassium RBC phenotype counterparts, which are often microcytic and normochromic.[10] A study determining RIs for Bernese mountain dogs revealed a slightly higher MCHC compared to the reference canine population; however, this slight change is not likely of clinical significance.[41]

The RDW represents the size variability of individual erythrocytes analyzed. Numerically, it represents the coefficient of variation of RBC volumes and can be graphically represented on a histogram. Increases in RDW indicate increased anisocytosis and may be more sensitive than the MCV in detecting early changes in erythrocyte size.[40]

LEUKOCYTES

Normal leukocyte morphology from several different canid species are shown in Figure 108.2. Neutrophils are the most abundant leukocyte in the peripheral blood of normal dogs (Figure 108.3). The mature neutrophil has an elongated nucleus which contains several distinct lobes separated by constricted areas, which may appear as narrowing of the nuclear material or form thin nuclear filaments. The nucleus stains dark purple and has a clumped chromatin pattern consisting of many areas of densely staining heterochromatin. Cytoplasm is typically clear and may be slightly basophilic upon Diff-Quik stain (Mercedes Medical, 12210 Rangeland Pkwy, Lakewood Ranch, FL 34211). Neutrophil granules in the dog are typically indiscernible, but may be faintly eosinophilic. Low numbers of immature, or band-shaped, neutrophils may be present in normal animals and are defined by elongated nuclei with parallel sides that lack distinct lobation and discrete constrictions. The nuclear chromatin of a band cell is often less condensed and lighter staining than that of a mature cell. Cytoplasm is similar to that of mature neutrophils (see Chapter 42).

Lymphocytes in the peripheral blood vary in size, with small lymphocytes being most abundant (Figure 108.3). Small lymphocytes have densely staining nuclei that are approximately the same size as an RBC which range from round to oval to cleaved. Nuclear chromatin is densely clumped. Small lymphocytes have a scant amount of cytoplasm, which is pale blue and may form a thin rim or crescent. Intermediate-sized lymphocytes have nuclei that are approximately twice the size of an RBC and have more abundant, light blue cytoplasm.

Reactive lymphocytes can be seen in blood smears from dogs responding to physiologic (e.g., exposure or vaccination) or pathologic antigenic stimulation. Reactive lymphocytes may be larger and have increased amounts of basophilic cytoplasm (Figure 108.3). Rarely, reactive B lymphocytes may appear plasmacytoid, with an eccentric nucleus and a perinuclear clear (Golgi) area. Large lymphocytes in peripheral blood may approach the size of neutrophils. Occasionally, lymphocytes will contain several small pink-purple cytoplasmic granules and are referred to as large granular lymphocytes (Figure 108.3). These granules may be confined to a small cluster near one side of the nucleus.

Monocytes are larger than neutrophils. Monocyte nuclei are extremely variable and may be round, multilobated, band-shaped, or S-shaped (Figure 108.3). Those that are band-shaped are generally wider and may have rounded, knob-shaped ends that differentiate them from band neutrophils. The nuclear chromatin is less condensed than that of a mature neutrophil. Their cytoplasm is moderate to abundant and gray-blue to deeply basophilic. Canine monocytes may have variable numbers of clear, distinct-walled cytoplasmic vacuoles. Occasionally, faint, dust-like azurophilic granules can be seen evenly spread throughout the blue cytoplasm (see Chapters 48 and 49).

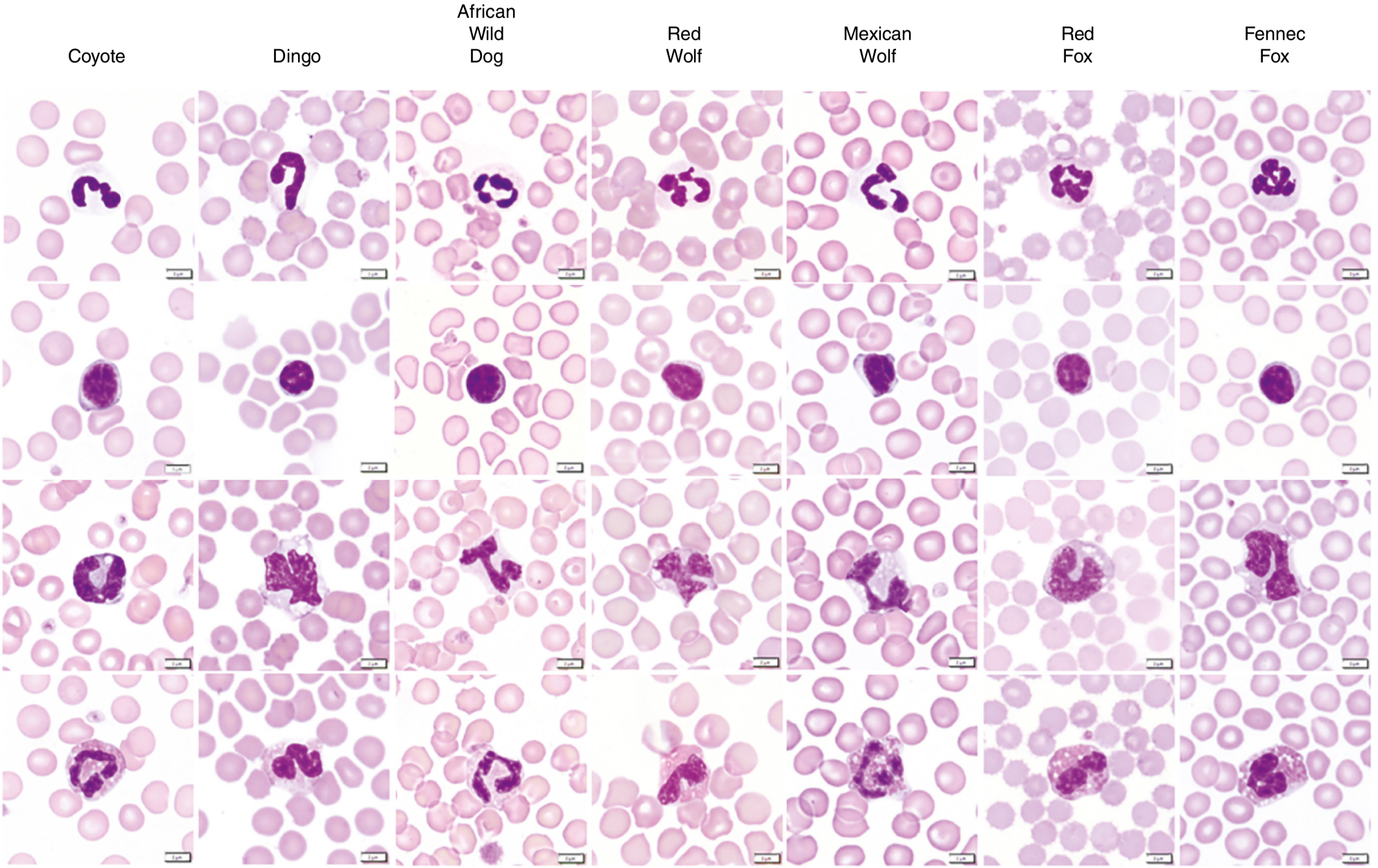


FIGURE 108.2 Leukocytes from seven species of nondomestic canids: coyote (*Canis latrans*), dingo (*Canis lupus dingo*), African wild dog (*Lycaon pictus*), red wolf (*Canis rufus*), Mexican wolf (*Canis lupus baileyi*), red fox (*Vulpes vulpes*), fennec fox (*Vulpes zerda*). Neutrophil, lymphocyte, monocyte, and eosinophil morphology (organized by row) is similar to that of domestic dogs. (Wrights and Diff Quik stains were used. Photo credit: Jennifer D'Agostino, Oklahoma City Zoo and Kari Velguth, Idexx Laboratories.)

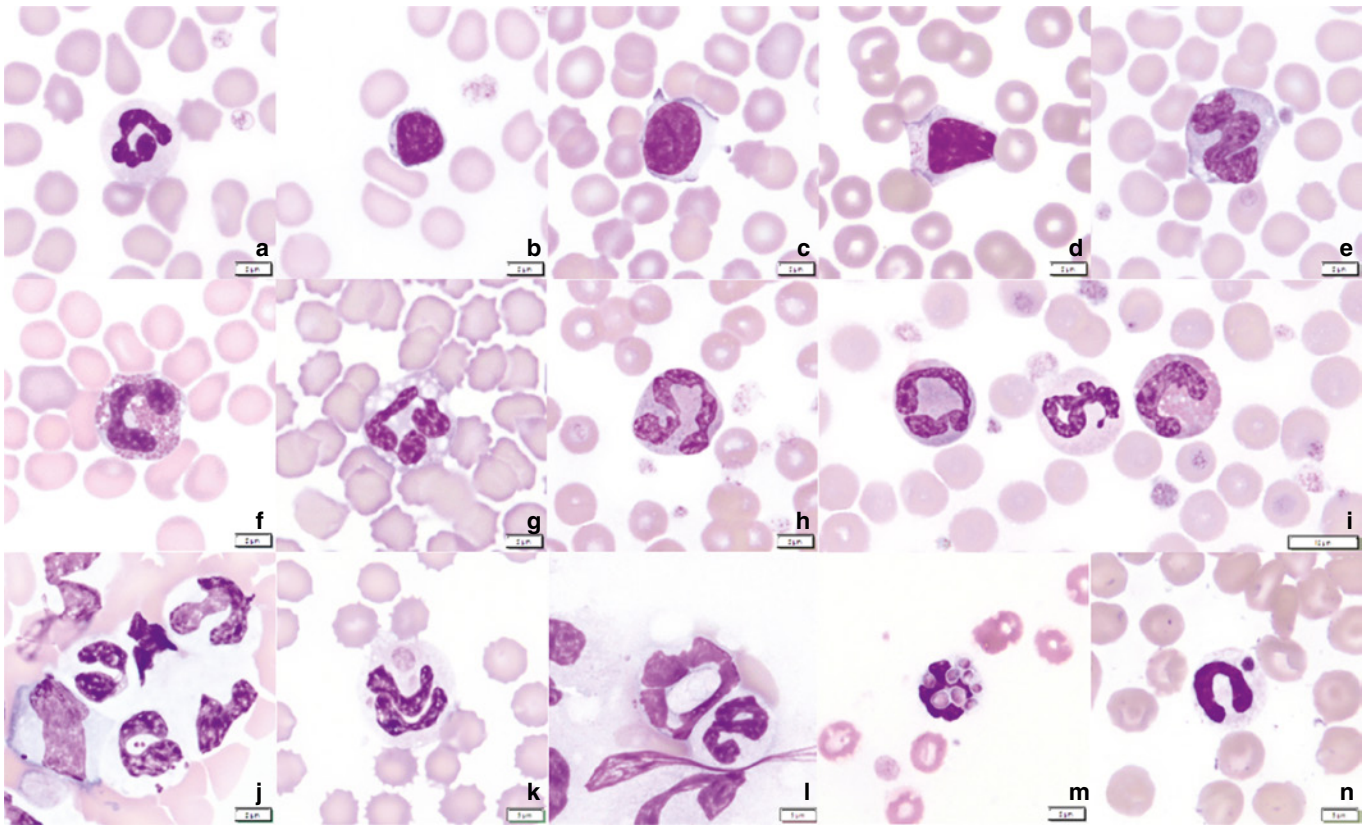


FIGURE 108.3 Canine leukocytes and infectious agents. Wright–Giemsa stain. (a) A mature segmented neutrophil with a segmented nucleus and mature chromatin; (b) A small lymphocyte with mature condensed chromatin and a scant rim of cytoplasm; (c) A reactive lymphocyte with an increased volume of cytoplasm; (d) A large granular lymphocyte with an increased volume of cytoplasm containing magenta-staining granules; (e) A monocyte with a S-shaped nucleus and basophilic cytoplasm containing several small vacuoles; (f) An eosinophil with round eosinophilic and clear cytoplasmic granules; (g) A gray eosinophil from a Greyhound dog with basophilic to gray cytoplasm and many clear vacuoles; (h) A single basophil with a segmented nucleus and pale purple-staining cytoplasmic granules; (i) All three canine granulocytes are seen side by side. From left to right: basophil, neutrophil, eosinophil; (j) Intracellular cocci are seen in a neutrophil from a dog with septicemia; (k) Three light magenta-staining amorphous canine distemper viral inclusions are seen within a neutrophil; (l) A single Hepatozoon gamont is seen as a poorly staining ovoid structure within a neutrophil; (m) Several *Histoplasma capsulatum* yeast organisms are phagocytized in neutrophilic cytoplasm; (n) A single Ehrlichia morulae within a neutrophil's cytoplasm.

Eosinophils can usually be found in low numbers on blood smears from healthy dogs. Eosinophils are slightly larger than neutrophils with the characteristic feature of prominent, round, eosinophilic granules. Eosinophil nuclei are less lobated than those of neutrophils, usually having only two or three lobes (Figure 108.3). In dogs, the number and size of the cytoplasmic granules are variable. Occasionally, an eosinophil may contain only one or two large granules, which could be mistaken for an inclusion body or parasite. Cytoplasm between the granules is lightly basophilic and may contain a few clear, nonstaining granules. Eosinophils of some adult greyhounds, Italian greyhounds, and whippets are unique in that they contain numerous clear granules and often lack distinct eosinophilic granules (Figure 108.3).[19] These "gray eosinophils" are fully functional with no ultrastructural or cytochemical differences when compared with normal eosinophils[28] (see Chapter 46).

Basophils are rare in blood smears from normal dogs and are not pictured in Figure 108.2. Basophils are larger than neutrophils and have a more elongated, convoluted nucleus. Cytoplasm is blue-gray to slightly purple. Compared to other species, canine basophils contain only scattered, small, purple to pink-purple cytoplasmic granules which may be difficult to discern on certain stains (Figure 108.3) (see Chapter 47).

Partitioning Factors

Physiologic leukopenia has been reported in healthy adult North American Belgian Tervuren dogs.[23] No difference in WBC values was found between Belgian Tervuren dogs and other dogs in a more recent study.[20] Study differences in North American Belgian Tervuren and Belgian Tervuren dogs were thought to reflect genetic or environmental factors.[20] Greyhounds, in addition to their varied RBC characteristics, have been

documented to have lower total WBC and neutrophil counts than the general dog population and breed-specific RIs should be used to interpret these values.[58] Bernese mountain dogs have been reported to have a higher upper reference limit for absolute eosinophil counts than the general dog population.[41]

There may be some mild age-associated change in WBC values. Fluctuations in total WBC and neutrophil counts have been noted in beagles during the first 2 months of life; however, with a few exceptions, values generally remained within the RI.[46] In one study, newborn animals (0–3 days) had band neutrophil counts that were slightly outside the adult RI, indicating a left shift. Occasional metamyelocytes were seen during this period without other evidence of disease. Band neutrophils decreased to within RIs by 7–10 days of life.[50] Lymphocyte counts are significantly higher in young dogs while they are establishing their immune system. Dogs <6 months of age typically have lymphocyte counts of at least 2000/μL, with some as high as 10,000/μL.[50]

After maturity, there are minor changes reported in hematologic values. Some studies have found no significant trends in WBC counts in dogs from 1.5 to 14 years of age.[14,35] More recently, senior and geriatric dogs were reported to have lower lymphocyte concentrations than adult dogs; however, absolute lymphocyte counts remained within the RI.[44] Senior and geriatric beagles and Labrador retrievers have a lower RI for absolute neutrophil counts, though geriatric beagles have a higher upper reference limit than the general dog population in one study due to unknown factors.[2]

Reported diurnal and seasonal variations in WBC counts are relatively minor and do not exceed the standard RIs.[34,54] In some geographic areas, dogs may have higher eosinophil counts in certain seasons due to environmental allergens and parasite load.

During pregnancy, total WBC counts may increase to levels that are slightly above RIs (approximately 19,000/μL) near parturition. White blood cell counts decrease during lactation and may not return to baseline values until the pups are weaned.[1] Dogs in diestrus had eosinophil counts 45% higher than dogs in other stages of the estrus cycle (absolute eosinophil count 0.3 vs. 0.2 × 10^3/μL).[57]

Obesity influences total WBC counts with overweight and obese dogs (body condition score ≥7) having a higher total WBC concentration due to higher neutrophil counts than normal control dogs; however, these counts remained within the RI.[45]

PLATELETS

Canine platelets are small, oval to round, anuclear structures on blood smears that are typically one-fourth to one-half the size of an RBC, though some platelets are larger than a RBC (Figure 108.1g,h). They have clear to light-gray cytoplasm and numerous small pink to purple granules. The granules may be dispersed throughout the platelet or aggregated into a central cluster. Occasionally, platelets become partially activated and have a spider-like appearance with cytoplasmic pseudopodia (Figure 108.1i). Activated platelets may form small clumps or a large, agglutinated mass. Platelet clumps are often found at the feathered edge of a smear (Figure 108.1j).

Reference intervals will vary between laboratories and analyzers. A RI determined utilizing the Sysmex XT-2000iV (Sysmex America, Inc., Lincolnshire, IL, USA) was reported to be 108,000–562,000 platelets/μL.[3] Lower platelet counts have been noted in healthy greyhounds (117,000–309,000/μL.[58]

Asymptomatic, inherited thrombocytopenia with enlarged platelets (macrothrombocytopenia) is common in cavalier King Charles spaniels and has also been documented in Norfolk and cairn terriers as well as Akita dogs.[18,26,43] In cavalier King Charles spaniels, Norfolk terriers, and cairn terriers, the condition is associated with a mutation in the β1-tubulin gene, although the exact mutation is different in cavaliers versus the terrier breeds.[11,18] The median platelet count from 105 cavalier King Charles spaniels was 92 × 10^3/μL (25–394 × 10^3/μL) with 56% of the spaniels having platelet counts <100 × 10^3/μL.[43] Affected Akita dogs present with macrothrombocytopenia and often display elongated platelets.[26] The cause of the macrothrombocytopenia in Akita dogs has yet to be elucidated. The few cases to date do not have a mutated β1-tubulin gene.[26] Analyzers that assess plateletcrit rather than impedance-based or optical-based particle counting have shown the total platelet mass in affected animals to be normal and provide clinically useful information when interpreting overall platelet density in these patients.[30,56]

Reference intervals established for dogues de Bordeaux reveal a higher mean platelet volume (MCV), but lower overall platelet counts when compared to the general dog population.[33]

REFERENCE INTERVALS

Table 108.1 provides published RIs for hematologic values of adult, male and female dogs generated in accordance with ASVCP guidelines. Reference intervals are designed to include values found in 95% of normal animals and will vary according to analyzer, population, and the definition of normal using partitioning factors. Many published RIs for canine hematologic values are derived from research animals and represent a single breed raised under controlled, confinement conditions. Such intervals may be narrower than would be applicable to a general patient population in which breed, age, and environmental variables are present. Alternatively, RIs that are derived from shelter dogs or clinically healthy animals brought to a veterinary clinic may be inappropriately wide because of subclinical disease, nutritional status, and physiologic or environmental influences (e.g., short-term alterations in WBC numbers due to an epinephrine- or corticosteroid-induced response in dogs that are not conditioned to blood collection) (see Chapter 140).

TABLE 108.1 Hematologic reference intervals for normal, adult, male and female dogs.

	Units	Reference intervals[a] for laboratory beagles (Advia 2120)	Reference intervals[b] from a blood donor database (Sysmex XT-2000iV)
RBC count	$\times 10^{12}$/L	6–8.3	5.9–8.4
Hgb	g/dL	14.4–19.4	14.2–20
Hematocrit	%	39–54	43–60
MCV	fL	62–71	65–79
MCH	pg	21.5–25.1	22.0–25.9
MCHC	g/dL	33–37	30.8–35.5
RDW	%	11.3–14.5	12.9–18.3
Reticulocytes	$\times 10^{9}$/L	Not reported	20–151
Leukocytes	$\times 10^{9}$/L	6–14.3	5.9–14.5
Neutrophils	$\times 10^{9}$/L	3.4–9.5	3.1–9.4
Lymphocytes	$\times 10^{9}$/L	1.5–4	1.5–4.7
Monocytes	$\times 10^{9}$/L	0.2–0.9	0.1–0.7
Eosinophils	$\times 10^{9}$/L	0.1–1.1	0.2–1.4
Basophils	$\times 10^{9}$/L	0.01–0.08	Not reported
Platelet count	$\times 10^{9}$/L	220–480	102–282
MPV	fL	Not reported	9.8–14
Plateletcrit[c]	%	0.13–0.40[c]	0.14–0.38

[a] Data from Bourges-Abella et al.[4]
[b] Data from Serra et al.[48]
[c] Data from Kelley et al.[30]

BONE MARROW

Bone marrow collection and canine hematopoietic cell morphology have been reviewed[25] (see Chapters 3 and 141).

Nucleated erythroid precursors recognizable in marrow aspirates include rubriblasts, prorubricytes, rubricytes, and metarubricytes. As cells mature, they become smaller, undergo nuclear condensation, and the color of the cytoplasm changes from deeply basophilic to red-orange (Figure 108.4a,b). Rubriblasts, normally present in low numbers, are large with a high N:C ratio. The nucleus is round with fine chromatin and one or more visible nucleoli (Figure 108.4a,b). The moderate amount of cytoplasm is deeply basophilic and is found in a concentric ring around the nucleus. Prorubricytes are slightly smaller with a lower N:C ratio. Prorubricyte nuclei lack visible nucleoli and have coarser chromatin that rubriblasts. The cytoplasm remains intensely basophilic. Rubricytes, while still potentially capable of mitotic activity, are significantly smaller with a lower N:C ratio. The nucleus has coarse chromatin and nucleoli are not visible (Figure 108.4b). Rubricyte cytoplasm matures from deeply basophilic (basophilic rubricyte) to polychromatophilic (polychromatophilic rubricyte) to orange (orthochromatic rubricyte), representing progressive production of Hgb. Metarubricytes are the smallest nucleated erythroid precursors, are characterized by a pyknotic nucleus that lacks a discernible chromatin pattern, and cannot replicate (Figure 108.4b). Progressive maturation results in polychromatophilic erythrocytes (reticulocytes), non-nucleated cells found in low numbers in the peripheral blood.

The granulocytic series exhibits a similar progression of relatively low numbers of early precursors to more numerous maturing cells (see Chapter 41). The recognizable stages of granulocytic maturation in the marrow are myeloblasts, promyelocytes (progranulocytes), myelocytes, metamyelocytes, band cells, and mature granulocytes. Myeloblasts are large cells with a high N:C ratio, fine chromatin, and one or more visible nucleoli. Compared to rubriblasts, the myeloblast exhibits more nuclear pleomorphism and has a lighter-staining blue cytoplasm. Promyelocytes are slightly larger than myeloblasts, but have a lower N:C ratio due to cytoplasmic enlargement. Nuclear chromatin is somewhat coarser and nucleoli may or may not be present. Numerous fine, azurophilic, cytoplasmic granules (primary granules) identify the promyelocyte's granulocytic origin (Figure 108.4c). Myelocytes are smaller than promyelocytes and are characterized by loss of visible primary granules. Round to oval nuclei have dense chromatin and no visible nucleoli. The cytoplasm becomes progressively lighter blue to clear and secondary granules may be present, identifying the cells as neutrophils, eosinophils, or basophils (Figure 108.4e,f). Secondary granules in neutrophilic cells are clear and indistinct. Metamyelocytes are characterized by the nuclear indentation resulting in a kidney bean or reniform shape. Cytoplasm is clear, with prominent secondary granules identifying the granulocytic precursor. The band cell has a rod- or sausage-shaped nucleus with parallel sides and no indentations or constrictions. The final stage is the mature granulocyte, characterized by nuclear constrictions giving rise to the classic polylobated appearance. Cytoplasm remains clear in neutrophilic precursors and has well-formed granules in the eosinophilic and basophilic cells.

Promegakaryocytes and megakaryocytes are typically identified on low magnification, as most are substantially larger than the other hematopoietic precursors. Megakaryoblasts are present in low numbers and have a high N:C ratio, single nucleus, and deeply basophilic cytoplasm. It is difficult to reliably differentiate them from progenitors of other cell lines. Promegakaryocytes are more easily identified. Endomitotic reduplication results in a large cell with two to four nuclei. These may appear to be separate, but are connected by nuclear strands. Promegakaryocytes have more abundant, deeply basophilic cytoplasm than the megakaryoblast. Further maturation with progressive reduplication of the nucleus gives rise to variably sized megakaryocytes that have a polylobated nuclear mass. As megakaryocytes mature, the cytoplasm becomes more abundant and changes from deeply basophilic to eosinophilic and granular, having an appearance similar to platelets in peripheral blood (Figure 108.4f) (see Chapter 75).

The monocytic series comprises a small percentage of cells in normal marrow. Mature monocytes/macrophages

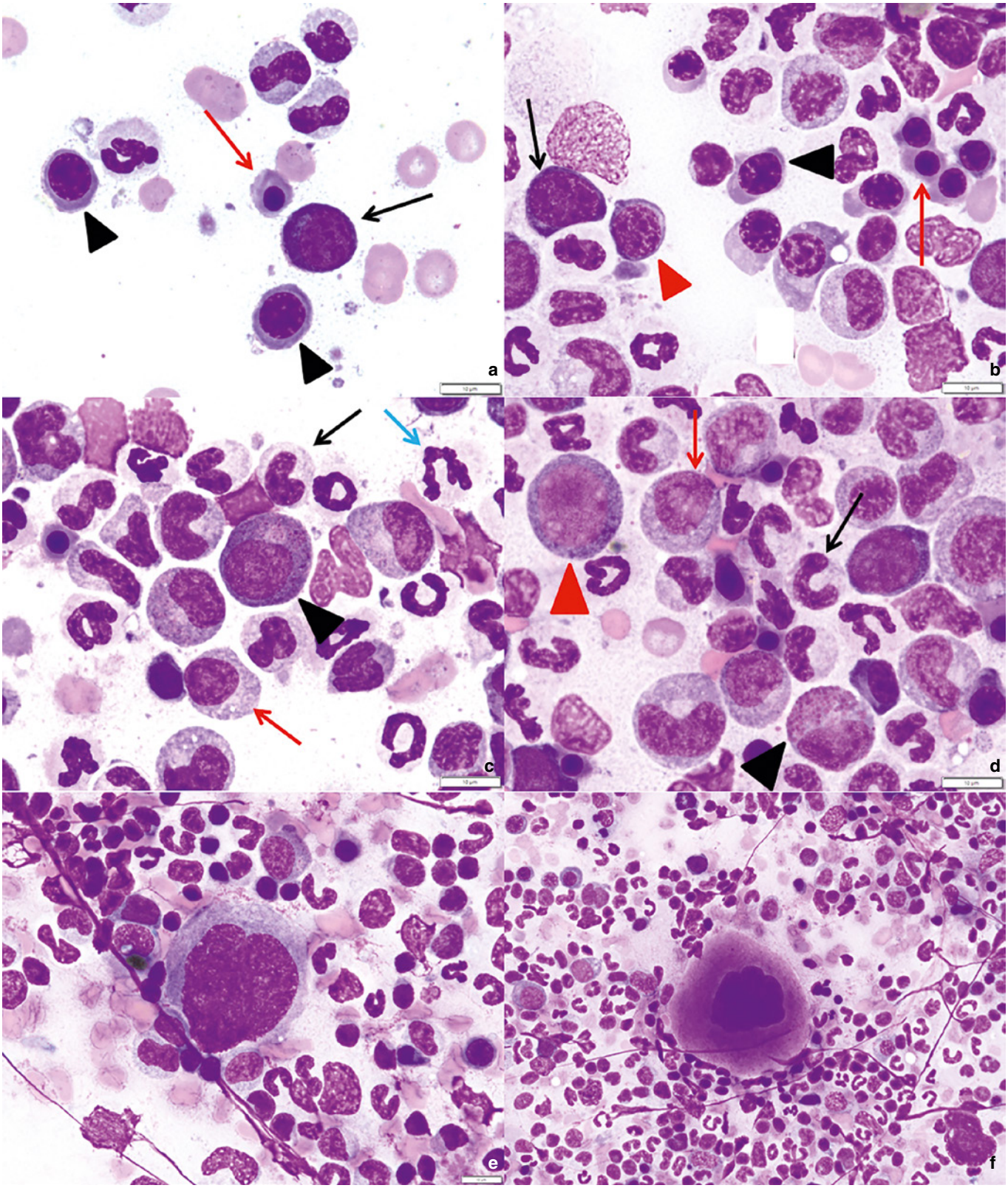


FIGURE 108.4 Morphology of canine bone marrow. Wright–Giemsa stain. (a) Several erythroid precursors including a rubriblast with a round nucleus, nucleoli, and deeply basophilic cytoplasm (black arrow), two rubricytes which are smaller with more condensed chromatin (black arrowheads), and one metarubricyte that is even smaller with a pyknotic nucleus and maturing, lightly polychromatophilic cytoplasm (red arrow); (b) Several RBC precursors including a rubriblast (black arrow), a prorubricyte which is smaller and has no nucleoli (red arrowhead), rubricytes (black arrowhead), and metarubricytes (red arrow); (c) Several myeloid precursors including a progranulocyte with magenta-staining primary granules (black arrowhead), a myelocyte which is smaller with a round nucleus (red arrow), a metamyelocyte with a slightly pinched nucleus (black arrow), and several segmented neutrophils (blue arrow); (d) Several myeloid precursors including a myeloblast with nucleoli and basophilic cytoplasm (red arrowhead), myelocytes (red arrow), an eosinophilic myelocyte with eosinophilic secondary granules (black arrowhead), and a band neutrophil (black arrow); (e) A large immature megakaryocyte with blue cytoplasm and a high N:C ratio; (f) A mature megakaryocyte with abundant purple, granular cytoplasm.

TABLE 108.2 Differential cell counts from bone marrow aspirates from 57 normal dogs.

Cell type		Mean ± SD (%)
Myeloid series		
	Myeloblasts and Promyelocytes	2.3 ± 1.28
	Myelocytes	2.1 ± 0.96
	Metamyelocytes	7.1 ± 3.16
	Neutrophils (bands and segmented)	32.5 ± 8.71
	Eosinophils (bands and segmented)	2 ± 1.32
Total myeloid series		45.3 ± 9.91
Erythroid series		
	Rubriblasts	0.7 ± 0.84
	Prorubricytes	2.1 ± 1.28
	Rubricytes	19.9 ± 5.87
	Metarubricytes	22.3 ± 8
Total erythroid series		46 ± 10.41
Myleoid:Erythroid ratio		1 ± 0.49
Other cells		
	Lymphocytes	6.3 ± 4.08
	Plasma cells	0.6 ± 1.13
	Macrophages/ Monocytes	0.7 ± 1.36

Source: Based on Turinelli et al.[55]

resemble those in peripheral blood. Monoblasts and promonocytes are difficult to clearly differentiate from granulocytic precursors. Their nuclear shape is more irregular and they lack the primary granules seen in promyelocytes (see Chapter 48).Mature lymphocytes may be present in moderate numbers and low numbers of plasma cells can be seen admixed among the hematopoietic precursors. Occasionally osteoclasts, osteoblasts, and stromal cells can be seen.

The relative proportion of hematopoietic cells found in bone marrow samples from normal dogs is given in Table 108.2.[55] The M:E ratio is reported as being between 0.7:1 and 2.5:1 (see Chapter 141). Mild variation in the M:E ratio may be found in samples from different anatomic locations.[12] Samples obtained from the iliac crest had a slightly higher M:E ratio than humeral samples collected in the same dog.[17] Repeated sampling of bone marrow at weekly intervals from the same anatomic location seemed to have little effect on differential cell counts.[37] However, repeated sampling within the same day may result in artifactually hypocellular samples. Neonatal dogs have a significantly increased lymphocyte percentage in bone marrow samples compared to adults.[15] Mean lymphocyte percentages peaked at 54% by 21 days of age, decreasing to 17% by 56 days of age in this older study. Bone marrow samples from normal adult dogs generally have less than 10% lymphocytes.

HEMATOLOGIC DISEASE

Changes in the Erythron

Anemia can be classified as regenerative or nonregenerative based on evaluation of polychromasia on a blood film or reticulocyte enumeration (see Chapter 25). Nonregenerative anemias are generally caused by decreased bone marrow erythropoiesis. Differential diagnoses for persistent nonregenerative anemia include inflammation, chronic disease, chronic renal disease, iron deficiency, or inherent bone marrow disease including immune-mediated disease, infectious disease, neoplasia, myelotoxicity, or myelophthisis (see Chapters 27, 33–35, 38–40, and 65–67).

Regenerative anemias in the dog are caused by either hemorrhage or hemolysis. These mechanisms lead to bone marrow stimulation and increased erythropoiesis, releasing immature erythroid stages into circulation which can be detected as polychromasia and reticulocytosis. Nucleated RBCs may also be released and quantified. Hemolytic anemia may be induced by a variety of etiologies including immune-mediated destruction (primary or secondary immune-mediated hemolytic anemia), erythrocytic parasites, oxidative damage, hereditary enzyme defects, metabolic derangements (e.g., hypoosmolality and hypophosphatemia), and diseases leading to intravascular RBC trauma (e.g., heartworm disease) (see Chapters 29, 30, and 32–35).

Changes in the Leukon

Three major leukogram patterns exist in the canine; physiologic, stress, and inflammatory (see Section IV). A physiologic leukogram is mediated by epinephrine (released during strenuous exercise, fear, or marked excitement) and induces leukocytosis, mild mature neutrophilia, and lymphocytosis. Epinephrine causes demargination of neutrophils, shifting them from the marginal to the circulating pool, resulting in neutrophilia. Lymphocytosis is due to a blockade preventing lymphocytes from entering lymph nodes and mobilization of lymphocytes from the thoracic duct. Monocyte and eosinophil counts may increase slightly. This response is more common in puppies than in adult dogs. When it does occur, the effects are immediate and transient with values returning to baseline within 30 minutes.[29,32]

A stress leukogram is caused by endogenous or exogenous corticosteroids, released during physiologic stress/illness, pain response, hyperadrenocorticism, or administered as therapy. Leukocytosis, mature neutrophilia, lymphopenia, monocytosis, and eosinopenia are induced in the dog. This effect is seen within 4–8 hours and resolution may occur 24 hours to several days after the event, depending on the duration of corticosteroid exposure. Corticosteroids cause demargination of

circulating neutrophils, release of storage pool neutrophils from the bone marrow, and decreased emigration of neutrophils to tissues leading to neutrophilia. Lymphopenia is due to redistribution of circulating lymphocytes and long-term steroid use can lead to lympholysis. Monocytosis is due to mobilization of marginated monocytes. Eosinopenia results from eosinophil sequestration in tissues and decreased release from the bone marrow.[29,32]

Inflammatory leukograms are caused by increased proinflammatory cytokine production due to stimuli, such as infectious or immune-mediated disease, tissue trauma/necrosis, or neoplasia (see Chapters 17–18). The time at which the blood is examined in relation to the onset of the inflammatory stimulus will affect interpretation. With peracute, overwhelming inflammation, as is seen with endotoxemia or sepsis, neutropenia will develop within 1–3 hours due to neutrophil margination, shortened neutrophil half-life, and increased neutrophil emigration to tissues. Acute inflammation (6–8 hours later) is characterized by neutrophilia with or without a left shift due to release of storage pool neutrophils (segmented and band neutrophils) from the bone marrow. Toxic changes may be present. A concurrent stress response is common causing lymphopenia and monocytosis in the dog. Chronic inflammation (days to weeks later) is characterized by a neutrophilia, often without a left shift, as the granulopoiesis has increased and there are adequate mature neutrophils produced and released to compensate utilization and sequestration. Within the bone marrow, there should be expansion of the proliferative, mature, and storage pools in chronic inflammation[29,32] (see Chapters 45 and 49–51).

With certain inflammatory conditions, there can be a severe neutrophilia (>50,000 WBC/μL), termed a leukemoid response, as this degree of neutrophilia mimics chronic granulocytic leukemias. The neutrophilia can be mature, but often there is an orderly left shift that may extend to myelocytes or promyelocytes. Toxic change may be present. Etiologies include localized pyogenic infections (pyometra, peritonitis, internal abscess), immune-mediated disease (immune-mediated hemolytic anemia), *Hepatozoon canis* infection, leukocyte adhesion deficiency, pancreatitis, bile peritonitis, and paraneoplastic reactions (renal tubular carcinoma, metastatic fibrosarcoma, metastatic pulmonary carcinoma, and rectal adenomatous polyp).[31,8,52,51,59]

Mechanisms of neutrophilia include neutrophil pool redistribution (e.g., physiologic or stress leukograms) or increased production (e.g., inflammation). Inflammation is one of the most frequent causes of neutrophilia. Other causes include physiologic or stress responses, hemolysis, hemorrhage, immune-mediated disease, and leukocyte adhesion deficiency. Mechanisms of neutropenia include redistribution from the circulating to marginating pool (e.g., endotoxemia, anaphylaxis), increased utilization or destruction in peripheral tissues (e.g., acute, severe inflammation often due to pyogenic infections, or immune-mediated destruction), or decreased production by the bone marrow. Decreased bone marrow production of neutrophils may be due to irradiation and chemotherapeutics, estrogen toxicity (endogenous or exogenous), parvoviral infection, idiosyncratic drug reactions, chronic ehrlichiosis, myelophthisis (myelonecrosis, myelofibrosis, osteopetrosis, neoplasia), and cyclic hematopoiesis of gray collies[29] (see Chapters 15–18 and 44).

Hypersegmented neutrophils have nuclei with more than five nuclear lobes. Hypersegmentation is often an aging change associated with prolonged neutrophil transit in the peripheral blood due to endogenous or exogenous corticosteroids. Hypersegmentation may also be caused by inherited (e.g., Poodles with congenital macrocytosis) and neoplastic (e.g., myelodysplastic syndrome and granulocytic leukemia) etiologies (see Chapters 65, 67, and 68).

Neutrophil hyposegmentation is often due to inflammatory left shift. Pelger–Huët anomaly, caused by a defect in the lamin B receptor gene, is a benign condition characterized by granulocyte hyposegmentation where neutrophils, eosinophils, and basophils have a band to peanut-shaped nucleus. Eosinophils can be used to identify the anomaly, if banded neutrophils due inflammation are present in the sample. The neutrophils have mature condensed chromatin and normal function. It has been identified in several breeds of dogs[29] (see Chapters 44 and 146).

Toxic changes in canine neutrophil cytoplasm include basophilia, Döhle bodies, vacuolation, toxic granulation, and gigantism. These are associated with proinflammatory cytokines, exposure to infectious agents, and maturational defects secondary to increased granulopoiesis.

Mild lymphocytosis may occur as part of the physiologic, adrenaline-induced leukogram or elicited by antigen during vaccinal response. Differential diagnoses include persistent antigenic stimulation in chronic inflammatory and infectious disease, especially viral and autoimmune disease, as well as lymphoid neoplasia. If the neoplastic population is composed of small mature lymphocytes (chronic lymphocytic leukemia or small cell lymphoma), further diagnostics, including flow cytometry or PCR for antigen receptor rearrangement (PARR), may be required to distinguish neoplastic from non-neoplastic lymphocytosis (see Chapters 56 and 65).

Lymphopenia is most commonly caused by glucocorticoids. Differential diagnoses include viral infections such as canine distemper and parvovirus, which lead to lymphocyte depletion and destruction, loss of lymph-rich fluid (protein-losing enteropathy, granulomatous enteritis, lymphangiectasia, chylothorax), and, rarely, congenital deficiencies of lymphocytes can occur (see Chapters 18, 55, and 146).

Monocytosis is most often either steroid-induced and/or induced by proinflammatory cytokines. Monocytopenia is not of clinical significance, as canine monocyte RIs approach zero (see Chapters 48 and 49).

The most common cause of eosinophilia in dogs is parasitism with a more pronounced response occurring with migrating endoparasites and heartworm. Differential diagnoses for eosinophilia include hypersensitivity,

fungal/algal disease, paraneoplastic disease (T-cell carcinoma, mast cell tumor), or primary neoplasia (eosinophilic leukemia, hypereosinophilic disease). Eosinopenia is not typically of clinical significance as clinically normal dogs can have eosinophil counts of 0/μL. However, when eosinophils approach zero, Addison's disease should be considered if clinical signs are supportive (see Chapter 46).

Basophilia is caused by similar mechanisms as eosinophilia including endoparasitism/ectoparasitism, hypersensitivity, paraneoplastic responses, and primary neoplasia, and the two are often found concurrently. Basophilic leukemia is rare, but has been reported. Basopenia is not of clinical significance as many clinically normal dogs have basophil counts of 0/μL (see Chapter 47).

Infectious Agents

Bacteria can occasionally be seen in neutrophil or monocyte cytoplasm indicating septicemia/bacteremia, typically resulting in degenerative changes (Figure 108.3j). Canine distemper virus is characterized by variably sized, amorphous, homogenous, magenta to basophilic cytoplasmic inclusions in both RBCs and WBCs (Figure 108.3k). These may be most easily visualized using Diff-Quik stain. *Hepatozoon canis* and *H. americanum* gametocytes are poorly staining to lightly basophilic and ovoid to rectangular in shape (Figure 108.3l). They can be present within neutrophils and monocytes, however, especially with *H. americanum*, the parasitemia can be low and organisms are often not identified. *Histoplasma capsulatum* can be identified within neutrophils and monocytes as a small, round to oval yeast with an eccentrically located crescent-shaped nucleus and a thin nonstaining capsule (Figure 108.3m). *Leishmania sp*. amastigotes present within neutrophils are small oval organisms with an oval nucleus and a rod-shaped, red kinetoplast (not pictured). Ehrlichia morulae can be present within neutrophil and monocyte cytoplasm. Species are morphologically indistinguishable requiring PCR for species identification (Figure 108.3n).

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CHAPTER 109

Hematology of Cats

DEANNA M. W. SCHAEFER

Erythron
  Normal Structure, Morphology, and Function
  Comparative Physiology
Leukon
Platelets
Blood and Bone Marrow Collection
Reference Intervals
Bone Marrow
Interpretation of the Erythron in Disease
Interpretation of the Leukon in Disease Response
  Neoplasia
  Inherited Changes in Leukocyte Morphology
Hemoparasites
Retrovirus-Associated Hematologic Abnormalities

Acronyms and Abbreviations

ALL, acute lymphoid leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; CTAD, citrate theophylline adenosine dipyridamole; DAT, direct antiglobulin test; FeLV, feline leukemia virus; FIV, feline immunodeficiency virus; IMHA, immune-mediated hemolytic anemia; MDS, myelodysplastic syndrome; MPV, mean platelet volume; PARR, PCR for antigen receptor rearrangements; SD, standard deviation; URL, upper reference limit; 2,3-DPG, 2,3-diphosphoglycerate.

ERYTHRON

Normal Structure, Morphology, and Function

Feline RBCs are anucleate, biconcave, and discoid, similar to those from other domestic mammals. Compared to dogs, domestic cat (*Felis catus domesticus*) erythrocytes have a shorter circulating life span (66–72 days)[6], are smaller (5.5–6.5 μm diameter),[19,27] have less central pallor, more commonly display rouleaux,[27] and have different surface antigens (see Chapters 96 and 99).

Feline Hgb contains eight reactive sulfhydryl groups, while most other species have two to four.[13,12,43] Sulfhydryl groups are readily oxidized, making feline Hgb particularly susceptible to oxidant damage manifesting as Heinz body formation. As a result, low numbers of Heinz bodies (<5% of erythrocytes) can be seen in some healthy cats.[13,12,27] The nonsinusoidal feline spleen is inefficient at removing Heinz-body-laden RBCs from circulation, so anemia may not develop in all cases of RBC oxidation.[12,13]

Cats are unique in that two types of reticulocytes can be identified on blood smears stained with a supravital stain such as new methylene blue: aggregate reticulocytes and punctate reticulocytes. Aggregate reticulocytes form shortly after extrusion of the nucleus. They have large aggregates of precipitated rRNA when stained with new methylene blue and appear polychromatophilic (blue-purple) when stained with a Romanowsky stain (Figure 109.1). Amounts of rRNA diminish as an aggregate reticulocyte matures until it becomes a punctate reticulocyte with only a few small dots of precipitated rRNA on new methylene blue staining and no detectable polychromasia on Romanowsky staining.[27] Maturation of punctate reticulocytes to mature erythrocytes occurs more slowly in cats than in most other species. Because they persist longer in circulation, punctate reticulocytes do not reflect a current increase in erythropoiesis in the marrow. Therefore, only aggregate reticulocytes should be included in the reticulocyte count that is used to determine the current degree of regeneration in cats.[27]

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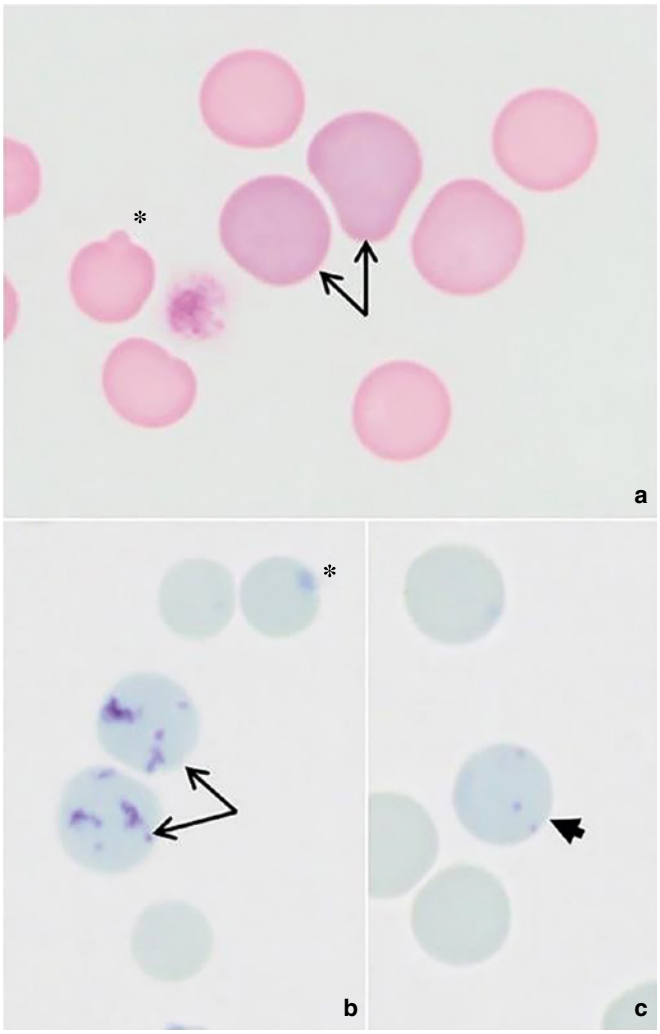


FIGURE 109.1 Reticulocytes and Heinz bodies from a cat with oxidant hemolytic anemia. (a) Polychromatophilic RBCs (arrows) and a pale pink Heinz body (*), Wright stain, 100× objective; (b) and (c) Aggregate reticulocytes with numerous large aggregates of dark blue precipitated rRNA (arrows), punctate reticulocyte with only a few very small dots of blue precipitated rRNA (arrow head), and an aqua blue Heinz body (*), new methylene blue stain, 100× objective.

Distinction of aggregate and punctate reticulocytes in cats is only relevant if reticulocytes are enumerated manually by evaluation of a blood smear stained with new methylene blue. The reticulocyte counts produced by automated hematology analyzers typically only include aggregate reticulocytes[27] (see Chapter 23).

Comparative Physiology

Mammalian Hgb oxygen affinity can be affected by multiple factors including the organic phosphate 2,3-diphosphoglycerate (2,3-DPG). In most mammals, the lower oxygen environment in non-lung tissue promotes reversible binding of 2,3-DPG to β-globin chains. This binding causes a conformational change that decreases oxygen affinity of Hgb, promoting oxygen delivery to tissues.[7] Cat RBCs, however, have low concentrations of 2,3-DPG, and β-chain structural differences cause their Hgb to have diminished responsiveness to 2,3-DPG. Instead, feline Hgb has intrinsically lower oxygen affinity than Hgb from other mammals, allowing adequate oxygen delivery to tissues without the requirement of 2,3-DPG.[7] It has not been established if this has any adaptive significance for cats (see Chapter 21).

LEUKON

The morphology and structure of feline leukocytes are similar to that of other domestic mammals. A compilation of segmented neutrophils, lymphocytes, monocytes, and eosinophils from domestic cats and from seven nondomestic felid species is provided (Figure 109.2). The microscopic appearance and cell size of neutrophils, lymphocytes, and monocytes are similar between domestic and nondomestic cats. Of note, healthy cats may sometimes have Döhle bodies within a few neutrophils or have neutrophil cytoplasm that is faintly blue rather than clear or light pink (Figure 109.2).[28] Therefore, the microscopist should take care in using these findings to define toxic change in cats.

Eosinophil morphology is similar between domestic cats and some nondomestic felids; however, the granules are larger and/or rounder in other species such as the lion, tiger, and leopard. Compared to other domestic species, feline basophil granules are often more elongated and lighter purple (Figure 109.3b,c). In some cases, the margins of the basophil granules can be somewhat indistinct, and the cells may resemble monocytes. On close inspection, though, the granules of the basophils are evident.

PLATELETS

As in other mammals, feline platelets are small anucleate granular cytoplasmic fragments of megakaryocytes. However, cat platelets are commonly larger and more variably sized than those from other domestic species (Figure 109.4). Platelets from dogs are typically 2–4 μm in diameter, with ≤5% of platelets larger than 4 μm. In contrast, cat platelets can be up to 6 μm or larger, with 10–25% of platelets being larger than 4 μm.[5] The β1-tubulin gene in Felidae has two sequence differences which may impact platelet microtubule assembly and contribute to larger and more variably sized feline platelets[5] (see Chapters 76 and 77).

There are important analytical and preanalytical considerations for automated platelet counts in cats. Because there is often overlap in size between feline erythrocytes and platelets, platelets counts obtained using impedance methodology may be inaccurate.[48,28] Additionally, platelet clumping is a frequent preanalytical problem in EDTA-anticoagulated blood samples from cats, and can lead to falsely decreased automated platelet counts.[28,48]

One study concluded that feline blood samples with marked platelet clumping (more than 10 large platelet

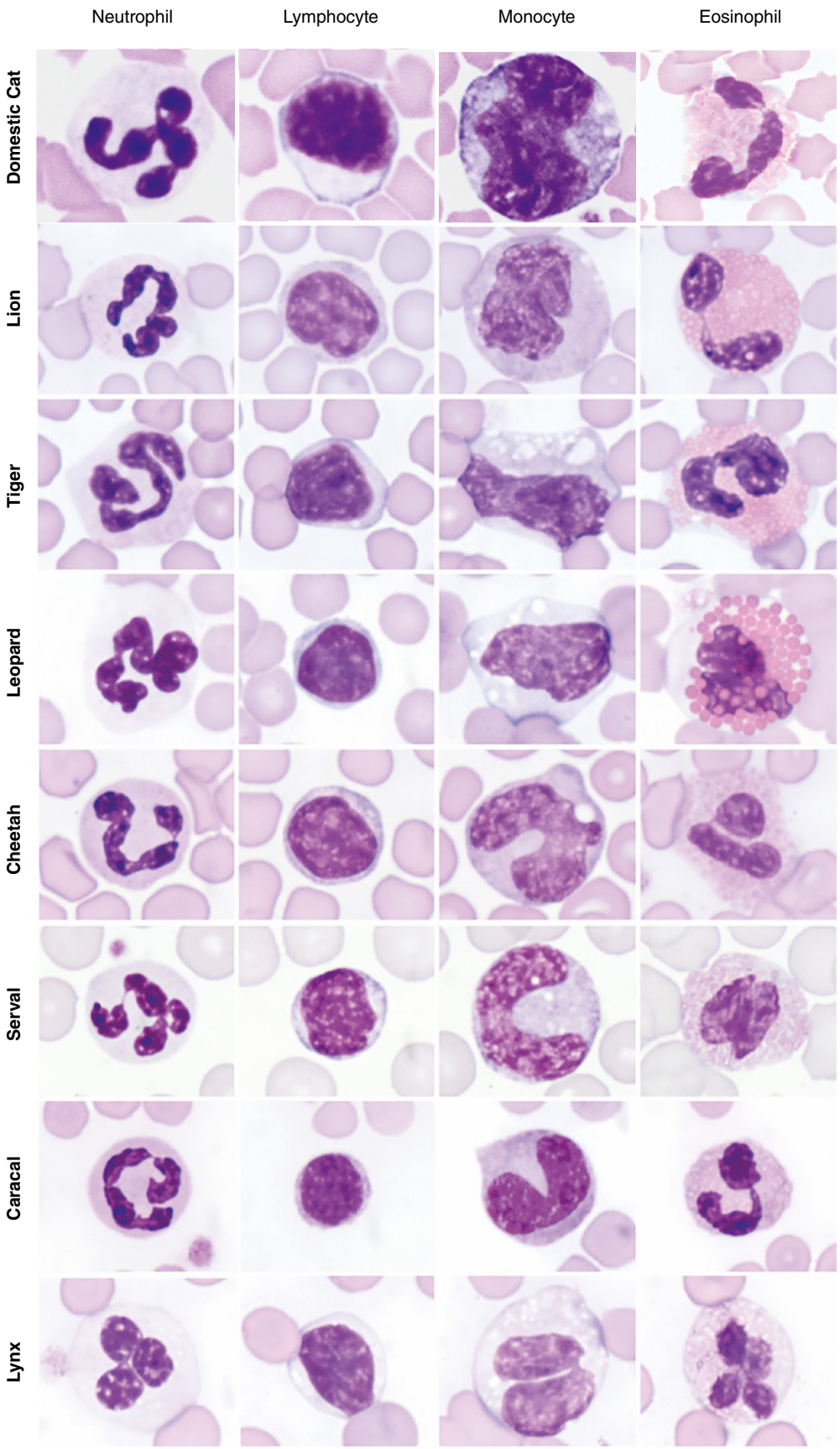


FIGURE 109.2 Leukocytes from selected feline species—the domestic cat (*Felis catus domesticus*) and from seven species of nondomestic felids: lion (*Panthera leo*), tiger (*Panthera tigris*), leopard (*Panthera pardis*), cheetah (*Acinonyx jubatus*), serval (*Leptailurus serval*), caracal (Caracal caracal), and lynx (*Lynx canadensis*). The leukocyte morphology for neutrophils, lymphocytes, and monocytes is similar between domestic and nondomestic cats. Eosinophil granules in some nondomestic felids in the Panthera genus, including lions, tigers, and leopards, are larger and/or more round than those from domestic cats. Wright stain, 100× objective.

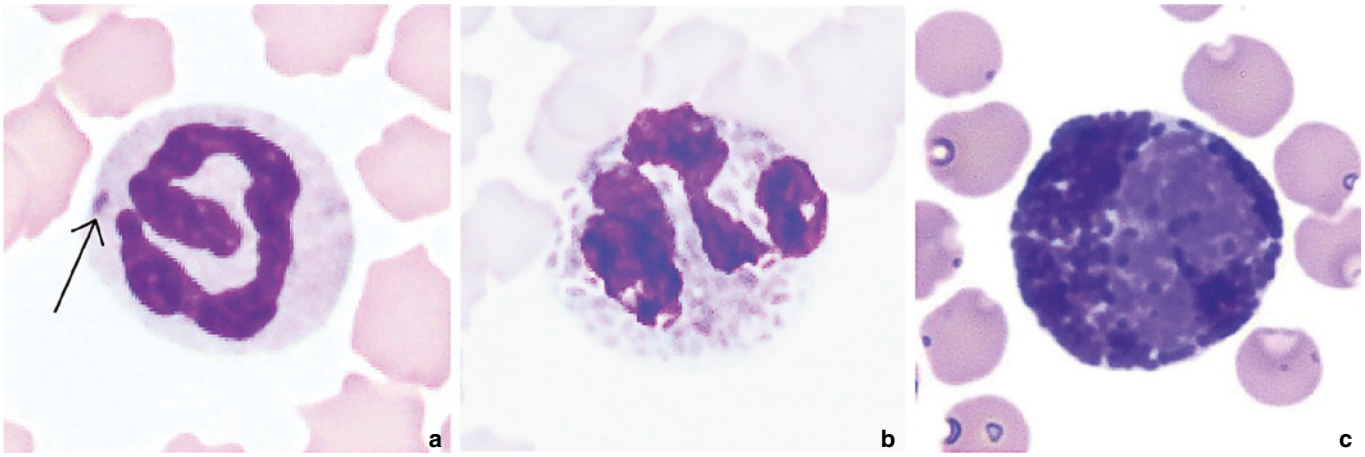


FIGURE 109.3 Neutrophil and basophil from domestic cat. (a) A segmented neutrophil from a healthy cat, which has a Döhle body (arrow) and faintly blue cytoplasm. Neutrophils from some healthy cats may have cytoplasmic features that mimic toxic change; (b) A basophil from a cat, with numerous lavender cytoplasmic granules; (c) A basophil from a horse, which by comparison has much darker purple cytoplasmic granules compared to the cat basophil. Wright stain, 100× objective.

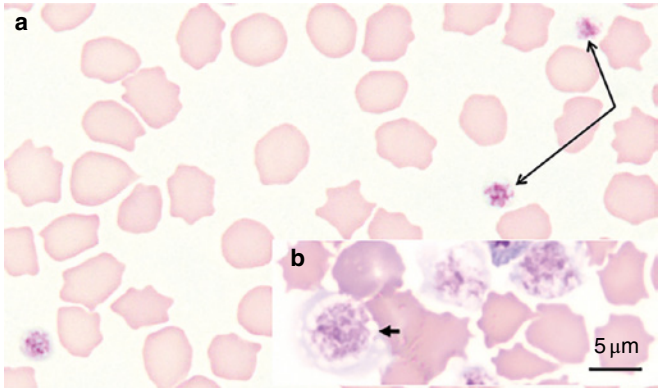


FIGURE 109.4 Feline platelets. (a) Smaller platelets, two of which are indicated by arrows; (b) Large platelets, one indicated by the arrowhead. Cats can commonly have large platelets, with 10–25% of platelets being larger than 4 µm in diameter.

aggregates at the feathered edge of the blood smear) are most likely to have artifactually decreased automated platelet counts.[48] In such cases, it is advisable not to report the automated platelet count, but rather include a comment on the CBC report that "platelets are clumped but appear adequate." If only small aggregates or if only a few large aggregates are noted at the feathered edge, then the error on the automated platelet count may not be significant.[48] The laboratory in such instances may choose to report the automated platelet count, but add a comment that "the automated platelet count may be falsely decreased due to clumping, but is a reasonable estimated minimum."

BLOOD AND BONE MARROW COLLECTION

Blood for hematologic evaluation from cats is commonly collected from a jugular, cephalic, or saphenous vein into a collection tube containing EDTA anticoagulant. Literature documenting differences in results due to differences in venous sites has been found in other species, but could not be found for felids. Collection tube additives such as prostaglandin E1, iloprost, or citrate theophylline adenosine dipyridamole (CTAD) may inhibit platelet aggregation and at least modestly reduce platelet clumping in feline blood samples, but at this time their use is largely restricted to research settings.[21,39,48]

The technique for marrow aspirate collection is similar in cats and dogs, and typical sites include the greater tubercle of the proximal humerus, iliac crest, or trochanteric fossa of the femur[8] (see Chapter 141).

REFERENCE INTERVALS

Hematology reference intervals (RIs) for adult cats using three modern hematology analyzers are provided in Table 109.1. Of note, the RI for reticulocytes was significantly lower on the ProCyte Dx than on the Sysmex XT-2000iV, even though these results were obtained using the same sample group.[21] Since these two analyzers use similar technology, the authors posit that the disparities may be due to differences in manufacturer calibration or software version. The reticulocyte RI established using the ADVIA 120 at the University of Georgia was also lower than that reported for the Sysmex XT-2000iV, but it is unclear if this is due to differences in analytic method or in the reference sample group. Older published studies of hematology results in healthy kittens were reviewed and found to have low sample sizes or use outdated methods.[1,16] These are not reported in this chapter.

BONE MARROW

Expected results from feline bone marrow aspirates are provided in Table 109.2. These values are adapted from multiple sources and should be used as guidelines only. Integrated interpretation of marrow findings should incorporate CBC results and clinical information from the patient.

TABLE 109.1 Hematology reference intervals for adult cats using whole blood collected into EDTA.

Reference	University of Georgia, CVM	Granat (2014)[21]	Granat (2014)[21]
Hematology analyzer	ADVIA 120	Sysmex XT-2000iV	ProCyte Dx
Sample size	120	81	81
Age range (years)	1–14	0.5–9.6	0.5–9.6
Statistical method	Nonparametric	Nonparametric	Nonparametric
Hematocrit (%)	33.6–49	29–51	30–54
RBC count (× 10^6/μL)	7.7–10.9	6.6–10.8 (O) 6.7–11.2 (I)	6.7–11.2
Hgb (g/dL)	11.3–16.2	9.9–16.6	9.4–16.1
MCV (fL)	39–52	34.4–49.3	37.3–52.6
MCHC (g/dL)	32–35	32.5–39.2	28.8–34.9
MCH (pg)	13–17.6	12.3–16.4	12–15.8
RDW (%)	13.7–18.4	19.7–29.3	20.2–30.2
RDW (fL)	NR	24.9–40.6	NR
WBC count (× 10^3/μL)	4.8–15.3	4–15.2	4–15.2
Leukocyte differential method	Manual	Automated	Automated
Neutrophil (× 10^3/μL)	1.9–10	1.7–8.8	1.7–8.8
Lymphocyte (× 10^3/μL)	0.8–6.4	1.2–10.2	1.2–10.2
Monocyte (× 10^3/μL)	0–0.6	0.1–0.6	0.1–0.6
Eosinophil (× 10^3/μL)	0–1.5	0.1–1.2	0.1–1.2
Basophil (× 10^3/μL)	0–0.1	NR	NR
Platelet count (× 10^3/μL)	206–465	87–610 (O) 33–516 (I)	87–610
MPV (fL)	8.9–23.3	NR	NR
Reticulocyte count (× 10^3/μL)	6–52.9	19.1–107.1	3.8–48.6
MCVr (fL)	50.5–72.4	NR	NR
CHr (pg)	13.3–19.5	NR	NR

CHr, reticulocyte hemoglobin content; I, impedance measurement; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MCVr, mean cell volume of reticulocytes; MPV, mean platelet volume; NR, not reported; O, optical measurement; RDW, red cell distribution width.

TABLE 109.2 Proposed guidelines for bone marrow cytology in healthy cats, adapted from multiple references.[19,31,26,52]

Particle cellularity (nucleated cells:fat)	25–75%
Myeloid:erythroid ratio	1.2–2.2
Lymphocyte (%)	<10 (<20 possible)
Plasma cell (%)	<2
Macrophage (%)	<1
Stainable iron deposits	Absent

Sources: Gilmore et al.[19]; Jain[31]; Harvey[26]; Haddad et al.[23]

Marrow samples from healthy cats can have a higher percentage of lymphocytes compared to dog marrow. Small lymphocytes comprise <10% of marrow nucleated cells in most cats, but occasional healthy cats may have up to 20% lymphocytes. Histologically, the lymphocytes may sometimes have follicular or nodular arrangements, particularly in cats with non-neoplastic causes of marrow lymphocytosis such as immune-mediated and infectious diseases.[23,52] Also in contrast to dogs, marrow from cats normally lacks microscopically detectable iron deposits. Marrow iron stores are typically only visible in cats with hemolytic anemia, anemia of inflammatory disease, dyserythropoiesis, or after blood transfusions.[10,23,26,45]

INTERPRETATION OF THE ERYTHRON IN DISEASE

The mechanisms for anemia in cats are the same as in other species: decreased production, hemorrhage, or hemolysis. Anemia is categorized as regenerative or nonregenerative based on the absolute reticulocyte count (Table 109.3). The causative mechanism for regenerative anemia is hemorrhage or hemolysis.

Feline Hgb structure makes RBCs particularly susceptible to oxidant injury, leading to Heinz bodies (Figure 109.3) and possibly methemoglobinemia and/or eccentrocytes. Eccentrocytes and erythrocytes with Heinz bodies have reduced deformability, which

TABLE 109.3 Classifying the regenerative response in anemic cats using absolute reticulocyte counts.

Regeneration	Reticulocyte count (× $10^3/\mu L$)
Nonregenerative	Within or below RI
Weakly to moderately regenerative[a]	URL to 190 × $10^3/\mu L$
Strongly regenerative[a]	≥ 200 × $10^3/\mu L$

Source: [a]Adapted from Korman et al.[33]
RI = reference interval; URL = upper reference limit.

contribute to a shortened RBC life span of 20–60%[13] (see Chapter 32). In some cats, ghost cells can be identified that appear as the outline of a RBC membrane with a visible attached Heinz body.[13] Hgb oxidation may occur in cats exposed to various oxidizing compounds in drugs or food, including acetaminophen, onions, propylene glycol, topical benzocaine, phenazopyridine, phenylhydrazine, methionine, and propofol.[2,13,15,27,29,30,50] Not all oxidants cause overt acute hemolytic anemia in cats, but they may shorten RBC survival time and exacerbate anemia caused by concurrent disease.

Heinz bodies are commonly also identified in cats with certain diseases that promote oxidation, and may be termed endogenous Heinz bodies. Diseases that are associated with erythrocyte oxidation and endogenous Heinz bodies in cats include diabetes mellitus (particularly when there is ketoacidosis), lymphoma, hyperthyroidism, and inflammatory disease.[11,12,53] Cats with endogenous Heinz bodies due to disease typically have low-normal to decreased erythron mass. The average PCV in cats with endogenous Heinz bodies was 28% and 30% in two studies.[11,12]

Immune-mediated anemia occurs in cats, although less frequently than in dogs, and may be idiopathic or secondary to infectious or inflammatory diseases (e.g., feline leukemia virus (FeLV), hemotropic mycoplasma, pancreatitis, cholangitis), neoplasia (e.g., lymphoma, leukemia), or drugs (e.g., propylthiouracil, methimazole)[4,45] (see Chapter 36). There is a wide age range for affected cats, but the majority are young (<3–4 years old).[4,45] As opposed to dogs, where immune-mediated anemia is reported more often in females, sex predilection has not been well-established in cats. Initial reports suggested that the diagnosis is more common in male cats, but a larger retrospective study found no sex predilection.[4,45] In recent studies, the majority of affected cats are negative for FeLV and feline immunodeficiency virus (FIV).[4,45] Some cats may have concurrent neutropenia and/or thrombocytopenia, but it is unclear if these cytopenias are also caused by immune-mediated cell destruction.[4,45]

Spherocytes cannot be identified reliably on feline blood smears, since cat erythrocytes are smaller with less distinct central pallor than dog erythrocytes, making the diagnosis of immune-mediated hemolytic anemia (IMHA) more difficult.[27,45] Diagnosis in cats may be based on detection of RBC agglutination, ghost cells in the absence of Heinz bodies, and/or a positive direct antiglobulin test (DAT) test (direct Coombs test).[27,45]

Precursor-targeted immune-mediated anemia (PIMA) is also recognized in cats and is characterized by anemia that is persistently nonregenerative for at least 5 days and phagocytosis of erythroid precursors in marrow.[4,45] Cases of PIMA in which the immune response targets early erythroid precursors, causing an essential absence of marrow erythroid precursors, are sometimes termed pure red cell aplasia. Coombs testing is negative for the majority of cats with PIMA. However, similar to canine PIMA, some cats may be positive, suggesting the immune target in those cases is also present on mature RBCs[4] (see Chapter 38).

The response to immunosuppressive therapy in cats with IMHA or PIMA is variable, but remission can be achieved. The time to remission may be longer for PIMA than IMHA (up to 42 days in one publication).[45,4] Relapses can occur, sometimes while the cat is still receiving immunosuppressive therapy.[4]

INTERPRETATION OF THE LEUKON IN DISEASE RESPONSE

Catecholamine-induced leukogram changes, termed physiologic leukocytosis, are more common in cats than many other domestic species. Characteristic features are a lymphocytosis and/or mature neutrophilia. The lymphocytosis is caused by altered lymphocyte distribution between blood, lymphatics, and lymphoid tissue, and the neutrophilia is induced by shifting from the neutrophil marginating pool to circulating pool.

The magnitude of the lymphocytosis and neutrophilia is usually mild to moderate, with the lymphocyte count commonly less than 20 × $10^3/\mu L$. However, the degree of lymphocytosis can be higher in cats than in other species with physiologic leukocytosis, rarely exceeding 30 × $10^3/\mu L$.[34] The alterations in leukocyte counts are transient, lasting only about 20–30 minutes after catecholamine release.

Increases in endogenous glucocorticoids or administration of exogenous glucocorticoids can lead to neutrophilia and/or lymphopenia, but monocytosis is infrequent in cats.[34] The lymphopenia is due to altered distribution of lymphocytes between blood and lymphoid tissue or, at high doses, lymphocyte cell death. The neutrophilia is due to increased release from the marrow maturation and storage pool and shifting in the blood from the marginated to circulating neutrophil pool. A neutrophilic left shift is not typically present unless the marrow maturation and storage pool is depleted[34] (see Chapter 43).

Inflammation in cats can cause similar leukogram changes as noted in other species, including a neutrophilic left shift and/or toxic change (see Chapter 45). Interpretation of toxic change in cats is unique in that Döhle bodies are common in cat neutrophils, and may be seen in some apparently healthy cats.[28] Thus, by themselves, Döhle bodies may not indicate toxic change.

Additionally, cytoplasmic granulation is a rare feature of neutrophil toxicity in cats and must be distinguished from the neutrophil granulation anomaly seen in cats breeds such as Birmans.[28]

A mild lymphocytosis can occur uncommonly in cats with certain infectious and inflammatory diseases, including FIV, FeLV, and IMHA.[3,20] In general, however, antigenic stimulation is more likely to result in the detection of reactive lymphocytes on blood smear review than lymphocytosis.

Hypereosinophilia (blood eosinophil count of $>2 \times 10^3/\mu L$) can be seen in a variety of disorders including parasitism and hypersensitivity responses.[35] Concurrent basophilia may or may not be present. In cats, hypereosinophilia is commonly caused by flea allergies, feline asthma, and eosinophilic granuloma complex. Other causes include paraneoplastic disease (e.g., mast cell neoplasia, lymphoma, and rarely carcinoma) and gastrointestinal disease (e.g., feline gastrointestinal eosinophilic sclerosing fibroplasia).[35,36,41] Rare cases of hypereosinophilic syndrome and chronic eosinophilic leukemia are reported in cats[35] (see Chapter 46).

Neoplasia

Circulating neoplastic cells may be identified in cats with leukemia or lymphoma. Chronic lymphocytic leukemia must be distinguished from non-neoplastic causes of lymphocytosis such as epinephrine responses. It is typically a disease of middle-aged to older cats, and the most common immunophenotype reported is CD4+/CD8−/CD3+/CD5+ T cells[9] (see Chapters 65 and 72).

A variety of acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL) subtypes occur in cats and may or may not be associated with retroviral infection.[17,37,47] In contrast to dogs, erythroleukemia (AML M6) is a common subtype of feline AML, and typically is preceded by myelodysplastic syndrome (MDS) with detectable dyserythropoiesis in blood.[17,37] Immunophenotyping may be performed by flow cytometry or immunohistochemistry, and cytochemical staining of blasts may also be helpful in some cases (see Chapter 61). Immunophenotyping of acute leukemia by PCR for antigen receptor rearrangements (PARR) is not advisable, since the false negative rate is reported to be fairly high.[47] Also clonal B or T cell rearrangements have been identified by PARR in humans and dogs with AML,[44] so it is possible this may occur in cats as well (see Chapter 62).

Circulating neoplastic cells can be detected in some cats with lymphoma. This may be particularly common in cats with small intestinal granular lymphocyte lymphoma, where circulating granular lymphocyte numbers occasionally are greater than $200 \times 10^3/\mu L$.[40] These granular lymphocytes may be small and morphologically mature; however, in some cases the cells are large (15–35 μm diameter) and/or have atypically large and coarse (1–2 μm diameter) cytoplasmic granules (Figure 109.5)[40] (see Chapter 65).

MDS is a clonal disorder of hematopoietic stem cells that is associated with peripheral cytopenias, normocellular or hypercellular marrow, intramedullary apoptosis

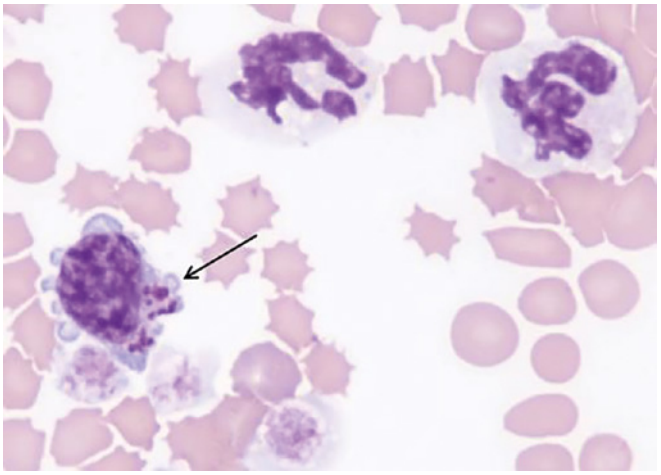


FIGURE 109.5 Circulating neoplastic granular lymphocyte (arrow) from a cat with intestinal lymphoma. In this case, the neoplastic cells were small and morphologically mature, but had atypical large coarse cytoplasmic granules. Wright stain, 100× objective.

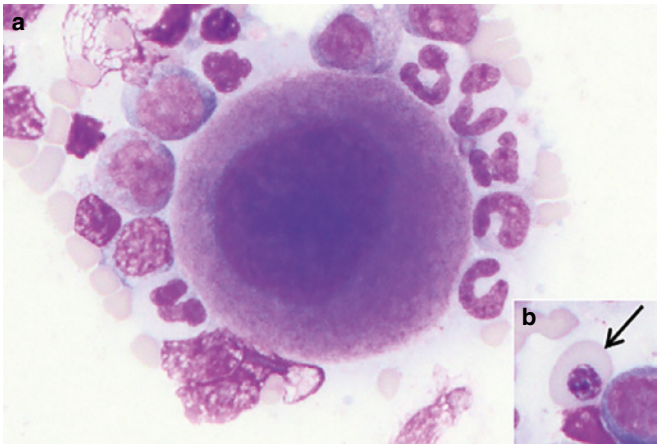


FIGURE 109.6 Bone marrow from a cat with myelodysplastic syndrome (MDS). (a) A dysplastic megakaryocyte with nuclear to cytoplasmic asynchrony evidenced as a hypolobulated (immature) nucleus with abundant pink granular (mature) cytoplasm; (b) A megaloblastic erythroid precursor (arrow), which is macrocytic with mottled, uncondensed chromatin and hemoglobinized cytoplasm. Wright stain, 40× objective.

of hematopoietic precursors, and dysmyelopoiesis (Figure 109.6).[22,32,37,51] Dysplasia may be morphologically evident in one or more hematopoietic lineages. Within megakaryocytes, features of dysplasia may include nuclear to cytoplasmic asynchrony (immature nonlobulated nucleus with mature pink granular cytoplasm, or mature polylobulated nucleus with immature scant dark blue cytoplasm), dwarf megakaryocytes (very small megakaryocytes that are not promegakaryocytes), and/or satellite micronuclei.[22,37,51] Dyserythropoiesis can manifest as nuclear to cytoplasmic asynchrony (immature uncondensed chromatin with mature hemoglobinized cytoplasm), megaloblastic cells (macrocytic cells with nuclear to cytoplasmic asynchrony), multinucleation, fragmented or blebbing nuclei, and other abnormal

nuclear shapes.[22,37,51] Possible dysplastic features in granulocytes include nuclear hyposegmentation or hypersegmentation, increased cell size, abnormal granulation, and bizarre nuclear shapes such as ringed nuclei[22,37,51] (see Chapter 67).

Myelodysplastic syndromes are reported more frequently in cats than dogs, and cats with MDS may subsequently develop AML.[37,51] The prognosis is generally poor. Most, but not all, cats with MDS are infected with FeLV.[22,32,51] MDS must be distinguished from nonclonal dysmyelopoiesis, which can occur secondary to a variety of disorders such as immune-mediated anemia, immune-mediated thrombocytopenia, lymphoma, or drug administration.[22]

Mast cells are identified rarely in feline blood or buffy coat smears.[18,38,42] In dogs, mastocytemia can occur due to a variety of non-neoplastic conditions as well as mast cell neoplasia. By contrast, mastocytemia in cats is almost always indicative of mast cell neoplasia.[38,42] One publication reported two cats with lymphoid neoplasia and one cat with multiorgan hemangiosarcoma that each had one to two mast cells identified per blood smear, so mastocytemia is not completely exclusive to mast cell neoplasia in cats[38] (see Chapter 47).

Inherited Changes in Leukocyte Morphology

Inherited Pelger–Huët anomaly occurs in many species and is rarely reported in cats. Neutrophils from affected cats have hyposegmented nuclei that may appear band-shaped, kidney-shaped, oval, or round. This is distinguished from an inflammatory left shift by detection of mature condensed nuclear chromatin.[28] Detection of nuclear hyposegmentation in both neutrophils and eosinophils can also support a diagnosis of Pelger–Huët. Acquired Pelger–Huët, also known as pseudo-Pelger–Huët, can occur in cats with infectious disease such as FeLV, with myeloid neoplasia, or during chemotherapy[28] (see Chapter 42).

Mucopolysaccharidoses VI and VII are associated with blue to purple cytoplasmic granulation in leukocytes, particularly neutrophils.[28] These granules must be differentiated from toxic granulation and from neutrophil granulation anomaly. Cats with mucopolysaccharidosis VII, GM1 gangliosidosis, GM2 gangliosidosis, α-mannosidosis, and Niemann–Pick can have distinct clear cytoplasmic vacuoles in lymphocytes.[28] Healthy cats of certain breeds, particularly Birmans, but also some Siamese and Himalayan cats, may have neutrophils with red cytoplasmic granules. These are not associated with any clinical disease, but must be morphologically distinguished from lysosomal storage disorders or toxic granulation[28] (see Chapter 44).

Chédiak–Higashi syndrome is a rare inherited disorder caused by a defect in formation of lysosomes and related organelles, causing abnormal granulation in multiple cell types. It has been reported in Persian cats and is associated with large granules in neutrophils and immune dysfunction. Affected cats can also have bleeding tendencies due to platelet granule defects[28,14] (see Chapter 44).

HEMOPARASITES

Three species of hemotropic mycoplasmas have been detected in cats: *Mycoplasma haemofelis* (formerly *Haemobartonella felis*), *Candidatus Mycoplasma haemominutum*, and *Candidatus Mycoplasma turicensis*.[27,46] These epicellular erythrocyte parasites have not yet been cultured, but may be detected by microscopic blood smear examination and/or PCR.[46]

M. haemofelis are small coccoid to ring-shaped organisms measuring about 0.6 μm and may form short chains of three to six organisms (Figure 109.7a). It is reported to be the least prevalent but most pathogenic of the three species and can cause hemolytic anemia which may be severe and life-threatening.[27,46] The number of organisms in blood can fluctuate dramatically within just a few hours, and visual detection of organisms has a low diagnostic sensitivity.[46] Additionally, care must be taken not to misidentify stain precipitate or Howell–Jolly bodies as mycoplasma.

M. haemominutum is about half the size of *M. haemofelis*, so is rarely detected during blood smear examination.[46] *M. turicensis* has only been identified by PCR, and to date there are no reports identifying the organisms microscopically on blood smears.[27,46] *M. haemominutum* and *M. turicensis* are commonly detected by PCR from nonanemic cats, so the pathogenicity of these organisms is questionable. Based on experimental inoculations, they may at least cause a transient mildly decreased HCT during acute infection in some cats or may exacerbate anemia caused by other diseases such as retroviral infections.[46]

Confirmatory PCR testing for feline hemotropic mycoplasmas is widely available and detects the bacterial 16S rRNA gene. Real-time PCR assays have an advantage over conventional PCR in that they may be able to provide information about parasite load. However, it is important to note that a positive PCR result does not define mycoplasma as the etiology of anemia in a patient, particularly for *M. haemominutum* and *M. turicensis*, since nonanemic cats can fairly commonly have positive PCR results.[46]

Cytauxzoon felis is an apicomplexan protozoal parasite of wild and domestic felids, and is endemic in the southern and southeastern United States.[49] Sporadic cases of cytauxzoonosis have also been reported in South America (particularly Brazil) and Europe.[49] The merozoites, termed piroplasms, can be identified on a blood smear within erythrocytes as small (1–2 μm) ring-shaped organisms with a peripheralized blue to purple nucleus (Figure 109.7b).[27,49] Macrophages containing *C. felis* schizonts can be found on cytology or histopathology of various organs or rarely on blood smears (Figure 109.7c).[27,49]

Bobcats are the most common natural host for *C. felis* and generally have subclinical or mild disease. In contrast, the infection was historically thought to be almost universally fatal in domestic cats, causing severe acute hemolytic anemia and organ failure.[49] However, these assumptions are now questioned because of increasing reports of nonfatal infections in domestic cats, as well as occasional fatal or severe cases in nondomestic felids.[49]

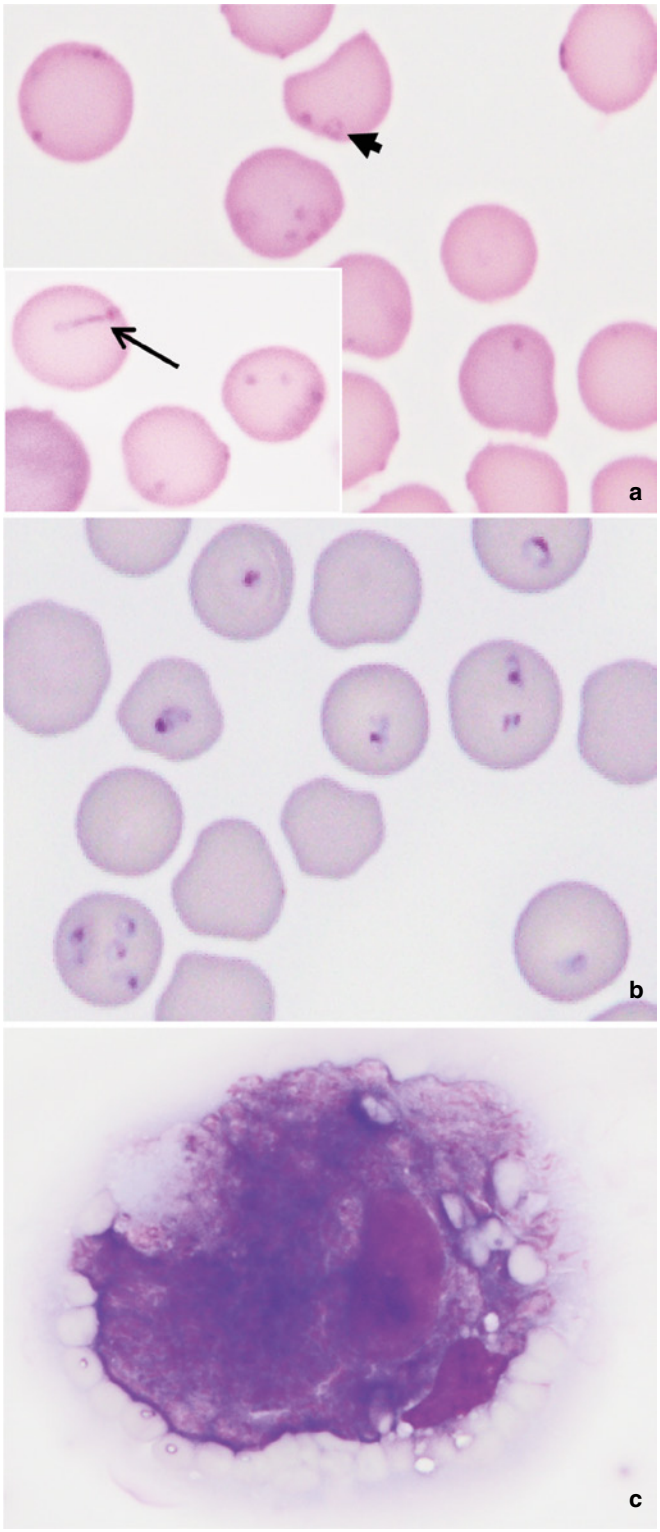


FIGURE 109.7 Feline hemoparasites. (a) Numerous *Mycoplasma haemofelis*, most of which are coccoid. A ring-shaped organism (arrowhead) and short chain of organisms (arrow) are also present; (b) Numerous *Cytauxzoon felis* merozoites, which appear as small ring-shaped organisms that are slightly larger than *M. haemofelis* and have a small peripheralized purple nucleus. Wright stain, 100× objective; (c) Schizont-laden macrophage at the feathered edge of the same blood smear as (b), Wright stain, 50× objective.

These findings suggest that there may be different strains of *C. felis* with variable virulence.[49]

Both small and large Babesia species have been reported in cats. Some species, particularly *Babesia felis*, can lead to severe hemolytic anemia.[27,25] The intraerythrocytic piroplasms in cases of small form Babesia can be morphologically indistinguishable from *C. felis*.[25,49] However, the geographic distribution of feline babesiosis is different, with most cases documented in South Africa.[25,27] Sporadic cases have been reported in domestic and wild felids in other regions.[25]

RETROVIRUS-ASSOCIATED HEMATOLOGIC ABNORMALITIES

Infection with FeLV or FIV can be associated with a variety of hematologic abnormalities in cats[20,24] (see Chapters 53 and 60). Numerous bone marrow disorders can be identified in FeLV-infected cats, including myelodysplasia, leukemia, lymphoma, myelofibrosis, and pure red cell aplasia.[20] These result from incorporation of FeLV provirus into bone marrow hematopoietic and stromal cells, which can alter gene expression, cell maturation, and marrow microenvironment.[24] Cats infected with FIV also have an increased likelihood of developing lymphoma or leukemia, but this is likely an indirect effect of immunosuppression rather than due to intercalation of FIV proviral DNA into host cells.[24]

In a study of cats naturally infected with FeLV or FIV, those with FeLV were more likely than retrovirus-negative cats to have abnormalities identified on CBC, including anemia, thrombocytopenia, neutropenia, and/or lymphocytosis.[20] Mechanisms for these hematologic abnormalities are multifactorial, including decreased marrow production and immune-mediated destruction. Cats with FIV had fewer CBC abnormalities compared to the cats with FeLV, but did have a higher incidence of neutropenia than control cats.[20] Infection with FIV is also associated with decreased numbers of CD4+ T cells in peripheral blood, which likely occurs through multiple mechanisms including decreased production and virus-induced apoptosis.[24]

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CHAPTER 110

Hematology of Equids

KATHLEEN P. FREEMAN, ALISON J. FARR, and ANNALISA BARRELET

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  Anemia
    Hemolytic Anemia in the Horse
    Oxidant-Induced Hemolytic Anemia
  Blood Parasites
    Other Blood Parasites
  Changes in the Leukon
    Neoplasia

Abbreviations and Acronyms

ACTH, adrenocorticotropic hormone; ASVCP, American Society for Veterinary Clinical Pathology; CV_A, analytical coefficient of variation; CV_I, intra-individual coefficient of variation; IRI, individualized reference interval; MPC, mean platelet component; MPV, mean platelet volume; PCDW, platelet component distribution width; RCV, reference change value; SAA, serum amyloid A.

INTRODUCTION

Equine hematologic results can be influenced by preanalytical, analytical, and postanalytical factors.

Preanalytical Factors

The equine spleen is a significant blood storage organ, estimated to store between 6 and 12 L of blood at rest, with the potential to release a large volume of blood with stimulation and catecholamine release, such as excitement or exercise.[24,31,34] The time required for hematologic results to return to resting values depends on the degree of the stimulation, and can vary from 40 minutes to several hours.[16] In contrast, tranquilizers or anesthetics can decrease circulating RBCs because of splenic sequestration.[3]

Venipuncture within 30 seconds of entering the stall in a calmly resting animal is suggested, since longer intervals before collection are more likely to be affected by splenic stimulation.[3,31,34] While this may not always be achievable, standardizing the venipuncture technique is recommended, keeping each horse as calm as possible and collection as rapid as possible. The prandial state should be considered, since the PCV has been reported to increase by 8–15% (e.g., a PCV of 40% may increase to 43–46%) within 1 hour of feeding hay and remain elevated for up to 2 hours.[3,18]

The duration between sample collection and analysis and the temperature at which the specimen is maintained prior to analysis may impact results. For ambulatory equine clinicians, there may be a delay in analysis because the clinician may be some distance from the clinic where specimens are analyzed or prepared for delivery to a commercial laboratory. It was found that delayed analysis (24–48 hours) resulted in minimal change in the WBC, RBC, Hgb, and Hct using the ADVIA 120 hematology analyzer.[7] However, increased numbers of normocytic, hypochromic RBCs, increased numbers of macrocytic, hypochromic RBCs, misclassification of granulocytes as mononuclear cells using the basophil reagent method, and pseudothrombocytosis due to misclassification of ghost RBCs as platelets occurred (later corrected by amended software). Many of the

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artifactual changes were identified by morphology flags, with cytogram changes that allowed recognition of artifactual changes which were less pronounced in samples stored at 24 °C (room temperature) than those at 4 °C (refrigerated).[7] Other authors recommend refrigeration if there is a delay in analysis.[3] Other analyzers may have different performance due to specimen aging and storage.

Age, training/exercise, sex, nutritional status, and presence of endemic parasites have all been found to influence equine hematology results.[3,14,30] While minor changes due to these factors may not appear to be significant based on general horse RI, they may be significant when considering individual animal reference intervals.

Analytical Factors

Manual versus automated methods and the instrument's make, model, and edition of veterinary software may produce different results. Automated determination of differential leukocyte counts has been reported for several hematology instruments using species-specific programs,[14] but confirmation of WBC differential cell counts by peripheral blood smear examination is recommended.[13,38] Morphologic evaluation is also helpful in identification of band cells and other immature cells, the presence of toxic changes, blood parasites, or other morphologic abnormalities.

Postanalytical Factors

Important postanalytical factors include whether the RIs are appropriately partitioned for the class of horse (hot-blooded, warm-blooded, or cold-blooded), breed, sex, age, and reproductive status.[14,36] Whether population-based RI or individualized reference intervals (IRI) (also called subject-based RI) based on biologic variation are used also should be considered.

Indications for Evaluation of Hematology in the Horse

Hematologic evaluation in the horse has traditionally been used to monitor young animals in training; with the hope of detecting changes indicative of emerging disease or positive or negative effects of training. The premise that the most benefit comes from "emphasizing the negative" findings[3] rather than positive findings has merit in the opinions of the authors. Hematologic evaluation is recommended as part of a minimum database, including biochemistry and urinalysis, in horses with nonspecific clinical signs, such as weight loss, failure to thrive in young growing animals, or failure to perform at expected levels. It has been suggested that the relationship of laboratory results to performance potential in healthy horses may be better assessed with blood collection immediately following a standardized submaximal exercise test, which produces a heart rate of >100 beats per minute, 30 seconds following the completion of the workout,[3] with RI established for sex, breed, and training stage. However, this has not been adopted as a standard approach in the authors' experience.[31]

In overtrained trotters with poor racing performance, red cell hypervolemia is associated with a decline in racing performance, despite high Hgb concentrations. Overtrained horses also have significantly lower basal plasma cortisol values and the response to adrenocorticotropic hormone (ACTH) administration may be attenuated, although evidence for this is variable.[3] ACTH response is improved following rest and removal of one to two gallons of blood.[3]

THE ERYTHRON

The life span of equine RBCs in circulation is approximately 140–150 days.[37] RBCs are primarily released from bone marrow as mature cells, have a mean diameter of 5–6 µm, and a MCV of 40–52fL.[14,19] Central pallor is not present. Equine blood contains few circulating reticulocytes compared with other species, even with mild to moderate anemia. This is likely due to the equine spleen's large RBC storage and buffering capacity.[1] Polychromasia is rarely observed in Romanowsky-stained blood smears. Young animals and healthy warm-blood breeds may have slightly higher reticulocyte numbers than other horses.

Rouleaux formation is a characteristic finding in healthy horses, due to weak surface charges on RBC membranes.[5] In most cases, the characteristic stack-of-coins appearance is sufficient to differentiate it from antibody-mediated agglutination, which has a three-dimensional grape-like cluster appearance. Saline dilution (up to 1:10) will disperse rouleaux, but not autoagglutinated RBCs.[14] Agglutination can cause erroneous MCV values and RBC concentrations determined by impedance because the aggregates interfere with electronic or optical evaluation of RBCs. Pretreating agglutinated blood from heparin-treated horses with trypsin can reverse heparin-induced agglutination and improve the accuracy of cell counts and indices.[14]

Howell–Jolly bodies (nuclear remnants in RBCs) may be seen occasionally in the peripheral blood from healthy horses, in regenerative anemias, or with splenic dysfunction,[14] but are not a consistent finding (Figure 110.1a).

THE LEUKON

Equine neutrophils contain sharply delineated nuclear lobation and coarsely clumped chromatin (Figure 110.1c). Their cytoplasm is clear to pale gray, depending on the stain used. Band neutrophils are less frequently seen in horses compared to dogs or cats, since horses do not exhibit marked left shifts in inflammatory states. Because of this, many hematologic profiles for horses have traditionally included fibrinogen, or more recently, serum amyloid A (SAA); increased acute phase protein concentrations are the most sensitive method to detect systemic inflammation[4] (see Chapter 144).

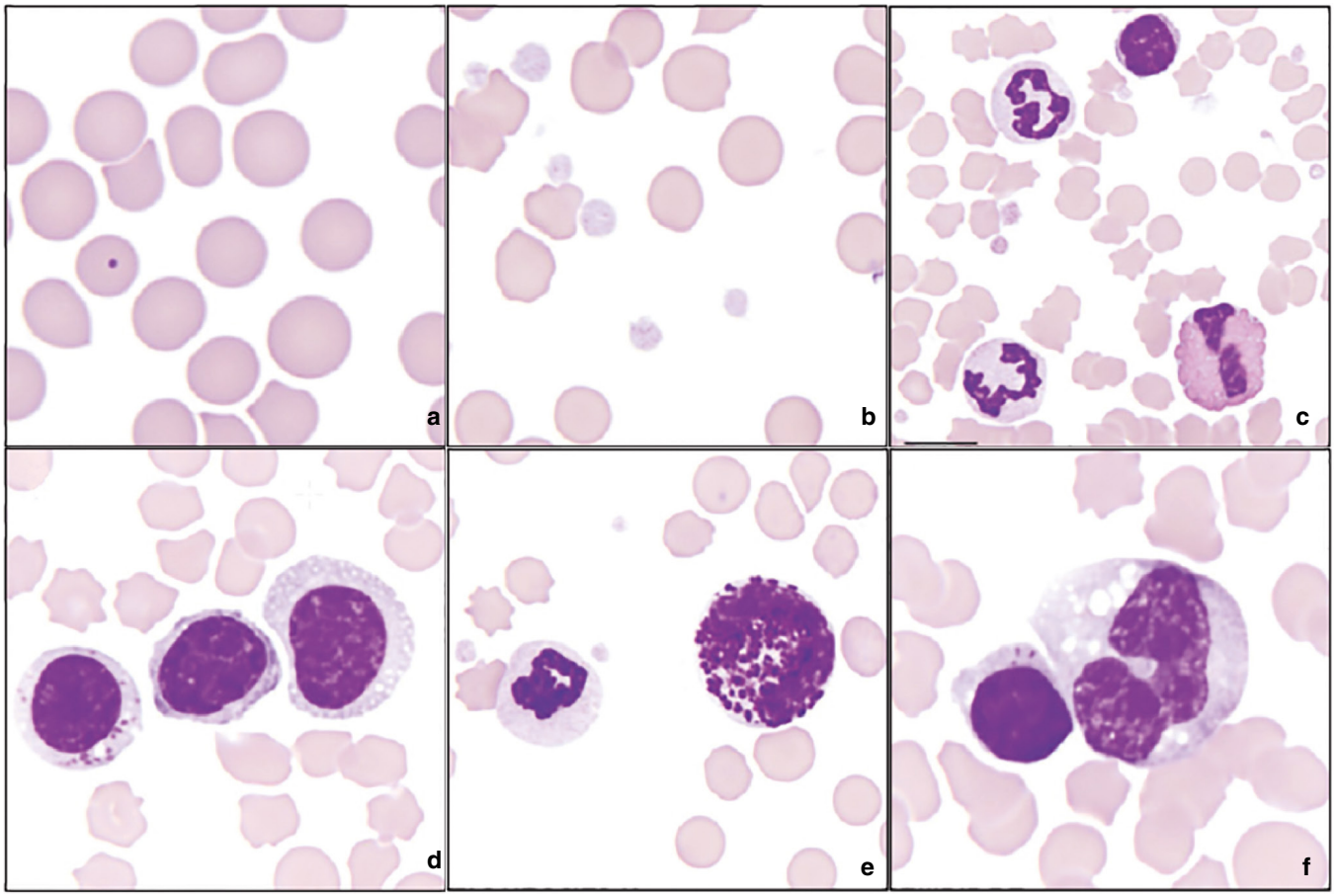


FIGURE 110.1 (a-f) Peripheral blood film from a horse. Modified Wright's stain: (a) Discocytes that lack a central pallor and an erythrocyte that contains a Howell-Jolly body. (b) Six light-staining platelets. (c) Two segmented neutrophils, one small lymphocyte, one eosinophil, erythrocytes and platelets. (d) Three lymphocytes and erythrocytes; small lymphocyte that contains cytoplasmic granules (left), small lymphocyte with scant cytoplasm (center), small lymphocytes with slightly more cytoplasm (right), (e) Segmented neutrophil, basophil, erythrocytes and platelets. (f) Small lymphocyte (with azurophilic granules), monocyte and erythrocytes.

Hypersegmented neutrophils, defined as having >5 nuclear lobes separated by filaments, are rarely seen in health. Prolonged blood storage may lead to the artifactual development of hypersegmented neutrophils. Idiopathic hypersegmentation has been found in quarter horses that lacked evidence of clinical disease.[14] Hyposegmented neutrophils have been reported in apparently healthy Arabian horses that were diagnosed with Pelger–Huët anomaly[14] (see Chapter 146).

Lymphocytes in the peripheral blood of normal adult horses are comprised of 38–66% T cells, 17–38% B cells, and small numbers of null cells.[14] Lymphocytes are smaller than granulocytes with dark-staining round to oval nuclei, coarse chromatin, and scant light blue cytoplasm (Figure 110.1c,d,f). In Wright-stained blood smears, small pink to magenta, irregularly shaped cytoplasmic granules may be present in small numbers of lymphocytes. Occasionally, larger lymphocytes that have smooth chromatin and moderately abundant pale blue cytoplasm are seen.[14] Reactive lymphocytes are rarely seen in smears from healthy, adult horses.[14] They are slightly larger than small lymphocytes, often have scalloped nuclear margins, and moderately aggregated chromatin, with infrequently discernible nucleolar rings, and scant to moderate amounts of intensely basophilic cytoplasm; sometimes, a palely stained Golgi zone will be apparent[14] (see Chapter 50).

The nucleus of the equine monocyte is large, variable in shape (i.e., oval, bilobed, horseshoe-shaped, trilobed, or irregular) and has lacy chromatin. The cytoplasm is gray-blue and may have small azurophilic granules and a few clear vacuoles (Figure 110.1f). Prolonged storage of blood resulted in an artifactual increase in the number of mononuclear cells detected by the ADVIA 120 hematology analyzer[7] (see Chapter 49).

Eosinophils have pale blue cytoplasm and contain large, pink-orange, specific granules that are usually tightly packed within the cytoplasm, and which may obscure the nucleus (Figure 110.1c). The lobulated nucleus seldom shows fine filamentation and often appears to be bilobed. Eosinophil degranulation is reflected by vacuolation and is rare in healthy animals (see Chapter 46).

Equine basophils have clear to light blue cytoplasm and contain variable numbers of purple granules (few to many) that vary in size and shape (Figure 110.1e). Basophil nuclei tend to have fewer lobes than those in mature neutrophils[14] (see Chapter 47).

THE THROMBON

Equine platelet concentrations are some of the lowest reported for mammals.[14] Finding 6–10 platelets/oil-immersion field (100× objective) in a peripheral blood film is consistent with an adequate platelet count, sufficient to prevent spontaneous hemorrhage. Equine mean platelet volume (MPV) has been reported to be 4.3–5.6 fL and mean platelet mass to be 0.47–0.96 × 10^6/fL.[14] Additionally, mean platelet component (MPC) concentration (26.2 ± 3.5 g/dL) and platelet component distribution width (PCDW) (7.9 ± 1.2 g/dL) have been reported,[14] but are not routinely included on most hematology profiles. MPC was found to decrease with disease processes that caused platelet activation. Plateletcrit has been suggested to be a better indicator of platelet mass than platelet count.[30]

Platelets are round, oval, or elongate, usually are 2.5–3.5 µm in diameter, and have light blue cytoplasm containing fine azurophilic granules. Pseudopodia may be noted projecting from activated platelets. Equine platelets are pale when stained with most Romanowsky stains and can be easily overlooked in blood smears (Figure 110.1b). However, platelets stain more heavily with Diff-Quik® stain. Giant platelets (greater than the diameter of an RBC) are associated with increased thrombopoiesis. Platelet clumping due to artifactual platelet activation and aggregation during blood collection can lead to erroneously low platelet counts. Prolonged sample storage can result in pseudothrombocytosis due to misclassification of ghost RBCs as platelets using the ADVIA 120.[7] EDTA-dependent pseudothrombocytopenia has been reported in a thoroughbred gelding.[15] Blood smear examination should be performed to assess platelet clumping and confirm diagnosis of thrombocytopenia.

REFERENCE INTERVALS

RIs are presented in Tables 110.1–110.3 and illustrate reference intervals obtained for various categories of horses based on age, breed, and use (racing, breeding), using different statistical approaches. RIs depend on the reference individuals, the instruments and methods, and the statistical methods applied. Environmental, nutritional, management, and exercise factors in horses should be considered. Partitioning of RI for an instrument/method by sex, age, and facility/management should be conducted.[3] Minor differences reported for age, breed, sex, and levels of training may be significant when evaluating individual animals. Table 110.1 presents partitioned reference intervals from healthy, adult horses in southern England using data from a single large veterinary practice. Health was determined by physical examination, history, and separated according to the animal's age and use. All samples were collected for routine health-monitoring purposes, at owner's or manager's request. The reference intervals have undergone RI transference validation by accumulation of results from healthy horses over an extended period and have shown to be satisfactory in continued use.

TABLE 110.1 Equine hematology reference intervals partitioned for age, breed, and training status generated or verified in accordance with ASVCP guidelines.

Hematology measurand (units)	Neonatal Thoroughbred foals (<36 hours old)	Thoroughbred foals (3–6 weeks old)	Yearling Thoroughbred horses	Thoroughbred horses in racing and training (2 years)	Thoroughbred horses in racing and training (3 years)	Adult Thoroughbred horses used for breeding	Adult non-Thoroughbred pleasure horses
RBC (× 10^{12}/L)	6.9–11.8	8.8–11.8	7.5–11.5	8.7–11.7	8.6–10.5	9.0–14.0	6.2–10.2
Hgb (g/L)	102–154	106–136	100–143	128–166	130–161	111–169	111–159
MCV (fL)	31.7–44.0	30.6–39.3	33.6–40.0	37.0–42.1	39.5–43.0	31.0–41.0	40.0–50.0
MCH (pg)	11.2–16.4	11.1–14.5	12.6–15.0	13.7–15.7	14.9–16.2	10.0–14.2	15.2–19.0
MCHC (g/L)	31.7–39.4	34.4–38.0	34.5–40.0	35.9–37.9	36.6–38.8	32.0–36.0	33.5–38.7
Hct (L/L)	0.30–0.44	0.30–0.38	0.30–0.41	0.34–0.45	0.35–0.43	0.35–0.47	0.31–0.43
Platelet (× 10^9/L)	140–315	133–325	118–243	127–206	127–206	127–206	100–250
WBC (× 10^9/L)	6.2–12.4	6.9–15.2	6.0–15.0	7.3–12.7	6.9–9.8	4.1–10.1	6.0–10.0
Neut (× 10^9/L)	4.1–9.5	4.1–9.1	3.7–5.4	4.0–6.0	3.9–5.2	1.4–5.8	3.4–5.4
Lymph (× 10^9/L)	1.0–3.1	0.9–5.9	3.5–4.9	2.7–4.4	2.3–3.4	1.4–4.7	2.0–3.2
Mono (× 10^9/L)	0.1–0.5	0.2–0.6	0.2–0.5	0.26–0.56	0.2–0.56	0.36–0.56	0.2–0.4
Eosin (× 10^9/L)	0.1–0.2	0.1–0.3	0.1–1.0	1.0–3.0	0.0–0.2	0.0–0.5	0.0–0.4
Baso (× 10^9/L)	Rare	Rare	Rare	Rare	Rare	Rare	Rare

Source: Based on Rossdale's Laboratories, Newmarket, CB8 8JS, United Kingdom.

TABLE 110.2 Hematology measurands, mean, within-individual coefficient of variation (CV_I), analytical coefficient of variation (CV_A), dispersion (D) (rounded to whole number below CV_I), critical number of specimens for estimating homeostatic set point, and reference change value (RCV) using data from the Biologic Variation Study performed at Charles Sturt University.

Measurand	Mean	Reference interval*	CV_I (%)	CV_A (%)	Dispersion (%) [$D < CV_I$]	Critical number[a]	RCV[b] (%)
RBC (× 10^{12}/L)	7.67	6.5–12.5	6.29	1.23	6	4.38 (5)	17.76
Hgb (g/L)	129.7	110–190	6.3	0.94	6	4.33 (5)	17.65
Hct (L/L)	0.37	0.32–0.52	6.18	1.19	6	4.23 (5)	17.46
Platelet (× 10^9/L)	154	100–500	16.54	9.85	16	5.56 (6)	53.36
WBC (× 10^9/L)	7.55	5.5–12.5	8.37	2.28	8	4.52 (5)	24.04
Neut (× 10^9/L)	4.55	2.5–8.0	11.50	5.10	11	5.02 (5)	34.86
Lymph (× 10^9/L)	2.65	1.5–5.5	15.45	8.18	15	5.22 (5)	48.46

* 95% population-based reference intervals from Charles Sturt University, Wagga Wagga, Australia.
[a] Critical number of specimens = $n = [Z \times [CV_A^2 + CV_I^2]^{1/2}/D]^2$ where Z is the number of standard deviations appropriate to the probability (1.96 is often used since this is the 95% probability [$p < 0.05$]); CV_A is the analytical precision appropriate for the homeostatic set point; CV_I is the intra-individual biological variation. CVs were obtained in the equine biologic variation study performed at Charles Sturt University.[27] D is the percentage deviation allowed from the true homeostatic set point.
[b] RCV = $Z \times 2_{1/2} \times (CV_I^2 + CV_A^2)^{1/2}$ where $Z = 1.96$ (95% probability, bidirectional change).
Source: Based on Overman.[27]

TABLE 110.3 Individualized RIs for two horses with different homeostatic set points for various hematologic measurands.

		RBC (× 10^{12}/L)	Hgb (g/L)	Hct (L/L)	Platelet (× 10^9/L)	WBC (× 10^9/L)	Neut (× 10^9/L)	Lymph (× 10^9/L)
	Reference change value (%)	17.76	17.65	17.46	53.36	24.04	34.86	48.46
	Population based reference interval*	6.5–12.5	110–190	0.32–0.52	100–500	5.5–12.5	2.5–8.0	1.5–5.5
1	Homeostatic set point estimate	11.0	185	0.48	300	10.5	8.0	4.5
	Individualized reference interval[a]	11 ± (0.1776)(11) = 11 ± 1.954 = 9.05–12.95	185 ± (0.1765)(185) = 185 ± 32.653 = 152–218	0.48 ± (0.17)(0.48) = 0.48 ± 0.0838 = 0.40–0.56	300 ± (0.53)(300) = 300 ± 160.08 = 140–460	10.5 ± (0.24)(10.5) = 10.5 ± 2.524 = 7.98–12.72	8.0 ± (0.35)(8.0) = 8.0 ± 2.789 = 5.21–10.79	4.5 ±(0.48)(4.5)= 4.5 ± 2.181 = 2.32–6.68
2	Homeostatic set point estimate	7.2	115	0.33	145	6.0	3.0	2.0
	Individualized reference interval	7.2 ± (0.1776)(7.2) = 7.2 ± 1.279 = 5.9–8.48	115 ± (0.1765)(115) = 115 ± 20.298 = 95–135	0.33 ± (0.1746)(0.33) = 0.33 ± 0.0576 = 0.27–0.39	145 ± (0.5336)(145) = 145 ± 77.37 = 68–222	6.0 ± (0.2404)(6.0) = 6.0 ± 1.442 = 4.56–7.44	3.0 ± (0.3486)(3.0) = 3.0 ± 1.046 = 1.95–4.05	2.0 ± (0.4846)(2.0) = 2.0 ± 0.969 = 1.03–2.97

[a] Homeostatic set point for the individual horse ± RCV (homeostatic set point for the individual horse).
* Reference interval used at Charles Sturt University, Wagga Wagga, Australia.

Other Equids

Donkeys (*Equus asinus*) are a separate equine species from the horses and ponies are kept commonly as companion animals or used as working animals across the world. Growing numbers of donkeys are also being kept in some parts of the world because of the increasing popularity of donkey milk. The RBCs, leukocytes, and platelets of donkeys appear similar to those of the horse on light microscopy and, as with horses, reticulocytes are rarely present in the peripheral blood. However, the physiology of donkeys is significantly different from horses and there is poor transferability of hematologic RI between horses and donkeys.[6] Donkeys have a higher MCV than horses, and lower RBC count and PCV have been recorded in some studies.[5] Reference intervals for donkeys are available in the literature, but they were not created using American Society for Veterinary Clinical Pathology (ASVCP) guidelines and it is notable that there may be poor transferability of RI between groups of donkeys, possibly due to variation in breed, geographical region, climate, season, age, gender,

reproductive status, nutrition, work load, parasite burden, and methods.

There is little literature regarding hematology from hybrid equids (mules and hinnies). In one study, the hybrids had hematologic results that were closer to those of horses than donkeys.[24] As with donkeys, specifically developed population-based RIs are likely to provide the most useful diagnostic information.

Individualized Reference Intervals Based on Biologic Variation

There has been recent resurgence in interest in the concept of biologic variation and within-subject biologic variation used to determine reference intervals for individual animals.[39] A recent study of privately owned horses in Australia determined the reference change value (RCV), the amount that a measurand must change in order to be of statistical significance with a stated probability (usually 95%). A RCV for Hgb of 17.65% was determined, which is reassuringly close to the 20% found historically.[3] Persson previously determined Hgb concentration on 7 consecutive days in a small number of horses and found that results varied by as much as 2.4 g/dL (42 g/L) or nearly 20% from the mean value for a given individual.[33]

Optimal use of RCV is obtained when the analytical coefficient of variation (CV_A), for the laboratory in which the specimen is analyzed is used in conjunction with the within-subject variation (CV_I) which can be obtained from the literature) or the veterinary biologic variation website (www.vetbiologicalvaration.org) (Table 110.2). The critical number of specimens is used to establish an individual reference interval (IRI = homeostatic set point ± RCV). The degree of dispersion desired may differ for various measurands. It may be preferable to determine the homeostatic set point very precisely if a small change is likely to be clinically significant, while other parameters where larger changes are more likely to occur without clinical significance may not require as precise an estimation. The authors suggest that the dispersion associated with the homeostatic set point should be < CV_I to ensure that the dispersion (range of uncertainty associated with the homeostatic set point estimate) does not add further unacceptable uncertainty to the interpretation. Equine individualized reference intervals calculation can be found in Table 110.3.

BONE MARROW

Indications for bone marrow aspirate and core biopsy in the horse include persistent anemia, leukopenia, thrombocytopenia, or diagnosis of suspected hematopoietic and lymphopoietic neoplasia. CBC and peripheral blood smear, bone marrow aspirate, and bone marrow core biopsy should be collected at the same time or at least within a few days of each other. If finances are limiting, the bone marrow core biopsy can be held, pending the results of the other evaluations, and examined if it may contribute to a diagnosis.

Bone marrow can be collected from the sternum (preferred site), ilium, and ribs in both adult and young horses, as well as from the tuber coxae in young horses. Care must be taken when collecting samples from the sternum or ribs to avoid inadvertent puncture of the thoracic cavity (see Chapter 141). Equine bone marrow cellularity can be routinely assessed on satisfactory bone marrow aspirates or bone marrow core biopsy specimens (Table 110.4). Bone marrow particles in the mature adult horse are typically composed of equal proportions of fat and hematopoietic cells, but young growing horses may have up to 70% hematopoietic cells. Older horses can have up to 70% fat.[14] One to several megakaryocytes are seen per 10× objective field in health; greater than 10–20 per 10× objective field are considered increased, and only finding a few after examination of multiple particles suggests decreased numbers.[31] Maturation of the erythroid series should be orderly and complete, with approximately 5% of the erythroid series consisting of rubriblasts and prorubricytes and 95% consisting of rubricytes and metarubricytes.[20] Maturation of the granulocytic series should be orderly and complete, with approximately 85% of the granulocytes being metamyelocytes, bands, or segmented cells and 15% being myeloblasts and progranulocytes. The M:E ratio varies from 0.9 to 3.8 (Rose Raskin, personal communication). This large range of the M:E ratio limits its usefulness in determining if increased erythropoiesis is present. Other cells present in healthy horses are small lymphocytes (2–9%), plasma cells (<2%), monocytes/macrophages (<1%), mitotic figures (<4%), and rare osteoclasts or osteoblasts.[20]

Stainable bone marrow iron is readily apparent in healthy horses and the amount increases with age.[20] Finding more than 5% reticulocytes in the bone marrow (demonstrated by new methylene blue staining) supports a regenerative response, with up to 60% reticulocytes in intense regenerative responses. Hemodilution can result in a falsely low bone marrow reticulocyte count.

HEMATOLOGIC DISEASE

Anemia

History, physical examination, and other testing should be considered in generating differential diagnoses for equine anemia. Increased reticulocyte counts may be observed in severe anemias, particularly hemolytic anemias; but have not been found to be of prognostic significance to date. Response to anemia in the horse is most often assessed by serial Hct evaluation.[28] Reticulocyte counts that are >5% in marrow aspirates in anemic horses are consistent with accelerated erythropoiesis.[37] Serial monitoring of the peripheral blood and/or bone marrow aspirates is most commonly used to assess whether a bone marrow response to anemia is present.[14] Increased MCV of up to 10–15 fL above the individual's baseline level supports increased erythropoiesis in routine hematologic evaluations.[16] Increased RDW also provides support for increased

TABLE 110.4 Cellular composition of equine bone marrow.

	Harvey (1993) (4 horses)[a]	Tschudi et al. (1975) (15 horses)		Franken et al. (1982a) (24 horses)	
Cell type	**Range (%)**	**Range (%)**	**Mean (%)**	**Range (%)**	**Mean (%)**
Myeloblast	0.3–1.5	0.3–2.0	1.2	0–5.0	1.0
Promyelocyte	1.0–1.9	0.0–3.0	1.3	0.5–3.5	1.7
Myelocytes					
Neutrophilic	1.9–3.2	1.0–5.0	3.3	1.0–7.5	3.2
Eosinophilic	Total eos below	0.0–0.3	0.1	Total eos below	–
Metamyelocytes					
Neutrophilic	2.1–7.3	5.0–11.0	7.91	1.5–15.0	5.6
Eosinophilic	Total eos below	0.0–0.3	0.1	Total eos below	
Basophilic	Total basos below	0.0–0.3	0.1	Total basos below	
Band neutrophils	6.8–14.7	–	–	6.0–26.5	15.7
Mature granulocytes					
Neutrophils	9.6–21.0	11.0–30.0	20.4	3–16.5	8.4
Eosinophils	2.8–6.8 (total)	0.0–0.6	0.2	0.0–5.0 (total)	1.8
Basophils	0.0–1.5 (total)	0.0–0.5	0.1	0.0–1.0 (total)	0.3
Total myeloid series			34.5		37.9
Rubriblast	0.6–1.1	0.6–4.0	2.2	0.0–2.0	0.7
Prorubricyte	1.0–2.0	2.0–9.0	5.8	1.0–9.5	3.6
Rubricyte	19.2–37.1	10.0–23.0	16.2	14.5–44.0	28.2
Metarubricyte	11.4–19.7	25.0–45.0	34.9	14.0–36.0	23.2
Total erythroid series			60.5		55.9
Monocyte	0–1.0	0.0–2.0	0.8	0.0–1.0	0.2
Lymphocyte	1.8–6.7	1.0–6.0	3.8	1.5–8.5	3.8
Plasma cell	0.2–1.8	0.0–2.0	0.7	0.0–2.0	0.6
Megakaryocyte	–	–	–	0.0–1.0	0.3
Mitotic figures	–	0.4–2.0	1.2	0.0–3.5	0.8
Myeloid:erythroid ratio	0.5–1.5	0.3–0.9	0.6	0.5–0.9	0.7

[a] Minimum to maximum ranges from healthy horses are presented.
Sources: Jain[16]; Jain.[17]

erythropoiesis.[11,19] The RDW is wider in healthy horses (14–25%) than in other species.[19] RBC creatinine concentrations have been reported to be higher in immature equine RBCs[40] and correlate well with the reticulocyte counts in bone marrow aspirates,[21] but this assay is not available commercially.

The response to hemolysis is generally greater than following hemorrhage, although the regenerative capability of horses is considered to be relatively poor compared with other species.[5] Recovery from very severe hemolytic or hemorrhagic anemia may take from 1 to 3 months.[22,23,36]

Peracute blood loss may result in splenic contraction, releasing stored RBCs into circulation and masking blood loss for at least several hours following hemorrhage. A decrease in total protein is an initial indicator of hemorrhage and occurs at 4–6 hours posthemorrhage. Decreased Hct occurs at 12–24 hours posthemorrhage. Continued decline with fluid fluxes may continue for up to 72 hours following a single episode of marked blood loss.[22] Chronic blood loss results in slowly developing anemia when blood loss exceeds the bone marrow's erythropoietic capacity. Internal hemorrhage may result in anemia, if severe, prolonged, or repeated.

Investigation of chronic blood loss resulting in anemia is highly dependent on a good physical examination, as well as laboratory tests, to determine if repeated trauma, parasitic disease (including ticks, lice, internal parasites), or ulcers (internal or external) may be contributing or underlying causes. Common causes of gastrointestinal hemorrhage include internal parasitism, gastric and/or duodenal ulcers, nonsteroidal anti-inflammatory toxicity, gastric squamous cell carcinoma, and swallowed blood from pulmonary bleeding. Causes of respiratory hemorrhage should not be underestimated and include guttural pouch mycosis, ethmoid hematoma, fungal rhinitis, nasal amyloidosis, severe pneumonia, pulmonary abscess with erosion of blood

vessels, pulmonary neoplasia, and exercise-induced pulmonary hemorrhage. Important causes of urogenital blood loss in the horse include urinary cystitis/urolithiasis, pyelonephritis, and vascular anomaly or rupture (especially postfoaling). Hemorrhage due to coagulopathy can result in hemorrhage at a variety of sites, depending on the underlying cause, e.g., trauma and environmental or individual factors. Considerations should include parasitic disease, gastrointestinal bleeding (ulceration, neoplasia), recent/prior surgery, thrombocytopenia, neoplasia, arterial rupture, moldy sweet clover toxicity, and hemophilia A, or other inherited coagulopathies[28] (see Chapter 146).

Hemolytic Anemia in the Horse

Hemolytic anemia in the horse may or may not be accompanied by icterus/hyperbilirubinemia. Total serum or plasma protein will likely be within normal limits (or increased compared to anemia from blood loss in which decreased total protein concentration is more common). There may be accompanying leukocytosis with neutrophilia, with or without a regenerative left shift.

Hemolytic anemia that is not associated with an immune response may occur due to RBC enzyme deficiency, chronic disseminated intravascular coagulation, and with any microangiopathic process including thrombosis or fibrinoid degeneration of small vessels. Intravascular hemolysis may occur in some horses with terminal hepatic or renal failure.[41]

Immune-mediated hemolytic anemia (IMHA) may be associated with macroscopic or microscopic autoagglutination. Microscopic autoagglutination may be recognized when macroscopic autoagglutination is not detected. Autoagglutination supports an immune-mediated mechanism for anemia. True autoagglutination must be differentiated from rouleaux, the stack-of-coins appearance which is an expected finding in equine blood. A saline agglutination test (1:10) is helpful in making this distinction. Immune-mediated anemia in the absence of autoagglutination is confirmed by a Coombs test. If autoagglutination is present macroscopically, a Coombs test is unnecessary. False negative Coombs tests may occur immediately after hemolytic crises, if corticosteroids have been administered, or if the test is not conducted with appropriate dilutions and controls. A negative Coombs test does not rule out the possibility of IMHA (see Chapter 143).

Neonatal isoerythrolysis (NI) caused by blood group incompatibility between the dam and foal is particularly important in the horse. Maternal antibodies against foal RBC are absorbed from colostrum and result in foal RBC destruction. Diagnosis may be presumptive in neonates with lethargy, anemia, and icterus during the first 4 days of life. Confirmation is accomplished with a hemolytic crossmatch test or jaundiced foal agglutination test. The condition is best prevented by early blood typing and prevention of colostrum ingestion by foals at risk for this condition (see Chapter 37).

Equine infectious anemia (EIA) is an important cause of hemolytic anemias and is a reportable disease in many countries, requiring euthanasia of confirmed cases, since there is no cure for this infectious disease. Thrombocytopenia is often the first indicator of infection and is a consistent finding. Leukopenia, lymphocytosis, monocytosis, and hypergammaglobulinemia are often present. There may be time periods in chronic carriers and affected individuals during which all hematologic variables are within normal limits.

Immune-mediated anemias also may be secondary to infections, especially *Clostridium perfringens*, injection site abscess or purpura hemorrhagica, drug administration (especially penicillin), as a paraneoplastic condition (especially lymphoma), or miscellaneous conditions (such as protein-losing enteropathy). Idiopathic immune-mediated hemolytic anemia is diagnosed after exclusion of other possible causes (see Chapter 37).

Oxidant-Induced Hemolytic Anemia

Oxidized Hgb results in the formation of Heinz bodies, eccentrocytes, and/or methemoglobinemia (see Chapters 23 and 32). Heinz bodies are aggregates of oxidized Hgb and are visible in peripheral blood smears as small, pale membrane projections or within RBCs. Heinz bodies appear as blue-green granules located near or protruding from the RBC membrane when stained with new methylene blue. Distinctive eccentrocytes may be present. Damaged hemoglobin is located to one side of the RBC with a thin erythrocyte membrane at the opposite side of the eccentrocyte. Icterus and hemoglobinuria are common sequelae in anemia due to oxidative damage.

Differential diagnoses of Heinz body anemia in the horse include phenothiazine, onion, red maple leaf, and petroleum toxicity. Phenothiazine toxicity is now uncommon due to reduced use as an equine anthelmintic, but may be seen as it is present in some ruminant supplements and salt blocks. There may be some individuals that are more sensitive than others to development of toxicity. Onion toxicity is caused by oxidation of a disulfide by wild or domestic onions. Horses usually have a distinctive onion odor present on their breath, urine, and feces. Red maple leaf toxicity results from the ingestion of dried red maple leaves (not fresh or viable leaves) which contain gallic acid and likely other toxins capable of inducing methemoglobin formation and hemolysis in equine erythrocytes.[7] These compounds are not unique to *Acer rubrum*, suggesting that ingestion of leaves from other maple species (e.g., *Acer saccharum* and *Acer saccharinum*) may result in clinical signs similar to red maple leaf toxicity.

Only the most common etiologies of nonregenerative anemia in this horse will be discussed here (see Section III). Anemia of chronic disease is the most common form of equine anemia encountered by the authors. It is usually a mild anemia (Hct >20%). Bone marrow iron is normal to increased, possibly due to inflammation. A special case of anemia of chronic disease is that associated with renal disease with a potentially defective marrow response to erythropoietin, since decreased erythropoietin levels have not been

demonstrated consistently in horses with renal-associated nonregenerative anemia.[8] There may be reduced RBC life span. Neoplasia may induce anemia of chronic disease due to shifts in cytokine production caused by the malignant cell population (see Chapter 39).

Myelophthisic diseases replace or destroy the normal bone marrow environment. Because these diseases do not selectively target RBCs, pancytopenia is often present. Neoplastic and inflammatory infiltration may cause equine myelophthisic disease (see Chapter 17). Myeloid neoplasia is the most commonly reported cause. Peripheral leukemia may or may not be present. When myeloid neoplasia involves the bone marrow but atypical cells are not visible in peripheral blood, this is termed aleukemic leukemia[27] (see Section V).

Iron deficiency is usually a problem of foals rather than adult horses and associated with chronic blood loss. An initial normocytic, normochromic anemia with decreased marrow iron progresses to microcytic, hypochromic anemia. Smaller, Hgb-deficient RBCs are the result of increased divisions of RBCs in an attempt to produce a critical intracellular concentration of Hgb. Serum ferritin assay is a better measure than total iron body stores to diagnose iron deficiency. Serum ferritin <45 ng/mL is suggestive of iron deficiency in horses. However, the assay is species-specific, not widely available, and may be falsely increased by concurrent inflammation, though less so than serum iron. Iron deficiency may be confirmed by bone marrow evaluation finding decreased iron stores and a predominance of rubricytes and metarubricytes with ragged cellular boundaries. Diagnosis of an underlying condition is also supportive with an improvement in the anemia upon treatment and resolution when iron supplements are administered (see Chapter 27).

Aplastic or hypoplastic anemia occurs when bone marrow stem cells fail to differentiate due to toxic, intrinsic, or immune-mediated damage and there is disruption of the bone marrow environment necessary for normal cellular production. This is an uncommon finding in horses, and is usually idiopathic, although rare cases have been reported with phenylbutazone administration. Other causes such as drugs, toxins, ionizing radiation, and immune-mediated destruction at the marrow level have been suggested. Diagnosis is based on finding peripheral anemia and/or pancytopenia with severe bone marrow hypoplasia/aplasia and replacement of marrow by fat. Because it may be difficult to judge if bone marrow aspirates of low cellularity are truly representative, bone marrow core biopsies from at least two sites are recommended for this diagnosis.

Blood Parasites

The most common blood parasites causing anemia in the horse are *Babesia caballi* and *Theileria equi* (formerly *Babesia equi*). These intraerythrocytic protozoan parasites are transmitted by ticks and may be visible in peripheral blood smears during febrile periods; but may be absent at the time of hemolytic crisis. Infection may result in death or chronic disease. Approximately 10% of apparently healthy standardbred horses in racing and training showed microscopic evidence of *B. caballi* and *T. equi* organisms in southern Italy.[31] This is similar to another Italian study which found 35% of Murgese horses were infested but did not show clinical or hematologic abnormalities compared to a group of healthy, noninfected horses.[35] Both studies hypothesized possible genetic resistance to clinical disease.[31,35] Following recovery from clinical babesiosis, horses may become carriers for an indeterminate period. Stress may induce clinical disease in carriers.

Other Blood Parasites

Setaria equina microfilaria have been reported in peripheral blood smears from an adult horse, but did not result in hematological abnormalities.[42] *Anaplasma phagocytophilum* morulae may be seen in granulocytes during the acute phase of disease and the chronic phase is often associated with thrombocytopenia.[30] Equine hemotropic mycoplasmas may be detected microscopically, but microscopic evaluation does not correlate well with findings of PCR testing. A positive correlation was found between PCR status and anemia in young horses (<1 year old), but no correlation between PCR status and anemia was found in horses >1 year old.[10]

Changes in the Leukon

Changes in the equine leukogram rarely constitute a primary diagnosis, but may provide clues as to underlying processes. Interpretation of leukocytosis depends on the degree and types of cells involved. Leukocytosis of up to $20 \times 10^3/\mu L$ is common and considered mild to moderate, while elevations of up to $30 \times 10^3/\mu L$ are considered marked. Elevations $>30 \times 10^3/\mu L$ are relatively uncommon and considered extreme. Leukocytosis with mature neutrophilia is the most common finding in the horse and is often associated with inflammation and/or stress.

The classic stress leukogram typically has slight to moderate leukocytosis (WBC usually $\leq 20 \times 10^9/L$) with neutrophilia and eosinopenia. Monocytosis may or may not be present. Band cells or less-mature leukocytes are not part of the stress leukogram and indicate release of immature cells from the bone marrow. Occasionally, a marked leukocytosis is seen with stress alone, but deserves additional consideration and investigation to rule out other inflammatory causes before attributed to stress alone. Endurance exercise can be associated with the development of a pronounced leukocytosis and neutrophilia, which frequently persist for up to 24 hours.[3] These changes are likely due to prolonged stress and corticosteroid release from the adrenal glands. More extreme stress leukograms are associated with more severely fatigued individual horses. Exhausted endurance horses have similar WBC counts to less-tired endurance horses, but showed a pronounced left shift. One study reported the mean

differential to include 8% band cells, and two horses with 22% band cells.[3] Bayly proposed that the N:L ratio may be used to assess the severity of stress experienced by a horse, with a value >10 compatible with approaching exhaustion.[3]

Band cells typically indicate an acute inflammatory response. In a regenerative left shift, mature neutrophils outnumber an increased population of band cells, which is usually a reflection of acute inflammation. In a degenerative left shift, increased numbers of band cells and other immature myeloid cells outnumber mature neutrophils. This indicates a severe inflammatory condition. In a leukemoid response, there may be difficulty in distinguishing severe inflammation from myeloid leukemia in which atypical immature myeloid cells appear in the peripheral circulation.[27]

Lymphocytosis be caused by epinephrine release when excited or scared, antigenic stimulation, or lymphoid neoplasia (lymphoid leukemia or stage 5 lymphoma).[29] Monocytosis is uncommon as a sole abnormality of the leukogram and may occur in conditions in which necrosis or chronic inflammation is present.[29]

Eosinophilia and/or basophilia are also uncommon as sole abnormalities of the leukogram. Considerations should include endoparasitic or ectoparasitic disease, hypersensitivity, dermatologic conditions, reaction to foreign material or antigenic stimulation, fungal disease, irritation of tissues rich in mast cells (such as skin, lung, gastrointestinal tract, or female genital tract), mastocytosis/mast cell neoplasia, myeloproliferative disease, or leukemia of these cell lines.[2,29] The absence of eosinophilia does not rule out these conditions.

Leukopenia may involve any or all of the cell lines. Mild decreases below reference interval in otherwise healthy horses are due to age or other partitioning factors and of little or no clinical significance.[29] These may represent horses with low homeostatic set points and associated physiological variation, compared to the population used for determination of reference intervals. Primary considerations for leukopenia include conditions resulting in WBC immigration to an inflammatory site (consumption) or decreased bone marrow production. Salmonellosis is a classic equine condition in which marked WBC migration into the intestine and increased fecal WBC found in profuse diarrhea result in leukopenia. There may or may not be a degenerative left shift. Combined immunodeficiency syndrome in Arabian foals and common variable immunodeficiency syndrome may cause lymphopenia.[9,12] Myelophthisic diseases typically result in leukopenia in conjunction with anemia and/or thrombocytopenia.

Neoplasia

Myeloid neoplasia may present with leukocytosis, leukopenia, or WBC that is within normal limits. Abnormal myeloid cells are present in the peripheral blood in most cases. Myeloid neoplasia involves neutrophils, monocytes, and/or eosinophils or has features of both myeloid and monocytoid (myelomonocytic) leukemia. No age predilection is apparent. Reports range from 10 months to 9 years old.[27] Reported clinical signs include weight loss, edema, anemia, mucosal petechiae, fever, peripheral lymphadenopathy hemorrhagic diathesis, and oral ulceration. Diagnosis is confirmed by bone marrow aspirate and/or biopsy of multiple organs.[27] There may be infiltration of many organs, including lymph nodes, spleen, and liver. Usually cellular morphology allows differentiation of malignant myeloid or monocytic cells from malignant lymphoid cells, but cytochemical typing or flow cytometry may be needed to determine the cell lineage involved[27] (see Chapters 64–67).

Acute lymphocytic leukemia results in peripheral lymphocytosis with atypical and immature cells. The most common clinical abnormalities in the horse are chronic weight loss, ventral edema, and regional lymphadenopathy. Chronic lymphocytic leukemia may result in large numbers of small lymphocytes in peripheral blood. These cells appear mature and well-differentiated.

Atypical lymphoid cells in peripheral blood are estimated to occur in 30–50% of horses with lymphoma, but are usually present in small numbers. Leukogram alterations are highly variable, but peripheral lymphocytosis with large numbers of circulating neoplastic lymphocytes is unusual and usually associated with bone marrow infiltration. There may be concurrent paraneoplastic immune-mediated hemolytic anemia, coagulopathy, thrombocytopenia, and/or paraneoplastic hypercalcemia. Common forms of lymphoma in the horse are generalized, intestinal, mediastinal, and cutaneous. Age of onset is usually 5–10 years, although lymphoma can be seen at birth or into old age. Histologic and/or cytologic evaluation of affected fluids or organs is used in diagnosis[26] (see Chapter 65).

Multiple myeloma, a tumor of plasma cells, is rare in horses and manifests in neoplastic proliferation of plasma cells in bone marrow, spleen, and lymph nodes. Hematologic changes are often nonspecific and may include pancytopenia. Focal osteolysis, a common finding in the dog, may or may not be present in horses. Diagnosis is made when there is plasmacytosis in bone marrow or soft tissue with invasion of surrounding tissue and monoclonal gammopathy (see Chapter 70).

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CHAPTER III

Hematology of Bovids

R. DARREN WOOD

Erythron
Leukon
Platelets and Coagulation
Reference Intervals
  Partitioning Factors
Blood and Bone Marrow Collection
Hematologic Response to Disease
  Anemia
  Leukocyte Responses
    Physiologic
    Inflammatory Response
  Hemoparasites

Acronyms and Abbreviations

ASVCP, American Society of Veterinary Clinical Pathologists; BLV, bovine leukemia virus; PT, prothrombin time; PTT, partial thromboplastin time.

ERYTHRON

The mature erythrocyte (RBC) of the adult bovine is biconcave, has a width of 5–6 μm, minimal central pallor, and a relatively long life span of 130–160 days.[64] Morphology and life span are similar in bison and water buffalo.[2,13] On a blood smear, anisocytosis is mild to moderate in most ruminants. Red blood cell shape is relatively uniform in adult animals, but poikilocytosis is common in blood smears from apparently healthy calves. This may be a reflection of a unique hemoglobin (Hgb) molecule, its association with the RBC membrane, or inadequate iron stores.[53] Water buffalo tend to have more rouleaux than cattle; but rouleaux formation of bovine RBCs is unusual unless inflammatory proteins are increased.[33] Polychromatophils are generally absent from the blood of healthy adult bovids.

The average Hct of domestic cattle is approximately 0.26 L/L (0.17–0.39 L/L). In water buffalo, the average PCV is approximately 42% (30–56%), more similar to bison than domestic cattle. The average PCV in bison is approximately 47% (24–61%). Compared with reported values for domestic cattle, the following variables tend to be higher in water buffalo and bison: RBC counts, PCV, and Hgb concentration. The average Hgb in bison is about 168 g/L (range, 82–230 g/L)[2,46,61,71] (Tables 111.1 and 111.2).

The bovine erythrocyte membrane, and that of other ruminants, has unique phospholipid composition in the external hemilayer. Instead of a predominance of phosphatidylcholine (PC), sphingomyelin (SM) is present in higher concentration.[18] This is thought to be due to the presence of anti-PC antibodies, formed due to evolutionary pressure of coexisting rumenal ciliates whose membranes contain a similar analog.[22] This variation may decrease rouleaux formation in this species.[63]

Ruminant Hgb is of particular interest because of the large amount of polymorphism that occurs between species, breeds, and even within the individual as it develops from embryo to adult.[33] This polymorphism is greatest in the β-chain. As in many other species, ruminants have two Hgb types. The embryonal type (HgbE) maintains a dam to in utero O_2 gradient, and the adult type (HgbA) for the ex utero environment. Transition from HgbE to HgbA begins in utero and may not be complete until months after birth. Like humans, domestic ruminants have a third type of hemoglobin, fetal hemoglobin (HgbF), which replaces HgbE in utero. As gestation progresses to the perinatal period, bovine HgbF is gradually replaced by HgbA. As prenatal erythrocytes live out their life span, HgbF-containing cells are replaced by HgbA-containing RBCs.

Bovine Hgb is a rich source of antimicrobial peptides.[50] A peptide isolated by Daoud et al. has antibacterial

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TABLE 111.1 Hematologic reference intervals for dairy and beef cattle of various ages and stages of production. Reference values are in compliance with the ASVCP reference interval guidelines.

	Hct (L/L)	Hgb (g/L)	RBC (× 10^{12}/L)	MCV (fL)	MCH (pg)	MCHC (g/L)	RDW (%)
1–9 days[55]	0.17–0.40	56.5–135.8	5.16–10.59	28.7–41.2	10.1–14.2	301.6–376.6	18.6–27.6
Dams 2–4 DIM[47]	0.25–0.38	93.3–137.8	5.3–8.3	40.1–50.3	13.7–19.5	328–374	14.1–19.7
Dams 27–33 DIM[47]	0.20–0.34	75.2–127.9	4.1–7.8	38.9–53.1	15–19.1	335–379	14.4–19
Dams 30–150 DIM[55]	0.21–0.31	73.5–119.8	4.34–7.40	35.4–50.6	13–19.1	345.3–399.5	17.2–23.2
Dams 30–150 DIM[70]	0.21–0.30	84–120	4.9–7.5	36–50	14–19	380–430	16–20
Dams mid-lactation[19]	0.22–0.33	85–122	5.1–7.6	38–50	14–18	360–390	15.5–19.7
Adult beef and dairy cows[32]	0.24–0.39	82–130	4.8–7.6	41.2–58.7	14.3–19.67	324–358	18–26

	WBC (× 10^9/L)	Neut (× 10^9/L)	Band (× 10^9/L)	Lymph (× 10^9/L)	Mono (× 10^9/L)	Eos (× 10^9/L)	Baso (× 10^9/L)
1–9 days[55]	3.84–19.55	0.82–14.38	–	1.43–8.16	0.02–1.25	0.03–1.19	0.02–0.22
Dams 2–4 DIM[47]	4.7–13.9	2.6–12.7	–	3.9–13.8	0–2.8	0–1.6	–
Dams 27–33 DIM[47]	5.4–14	1.5–9.0	–	1.6–7.7	0–1.1	0–1.3	–
Dams 30–150 DIM[55]	4.9–12.45	1.47–7.57	–	1.17–4.83	0.18–1.06	0.06–0.81	0.03–0.11
Dams 30–150 DIM[70]	5.1–13.3	1.7– 6.0	0–0.2	1.8–8.1	0.1–0.7	0.1–1.2	0–0.2
Dams mid-lacation[19]	4.9–12	1.8–6.3	Rare	1.6–5.6	0–0.8	0–0.9	0–0.3
Adult beef and dairy cows[32]	4.4–10.8	0.8–5.0	–	1.8–4.9	0.3–1.2	0.1–2.1	0–0.1

Sources: Panousis et al.[55]; Moretti et al. [47]; Wood et al. [70]; George et al. [19]; Herman et al. [32]

TABLE 111.2 Hematologic reference intervals for water buffalo (*Bubalus bubalis*) of various ages, sexes, and stages of production. Reference values are in compliance with ASVCP reference interval guidelines.

	PCV (%)	Hgb (g/L)	RBC (× 10^{12}/L)	MCV (fL)	MCH (pg)	MCHC (g/L)
Male calves[56]	0.36–0.40	119–131	8.19–10.75	35–44	13.5–16.2	315–399
Female calves[56]	0.37–0.42	118–149	9.06–11.3	35–44	11.7–14.9	334–386
Bulls[56]	0.42–0.47	154–179	11.39–13.51	34–55	12.9–15.5	305–360
Pregnant lactating cows[56]	0.31–0.37	107–137	6.93–8.73	33–53	15.2–16.7	318–389
Non-pregnant lactating cows[56]	0.29–0.36	98–131	6.34–7.62	38–57	15.1–18.2	308–364
Pregnant dry cows[56]	0.33–0.38	120–145	6.68–8.60	40–47	14.4–19.1	307–374
Non-pregnant dry cows[56]	0.30–0.37	102–121	6.02–7.50	39–43	15.6–18.7	330–406

	WBC (× 10^9/L)	Neut (%)	Lymph (%)	Mono (%)	Eos (%)	Baso (%)
Male calves[56]	6.88–9.44	21–29	62–72	3–5	2–5	0–1
Female calves[56]	7.45–10.45	27–35	64–74	3–6	2–5	0–1
Bulls[56]	9.78–12.5	29–39	56–67	3–6	3–7	0–1
Pregnant lactating cows[56]	8.02–9.78	31–37	57–67	3–6	2–7	0–1
Non-pregnant lactating cows[56]	7.4–8.92	31–36	59–68	2–6	3–5	0–1
Pregnant dry cows[56]	8.75–10.87	28–34	58–71	3–6	4–5	0–1
Non-pregnant dry cows[56]	7.74–9.34	29–37	59–68	3–5	2–6	0–1
Post-lactating adult females[67]	7.1–13.7	–	–	–	–	–

Sources: Patel et al. [56]; Torres-Chable et al. [67]

activity against several organisms including *Escherichia coli* and *Salmonella enteritidis*.[16] Another group isolated four distinct peptides with similar activity, three of which corresponded to the bovine α-chain and one to the β-chain.[51]

Bovine Hgb has also been used extensively as an oxygen-carrying substitute in humans and in small animals.[12,23]

The metabolism of domestic animals' RBCs was reviewed by Harvey.[30] Glucose consumption by bovine

RBCs is approximately 0.6 μmol/hour/mL cells. Most cattle have moderate to high Na^+ and low K^+ erythrocytes. Some cattle with specific autosomal recessive phenotypes have high intraerythrocytic K^+ associated with certain blood groups.[30]

The ferrous iron of oxyhemoglobin (OxHgb) is constantly being oxidized to the ferric iron of methemoglobin (MetHgb) by intracellular metabolic products.[17] Erythrocytes normally contain a low concentration of MetHgb that is cyclically reduced back to OxHgb by the intraerythrocytic antioxidant enzyme, NADH methemoglobin reductase. When the quantity of MetHgb reaches a certain point, polymerization occurs, and the precipitated Heinz body becomes associated with the cell membrane. Accumulation of excessive Heinz bodies may result in both intravascular and extravascular hemolysis.

Erythrocytic 2,3-diphosphoglycerate (2,3-DPG) accumulation modifies the Hgb of some species, but not in cattle.[25] Ruminant fetuses and neonates have greater amounts of erythrocytic 2,3-DPG than adults. However, unlike many other species, 2,3-DPG is not bound as well to ruminant HgbF and HgbA.[25] Bovine HgbF has sufficient inherent oxygen affinity to maintain fetal Po_2.

The copper and zinc metalloenzyme superoxide dismutase was originally identified in cattle.[44] Although its value as an in vivo antioxidant is not entirely known, activity of this enzyme is increased in neonatal calves, presumably due to increased oxidative stress associated with parturition.[18] Another RBC metalloenzyme of clinical diagnostic significance is selenium-containing glutathione peroxidase (GPx). There is a direct correlation between selenium deficiency and enzyme activity. Although erythrocytic GPx deficiency is directly related to white muscle disease, it is inconsistently accompanied by methemoglobinemia. Hemolytic anemia does not frequently occur with selenium deficiency in ruminants. Heinz bodies in selenium-deficient Florida cattle were resolved by selenium treatment.[43]

Differences in hemoglobin types and RBC metabolism of cattle do not appear to make bovid RBCs more susceptible to oxidation. Cattle have intermediate activity of the RBC pentose phosphate pathway's limiting enzyme, glucose phosphate dehydrogenase.[30] When plants containing large amounts of nitrates or fertilizer nitrates are consumed, rumenal microflora reduce nitrate to nitrite, which when absorbed oxidizes OxHgb to MetHgb without Heinz body formation. Sulfoxide-containing kale and other *Brassica sp*. are converted by rumen microflora to an oxidant that, when absorbed, produces Heinz body hemolytic anemia. Methemoglobin with or without Heinz body formation and hemoglobinuria is associated with consumption of onions and ryegrass and may also occur with copper deficiency, toxicosis, and hypophosphatemia.[15,52]

LEUKON

The traditional leukogram is predominantly used to assess leukocyte (WBC) concentrations.[59] Specific cell subset profiles, blood and organ distribution, and cytokines are the current subjects of clinical research and development and may one day supplement or replace the conventional leukogram.[57] Acute phase protein assessment, specifically haptoglobin in bovids, is also frequently used, as it is the most sensitive indicator of systemic inflammation[47] (see Chapter 144).

Cattle have less granulocytic bone marrow reserve than many other species, which is reflected by a myeloid to erythroid (M:E) ratio of about 0.5–1:1.[10] This smaller granulocytic reserve is suspected to be part of the reason why neutropenia develops during the early granulopoietic response to suppurative inflammatory disorders in cattle. Once granulopoiesis is accelerated, a regenerative left shift and neutrophilia develop.

Bovine neutrophils have three types of cytoplasmic granules. The third type of neutrophil granule is larger than the primary and secondary granules and occupies twice the cytoplasmic space as in sheep and other domestic species, giving the bovine neutrophil its characteristic pink cytoplasm (Figure 111.1a).[10] The bovine's large neutrophil granules have more antimicrobial activity than the cytoplasmic granules of most nonruminant species.[8] Cytochemical and immunocytochemical staining reveals a range of enzymes and substrates in ruminant neutrophils.[3,30] Lysozyme, an enzyme common to many species, is not found in the neutrophils of cattle. Immunocytochemical cell membrane antigens add to the specificity of neutrophil characterization[57] and proteomic studies assist in further characterizing the neutrophils' molecular profile.[42]

Neutrophils of water buffalo vary morphologically from those in cattle. They have a mean size of 13.2 ± 0.7 μm and are multilobed or may have decreased lobation in samples from apparently healthy animals. Multilobed neutrophils have an irregular nuclear outline with two to four lobes and a dense chromatin pattern.[33] Monolobed nuclei may have one to two indentations, with decreased amounts of condensed chromatin compared to multilobed neutrophils. The cytoplasm is pale with fine indistinct, sometimes brown granules.[13,33] Band neutrophils are rarely found in healthy water buffalo, but have a smooth nuclear outline with no nuclear constrictions.[33] The neutrophils of bison appear morphologically similar to those in cattle (Figure 111.1f).[2]

Bovine lymphocytes are 8–15 μm in diameter and three sizes are described. Small lymphocytes have a round, centrally located nucleus up to two times the size of a RBC with a densely staining chromatin pattern in a small volume of clear to gray-blue cytoplasm (Figure 111.1b). The paucity of cytoplasm and chromatin density suggest that the small lymphocyte may be metabolically dormant or less active than the other two forms of lymphocytes. Medium lymphocytes have larger, round to indented nuclei in which the same amount of chromatin is spread over a greater area, revealing lighter-staining parachromatin adjoining areas of condensed chromatin. The cytoplasmic volume is also greater and encircles the central to eccentrically placed nucleus. Large lymphocytes have an even larger, lighter-staining nucleus and more cytoplasm than the medium-sized lymphocyte. Large lymphocytes have a round, indented,

or deeply cleaved centrally placed nucleus and are frequently confused with monocytes. Like monocytes, the cytoplasm of large lymphocytes stains blue to gray and sometimes contains small clear vacuoles. Variable shapes, sizes, and numbers of magenta cytoplasmic granules are frequently seen in a focal arrangement and may serve to identify the large bovine lymphocyte. Agranular large lymphocytes and monocytes are ultrastructurally inseparable, and cytochemical and immunocytochemical staining are needed to separate these two cell types. Chromatin centers, nucleolar rings, and nucleoli are seen with low frequency in Wright-stained normal bovine lymphocytes and should not be mistaken for evidence of neoplasia. Lymphocytes in water buffalo and bison are similar in appearance to those in cattle (Figure 111.1g).[2,13]

The bovine monocyte is a round to convoluted-shaped cell 13–19 μm in diameter. It has a large indented, bilobed to ameboid nucleus containing a diffuse chromatin pattern in a blue-gray cytoplasm containing fine, small, indistinct, magenta to pink granules. Vacuoles are frequent and more irregular in shape than those seen in some large lymphocytes (Figure 111.1c). Monocytes in water buffalo and bison are similar in appearance to those in cattle (Figure 111.1h).[2]

The ruminant eosinophil has a band to bilobed nucleus, which is surrounded by numerous, small, round, intensely stained, red cytoplasmic granules in basophilic cytoplasm (Figure 111.1d). Eosinophils may be increased with hypersensitivity disorders and tissue parasitism such as that caused by *Sarcocystis* and *Hypoderma* warbles.[54] Eosinophils in water buffalo and bison are similar in appearance to those in cattle (Figure 111.1i).[2]

The bovine basophil is a rare cell about the same size as the eosinophil, with electron-dense, numerous, small, intensely blue-staining granules that sometimes mask the nucleus (Figure 111.1e). Basophils occur at such low concentrations that they are not reliably quantified by the 100 differential cell method. Basophils in water buffalo and bison are similar in appearance to those in cattle (Figure 111.1j).[2,13]

PLATELETS AND COAGULATION

Bovine platelets in Wright-stained peripheral blood are distributed singly or in aggregates, are generally round but can vary in size and shape, and often contain numerous prominent azurophilic granules (Figure 111.1a,c). Cytoplasmic projections are rare. Platelets are small but numerous with a normal circulating survival time of 5–10 days. Enlarged platelet forms and pseudopodia are associated with activation and accelerated production in the bone marrow. Ultrastructure of bovine platelets reveals extensive invaginations of the surface membrane.[45] At the Animal Health Laboratory at the University of Guelph, reference values for platelets (160–650 × 10^9/L) were created in accordance with American Society of Veterinary Clinical Pathologists (ASVCP) guidelines with 28 proven healthy adult cattle. Platelets in water buffalo and bison are similar in appearance to those in cattle (Figure 111.1f).[2,13]

Blood clotting times for bovids are rarely reported in the literature. At the Animal Health Laboratory at the University of Guelph, reference values were created from 28 clinically healthy adult animals of various breeds resulting in the following: prothrombin time (PT) 18.4–23.1 seconds, partial thromboplastin time (PTT) 22.8–35.8 seconds, fibrinogen concentration 2.08–3.45 g/L.[1] Clotting times for water buffalo and bison have not been reported.

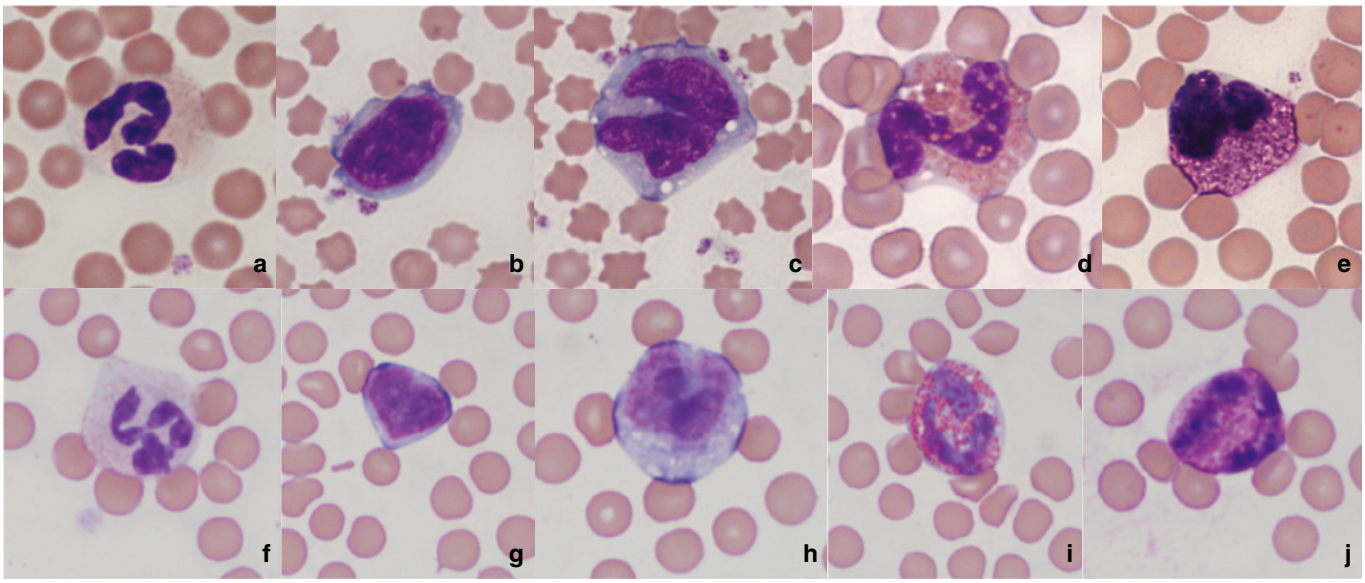


FIGURE 111.1 Blood cells of selected bovids. Domestic cattle (*Bos taurus*). (a) Segmented neutrophil; (b) Medium lymphocyte; (c) Monocyte; (d) Eosinophil; (e) Basophil. Erythrocytes and platelets are also present. North American bison (*Bison bison*). (f) Neutrophil; (g) Lymphocyte, and a small hemoglobin crystal; (h) Monocyte; (i) Eosinophil; (j) Basophil. Wright's stain; magnification 1000×.

REFERENCE INTERVALS

Bovine neutrophil counts have increased over the last 50 years, and now the N:L ratio can be equal to or >1 in some healthy cattle. It was previously suggested that a N:L ratio >1 could be used to indicate inflammation, but this is no longer recommended, particularly in the absence of the animal's bovine leukemia virus (BLV) status. Lymphocyte and eosinophil counts have also decreased in this time period.[19,66] Increased diligence to exclude BLV-positive animals from reference value cohorts may be resulting in overall lower lymphocyte counts.[66]

Partitioning Factors

There are many factors that influence hematologic values in cattle that should be considered when interpreting results against reference intervals. Examples include age, breed, sex, production status, geographical location, and diet.[55] Domesticated bovids may have little or no direct physical contact with humans; therefore, when sampling occurs, the animals must be physically restrained and become excited. Reports of reference intervals seldom include consideration of such variables as physiologic state, history, form of restraint, ambient temperature, hydration status, BLV status, or parasite burdens. As in most species, the ruminant neonate has fewer lymphocytes than granulocytes. In a matter of weeks, the lymphocyte concentration increases to values similar to neutrophils, or higher. During adulthood, a slow progressive decline in lymphocyte concentration occurs. These age-related fluctuations in lymphocyte concentration exemplify the need for age-related reference intervals.

During gestation and at birth, Hgb types change and the erythroid compartment increases. At birth, about 60–90% of the neonatal calf's Hgb is HgbF and up to 9% of RBCs are reticulocytes. Fetal calf RBCs are less fragile and larger than adult bovine RBCs. In late gestation, bovine fetal RBCs have a mean corpuscular volume (MCV) of approximately 54 fL, but by birth it decreases to about 46 fL. From birth to about 8–12 weeks of age, the MCV continues to decrease to approximately 37 fL as HgbF-containing RBCs are replaced by cells containing HgbA. Compared to adults, the RBC count and MCV are lower in calves, and then gradually increase up to 15–19 months of age. Erythropoiesis takes about 5–6 days.[14] Veal calves raised solely on a diet of milk or milk replacer have lower RBC values than conventionally raised calves due to milk's low iron content.[4]

Recent studies have generated hematologic reference intervals for water buffalo, which are relatively similar to those for cattle and bison.[32,56,67] Bison calves have significantly higher RBC counts than do adult bison, which is also the case with calves of domestic cattle.[27] Average oxygen-carrying capacity was 17 mL/100 mL from calf blood and 22.2 mL/100 mL blood from adult blood; Hgb concentration averaged 13.6 g/dL in calves and 17.1 g/dL in adults.[27] Other age-related variations in bison CBC results include: RBC, monocyte, Hct, and neutrophil numbers which are negatively correlated, while lymphocyte numbers are positively correlated with increasing age.[2]

Bison raised and/or maintained under ranch conditions comparable to beef cattle operations have similar hematologic values as free-ranging bison living on range lands or in national parks.[61] However, an increased PCV is seen in free-ranging wild bison impounded for 24 hours before blood collection.[28] The authors suggested this may have been due to dehydration and/or excitement. Seasonal variation in PCV, mean corpuscular hemoglobin concentration (MCHC), MCV, and Hgb values may be a factor in establishing reference intervals.[21]

BLOOD AND BONE MARROW COLLECTION

Venipuncture sites for cattle include the jugular and coccygeal (tail) veins. Blood collection can usually be accomplished in an unsedated animal restrained with a halter or in a squeeze chute. A short period of acclimation is recommended to minimize the effects that epinephrine and/or cortisol may have on the hemogram. Blood is collected into tubes containing EDTA anticoagulant followed by two to three tube inversions to ensure proper mixing. Samples should be analyzed within 2–3 hours at room temperature or 12–24 hour if refrigerated (4 °C).[59]

The preferred sites for bone marrow collection in cattle are the sternum and the dorsal ends of the ribs. A unique feature of bovine bone marrow is the lower M:E ratio of 0.5–1.5 compared with other species. Harvey reports a ratio as low as 0.31.[29] The lower bovine bone marrow M:E ratio corresponds with the low–moderate neutrophil concentration in peripheral blood in some cattle and the rapid rate at which neutrophils are depleted during acute inflammation (see Chapters 112 and 141).

HEMATOLOGIC RESPONSE TO DISEASE

Anemia

Anemia can be due to blood loss, hemolytic disease, or decreased bone marrow erythropoiesis. Regenerative anemia occurs due to hemorrhage or hemolysis. Causes of blood loss in bovids include trauma, blood-sucking parasites, gastrointestinal ulcers, coagulopathy, and hemorrhagic enteritides (such as with bovine viral diarrhea virus (BVDV)). Immediately after blood loss, RBC variables remain unchanged until sufficient volume loss occurs to elicit fluid shifts into the intravascular space. Reticulocytosis may be observed as soon as 2–3 days, but the peak response may not occur until the following weeks. Regeneration is at most moderate compared with small animals. When severe regenerative anemia occurs, accelerated erythropoiesis is evidenced by moderate reticulocytosis, which results in macrocytosis, polychromasia, and basophilic stippling of RBCs, indicating accelerated erythropoiesis. Basophilic stippling is common during accelerated erythropoiesis in ruminants and should be interpreted as evidence of a

regenerative response. Healthy cattle typically have no polychromatophils in circulation, but they are released during a regenerative response. If hemorrhage becomes chronic and is of sufficient volume, the regenerative response can decrease as iron stores become depleted, and RBCs released from the bone marrow are microcytic and hypochromic. Veal calves fed all-milk diets will develop iron deficiency anemia.[41]

Hemolysis in bovids occurs with toxins, drugs, heavy-metal imbalances, osmolality abnormalities, immune-mediated, and parasitic and bacterial infectious agents. Some common hemolytic toxins include plants such as *Brassica sp.*, *Allium sp.*, and red maple (*Acer rubrum*). Deficiency of or excess copper may result in hemolytic anemia. Phosphorus deficiency in cattle has been linked to postparturient hemoglobinuria in early lactating cows, although not all animals with hypophosphatemia develop hemolysis, so other factors may contribute to this disease.[24] Water intoxication leading to hyponatremia and hypoosmolality with resultant hemolysis can occur in calves, but perhaps not in adult cattle.[20,35] Coombs-test-positive immune-mediated hemolysis in cattle has been described and is most often secondary to underlying infectious diseases or drug administration.[49]

Nonregenerative anemia most commonly occurs secondary to chronic inflammation when cytokines signal iron sequestration in macrophages, making it less available for full, robust erythropoiesis. The anemia of inflammation is usually mild to moderate, and normocytic and normochromic.[65] A similar mechanism likely occurs with neoplasia. Other causes of nonregenerative anemia include chronic kidney disease, endocrine diseases, and nutrient imbalances such as vitamin B12 or folic acid deficiency (usually macrocytic and normochromic) and iron deficiency (usually microcytic and hypochromic). Macrocytic normochromic nonregenerative anemia has also been described with congenital dyserythropoiesis in polled Hereford calves[64] (see Chapters 37, 29, and 146).

Leukocyte Responses

Physiologic

Transient physiological neutrophilia and lymphocytosis can occur during periods of excitement or fear due to the effects of epinephrine, particularly in younger animals. Mildly increased lymphocyte concentration may occur after vaccination. Endogenous or exogenously administered corticosteroids cause demargination of neutrophils and enhance release from the bone marrow producing neutrophilia, lymphopenia, and possibly monocytosis that is part of the stress leukogram. Leukocyte responses due to corticosteroid injection have been evaluated in buffalo calves. A single injection of corticosteroid, either prednisolone or dexamethasone, resulted in a leukocytosis with neutrophilia and no left shift, lasting from 2 to 24 hours. Prednisolone administration also caused an initial monocytosis 2–8 hours postadministration and an eosinopenia and basopenia by 6 hours postadministration. Interestingly, lymphopenia was not evident after prednisolone administration. Inconsistent changes were noted in lymphocyte, monocyte, eosinophil, and basophil numbers after dexamethasone administration. The lack of lymphopenia with steroid administration is dissimilar to many other species.[34]

Inflammatory Response

In neonatal calves, the inflammatory leukocyte response comprises neutrophilia and a left shift, similar to small animals. Thereafter, as neutrophil concentration declines somewhat,[9] the typical response to severe acute inflammation in cattle is neutropenia with a left shift, similar to horses. Mature cattle have a smaller granulocytic bone marrow reserve, so it may take 3–5 days for rebound from initial neutropenia. Serial leukograms can help determine if the bone marrow is responding adequately, in conjunction with clinical condition. Persistent neutropenia, particularly if accompanied by a degenerative left shift, warrants a more guarded prognosis. Toxic changes in neutrophils are frequently present with acute inflammatory conditions in cattle.

Chronic inflammatory stimulus results in neutrophilia, once granulocytic hyperplasia occurs in the bone marrow. Neutrophilia in cattle rarely exceeds $30 \times 10^9/L$ even with long-standing lesions and closed-cavity processes, such as an abscess or peritonitis. An exception to this occurs with bovine leukocyte adhesion deficiency, where concentration may approach $100 \times 10^9/L$[36] (see Chapters 43 and 44).

Neutropenia can also occur with viral diseases such as BVDV,[69] infection with rickettsial organisms (*Anaplasma phagocytophilum*),[62] toxins, drug reaction, and bone marrow disorders. If persistent neutropenia is accompanied by nonregenerative anemia and thrombocytopenia, bracken fern toxicity should be considered in the differential diagnosis list.[58]

Lymphocytosis that persists on repeat evaluation should prompt suspicion for BLV infection. Animals with persistent lymphocytosis due to BLV infection usually have no clinical signs. Rarely, a BLV-infected animal may develop lymphoma or lymphocytic leukemia. Because of the high prevalence of subclinical BLV infection in some regions, data from apparently healthy cattle may have been incorporated into reference values, thereby falsely increasing expected lymphocyte concentration.[66] Lymphoproliferative diseases are rare in bovids, but are another possible cause of lymphocytosis. Since bovine lymphocyte morphology is so variable, even in healthy cattle, presence of immature cells alone should not be used as the sole determining factor when diagnosing lymphocytic leukemia. Lymphopenia may occur with viral and bacterial disease as well as in the stress response. Congenital immunodeficiency may result in lower lymphocyte concentration.[7]

Monocytosis will occur with chronic inflammatory conditions. Corticosteroid therapy will also induce this change, but may be less common in cattle. Monocytopenia has limited clinical relevance.

Eosinophil counts are low in newborn calves and increase as the animal matures. Eosinophilia typically

occurs with hypersensitivity (especially type 1) and with ectoparasitism and endoparasitism. Blood eosinophilia was observed in a heifer with nasal granuloma caused by *Nocardia sp*.[60] Rarely, eosinophilia might be associated with neoplasia. Eosinopenia has minimal interpretive relevance.

Basophils are rare in cattle, but may be increased in conjunction with eosinophilia. Basophilia has been observed with allergies and parasitic disease. Similar to eosinopenia, basopenia has minimal interpretive relevance.

Hematologic changes are observed in response to endotoxin injection and are dose-dependent. Low-dose endotoxin injection (10 µg) resulted in mild leukopenia, mild lymphopenia, and moderate neutrophilia 6 hours postadministration. A trend toward a more significant leukopenia, neutropenia, and lymphopenia was noted with higher doses of endotoxin. A rebound neutrophilia was not observed after endotoxin administration, although neutrophil numbers returned to normal by 6 hours for endotoxin doses <50 µg and within 24 hours for doses of 100 µg endotoxin.[34]

Hemoparasites

Bovine hemoparasites may cause variable hematologic abnormalities dependent on the specific agent, pathogenic mechanisms, host specificity, and overall health status of the animal. Protozoal organisms include *Babesia sp*.,[11] *Theileria sp*.,[48] and various trypanosomes,[6] and bacteria include *Anaplasma marginale*.[5] Hematologic abnormalities in bison have been reported for infection with *Theileria annulata*, *Trypanosoma theileria* and *evansi*, *Mycobacterium bovis*, and *A. marginale*.[26,38,40,46] Water buffalo can be infected with many of the same organisms as tick vectors are similar.[30,39] Hemoparasitic infestation of WBCs and platelets does not occur frequently or has not been described well in bovids.

Babesiosis results in direct infection of RBCs by the parasite, and subsequent intravascular hemolysis when division is complete. Hyperbilirubinemia and hemoglobinuria are usually present. Reticulocytosis is usually evident. Disease is most often caused by *Babesia bovis* or *Babesia bigemina*.[11]

Theileria are closely related to *Babesia*, but differ by first developing in WBCs before infecting RBCs. Hemolytic anemia ranges from mild to severe depending on the specific organism and host factors. *Theileria parva* and *T. annulata* are two of the more common agents infecting cattle.[48]

Disease-causing trypanosomes include *Trypanosoma congolense*, *Trypanosoma vivax*, and *Trypanosoma brucei*. Anemia is usually present, and organisms may be observed in a blood smear during active infection. Nonpathogenic trypanosomes can occur in cattle in many parts of the world, such as *T. theileri*.

Infection with the intraerythrocytic rickettsial bacterium *A. marginale* typically causes an extravascular hemolytic anemia in affected cattle.[5] Hemoglobinemia and hemoglobinuria are usually absent. Organisms can be observed on a blood smear during the acute phase of infection, but not during the carrier state.

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CHAPTER 112

Hematology of Sheep and Goats

JASON STAYT

Erythron
  Molecular Structure
  Blood Types
Leukon
Platelets
Plasma Proteins
Bone Marrow
Reference Intervals
  Partitioning Factors
Hematologic Disease
  Physiologic
  Anemia
  Changes in the Leukon

Acronyms and Abbreviations

2,3-DPG, 2,3-diphosphoglycerate; A1AGP, alpha-1-acid glycoprotein; APP, acute phase protein; Auto fib, automated fibrinogen concentration; EPO, erythropoietin; Fib %, percentage fibrinogen measured by the heat precipitation method; Fib sub, subtracted fibrinogen measured by the heat precipitation method; GMT, gelatinous marrow transformation; Hp, haptoglobin; HbA, hemoglobin A; HbAB, hemoglobin AB; HbAD, hemoglobin AD; HbB, hemoglobin B; HbC, hemoglobin C; HbD, hemoglobin D; HK, high potassium; LK, low potassium; SAA, serum amyloid A.

In 2020, worldwide populations of sheep (Ovis aries) and goats (Capra hircus) were estimated at 1.4 and 1.2 billion, respectively, and are economically important livestock species in many countries, including in the Middle East, Asia, and Africa. Furthermore, sheep are extensively used in biomedical research as models for human disease. Despite the widespread use of sheep and goats in research, some variation exists for normal hematologic reference intervals due to differences in methods of restraint, blood collection, laboratory methodologies, and physiologic factors such as age, reproductive status, and breed.

Blood collection is accomplished with the animal restrained in a standing position or tipped onto the hind quarters. Blood for hematologic analysis is obtained from the jugular vein. Animals should be at rest and handled gently to minimize stress.[10]

ERYTHRON

Goat RBCs are the smallest of the domestic species with an average diameter of 3.2–3.4 µm and an average mean corpuscular volume (MCV) of 19.5 fL. Sheep RBCs are slightly larger with an average diameter of 4.4–4.5 µm and an average MCV of 34 fL (Tables 112.1 and 112.2). Because of their small size, sheep and goat RBCs may not be correctly classified by impedance-based analyzers. Anisocytosis and poikilocytosis are prominent common features observed in normal blood films of juvenile and adult goats; RBCs can be round to flat (with a very shallow central depression) to oval or pear-shaped to spindled/fusiform in shape. Spindled/fusiform RBCs are particularly prominent in the Angora goat breed and in some breeds of British sheep (Figure 112.1). This is an innocuous change due to hemoglobin polymerization.

The majority of sheep RBCs appear as round biconcave discs with a shallow central depression and typically show slight anisocytosis. Small wart-like protuberances on the surface of sheep RBCs have also been observed. Since the degree of biconcavity in sheep and goat RBCs is less than in other domestic species, their ability to deform as they circulate is less.[22–24,30,43] Polychromasia and reticulocytes are not normally present in the circulation of adult goats and sheep. Their presence therefore indicates some degree of regeneration.

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TABLE 112.1 Normal ovine hematologic reference intervals and other blood values.

	Merino lambs
	Reference interval
Hemoglobin (g/L)	105–137
RBC count (×10^{12}/L)	9.2–13
Hct (L/L)	0.28–0.39
PCV (L/L)	0.30–0.43
MCV (fL)	28–35
MCHC (g/L)	332–392
Platelets (× 10^9/L)	426–1142
WBC count (× 10^9/L)	5.1–15.9
Neutrophils (× 10^9/L)	0.8–6.3
Lymphocytes (× 10^9/L)	2.1–10.2
Monocytes (× 10^9/L)	0.1–0.8
Eosinophils (× 10^9/L)	0–0.2
Basophils (× 10^9/L)	0–0.2
N:L ratio	0.1–1.2
Fib % (g/L)	1.6–4.1
Fib sub (g/L)	1–4
Auto fib (g/L)	1.2–2.6
Haptoglobin (g/L)	0.06–0.12
SAA (μg/L)	0–2
Plasma proteins (g/L)	60–75
RBC life span (days)	140–150

Auto fib, automated fibrinogen concentration; Fib %, percentage fibrinogen measured by the heat precipitation method; Fib sub, subtracted fibrinogen measured by the heat precipitation method; SAA, serum amyloid A. **Sources:** Lepherd et al.[29]; Oikonomidis et al.[36]; Byers and Kramer.[9]

TABLE 112.2 Hematologic reference intervals in healthy Shami goats (age 4 months to adults).

	Female	Male	Female and male combined
RBC count (× 10^{12}/L)	9–16	8–15	8–16
Hemoglobin (g/L)	60–130	60–110	60–130
PCV (L/L)	0.19–0.39	0.22–0.38	0.19–0.39
MCV (fL)	17–27	20–29	17–29
MCH (pg)	6–10	6–11	6–11
MCHC (g/L)	240–410	230–420	230–420
WBC count (× 10^9/L)	2–19	4–12	2–19
Seg neutrophils (× 10^9/L)	1–9	1–7	1–9
Lymphocytes (× 10^9/L)	1–12	0.9–6	1–12
Monocytes (× 10^9/L)	0–1	0–0.5	0–1
Eosinophils (× 10^9/L)	0–1	0–2	0–2
Basophils (× 10^9/L)	0–1	0–0.02	0–1

Source: Based on Al-Rukibat and Ismail.[43]

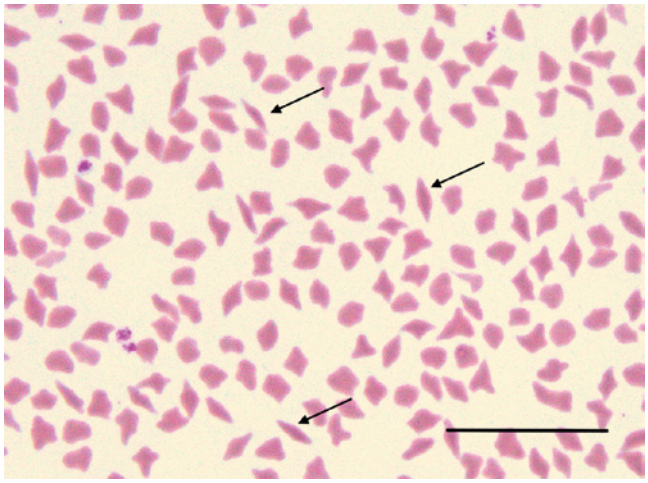


FIGURE 112.1 Blood film from an Angora goat. Poikilocytosis is a normal feature observed in many kids and adult goats. In the Angora goat, fusiform-shaped RBCs (black arrows) are a normal finding and, in this image, can be seen in low numbers admixed with other RBCs. The fusiform morphology results from hemoglobin polymerization (Wright–Giemsa, scale bar 20 μm).

Molecular Structure

The phospholipid classes found within the membrane of sheep and goat RBCs are different to nonruminant species. Membranes in sheep and goat RBCs lack phosphatidylcholine and have relatively higher amounts of phosphatidylinositol. This feature is thought to be dietary-related, reflecting the high level of inositol compounds present in the leaves of plants and grains.[34]

Two types of hemoglobin (Hgb) are present in normal adult sheep, HbA and HbB, which give rise to three phenotypes, HbA, HbAB, and HbB. In goats, three adult hemoglobins have been identified, HbA, HbB, and HbD. Polymorphisms occur with adult goat hemoglobin with four phenotypes HbA, HbB, HbAB, and HbAD being most common. Another hemoglobin type, HbC, demonstrates decreased oxygen affinity and is not readily seen in normal adult sheep, but is present in low concentrations in adult goats, accounting for approximately 5–10% of adult hemoglobin. HbC replaces fetal hemoglobin (HbF) and this remains the predominant hemoglobin in lambs and goat kids up to 4–5 months of age. In severely anemic animals or in animals living at high altitudes, the erythroid line can switch to synthesize HbC.[4,43] General RBC physiology and metabolism are described in Chapters 20 and 21.

Blood Types

Seven blood types are currently recognized in sheep, including A, B, C, D, M, R, and X. The C type is homologous to the C type in cattle and blood types B, C, and M are complex and highly polymorphic.[38–40] The M system is involved in RBC potassium polymorphism resulting in RBCs with high potassium (HK-type cells) or low potassium (LK-type cells) concentrations. In HK-type RBCs, increased (Na^+–K^+) ATPase activity results in increased potassium transport.[12,41] In lambs under 7 weeks of age, RBC potassium levels are higher than

adult levels, irrespective of their potassium genotype, and adult RBC potassium levels are achieved following the full development of M antigens.[47] The X type resembles the F type in cattle and the R type is similar to the soluble J type observed in cattle.[41]

Using sheep reagents, five blood types are recognized in goats, including A, B, C, M, and J, although other reports have described up to thirteen types. Regardless of the study, the B type has been found to be complex, highly polymorphic, and is comparable to that of sheep. The J type is similar to cattle and comprises a soluble antigen.[33,35,37,48]

LEUKON

Changes in the leukon are often evaluated for evidence of inflammation. Both total WBC count and differential cell count should be undertaken, as changes in individual cell types can occur without leukocytosis or leukopenia. Visual examination of leukocyte morphologic changes, such as toxic change, will assist in determining inflammatory conditions and septic etiologies.

Ovine and caprine neutrophils are similar and will be described together. Mature segmented neutrophils are approximately 10–15 μm in diameter and contain a large multilobulated nucleus (Figure 112.2a,g). Segmented neutrophils are similar to other ruminant and nonruminant species; although a third granule population, tertiary granules, is present. Unlike cattle whose tertiary granules are numerous and prominent, tertiary granules in sheep and goat neutrophils only occupy a small amount of cytoplasm; giving the cytoplasm a pale eosinophilic and finely granular texture when stained with Wright- or Giemsa-based stains.[9] In goat neutrophils, granules are electron-dense and markedly pleomorphic when viewed under transmission electron microscopy. The majority appear round to rod-shaped to dumbbell-shaped with fewer granules appearing large and ovoid to rod-shaped.[3] Granule enzymes in sheep and goat neutrophils are similar to other species; however, they lack lysozyme, as do bovine neutrophils.[46]

Ovine and caprine lymphocytes are typically small to intermediate in size (6–9 μm) and are easily distinguished from monocytes on Wright- or Giemsa-stained blood films (Figure 112.2b,h). Cells have scant pale blue to moderately blue cytoplasm and high to moderate N:C ratio. Some lymphocytes may also have low numbers of small azurophilic granules typical of natural killer cells (Figure 112.2n). Lymphocyte nuclei are round to oval with a clumped chromatin pattern. When antigenic stimulation is present in an individual, circulating reactive lymphocytes may be observed (Figure 112.2o).

As in most nonruminant and ruminant species, the monocyte is the largest of the circulating leukocytes

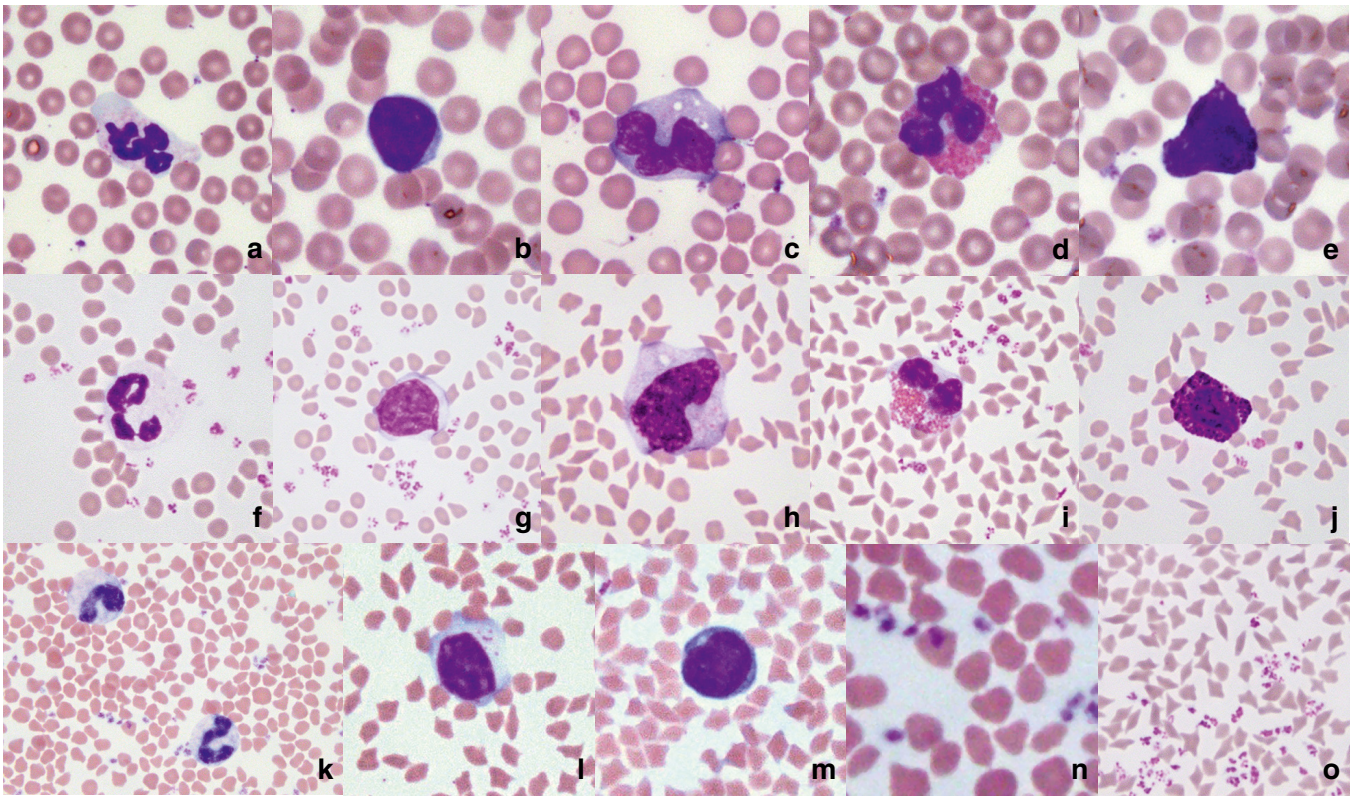


FIGURE 112.2 Comparative leukocytes and platelets. Ovine leukocytes and platelets: (a) Segmented neutrophil; (b) Lymphocyte; (c) Monocyte; (d) Eosinophil; (e) Basophil; (f) Segmented neutrophil. Caprine leukocytes and platelets: (g) Lymphocyte; (h) Monocyte; (i) Eosinophil; (j) Basophil; (k) A toxic band neutrophil demonstrating cytoplasmic vacuolation and basophilia; (l) Granular lymphocyte. Additional caprine leukocytes: (m) Lymphocyte exhibiting features of reactivity including dark blue cytoplasm with a small pale juxtanuclear Golgi area. Wright–Giemsa. (n) Ovine platelets. (o) Caprine platelets.

(13–19 μm). When in circulation, monocytes are round to oval in shape and have pale blue gray cytoplasm which may contain small vacuoles and small azurophilic granules (Figure 112.2c,i).

Eosinophils are slightly larger than neutrophils and basophils (10–15 μm) and have a bilobed or band nucleus which may be partially obscured by granules. Eosinophil granules in sheep and goat are numerous, round, refractile, and stain intensely red (Figure 112.2d,j).

Circulating basophils are infrequently observed in blood films from healthy sheep and goats. Basophils are typically round, measure approximately 8–10 μm, and have a less segmented nucleus than neutrophils. The cytoplasm is generally pale blue and contain numerous basophilic granules which typically obscure the nucleus (Figure 112.2e,k).

PLATELETS

Ovine and caprine platelets are small compared to other domestic species, with mean diameters of 2.6 and 2.2 μm, respectively, and, in the goat, a mean volume of 4.2 fL.[22] In both species, platelet numbers and platelet mass are high when compared to other domestic species.[30] In Wright-stained blood films, ovine and caprine platelets are distributed individually and in aggregates (Figure 112.2f,l). Surface features of ovine and caprine platelets are similar to other domestic species. Scanning electron microscopic studies reveal a discoid shape with a smooth surface and occasional small surface depressions.[22] Pseudopod formations are uncommon. Caprine platelets contain an open canalicular system with dense glycogen particles and small dense bodies which are sometimes surrounded by a clear vacuole. A circumferential microtubule system is also present. Both ovine and caprine platelets readily aggregate with ADP and thrombin, but not with epinephrine, and sheep platelets are unable to adhere to fibrinogen[30] (see Chapters 76, 77, and 79).

PLASMA PROTEINS

Ovine and caprine plasma is colorless independent of diet. Discolored plasma may be indicative of an underlying pathologic disorder including intravascular or in vitro hemolysis (red) and intra and extravascular hemolysis or hepatic disease (yellow).

The major acute phase proteins (APPs) in the sheep and goat are haptoglobin (Hp) and serum amyloid A (SAA). Concentrations increase rapidly following the onset of infectious and noninfectious inflammatory conditions, often peaking within 24–48 hours. In goats, Hp response can decrease in pregnancy secondary to systemic immunosuppression. Therefore, Hp concentrations in goats with unknown pregnancy status should be cautiously interpreted. Similar to other ruminant and nonruminant species, albumin is a negative APP, decreasing in concentration following an inflammatory stimulus.[15,16,21]

Fibrinogen is commonly measured as an indicator of inflammatory disease in ruminants; however, this is only a moderate APP in the goat and a minor APP in the sheep. Furthermore, the hydration status of the animal may affect plasma concentrations. Hypofibrinogenemia may be observed with liver disease and consumptive coagulopathies. A heritable afibrinogenemia has been reported in Saanen goat kids.[8]

BONE MARROW

In the commercial setting, bone marrow collection from sheep and goats for diagnostic purposes occurs infrequently. Bone marrow may be analyzed using one or a few individuals when herd health is of concern, such as cytopenia induced by infections, nutritional disorders, toxicosis, and other less common etiologies such as neoplasia. Under these circumstances, sacrificial euthanasia is often performed. Domestic pet and companion sheep and goats may have diagnostic bone marrow examinations performed for similar indications as those in nonruminant species.

Prior to sample collection in the live animal, sheep and goats should be heavily sedated or placed under general anesthesia and the surgical site aseptically prepared. Hematopoietic tissue is fragile and lyses easily. Therefore, postmortem bone marrow specimens should be collected shortly after euthanasia to minimize any morphologic changes. Bone marrow aspirates and core biopsies are most easily obtained from the iliac crest, although other sites, including the ribs, femur, and sternebrae, may also be used. In goats, however, the latter site is least favored due to the potential for chronic abscesses in this region[10,43] (see Chapter 141).

Bone marrow cell differential values in sheep and goats and cytochemistry of bone marrow cells in sheep have been reported (Tables 112.3 and 112.4). As in other ruminant species, the M:E ratio in sheep and goats is lower than that of nonruminant species and the marrow stores of mature neutrophils are also lower. In sheep and goats, the M:E ratio is currently reported to range from 0.8 to 1.7 and 0.5 to 0.8, respectively.[1,9,43]

REFERENCE INTERVALS

There are several reference interval manuscripts for domestic and wild sheep and goats that are not created in accordance with the ASVCP guidelines and may not have outliers removed.[6] These should be used with caution as misdiagnosis may result.

Partitioning Factors

Red blood cell parameters in sheep and goats may vary widely and are influenced by several physiologic and environmental factors such as collection, storage, age, species, breed, gender, nutrition, seasonal variation, heat stress, and reproductive status.[2,18,20,42]

TABLE 112.3 Cytochemical reactions of bone marrow cells in clinically normal sheep.

Cell type	May–Grunwald–Giemsa stain Mean (%) ± SD	Peroxidase reaction	Sudan black B reaction	Periodic acid-Schiff reaction
Myeloid series				
Myeloblasts	0.96 ± 0.15	–	–	–
Promyelocytes	2.04 ± 0.23	+	+	+
Neutrophilic myelocytes	4.52 ± 0.61	+	+	+
Eosinophilic myelocytes	0.80 ± 0.61	+	+	+
Basophilic myelocytes	0.04 ± 0.08	–	+	+
Neutrophilic metamyelocytes	7.27 ± 1.47	+	+	+
Eosinophilic metamyelocytes	2.16 ± 1.08	+	+	+
Basophilic metamyelocytes	0.04 ± 0.08	–	+	+
Neutrophilic bands	14.68 ± 1.83	+	+	+
Eosinophilic bands	3.20 ± 1.59	+	+	+
Basophilic bands	0.16 ± 0.23	–	+	+
Neutrophils	3.52 ± 1.50	+	+	+
Eosinophils	1.20 ± 0.73	+	+	+
Basophils	0.16 ± 0.19	–	+	+
Total myeloid series	40.75 ± 2.95			
Erythroid series				
Rubriblasts	0.68 ± 0.16	–	–	–
Prorubricytes	1.36 ± 0.29	–	–	–
Basophilic rubricytes	5.08 ± 0.50	–	–	–
Polychromatic rubricytes	31.96 ± 1.91	–	–	–
Metarubricytes	16.65 ± 2.26	–	–	–
Total erythroid series	55.73 ± 2.54			
Myeloid:erythroid ratio	0.73 ± 0.08			
Lymphocytes	1.88 ± 0.59	–	–	+ or –
Plasma cells	0.24 ± 0.15	–	–	–
Monocytes	0.60 ± 0.28	+ or –	+ or –	+ or –
Megakaryocytes	0.32 ± 0.20	–	–	–
Mitotic cells	0.48 ± 0.41	–	–	–

+ = positive reaction; – = negative reaction; + or – = weak reactivity was observed in a few cells while others were negative.
Source: Al Izzi et al.[1]

Artifactually increased PCV can occur with prolonged storage.[20] At birth, the PCV is high, but this soon diminishes following ingestion of colostrum.[10] Juvenile lambs and kids generally have lower RBC numbers, increased MCV, and decreased mean cell hemoglobin concentration (MCHC) values.[9,13,26] Seasonal variations in RBC parameters have been reported; however, results are highly variable and likely associated with differences in geographic location, breed, and study design.[13,31,42]

There is limited information regarding maternal anemia during gestation in sheep and goats. Anesthetized pregnant sheep have demonstrated low RBC values, including a low PCV and Hgb concentration; however, this has not been found in pregnant sheep kept in a farm environment.[31]

At birth, the total WBC count in sheep and goats is slightly less than or similar to that observed in adults and numbers remain steady for the first 14 days. During this postnatal period, neutrophils are the predominant leukocyte and in kids they account for approximately 60% of the WBC population. By approximately 3–4 months of age, lymphocytes become the predominant leukocyte resulting in neutrophil:lymphocyte (N:L) ratios of 0.5 in lambs and 0.6 in kids. In adult sheep, the N:L ratio is approximately 0.5 and in adult goats it is approximately 1:1[2,9,13,17] (Tables 112.1 and 112.2).

TABLE 112.4 Proportions of bone marrow cells in the goat. This is presented as a rough guide to what may be found in the healthy goat.

Cell type	% cells
Myeloblasts	0.8
Promyelocytes	2.7
Myelocytes	8.3
Bands	8.9
Segmented neutrophils	10.0
Eosinophils	1.8
Monocytes	>0.1
Lymphocytes	7.5
Nucleated RBCs	56.3
Myeloid:erythroid ratio	0.6

Source: Based on Smith and Sherman.[44]

HEMATOLOGIC DISEASE

Physiologic

Neutrophilia and lymphopenia may be secondary to corticosteroid administration and are commonly seen as a response to stress associated with restraint for venipuncture or concomitant disease.

Anemia

Anemia is the most common and significant hematologic abnormality of sheep and goats and has many causes. Regenerative anemia occurs secondary to hemorrhage or RBC destruction due to predation, other trauma, internal parasitic disease (anaplasmosis, babesiosis, theileriosis, trypanosomiasis; *Mycoplasma sp*., coccidiosis; *Haemonchus sp*., schistosomiasis, etc.), bacterial endotoxemia (*Clostridium perfringens* type A; leptospirosis), ingestion of toxic plants (*Brassica sp*.; bracken fern), copper toxicosis, phosphorus deficiency, and external parasitic disease (ticks, lice). Neonatal isoerythrolysis is not known to naturally occur in sheep or goats; however, neonatal anemia and death have occurred in lambs and goat kids administered bovine colostrum. Hemolytic disease has been experimentally induced in the goat.[32,45,50]

Polychromasia, macrocytosis, reticulocytosis, metarubricytosis, and basophilic stippling in RBCs usually appear within a day or two of an anemic event. The reticulocyte response to anemia in the sheep and goat is mild to moderate and most obvious when severe anemia is present. The reticulocyte response is usually maximal by days six to eight.[9,10,26] In the acute to subacutely anemic animal, indicators of accelerated erythropoiesis can be observed in bone marrow aspirates, including hypercellularity, decreased M:E ratio, left shifting of the erythroid series, an increase in macrophage activity, and in some conditions (i.e., hemolytic anemias) increased iron turnover.[1,9,44]

Anemias associated with decreased bone marrow production can occur with chronic disease (e.g., *Mycobacterium paratuberculosis*), malnutrition/starvation and nutritional deficiencies (iron, copper, cobalt), and toxicoses (e.g., fluorosis, bracken fern).[10,11,44] Chronic systemic illness in sheep and goats of any age can result in a reduction in bone marrow cellularity with resulting cytopenias. In cases where these conditions lead to malnutrition and emaciation, gelatinous marrow transformation, also known as serous atrophy, is evidenced by focal loss of hematopoietic cells and fat cell atrophy with concomitant deposition of extracellular gelatinous substances (e.g., mucopolysaccharides).[5,44,49]

Erythrocytosis is commonly observed in the dehydrated animal or rarely in those affected by conditions causing hypoxemia, such as chronic pulmonary disease or right-to-left cardio vascular shunting, and animals grazing at high altitudes. Erythrocytosis due to neoplasia has been reported in a sheep with cholangiocellular carcinoma.[7,10]

Stomatocytic and echinocytic changes following administration of chlorpromazine and lysolecithin, respectively, have been reported in the goat.[25] Fusiform RBCs, however, appear to be less affected.

Changes in the Leukon

Mature neutrophilia and a reversal in the N:L ratio often occur with mild to moderate inflammatory stimuli. As the granulopoietic response continues, circulating immature neutrophils may be observed, which may or may not be accompanied by toxic changes (Figure 112.2m). Because of the small granulocyte reserve in the bone marrow, neutropenia is often observed with severe acute inflammatory conditions such as septicemia (often associated with Gram-negative bacteria), peritonitis, pericarditis, pneumonia, polyarthritis, endotoxemia, coccidiosis, and others.[10,24] *Anaplasma phagocytophilum* and *Anaplasma ovis* infection may also cause neutropenia without a left shift.[10,44]

Pathologic lymphocytosis is infrequent, but can occur secondary to various chronic infectious and noninfectious inflammatory disorders. While lymphoma has been described in both sheep and goats, an associated leukemia has not been reported.[27,28] Lymphocytic leukemia has, however, been induced in sheep inoculated with bovine leukemia virus.[14]

Eosinophilia is observed in various hypersensitivity-mediated and other inflammatory conditions including parasitic, bacterial, fungal, and protozoal infections. When present, basophilia often accompanies eosinophilia, particularly in response to the release of chemical inflammatory mediators such as histamine and heparin.[9,10,26]

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CHAPTER 113

Hematology of Pigs

CATHERINE E. THORN, ANDREW S. BOWMAN, and DAVID ECKERSALL

Erythrocytes
Leukocytes
Platelets and Coagulation
Bone Marrow
Reference Intervals
  Partitioning Factors
  Pregnancy, Parturition, and Lactation
Hematologic Disease
  Physiologic Change
  Pathologic Change
    Anemia
    Inflammation
    Hemoparasites

Acronyms and Abbreviations

APP, acute phase protein.

Blood collection in pigs can be difficult for unpracticed individuals. Pigs are quite excitable and need to be restrained or sedated for animal and human safety. Additionally, the major blood vessels cannot be visualized because they lie below a fair amount of fat and muscle, which makes site identification reliant upon anatomic landmarks. However, adequate blood volume can be obtained from a number of sites.

The jugular vein is the preferred site, as it allows for large volumes of blood to be quickly collected from any aged animals with minimal restraint. The cephalic vein on the foreleg can be used in pigs of any age for up to 5 mL of blood. The lateral auricular vein, or ear vein, can be used for collection of <5 mL of blood. This vein is large and visible, but collapses easily unless a butterfly catheter is used. Access to the anterior vena cava can be technically difficult and may require a cut down, but large volumes of blood may be obtained from this vessel. The coccygeal or tail vein can be problematic, since it is not easily visible, especially in young or small pigs, and lymph dilution is possible. The cranial abdominal vein (external mammary or milk vein) can be used to rapidly obtain 5–20 mL of blood in older animals.[27,58,63]

EDTA anticoagulant and a Romanowsky-type stain are preferred for optimal hematologic evaluation. Because the porcine RBC is relatively fragile, excess turbulence or improper sample handling often results in hemolysis. Routine hemogram values, except the differential cell count, are stable at 20 °C or 4 °C for 36 hours,[34] whereas the WBC differential count becomes less reliable within 12 hours after collection.[15]

ERYTHROCYTES

Porcine RBCs range in diameter from 4–8 μm with an average of 6.0 μm. Spiny crenation and rouleaux formation are common in health (Figure 113.1). Central pallor is not visible in every cell. Anisocytosis is seen in adults, but is more prominent in younger pigs. Blood from young pigs contains many large polychromatic cells, nucleated RBCs, and Howell–Jolly bodies.

The pig erythrocyte is highly susceptible to hemolysis by hypotonic saline and is more fragile than that of other species. Porcine RBCs display a bimodal pattern, with adult and fetal populations being more resistant to lysis than RBCs from weaned pigs. Osmotic resistance is temperature-, pH-, and time-dependent.[36,37] The sedimentation rate is faster than that of other domestic animals.[64]

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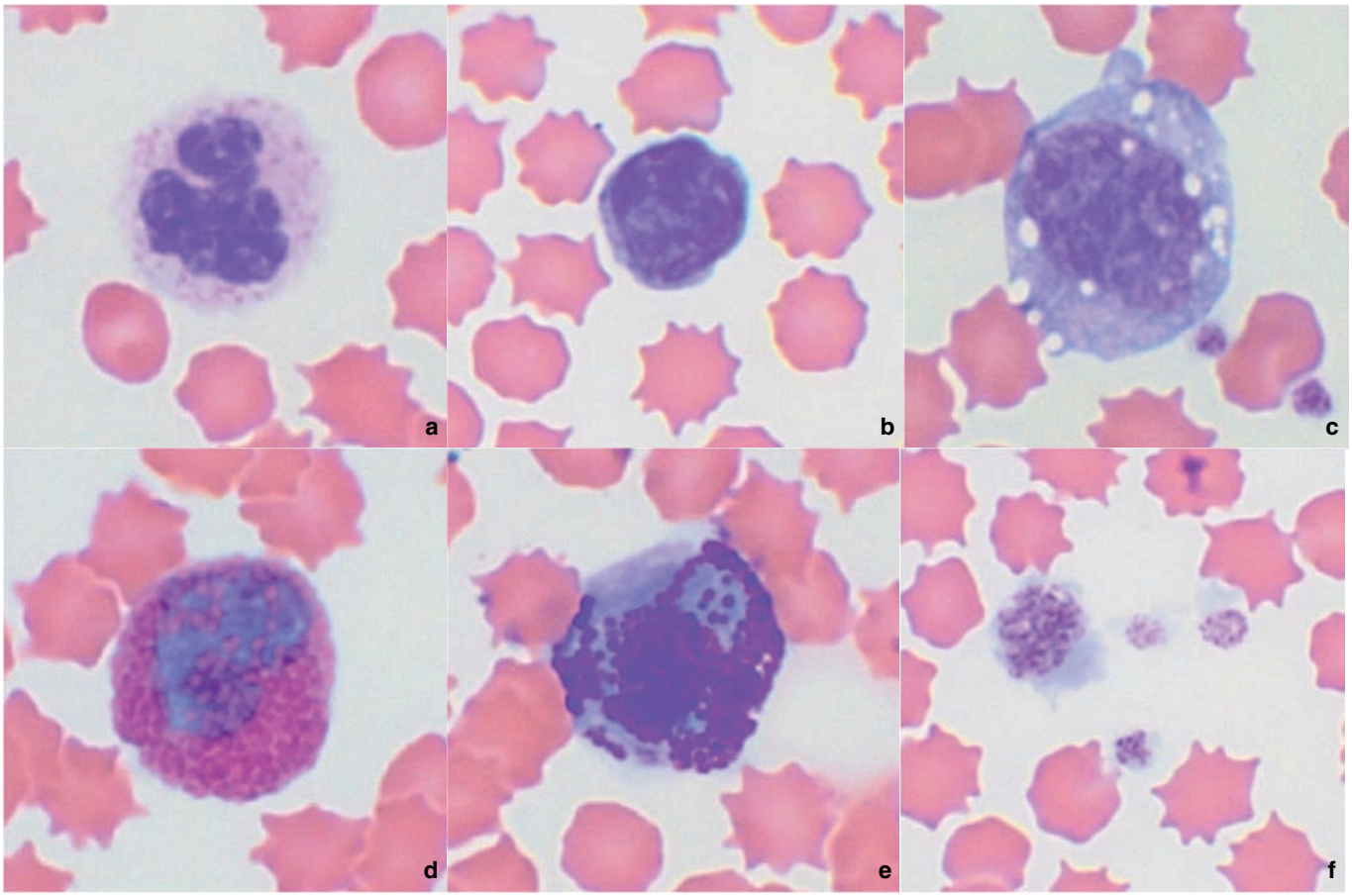


FIGURE 113.1 Photomicrographs from an apparently healthy Vietnamese pot-bellied pig (Sus scrofa). (a) Neutrophil; (b) Lymphocyte; (c) Monocyte; (d) Eosinophil; (e) Basophil; (f) Variation in platelet size and ecchinocytosis found in normal pigs. 1000×. Modified Wright–Giemsa stain. (**Source:** Volu-Sol, Salt Lake City, UT.)

LEUKOCYTES

Porcine WBC are generally similar in morphology to other domestic mammals. The mature neutrophil is 12–15 µm in diameter. It has an irregular nuclear membrane and moderately coarse chromatin with well-defined lobes (Figure 113.1a). The cytoplasm stains a pale pink or blue and may contain a few pink to purple granules. Band neutrophils have a U-shaped nucleus, but are otherwise similar to the mature form. Low numbers of band cells may be present in the healthy animal.

Lymphocytes are the dominant cell type in peripheral pig blood (Figure 113.1b). Small lymphocytes are 7–10 µm in diameter and have a round to oval nucleus with condensed chromatin and a small rim of pale blue cytoplasm. Large lymphocytes are 11–15 µm in diameter. The chromatin pattern is more diffuse and does not stain as intensely as the small lymphocyte. The cytoplasm is slightly more abundant and stains pale blue. Large lymphocytes may contain azurophilic granules as in other mammalian species.

Monocytes are 14–18 µm in diameter and have a convoluted, bean-, or horseshoe-shaped nucleus. The nucleus has lacy chromatin, often with areas that are more condensed or clumped (Figure 113.1c). The abundant blue-gray cytoplasm may contain vacuoles. Some monocytes may be difficult to distinguish from a large lymphocyte or immature, toxic neutrophil.

Eosinophils are similar in size to neutrophils, but the round to oval cytoplasmic granules stain a pale orange to pink color and tend to fill the cytoplasm (Figure 113.1d). Eosinophil nuclei are poorly segmented and may appear immature. The basophil nucleus stains lavender to dark purple and has a smooth chromatin pattern (Figure 113.1e). The basophilic cytoplasmic granules are coccoid to dumbbell-shaped and stain similarly to or more intensely than the nucleus, which they may obscure.

PLATELETS AND COAGULATION

Porcine platelets are variable in shape and size (Figure 113.1f). They are generally small, 1–3 µm in diameter, with a mean volume of 6.9–8.9 fL.[13] Pliszczak-Król reported a slightly higher range of 8.4–9.75 fL. They are anucleate and have deeply staining purple cytoplasmic granules. Clumps are frequent. The plateletcrit in pigs has been reported as 0.009–0.395%.[51]

Coagulation of pig blood not collected into anticoagulant is rapid. Coagulation tests and platelet function analyses in pigs are seldom used in clinical situations. Kostering reported a prothrombin time of 14.7 seconds for adult Hanover domestic pigs. Pliszczak-Król reported similar findings of 12.2–16.3 seconds for pigs at 24 weeks of age. Thrombin time ranges between 16.7 and 24.5 seconds.[27,35] Activated partial thromboplastin time has a biphasic course, being relatively short before week 5 of age (17.8–19.9 seconds) and then becoming much longer (28.7–52.5 seconds). Coagulation profiles are utilized in research settings.[53,62] Flow cytometry has been used for measurement of platelet activation and assessment of clot quality[30] (see Chapter 118).

BONE MARROW

Similar to blood collection, bone marrow is more frequently collected under research conditions. The iliac crest (tuber coxae) is a common site for collection.[6] However, cortical thickness of this site in swine is much greater than that of other species. Except in neonatal swine, collection of marrow cells from the sternum is possible. The proximal tibia is also accessible, this area has less cortical thickness and is free of muscle.[58] Myeloid to erythroid ratios of 1.2–2.2:1 have been reported[58] (see Chapter 141).

REFERENCE INTERVALS

Many reference intervals for porcine blood have been published, but are not in compliance with American Society of Veterinary Clinical Pathlogy guidelines.[13,23,27] Although these are comparable, the ranges for most elements of porcine blood are quite wide. Variability occurs with age, breed, growth rate, diet, age, stage of gestation or lactation, feeding method, management practices, or season. Interpretation of porcine hematology data requires consideration of these partitioning factors; however, most sex differences are not biologically important.

Partitioning Factors

Many RBC changes take place after birth.[11,35] Within a few days of age, RBC number and hemoglobin (Hgb) concentration drop 30–38% after expansion of plasma volume. Cell size increases soon after birth, decreases to its smallest volume at 2–6 months of age, and then increases again to adult size. In suckling pigs, reticulocyte counts of 3–8% and nucleated red cell counts of 5% are common. These decrease as the pig matures. Changes in hematologic parameters of piglets farrowed and kept on concrete for 10 days and then transferred outside to soil are presented (Table 113.1).

Polychromasia, Howell–Jolly bodies, crenation, rouleaux, and poikilocytes are often seen in the blood of normal young pigs. Red cell count and Hgb concentration increase to reach adult levels at about 5 months of age.

Leukocyte numbers increase over the first 12 hours of birth. Rolinec reported a mean of 5.26×10^3 WBC/μL at birth; this increased to an average of 10.82×10^3 WBC/μL at 12 hours but with a very wide interval ($6.36–17.74 \times 10^3$ WBC/μL). Colostral leukocytes appear in the blood between 5 and 7 hours after colostrum intake.[7] At birth, neutrophils predominate with fewer lymphocytes. By 12 hours, due to colostrum intake, the ratio reverses and most of the cells are lymphocytes. The absolute number of lymphocytes has a large interval at 12 hours ($2.39–17.47 \times 10^3$ μL), and is affected by many factors such as age at weaning, disease, nutrition, stress, social status, and housing. Some apparently healthy, individual animals may not have a neutrophil:lymphocyte reversal until a week of age. Total WBC count wains shortly after birth, then increases at about the fifth week of life.

Platelet numbers are variable and fluctuate markedly in the first few hours and weeks of life, but stay within the reported reference interval of $211–887 \times 10^9$/L.[23,51] After 24 weeks of age, platelet number becomes more stabilized at about $400–450 \times 10^9$/L.[45] The mean platelet volume tends to increase insignificantly over the first 12 hours of life, but stabilizes quickly after that.[51]

The rapid growth rate of young pigs places a large demand on iron requirements that cannot be met by milk alone. Unless supplemental iron is provided, piglets develop a severe microcytic, normocytic to hypochromic anemia. Early contact with soil or intramuscular injection of iron dextran is recommended. Spontaneous recovery occurs about the fifth week of life when the pigs start to derive nutrients from other sources. The effect of low iron on WBC counts is variable;[56] however, iron deficient piglets have less developed immunocompetence.[57]

Pregnancy, Parturition, and Lactation

Hematologic changes occur with pregnancy, parturition, and lactation. Approximately 2 weeks before parturition, RBC values decrease and stay low until the end of lactation. Reticulocytes may be seen during this period. Total WBC number decreases during gestation, and anemia during pregnancy has been documented. Neutrophilia, left shift, and lymphopenia often occur at parturition, likely due to endogenous corticosteroids. Within 24 hours of farrowing, the neutrophil:lymphocyte ratio reverses.

HEMATOLOGIC DISEASE

Physiologic Change

Sampling stress is one of the largest sources of hematologic variation.[9] Pigs are easily stressed through handling and restraint, and the stress response develops within 2 minutes, rapidly affecting the leukogram. The total WBC and neutrophil count can increase by two to threefold. There are also reported elevations in Hgb

TABLE 113.1 Influence of age and husbandry on the hematology of young Duroc-Jersey pigs. Mean and minimum/maximum range for a single litter of five males and four females. Pigs kept on concrete until 10 days of age and then placed on soil.[a] No outliers were removed and this does not represent a reference interval.

Age		Wt.	RBC	Hgb	PCV	MCV	MCHC	MCH	Retic.	nRBCs	Sed Rate	WBC
(days)	Value	(lb)	(× $10^6/\mu L$)	(g/dL)	(%)	(fL)	(%)	(pg)	(%)	/100 WBC	(1 hr)	(× $10^3/\mu L$)[b]
1	Min.	1.7	4.3	8.4	27	57	28.9	18	4.5	0.5	0	7.6
	Max.	3.3	6.4	12.3	42.5	71	31.3	21	10	4.0	4	15.3
	Ave.	2.4	5.3	10.5	35	67	30.5	20	6.7	2	2	11.5
3	Min.	2.4	3.3	7.8	26.5	70	29.1	21	6.9	7	2	6.3
	Max.	4	5.2	11	36.5	81	30.3	24	16.6	57	12	13.4
	Ave.	3.2	4.5	9.8	33	73	29.5	22	12	17	5	9.4
6	Min.	3.5	3.4	6.4	22	60	26.4	17	4.5	5	12	7.4
	Max.	5	4.7	9.4	31	74	30.9	23	13	54	33	10.5
	Ave.	4.5	4	8	26.7	67	29.1	20	7.7	14	22.6	8.2
10	Min.	5.2	2.1	4.2	15	62	29	19	6	3	1	5.6
	Max.	7.1	4.3	8.7	20	78	31	24	12	30	35	19.1
	Ave.	6.4	3.5	7	24	68	29.6	20	10	11	12	10.9
20	Min.	8.5	4.4	9	35.5	70	26	19	9	1	0	6.2
	Max.	11.5	5.3	11.2	40.5	82	29	23	13	25	1	10.5
	Ave.	10.5	4.9	10.2	37	76	27.6	21	10.6	11.5	0	7.7
36	Min.	–	5.9	11.3	37	62	28	18.8	1.6	0	0	12.7
	Max.	–	6.8	13.3	44	68	32	20	6.8	1	2	20.9
	Ave.	–	6.2	12.1	39.7	64	30.5	19.4	3	0.5	0.5	16.3

[a] These data were developed in cooperation with Dr. Otto Straub.
[b] Corrected for nucleated red blood cells (nRBCs).

concentration and hematocrit (Hct). Long-term stress including transport,[4] disease,[12,32,34] infection, or living conditions[14] can affect hematological values.

Pathologic Change

Anemia

Anemia is a common problem in pigs. Acute and chronic blood loss can lead to hemorrhagic anemia. Active bleeding and injury are possible as group-housed pigs commonly fight to establish social dominance. Gastric ulcers are common in pigs and have been associated with several etiologies such as finely ground feed, stress caused by management failures, and disruptions in feed intake as a result of other diseases. Proliferative hemorrhagic enteropathy caused by infection with *Lawsonia intracellularis* and hemorrhagic bowel syndrome can cause blood to accumulate in the intestinal tract. Internal and external parasites (nematodes and lice), swine dysentery, rectal prolapses, gastric or intestinal torsions, and warfarin poisoning are other potential causes of blood loss.

Nutritional deficiency of iron is a well-known cause of anemia in young pigs, which have limited iron stores, grow quickly, and are usually raised indoors with no access to iron-rich soil. Iron deficiency anemia has been largely alleviated by the ubiquitous management practice of administering supplemental iron to neonatal piglets; however, iron supplementation should not be assumed, especially in small and unconventional herds. Copper deficiency may also cause defective erythropoiesis, as it is needed for the incorporation of iron into the hemoglobin molecule.

Several infectious diseases can also cause anemia directly or secondarily through inflammatory cytokines. In general, recovery from the primary disease leads to resolution of the anemia. Porcine reproductive and

respiratory syndrome virus which infects all age swine, can induce anemia through acute bone marrow failure. Hemolytic anemia and microangiopathic hemolysis can be seen with African swine fever and chronic classical swine fever. Schistocytes are common in blood smears from these animals.

Inflammation

Leukocytosis is a common response in pigs, similar to other animals as a reaction to stress or infection. Similar to parvovirus infections in other species, porcine parvovirus causes a transient leukopenia, usually within 10 days of infection. However, infections with porcine parvovirus are often subclinical except in developing fetuses where the outcome of infection is dependent upon gestational age.

The acute phase protein (APP) response to infections, inflammation, and trauma has been recognized in pigs for over two decades with initial recognition that c-reactive protein, haptoglobin, and α_1-acid glycoprotein were biomarkers of the porcine acute phase response.[10,31] An additional porcine-specific APP named major acute phase protein (pigMAP) also has potential as a biomarker and is analogous to kallikrein-sensitive PK-120, otherwise known as inter-alpha-trypsin inhibitor H4 in human plasma.[1,17] The role of the APP in assessment of health and welfare of pigs, including their use as experimental models of human disease, has been reviewed.[3,40,59]

The porcine APP responds to a variety of infections with a selection of diseases being examined for their role in stimulating c-reactive protein, haptoglobin, α1-acid glycoprotein, pigMAP, and serum amyloid A (SAA) including bacterial (*Actinobacillus pleuropneumoniae*, *Streptococcus suis*, *Mycoplasma hyosynoviae*), parasitic (*Toxoplasma gondii*), and viral (porcine respiratory and reproductive syndrome virus) infection.[24] Increased haptoglobin concentration has been documented in porcine respiratory coronavirus infection, which is a possible model for covid-19 infection of humans.[61] APP responses to single and concurrent infections[46-48] can identify an activated innate immune reaction to infection and, though increases are variable between pathogens, the response reflects active stages of disease with falling concentrations demonstrating recovery or response to treatment.

The use of porcine APP to detect and monitor the health and welfare of pigs in production has also been the subject of extensive research[38,43]with studies examining the change in APP concentration in wasting disease,[49] pleuritis, and cranioventral pulmonary consolidation.[52] Haptoglobin concentrations at slaughter can provide valuable diagnostic information regarding rearing conditions and respiratory disease.[2] Further study of haptoglobin and SAA at slaughter has demonstrated the value of monitoring APP as an aid to meat inspection.[5,39] Haptoglobin has been shown to be a biomarker for stress in pigs, both as a result of transport[22,44] and food deprivation.[41]

Porcine APP concentration has been assessed in alternate biological fluids to determine the presence of an acute phase response without the need for blood sampling, which can be difficult and traumatic for the animal. Haptoglobin and SAA have been examined in porcine saliva,[18,19,21] while the estimation of APP in meat juice[20,25,42] at slaughter has been suggested to assist in meat inspection and the elimination of carcasses from unhealthy animals from the food chain.

Hemoparasites

There are two recognized hemotropic hemoplasmas that infect swine, *Mycoplasma suis* and *Mycoplasma parvum*. A novel hemoplasma has recently been reported in China.[16] *M. suis* is distributed worldwide and where pigs are managed intensively; it causes only sporadic disease across all ages.[26,50] In affected piglets, it presents as life-threatening hemolytic anemia, icterus, fever, anorexia, general ill-thrift, and acute death. In sows, acute *M. suis* infections may cause sudden death due to hypoglycemic coma, while milder acute forms of disease cause decreased fertility.[55] *M. suis* rarely causes spectacular death losses. It is often associated with stress, outbreaks are more likely in the summer along with other concurrent infectious diseases, parasitism, or management problems. *M. suis* is an obligate, 0.8–1.0 µm parasite that attaches to the surface membrane of RBCs and endothelial cells. It is predominantly coccoid-shaped, but may appear rod- or ring-shaped and may be found in chains, solitary, or clustered. Parasitemia is transient, making evaluation of blood smears poorly sensitive and likely underdiagnoses infection.[55] More recently, *M. suis* is diagnosed using PCR or serologic examination of relevant animal groups (whole cell ELISA or recombinant ELISAs).[26] Using quantitative PCR methods, *M. suis* prevalence of 14% and 10% was determined for weaned piglets,[50] 14% for presuckling piglets, and 31% for sows at farrowing.[54] Transmission of *M. suis* occurs with transfer of infected blood during iatrogenic or zootechnical procedures or wounds associated with infighting.[5] Vertical transmission has been recently demonstrated.[54]

M. parvum is similar to *M. suis*, but is a smaller (0.2–0.5 µm), coccoid- to rod-shaped organism, which is observed individually or in short chains. Despite heavy parasitism, *M. parvum* causes few clinical signs.[33]

There are two forms of Babesia that can infect swine. *Babesia trautmanni* is a large Babesia found in the RBCs of infected pigs. The merozoites (which are usually paired) measure 2.5–4 × 1.5–2 µm and are oval or pyriform in shape. Single forms, probably trophozoites, are ameboid or round. The host cell usually contains between one and four parasites. *Babesia perroncitoi* is smaller than *B. trautmanni*, the majority of parasites being pleomorphic, annular, and measuring 0.7–2.0 µm in diameter. *B. trautmanni* may cause severe disease in pigs from Europe, Africa, and China. *B. perroncitoi* is of similar pathogenicity, but has a limited distribution in the areas mentioned in the earlier text. The vectors of these Babesia have not been clarified, although *Rhipicephalus sp*. have been shown to transmit *B. trautmanni*.[60]

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CHAPTER 114

Hematology of Rodentia

AMY L. MacNEILL

Introduction
Erythron
Leukon
Platelets and Coagulation
Reference Intervals
  Partitioning Factors
Blood Collection
Bone Marrow Collection and Evaluation
Hematologic Disease
  Physiologic
  Pathologic
Hemoparasites

Acronyms and Abbreviations

ASVCP, American Society for Veterinary Clinical Pathology; PV, mean platelet volume; RDE, red cell distribution width.

Species: Mouse (*Mus musculus*); Rat (*Rattus* sp.); Gerbil (Genera: *Ammodillus*, *Brachiones*, *Desmodilliscus*, *Desmodillus*, *Dipodillus*, *Gerbilliscus*, *Gerbillurus*, *Gerbillus*, *Meriones*, *Microdillus*, *Pachyuromys*, *Psammomys*, *Rhombomys*, *Sekeetamys*, *Tatera*, *Taterillus*); Hamster (Genera: *Mesocricetus*, *Phodopus*, *Cricetus*, *Cricetulus*, *Allocricetulus*, *Cansumys*, *Tscherskia*); Chinchilla (*Chinchilla* sp.).

INTRODUCTION

There are over 2250 known species of mammals classified within the phylogenetic order Rodentia. Individual rodent species have adapted to several vastly different environments, eat varied diets, and have species-specific behavioral traits. This chapter reviews hematologic findings in a small handful of rodent species that are commonly seen in veterinary practice. There are at least 14 species of gerbils and four subgenera. Most studies evaluating hematology in gerbils have been done using *Meriones unguiculatus*. Over 20 species of hamsters are recognized. Hematology indices for Chinese (*Cricetulus griseus*) and Syrian/golden (*Mesocricetus auratus*) hamsters are most commonly reported. There are two species of chinchilla, short-tailed chinchillas (*Chinchilla chinchilla*) and long-tailed chinchillas (*Chinchilla lanigera*). Note that, even among the small sampling of rodents discussed in this chapter, there are significant species differences in hematologic parameters. This must be taken into account when interpreting hematologic data for diagnostic purposes. Reference intervals used for diagnostic purposes should be generated or verified in accordance with the American Society for Veterinary Clinical Pathology (ASVCP) reference interval guidelines.

ERYTHRON

Rodent RBCs are anucleate; biconcave discs approximately 5–7 μm in diameter and 2 μm wide, though additional variation across different-sized species occurs. RBCs of mice, rats, gerbils, and hamsters circulate for 41–52, 42–69, approximately 30 days, and 50–70 days, respectively.[6,8,9,10,43] To the author's knowledge, chinchilla RBC life span has not been reported.

Reticulocytes comprise up to 20% of peripheral RBCs in healthy young rodents, but decrease to 2–5% in adults.[41] Polychromasia and basophilic stippling, indicative of reticulocytes, are common findings in Romanowsky-stained peripheral blood smears of healthy gerbils up to 20 weeks of age.[3,41] Low numbers of Howell–Jolly bodies and nucleated RBCs also may be observed in blood smears from normal rodents. In rats, reticulocyte life span in the peripheral blood is 1–1.5 days.[10]

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LEUKON

Approximately 70–80% of peripheral blood leukocytes (WBC) in most rodents are lymphocytes. Neutrophils (or heterophils in hamsters) typically comprise approximately 20–30% of the relative differential cell count in healthy rodents. Monocytes, eosinophils, and basophils are present in very low percentages in healthy rodents. Band neutrophils, earlier neutrophil precursors, and mast cells are not typically observed in the peripheral blood of healthy rodents. A diurnal variation in leukocyte concentration has been reported in rodents;[41] however, the time of blood collection is rarely indicated in published reference intervals or noted on sample submissions. Possible seasonal variations also should be considered; e.g., WBC concentration and lymphocyte concentrations are higher in chinchillas in late winter.[22] The appearance of WBCs in rodents is similar to that of other mammals (Figure 114.1).

Rodents have numerous small, fewer intermediate-sized, and rare large lymphocytes in circulation. In Romanowsky-stained blood smears, lymphocytes have scant, lightly basophilic cytoplasm and a round nucleus with dense to clumped chromatin. The nuclear diameter of small lymphocytes ranges from approximately 6–8 µm. The life span of a small lymphocyte in rat peripheral blood is 60–100 days.[10] Intermediate-sized lymphocytes have a 10–12 µm nuclear diameter. Low numbers of intermediate-sized lymphocytes that contain magenta cytoplasmic granules can be observed in rodent blood smears. Intermediate-sized lymphocytes with more deeply basophilic cytoplasm are occasionally observed and are often considered reactive lymphocytes. Large lymphocytes can have nuclei up to 15 µm in diameter.

The predominant granulocyte in mice, rats, chinchillas, and gerbils are neutrophils. Neutrophils are approximately 10–12 µm in diameter and have abundant clear cytoplasm with small nonstaining to lightly eosinophilic cytoplasmic granules. Murine neutrophils do not contain alkaline phosphatase activity.[14] The nucleus of rodent neutrophils is often segmented, although hyposegmented forms and ring-shaped nuclei can be observed. Mature neutrophil nuclei have clumped chromatin. Neutrophils circulate for approximately 6 hours in rats.[10] In hamsters, the predominant granulocytes are heterophils. Heterophils are similar in size and appearance to neutrophils, but contain small, thin, rod-shaped, eosinophilic to variably staining, cytoplasmic granules. Toxic neutrophils/heterophils can circulate if inflammation is occurring. Toxic neutrophils and heterophils can have more basophilic cytoplasm or may contain increased numbers of eosinophilic cytoplasmic granules.

Monocytes are larger than neutrophils (approximately 15 µm in diameter) and have abundant light blue-gray cytoplasm. Clear cytoplasmic vacuoles may be seen in normal animals. The nucleus tends to be ovoid to horseshoe-shaped with finely stippled chromatin. In rats, monocytes remain in the peripheral blood for approximately 18 hours.[10]

Eosinophils are approximately the same size as monocytes (15 µm). They have pale, basophilic cytoplasm filled with eosinophilic granules and a segmented or bilobed nucleus with clumped chromatin. In mice, granules often clump together making the borders of the granules difficult to discern. Eosinophil granules are rounded in mice and rats. They are ovoid, short, thick, and brightly eosinophilic in hamsters. Rat eosinophils circulate for 18–23 hours.[10]

Basophils are similar in size to monocytes and eosinophils. They have pale, basophilic cytoplasm filled with dark purple granules and a segmented or bilobed nucleus with clumped chromatin.

PLATELETS AND COAGULATION

Much of the platelet and hemostasis research provided in Sections VI and VII of this text was performed using rodents. Rodent platelets are 1–4 µm diameter, anucleate, rounded to ovoid fragments of clear to lightly granulated cytoplasm. Platelet granules can stain basophilic to purple with Romanowsky stains. Platelet life span in mouse peripheral blood is approximately 4–5 days.[30] Whereas in rats, platelet life span is 3.7–8 days.[10] Platelet volume in CD1 mice ranged from 4.5–11.6 fL and in rats from 5.7–10.1 fL.[36] Storage of murine blood at 4 °C for 24 hours can increase mean platelet volume (MPV), likely due to swelling.

Clotting times (2–10 minutes), prothrombin times (7–15 seconds), and partial thromboplastin times (55–110 seconds) for mice are published;[17] however, critical factors such as site of blood collection, age, strain, and sex were not indicated. Similar clotting times and prothrombin times are seen in hamsters[41] and rats,[28] but partial thromboplastic times appear to be shorter. Due to tissue trauma, prothrombin time and partial thromboplastic time may be increased when blood is collected from the retro-orbital sinus. The bleeding time in mice is reported to be 50–60 seconds.[35] Bleeding time may be twice as long in other hamsters[12] and rats, but this should be replicated and confirmed.[28]

REFERENCE INTERVALS

Hematologic reference intervals vary greatly between members of the rodent family and are affected by differences between individual animals, sample collection and handling techniques, hematology analyzers, and any concurrent treatments the animal is receiving. Published reference intervals rarely include details about how samples were handled, instrumentation used, or even number of animals sampled and should be used with extreme caution when interpreting data. Few reference intervals for rodent species have been generated or verified using ASVCP guidelines, including current data available online from research companies supplying rodent species for experiments. Diagnostic interpretation of rodent

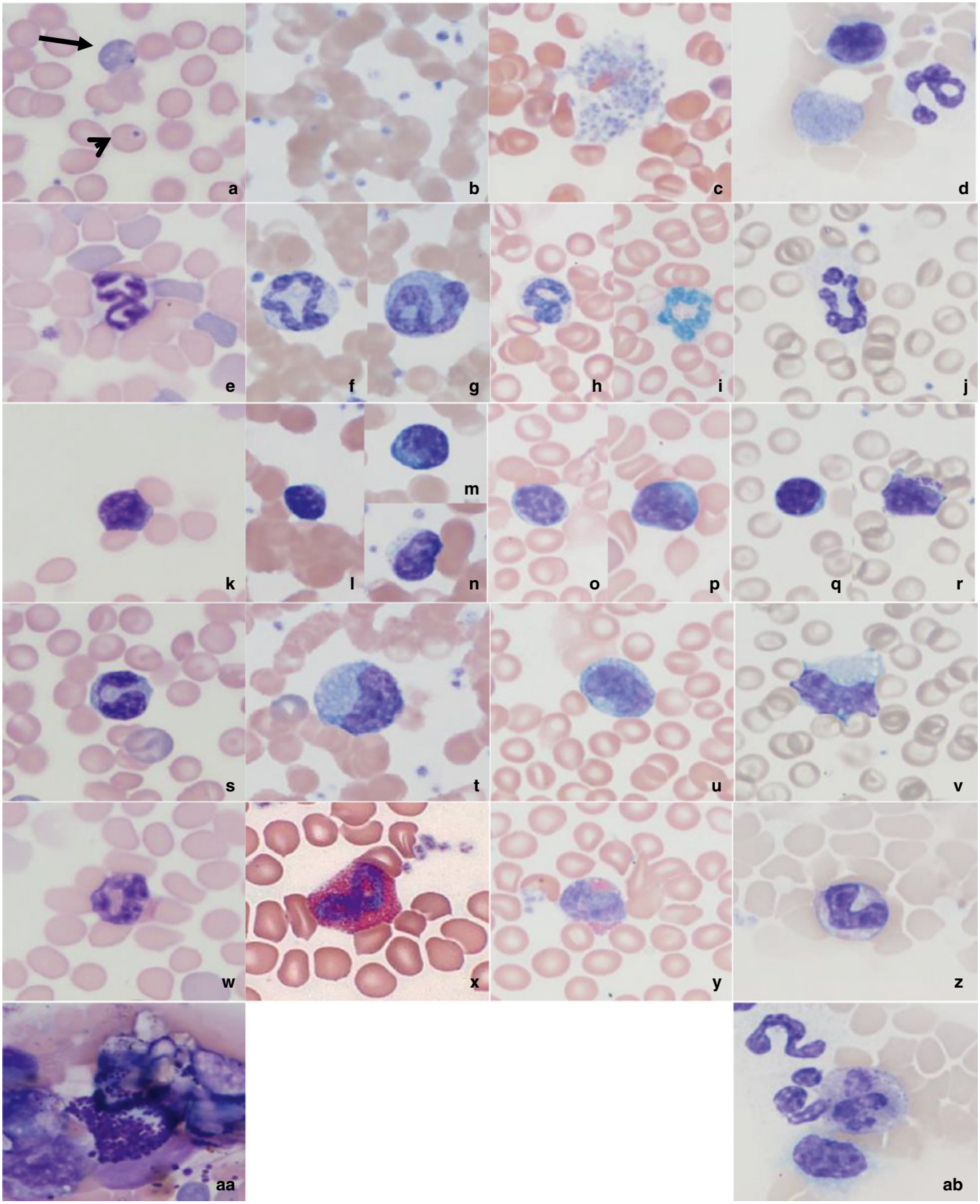


FIGURE 114.1 Representative leukocytes from a mouse (*Mus musculus*), rat (*Rattus* sp.), hamster (unknown genera), and chinchilla (*Chinchilla* sp.) (Wright–Giemsa stain, 100× objective magnification). Erythrocytes and platelets are present in most images. (a) Mouse RBCs and platelets with a Howell–Jolly body (nuclear remnant, open arrowhead) and a polychromatophil (arrow); (b) rat RBCs and platelets; (c) hamster RBCs and a platelet clump; (d) chinchilla RBCs and an atypical giant platelet (approximately 15 μm in diameter, to the left of the neutrophil and below the lymphocyte); (e) mouse segmented neutrophil; (f) rat segmented neutrophil; (g) rat toxic neutrophil with cytoplasmic basophilia; (h) hamster segmented neutrophil; (i) hamster toxic neutrophil with prominent eosinophilic cytoplasmic granules; (j) chinchilla segmented neutrophil; (k) mouse lymphocyte; (l) rat lymphocyte; (m) rat reactive lymphocyte; (n) rat granular lymphocyte; (o) hamster lymphocyte; (p) hamster reactive lymphocyte; (q) chinchilla lymphocyte; (r) chinchilla granular lymphocyte; (s) mouse monocyte; (t) rat monocyte; (u) hamster monocyte; (v) chinchilla monocyte; (w) mouse eosinophil; (x) rat eosinophil; (y) hamster eosinophil; (z) chinchilla eosinophil; (aa) mouse basophil; (ab) chinchilla basophil.

hematology is hindered by the lack of appropriate reference intervals to date.

Some published platelet reference intervals are quite broad (100,000–1,000,000 platelets/μL of blood in mice) and should likely not be used.[17] For rats and gerbils, most publications report 400,000–800,000 platelets/μL. Hamsters are reported to have lower platelet concentration reference intervals than other rodents (200,000–500,000 platelets/μL) and platelet concentration is decreased during pseudohibernation.[32]

Partitioning Factors

It is very important to use reference intervals appropriate for the age, sex, and strain of mice (*Mus musculus*) and in rats (*Rattus* sp.). Charles River (criver.com) provides some strain-specific hematology data ranges for mice and rats that should be interpreted with caution. Compliance with ASVCP guidelines should be assessed prior to use of any reference intervals.

Variations in several rodent RBC indices occur with age. RBC concentrations tend to be lower in young animals and reach adult values by 3 months of age in mice and 4 months in rats.[28] In C57BL/6 mice, age-related changes in Hgb and RBC concentrations reflect the reticulocytosis observed in young mice of all strains: Hgb and RBC concentrations increase while mean cell volume (MCV) decreases between days 0 and 35 of life.[42] The RBC concentration of newborn Mongolian gerbils is approximately half that of adults and Hgb concentration is high; these values adjust to adult reference intervals by 8 weeks of age.[39] As expected, MCV of newborn gerbils also is high and gradually decreases until 12 weeks of age, likely due to decreasing reticulocytosis. The observation that Hgb concentration increases in mice but decreases in gerbils as they age may be due to differences in the amino acid structure of the Hgb molecules in the different species.[23]

There are published sex-specific hematologic values for different species of rodents.[41] Four-week-old, male, Sprague–Dawley rats have higher Hgb concentration, Hct, RBC concentration, RDW, MCV, and MCH, and lower MCHC than age-matched females.[20] However, both MCH and MCHC are lower in male Wistar rats as compared to females.[26] Male Mongolian gerbils have higher MCV, Hct, and Hgb concentration than females.[39]

In some species of rodents, RBC indices differ in pregnant females as compared to males or nonpregnant females. In Wistar Hannover rats pregnant for 18–19 days, Hct, RBC concentration, and Hgb concentration were decreased, while MCH, MCHC, and reticulocyte concentrations were increased, as compared to nonmated, age-matched, female rats.[27] In pregnant chinchillas, Hct and MCV increased gradually between 0 and 112 days of gestation.[19]

RBC indices in rodents also are affected by seasonal changes. During pseudohibernation of adult hamsters in the winter, RBC life span, RBC concentration, and Hgb concentration are increased.[8,35] RBC concentration, Hgb, Hct, MCV, MCH, and MCHC are all higher in chinchillas during late winter.[22] It is possible that dehydration due to pseudohibernation could be contributing to some of these differences.

Leukocyte reference intervals also vary with age, sex, and reproductive cycle, among other partitioning factors. The percentages of neutrophils and monocytes decrease, and lymphocytes increase as C57BL/6 mice age from 0–35 days.[42] The lymphocyte to neutrophil ratio (L:N) decreases in adult rats as they age.[41] As compared to female rats, male rats have a higher WBC, neutrophil, lymphocyte, and monocyte concentration, but a lower eosinophil concentration.[20] Pregnant Wistar Hannover rats were shown to have higher WBC, neutrophil, lymphocyte, and monocyte concentrations when compared to age-matched, nonmated rats.[27] Gerbils tend to have high WBC concentrations at birth, then a relative leukopenia at 4 weeks of age, and then adult values by 8 weeks of age.[39] Relative numbers of neutrophils/heterophils increase during inflammation in all rodent species and following pseudohibernation in hamsters.[35] Increases in WBC, neutrophil, and lymphocyte concentrations are observed during the first 2 months of pregnancy in chinchillas, but then decrease back to nonpregnant female values.[1,19]

As with other hematologic indices, several factors should be considered when evaluating platelet reference intervals. Platelet concentration increases between 0 and 35 days of age in C57BL/6 mice.[42] Female Sprague–Dawley rats have a higher plateletcrit and platelet concentration than their male counterparts.[20] Pregnant female Wistar Hannover rats have a higher platelet concentration than nonmated females.[27]

BLOOD COLLECTION

Blood collection from rodents can be difficult due to the small size of the animals. The recommended blood volume to collect is 1% of the patient's body weight 0.01 mL blood/g body weight and, whenever possible, blood should not be collected more than once every 2 weeks from small rodents. This means that diagnostic tests must be carefully selected to best utilize blood collected from mice, gerbils, and hamsters. Typically, mice reach puberty between 28 and 49 days of age, adult males weigh 20–40 g, and females weigh 18–35 g.[17] The reported adult murine blood volume is 6.3–8 mL/100 g body weight.[13] Gerbils tend to be slightly larger than mice with adult males reaching 65–100 g and adult females weighing 55–85 g.[4] Body weight of Djungarian hamsters (*Phodopus sungorus*) ranges from 18–25 g, Chinese hamsters (*C. griseus*) 39–46 g, Syrian/golden hamsters (*M. auratus*) 110–140 g, and European hamsters (*Cricetus cricetus*) 300–400 g.[16] Blood collection from rats and chinchillas is slightly easier than most mice, gerbils, and hamsters due to the larger size of these rodents. Rats reach puberty at 40–60 days, adult males weigh 300–500 g, and females weigh 250–300 g.[2] Rat blood volume is 5–7.1 mL/100 g body weight.[28] Chinchilla body weight is 400–500 g for males and 400–600 g for females.[16]

Differential cell counts should be determined using fresh whole blood, as WBCs can degrade during storage. In mice and rats, monocyte concentration was significantly decreased after storage of EDTA-blood for 24 hours at 4 °C.[1] Dipotassium ethylenediaminetetraacetic acid (K_2EDTA) is the preferred anticoagulant for most hematologic indices. Due to the small blood sample volume that can be collected from rodents, the needle and syringe used to collect blood can be coated with 7.5% K_2EDTA before sampling to avoid dilution of blood with the excessive anticoagulant volume provided in larger blood collection tubes. Citrated plasma is needed for most coagulation tests. Note that hemolysis, which often occurs during traumatic blood collection, will alter coagulation factors and tends to increase MCH and MCHC; and decrease platelet concentration, RBC concentration, and PCV.

There are numerous rodent blood collection sites that have been described (Table 114.1). Many techniques that lance vessels release tissue factors that affect platelet and coagulation indices. In most rodents, the lateral tail vein is the preferred site for blood collection. The tail can be warmed to 104 °C to dilate vessels. The lateral tail vein is either lanced or blood is collected directly with a needle. If the vein is lanced, the first drop of blood should be discarded, and then subsequent drops can be collected using a microhematocrit tube. Do not squeeze the tail to extrude blood, as this will release tissue factors and alter coagulation measurands.

Sample storage may induce similar changes in hematologic values found in other mammalian species. Blood from rats stored at 4 °C for 24 hours had increased MCV, and decreased MCHC and corpuscular hemoglobin concentration mean. The same was found for murine blood, although RDW also was increased.[1]

BONE MARROW COLLECTION AND EVALUATION

Bone marrow evaluation is beneficial in patients with refractory cytopenias or suspicion of leukemia. However, bone marrow collection is a terminal procedure in the mouse, gerbil, and hamster (see Chapter 141).

Megakaryocytes can be present in high numbers in healthy rodents. In rats, megakaryocytes may have nuclear fragments and variable nuclear to cytoplasmic (N:C) ratios.[7] Erythroid and granulocyte maturation in rodents are cytologically similar to other mammals; however, in neutrophils and eosinophils, ring-shaped nuclei are commonly observed. In apparently healthy gerbils, ring-shaped nuclei can comprise a third of the granulocytes in the bone marrow.[40] Lymphocytes comprise 7–21% of nucleated cells in rodent bone marrow.[7] Plasma cells and histiocytic cells are seen in low percentages. Mast cells, osteoblasts, osteoclasts, and endothelial cells may be observed rarely. No differences in bone marrow differentials were observed when male and female chinchillas were compared.[38]

Myeloid to erythroid ratios (M:E) overlap in most rodent species. The reported M:E ratio in mice is wide and ranges from 0.8–2.4.[31] Rats have a M:E approximately equal to one normally.[21] In gerbils, M:E ranges from 0.6–3.6.[40] Chinchillas are reported to have a M:E approximately equal to 0.5.[38] Hamsters reportedly have a very high M:E (8–10),[12] but the authors believe this count may be artificially increased by inclusion of lymphocyte precursors in the myeloid count.

Hematopoiesis occurs in both the bone marrow and spleen of rodents. Two to four phases of hematopoiesis are described in embryonic and fetal rodents. In mice, two phases are described with primitive hematopoiesis occurring in the yolk sac (embryonic day 7) and definitive hematopoiesis in the liver (embryonic day 10).[24] Mice are born after embryonic day 21. In gerbils, the four-phase description includes: (1) a vitelline phase (embryonic day 12), (2) a hepatic phase with thymic development (embryonic days 14–19), (3) a splenic phase (embryonic days 16–23), and (4) a medullary phase with development of secondary lymphoid tissues (embryonic days 22–24).[37] Gerbils are born after embryonic day 24.[37]

HEMATOLOGIC DISEASE

Physiologic

A common physiologic hematologic change of note is leukopenia associated with endogenous or exogenous glucocorticoids in some rodents.[41] Stress (glucocorticoids) causes lymphopenia and neutrophilia in many rodents, with a marked decrease in circulating lymphocytes, potentially resulting in an overall leukopenia. Alternately, excitement associated with blood collection in rats can alter total WBC concentrations, causing an immediate leukocytosis and increased circulating lymphocytes.

Pathologic

Hematologic diseases in rodents are diagnosed infrequently in general practice. This is likely because a hemogram is rarely performed in small rodent species. In pet rodents, the pre-anesthetic workup often includes evaluation of Hct, but may not include WBC evaluation.

Changes in the hemogram in response to disease conditions are similar to other species; e.g., anemia in laboratory rats fed an iron-deficient diet.[25] In gerbils with *Helicobacter pylori*-induced iron deficiency anemia, findings included decreased MCV and Hgb concentration, along with leukocytosis and thrombocytosis resulting from a systemic inflammatory response.[5] The mouse is commonly used as a model for anemia of inflammation; multiple bacterial infections reliably produce this condition. Exposure to cell wall components of Gram-positive bacteria produces the same in experimental rats.[33] Spontaneous renal disease in pet rodents may result in nonregenerative anemia, and a

TABLE 114.1 Summary of anatomic sites for blood collection in rodents.

Site	Mouse	Rat	Hamster	Gerbil	Chinchilla
Jugular vein	√	√	√	√	√
Medial saphenous vein	√	√	√	×	×
Lateral saphenous vein	√	√	√	√	√
Cephalic vein	×	×	×	√	√
Lateral tail vein	√	√	√	√	×
Middle caudal tail vein	√	√	×	×	×
Superficial temporal vein	√	×	×	×	×
Sublingual vein	√	×	×	×	×
Submandibular vein	×	×	×	√	×
Metatarsal vein	×	×	+	×	×
Lateral tarsal vein	×	×	+	×	×
Dorsal metatarsal vein	×	+	×	×	×
Transverse sinus[a]	×	×	×	×	+
Toenail clip	*	*	*	*	*
Tail clip	*	*	*	*	*
Retro-orbital sinus	*	*	*	*	*
Dorsal tail artery	+	×	×	×	×
Ventral tail artery	+	+	×	×	+
Femoral vein	+	+	×	×	+
Femoral artery[a]	†	×	×	×	×
Heart[a]	†	†	†	†	†
Abdominal aorta[a]	†	†	†	†	×
Abdominal vena cava[a]	†	†	†	†	×
Cranial vena cava[a]	×	×	×	×	†
Decapitation[a]	†	†	×	×	×

+ = Described.
√ = Described and recommended.
* = Described but not recommended due to tissue damage.
† = Terminal procedure.
[a] = Anesthesia required.
× = Not described

CBC should be part of the diagnostic workup for suspected cases.[15] Other reported causes of anemia in rodents include flea infestation, intestinal bleeding, blood loss, immune-mediated hemolysis, renal disease, myelodysplastic disease, and genetic disorders. CBC values in mice, rats, hamsters, and gerbils with intestinal parasites are usually within normal ranges, but the PCV may be elevated if the animal is dehydrated from diarrhea.

Relative numbers of neutrophils/heterophils increase during inflammation in all rodent species.[35] When neutrophils are released from the bone marrow in response to peripheral inflammation, band neutrophils and earlier neutrophil precursors can be observed. Toxins and bone marrow damage can induce changes in neutrophil cytoplasm so that Döhle bodies, cytoplasmic basophilia, and/or cytoplasmic vacuolation are seen. As in other mammals, increased platelet concentrations have been associated with inflammation in rodents. In rodents with pneumonia, the total WBC concentration is not usually elevated, but neutrophilia/heterophilia and lymphocytopenia often are observed. However, neutropenia can be observed if septicemia occurs. The CBC in rats with a chronic respiratory tract infection (e.g., murine respiratory mycoplasmosis) may be normal or consistent with chronic inflammation with neutrophilia and monocytosis.

Eosinophilia is observed in rodents with ectoparasites. Additionally, lice and mites may predispose the animal to a secondary bacterial infection that may induce leukocytosis, monocytosis, eosinophilia, and basophilia.

Hematopoietic neoplasms are recognized in rodents. In aged Fischer 344 rats, large granular lymphocytic leukemia with leukocytosis and hemolytic

anemia can develop spontaneously. Hamster polyomavirus causes a multicentric lymphoma in hamsters that affects a wide range of organs and tissues. During advanced stages of this disease, the CBC is characterized by anemia and leukocytosis with circulating neoplastic cells.[29]

HEMOPARASITES

In healthy rodents, most hemoparasites appear to be nonpathogenic. *Mycoplasma haemomuris* can be observed on mice and rats' RBCs.[34] This organism is endemic in free-ranging rats (*Rattus norvegicus*) in Brazil.[11] Trypanosome trypomastigotes can be observed in peripheral blood samples from *R. norvegicus*. Recently, zoonotic trypanosomes, *Trypanosoma lewisi* and *Trypanosoma cruzi*, have been detected in fleas of rats living in close association with humans in Venezuela, which may put endemic populations at risk for re-emergence of trypanosomiasis.[18]

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CHAPTER 115

Hematology of Mustelids

STACY CLOTHIER and CATHY JOHNSON-DELANEY

Blood Collection and Sample Handling
Erythron
Leukon
Platelets and Coagulation
Bone Marrow Collection and Hematopoietic Evaluation
Reference Intervals
Hematologic Disease
  Anemia
  Leukogram Changes
  Aleutian Disease Virus
  Hemoparasites
Hemostatic Disorders

Acronyms and Abbreviations

ASVCP, American Society for Veterinary Clinical Pathology; ADV, Aleutian disease virus; ADP, adenosine diphosphate; EMH, extramedullary hematopoiesis; CHS, Chédiak–Higashi syndrome; FHS, ferret hemorrhagic syndrome; PT, prothrombin time; PTT, partial thromboplastin time; TT, thrombin time.

The domestic ferret (*Mustela putorius furo*), mink (*Mustela vison*), and otters (subfamily *Lutrinae*) are members of the mammalian order Carnivora and the Mustelidae family. Mustelid hematology literature comprises predominantly research and publications related to ferret hematology, given their increasing popularity as pets as well as their utility as animal models of human disease. Hematologic parameters of both captive-bred and wild-caught mink are generally similar to those reported in common ferrets. The main otter species for which hematologic data are available are wild-caught sea otters, Eurasian otters, and river otters. Otters are uniquely adapted to a marine environment, and, consequently, certain species have hematologic profiles that are more similar to those of unrelated aquatic mammals than terrestrial mustelids.[40]

BLOOD COLLECTION AND SAMPLE HANDLING

The most common locations for venipuncture in ferrets when obtaining larger volumes of blood (1–3 mL) are the jugular vein and the cranial vena cava.[10] Other commonly described and easily accessible phlebotomy sites include the lateral saphenous vein and cephalic vein that yield smaller blood volumes still adequate for a CBC or PCV.[33] Uncommon venipuncture sites that have been described include retro-orbital bleeding and cardiac puncture, which can be highly traumatic and should only be performed with heavy sedation, anesthesia, or during euthanasia.[14] The total blood volume in ferrets is approximately 5–7% of the body weight, or approximately 75 mL/kg.[14,35] Healthy adult female ferrets weighing 750 g have an approximate blood volume of 40 mL, while adult male ferrets weighing up to 1 kg have 60 mL.[14]

Blood can be collected from otters using jugular venipuncture or the proximal aspect of the femoral vein.[38,40] Published total blood volumes in sea otters range from 91 mL/kg to 173.5 ± 9.85 mL/kg of body mass.[8]

There are no recent published studies on blood volumes in mink. Blood collection sites and procedures in mink are the same as in the domestic ferret. Blood is commonly obtained from jugular venipuncture with cranial vena cava or cardiac puncture (during necropsy) rarely utilized.[32,39]

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ERYTHRON

Erythrocytes in ferrets are anuclear biconcave disks with central pallor, similar to other mammalian species, with an average diameter of 5.9 µm in males and 6.3 µm in females (range for both sexes, 4.6–7.7 µm).[26,37] Howell–Jolly bodies are observed in approximately 5% of male and female ferrets.[37] The hematocrit (Hct), total RBC count, hemoglobin (Hgb) concentration, and reticulocyte percentage are generally higher in ferrets than in dogs and cats, and the Hct and Hgb are higher than in rats.[26,37] Siperstein reported a shorter RBC life span with increased polychromasia when compared to dogs and cats.[33] Albino ferret mean relative reticulocyte counts are 4% (1–12%) in males and 5.3% (2–14%) in females.[37] The sedimentation rate of RBCs is negligible in ferrets, so longer centrifugation times (20% longer) are required.[14,37]

Mink RBCs are described as biconcave disks with varying degrees of central pallor.[13] Large singular Heinz bodies are observed in <1% of 1000 RBCs.[39] Aggregate reticulocytes range from 0–4%, consistent with the degree of polychromasia observed.[13,39] Nucleated RBCs are rarely recognized in normal mink, but can be a normal finding in postnatal mink pups.[13,21]

River otter RBCs are similar in appearance to those described for other terrestrial mammalian species.[38] Nucleated RBCs are rarely observed in river otters.[38] While some information from Tocidlowski et al. is included in this chapter, no reference intervals are presented, as it is apparent that the reference population includes outlier values which were not eliminated.[38] Sea otters have hematologic parameters more similar to pinnipeds and cetaceans than to mustelids, due to adaptation to a marine environment.[40] This includes reportedly higher Hct and Hgb values together with lower RBC counts.[40]

LEUKON

The morphology of mustelid neutrophils, lymphocytes, monocytes, eosinophils, and basophils are comparable to those of other mammals. Normal ferrets can have lower WBC counts than other mammalian species.[16] Neutrophils and lymphocytes are the most numerous cell types in mustelid species, with neutrophils outnumbering lymphocytes.[13,33,38] However, Hein and colleagues observed lymphocyte predominant leukograms in ferrets.[16] Ferret neutrophil–lymphocyte ratios range from 0.7:1 to 1.7:1.[26] Neutrophil to lymphocyte ratios are approximately equal to unity in mink, although in some animals lymphocytes predominate, and in other animals neutrophils are the dominant cell type.[13,39]

Ferret neutrophils (Figure 115.1a) have a mean diameter of 11.2 µm (10.0–13.1 µm) in males and 9.6 µm (9.2–10.0 µm) in females.[37] They have lobulated nuclei, small occasionally polychromatic cytoplasmic granules, and infrequent vacuoles, though the granules may be less prominent than in dog or cat neutrophils.[33,37] Left-shifting to band neutrophils is uncommon in healthy ferrets.[20]

The mean diameter of small lymphocytes in ferrets is 7.7 µm (6.2–9.2 µm) in males and 8.7 µm (7.7–10.0 µm) in females[37] (Figure 115.1b). Large lymphocytes are similar in size to neutrophils, with mean diameter of 11.1 µm (9.2–13.1 µm) in males and 11.9 µm (9.2–10.0 µm) in females.[37] However, this gender difference is likely not clinically significant. River otters have reactive lymphocytes with increased size, basophilia, and less-clumped chromatin, similar to those of other species.[38]

Ferret monocytes measure 12–18 µm in diameter.[37] The nuclei are round, lobulated, or indented, similar to other species. The cytoplasm is pale basophilic and may contain frequent vacuoles[37] (Figure 115.1c).

Eosinophils have a mean diameter of approximately 12.7 µm in male and female ferrets.[37] Ferret eosinophils have a one- or two-lobed nucleus and many small cytoplasmic granules which are rounder compared to neutrophils[33,37] (Figure 115.1d). Mature eosinophil granules in mink are different from mammals in that they lack a crystalline component based on ultrastructural analysis.[9] Sea otter eosinophils typically have far less pink granulation than ferrets with just a dusting of eosinophilic granules (Figure 115.1j).

Basophils have a segmented nucleus in ferrets, similar to that seen in dogs, and a mean diameter of 13.5 µm in males and 13.8 µm in females[37] (Figure 115.1e). One study in mink reported a morphologically unique ultrastructural characteristic in late-stage basophils.[9] A second population of narrow and dense cytoplasmic granules was observed in the late basophils and interpreted as secondary granules.[9]

PLATELETS AND COAGULATION

In observations by Thornton et al., platelets had a mean diameter of 1.7 µm (1.5–2.3 µm), and mean platelet volume of 19.8 µm[337] (Figure 115.1f,k). Platelets are rarely as large as RBCs (<1% of total platelets).[37] It is unknown if the large platelets (megaplatelets) represent immature cells as is assumed in some domestic animals.

In a study characterizing von Willebrand factor (vWF) in ferrets, the findings were comparable to those of other mammalian species in that a similar differential distribution of von Willebrand factor was observed along the vascular tree.[14,19] Two unique differences are noted in ferret vWF compared to other species.[14,19] Rather than ciliated bronchial epithelial cells, the bronchial mucous goblet cells stained positive for vWF.[14,19] In addition, the multimeric distribution of platelet vWF was skewed toward the smaller–molecular-weight multimers and larger-molecular-weight multimers were not released following 1-deamino-8-D-arginine-vasopressin administration.[14,19] There are few published reports on mustelid coagulation values (Table 115.2). Mustelid coagulation pathways are reported to be similar to those of other mammalian species.[5,24,36]

A study on mink platelet function found that normal mink have unique platelets that contain less ADP than

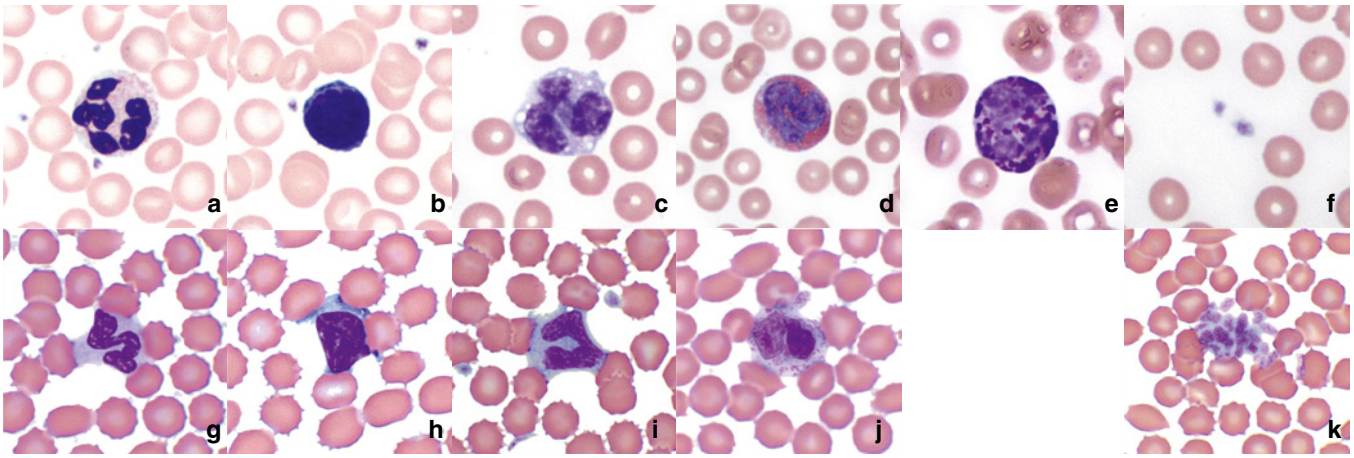


FIGURE 115.1 Comparative WBC and platelet images from selected mustelids. Modified Wright stain on 100× objective unless otherwise noted. Ferret (*Mustela putorius furo*): (a) neutrophil; (b) small lymphocyte; (c) vacuolated monocyte; (d) eosinophil; (e) basophil; and (f) individual platelets. Sea otter (*Enhydra lutris*): (g) neutrophil; (h) lymphocyte; (i) monocyte; (j) eosinophil; (k) platelet aggregate. All slides were stained using a Midas III Automated Slide Stainer with Harleco Wright–Giemsa stain. Siemens Autoslide Buffer with Harleco stain and tap water were used for ferret slides. Buffer pH 6.4 (Merck, Germany) with Harleco stain and distilled water were used for otter slides. (**Source:** Sea otter photos courtesy of Dr. Kendal Harr.)

humans and pigs, but more than in guinea pigs, rabbits, and cattle.[30] Normal mink platelets aggregated in response to stimulation with collagen, exhibited irreversible aggregation with ADP, were nonresponsive to epinephrine, and secreted more ATP than ADP when treated with thrombin.[30] After administration of serotonin and arachidonate, only shape change was observed.[30]

BONE MARROW COLLECTION AND HEMATOPOIETIC EVALUATION

The proximal femur is the most common site for bone marrow aspiration or core biopsy in ferrets; other sites of collection include the proximal humerus and the wing of the ilium.[14,35] Mink bone marrow can be obtained from the femur or the humerus, as in the domestic ferret.[9,21] In sea otters, bone marrow can be collected from the pelvis or femoral trochanter[7] (see Chapter 141).

The M:E ratio of healthy ferrets is 1.1 to 3.4:1.0. Unique morphologic features or maturation patterns of the erythroid, myeloid, and megakaryocytic lineages have not been reported in normal mustelids at the light microscope level.[21] Characteristic ultrastructural features of granulopoiesis are reported in mink.[19]

Extramedullary hematopoiesis (EMH) occurs in almost all ferrets older than 2 years, and in some can lead to splenomegaly. It is a compensatory reaction in response to either inadequate bone marrow function or increased blood cell needs, although the exact process is often undetermined. Splenomegaly found prior to anesthetic administration should be noted as a clinical finding and etiologies pursued, as splenic cysts, nodules, splenitis, and neoplasia are also etiologies. Splenectomy should not be undertaken without bone marrow evaluation, as the spleen may be the sole source of blood cells.[14,35]

REFERENCE INTERVALS

Complete blood count and coagulation data from various mustelid species are presented in Tables 115.1 and 115.2. Many factors can affect the results of hematologic profiles of normal mustelids including stress, age, gender, reproductive status, anesthesia, nutrition, and environment.

Ferrets exhibit an increase in neutrophils and a decrease in lymphocytes as they age, similar to age-related changes in other species.[20] In general, ferrets younger than 5–7 months can normally have lymphocyte predominant hemograms.[20] Younger ferrets have lower PCVs than older ferrets, similar to the known lower PCVs in young animals.[16,20] Male ferrets can have higher Hgb levels than female ferrets.[37] Young male ferrets aged 10 weeks to adult age were observed to have an age-related increase in RBC count, Hgb concentration, and Hct.[14] Young female ferrets exhibited a PCV decrease from 12 to 18 weeks old potentially due to reproductive maturation.[20] Mink experience age-related changes in the total WBC count and differential similar to other animals.[21] The total WBC count, neutrophil, and monocyte counts are higher in adult river otters than juveniles, although capture stress may contribute.[38] Adult Eurasian otters are thought to have higher platelet counts than juveniles.[12]

While one study did find increased mean PCV and Hgb concentration in male mink, no significant differences in PCV, RBC count, and Hgb concentration were observed between males and females in more recent

TABLE 115.1 Hematologic values of male and female ferrets and mink.

Parameter	Mean ± SD	Median	Reference interval	Animal
RBC (× 10^6/μL)	7.90 ± 0.59	–	–	Mink[a]
	9.23 ± 1.67	–	–	Mink[b]
	–	10.5	7.4–13.0	Ferret[c]
	–	–	4.8–7.8	Ferret[d]
	–	–	6.6–12.2	Ferret[e]
Hgb (g/dL)	15.55 ± 1.0	–	–	Mink[a]
	15.72 ± 2.68	–	–	Mink[b]
	–	17.89	13.9–21.9	Ferret[c]
	–	–	9.6–13.8	Ferret[d]
	–	–	10.8–19.7	Ferret[e]
Hct or PCV (%)	46.6 ± 3.0	–	–	Mink[a]
	49 ± 9.0	–	–	Mink[b]
	–	60.0	40–70	Ferret[c]
	–	–	27–39	Ferret[d]
	–	–	35–58	Ferret[e]
MCV (fL)	59.05 ± 2.05	–	–	Mink[a]
	52.99 ± 1.35	–	–	Mink[b]
	–	54.3	49.6–60.6	Ferret[c]
	–	–	47.8–57.6	Ferret[d]
	–	–	42–60	Ferret[e]
MCHC (g/dL)	33.35 ± 0.76	–	–	Mink[a]
	32.30 ± 1.0	–	–	Mink[b]
	–	31.1	28.68–33.68	Ferret[c]
	–	–	34.7–37.0	Ferret[d]
	–	–	28.4–37.9	Ferret[e]
PLT (× 10^9/L)	785.14 ± 192.38	–	–	Mink[a]
	274.38 ± 97.34	–	–	Mink[b]
	–	807.0	171.7–1280.6	Ferret[c]
	–	–	382–830	Ferret[d]
	–	–	300–900	Ferret[e]
WBC (× 10^9/L)	5.88 ± 1.85	–	–	Mink[a]
	5.22 ± 1.84	–	–	Mink[b]
	–	7.2	3.0–16.7	Ferret[c]
	–	–	5.3–12.6	Ferret[d]
	–	–	2.2–11.3	Ferret[e]
Bands				
Neutrophils (× 10^9/L)	2.48 ± 1.13	–	–	Mink[a]
		3.0	0.9–7.4	Ferret[c]
	–	–	1.5–6.5	Ferret[d]
		–	0.7–7.4	Ferret[e]
Lymphocytes (× 10^9/L)	2.74 ± 0.93	–	–	Mink[a]
	–	3.4	0.6–10.5	Ferret[c]
		–	1.8–8.6	Ferret[d]
		–	0.5–5.6	Ferret[e]

(*Continued*)

TABLE 115.1 (Continued)

Parameter	Mean ± SD	Median	Reference interval	Animal
Monocytes (× 10^9/L)	0.185 ± 0.12	–	–	Mink[a]
		0.2	0–0.5	Ferret[c]
	–	–	0.1–0.5	Ferret[d]
		–	0–0.5	Ferret[e]
Eosinophils (× 10^9/L)	0.425 ± 0.46	–	–	Mink[a]
		0.1	0–0.7	Ferret[c]
	–	–	0.1–0.7	Ferret[d]
	–	–	0–0.6	Ferret[e]
Basophils (× 10^9/L)	0.04 ± 0.29	–	–	Mink[a]
		0	0–0.2	Ferret[c]
	–	–	0–0.1	Ferret[d]
		–	0–0.1	Ferret[e]

[a] Anesthetized, healthy, adult, captive, brown mink (n = 160). Data acquired using a Coulter S-Plus IV counter with manual differentials using Wright staining.[39]
[b] Anesthetized, healthy, 6-month-old black mink (n = 20). Data acquired using a Sysmex KX-21 without manual differentials. Results reported as mean ± standard error.[32]
[c] Eleven weeks to 9-year-old, mixed sex, unanesthetized, healthy pet ferrets (n = 106). Data acquired using a Cell-Dyn 3500 with manual differentials using modified Wright staining. Statistics and RI generation done in accordance with ASVCP guidelines.[15,16] Significant differences in age, sex, and fasted status were noted by the authors, but details were not provided.
[d] Ferrets 10–12-week-old, mixed sex, anesthetized, using cardiac puncture. Data supplied by Marshall Farms (Marshall BioResources, North Rose, NY).[14]
[e] Adult, mixed sex, apparently healthy ferrets (n > 500). Anesthetic status unknown; however, from the comparatively decreased PCV and WBC, it is possible that many of the ferrets were anesthetized. Data acquired using Sysmex XN technology, with manual differentials and PCV. RI generated in accordance with ASVCP guidelines and verified[15] (Harr, personal communication).

TABLE 115.2 Coagulation values of ferrets and mink using select methods.

Parameter	Mean ± SD	Range	Animal
Clotting time (seconds)	120 ± 0.5[a]	–	Ferret[24]
	240 ± 1.0[a]	–	Mink[24]
Bleeding time (minutes)	5.0 ± 1.5[e]	–	Mink[30]
Coagulation time (minutes)	7.0 ± 1.25[e]	–	Mink[30]
PT (seconds)	10.3 ± 0.1	–	Ferret[24]
	6.9 ± 0.6	–	Mink[24]
	12.3 ± 0.3[b]	11.6–12.7	Ferret[5]
	10.9 ± 0.3[c]	10.6–11.6	Ferret[5]
	11.3 ± 0.4	10.9–12.0	Ferret[36]
PTT (seconds)	18.4 ± 1.4	–	Ferret[24]
	15.8 ± 1.2	–	Mink[24]
	20.4 ± 0.8[b]	18.9–22.3	Ferret[5]
	20.0 ± 1.0 [c]	18.6–22.1	Ferret[5]
	18.7 ± 1.0 [b,d]	17.5–21.1	Ferret[5]
	18.1 ± 1.1 [c,d]	16.5–20.5	Ferret[5]
	17.0 ± 1.2	15.3–18.7	Ferret[36]
Fibrinogen (mg/dL)	107.4± 19.8[c]	90.0–163.5	Ferret[5]
	486.7 ± 97.9	382.5–658.3	Ferret[36]
TT (seconds)	13.7 ± 2.7	9.4–16.9	Ferret[36]
Antithrombin (%)	96 ± 12.7[c]	69.3–115.3	Ferret[5]

[a] Glass. Ferrets (n = 6) and mink (n = 3) of unspecified age, sex, and anesthetic status.[24]
[b] Fibrometer (coagulation analyzer, Becton Dickinson, Franklin Lakes, NJ). Anesthetized, healthy, adult, mixed sex ferrets (n = 18), using cranial vena cava venipuncture.[5]
[c] ACL 3000 (coagulation analyzer, Beckman Coulter, Brea, CA). Anesthetized, healthy, adult, mixed sex ferrets (n = 18), cranial vena cava venipuncture.[5]
[d] Ellagic acid added. Anesthetized, healthy, adult, mixed sex ferrets (n = 18, cranial vena cava venipuncture.[5]
[e] Lee–White method. Adult, healthy, anesthetized mink (n = 6). Sex unknown.[30]

studies.[13,32,39] In a reference population of 160 healthy, adult, captive mink, there was more variability in females: 729.58 ± 125.40 × 10^3/μL in males and 840.70 ± 259.36 × 10^3/μL in females.[39] No notable sex differences were observed in the hematologic profiles from river otters, despite trapping season coinciding with postbreeding or early gestation.[38] Moran et al. observed higher platelet counts in male Eurasian otters than females, though this was not clinically relevant.[12] Capture stress likely contributes to lymphocytopenia observed in Eurasian otters.[12]

Hematologic changes are observed in response to sedation and restraint. Ferrets anesthetized with isoflurane have significant decreases in Hct, RBC count, Hgb concentration, and WBC count; isoflurane causes splenic sequestration of RBCs.[27] The Hct is reduced by approximately 35% using isoflurane, and all of the values return to within 7–14% of baseline within 45 minutes after anesthesia.[27] Sevoflurane causes a similar drop in PCV, likely caused by a similar mechanism.[23] Weiss et al. observed that Hct, Hgb concentration, and RBC counts are 10–20% lower in anesthetized mink when compared to previously reported unanesthetized mink.[39]

The nutrition and environment of the different mustelid species also play a role in hematologic difference. The addition of a dietary antioxidant, phenozan, results in increased Hgb content of RBCs as well as a higher RBC count; although the exact mechanism is unclear, nutrition plays an important role in reducing oxidative stress levels in mink, which is important for fur quality.[3] In another study, river otters adapted to high altitudes are observed to have higher Hct, total Hgb, and RBC counts than sea-level populations.[8] Although Eurasian otters have similar diving behavior to river otters, their RBC counts are reported to be lower than river otters, and their MCV values are also higher.[12]

Platelet reference intervals are variable depending on the study possibly due to the relatively quick clotting time in ferrets of 1–3 minutes, as well as several publications that are not in accordance with American Society for Veterinary Clinical Pathology (ASVCP) guidelines. Platelet values in 106 clinically healthy, nonanesthetized, pet ferrets are 171.7–1280.6 × 10^9/L using a Cell-Dyn 3500.[16] This has more variation than earlier literature reporting ranges from 245–910 × 10^9/L in fitch and albino ferrets, 297–910 × 10^9/L in male and female albino ferrets, and 297–730 × 10^9/L.[20,37] Rui et al. presented a mean platelet value of 274.38 ± 97.34 × 10^9/L in male and female healthy minks.[32] There are a few published reports on mink coagulation values[24,30] (Table 115.2).

HEMATOLOGIC DISEASE

Anemia

Aplastic anemia (pancytopenia) is commonly associated with hyperestrogenism in ferrets due to prolonged estrus in females, exogenous estrogen administration, or in ferrets with estrogen-secreting adrenal neoplasms.[14] Bone marrow suppression of the erythroid, myeloid, and megakaryocytic lineages occurs secondary to estrogen-induced damage, and, consequently, anemia, neutropenia, and thrombocytopenia are common hematologic findings.[22] Normocytic normochromic nonregenerative anemia is a consistent initial finding in estrogen-induced anemia in ferrets. [22] Aplastic anemia was observed in 100% of ferrets in estrus without copulation in one study, with a 100% mortality rate.[22] In this same study, hemorrhages were noted in 83% of ferrets due to severe thrombocytopenia.[22] Females with severe anemia may have platelet counts <20 × 10^9/L, and one ferret had a platelet count as low as 7 × 10^9/L.[20,22]

A juvenile ferret was reportedly diagnosed with immune-mediated pure red cell aplasia.[25] The ferret presented with a severe nonregenerative anemia, lymphocytosis, and a hypercellular bone marrow with an erythroid maturation arrest.[25] In the editor's experience, this is seen with some frequency predominantly in Marshall Farms ferrets. The hypercellular description of the marrow in Malka's report is incongruous with aplasia. The editor typically interprets this fairly common presentation as nonregenerative immune-mediated anemia (NRIMA) and describes the erythroid population as having a maturation arrest to be consistent with diagnosis in dogs[34] (Figure 115.2d–f).

Splenic extramedullary hematopoiesis often plays an important role in ferrets with anemia and is a common diagnosis from splenic aspirates, particularly in ferrets with splenomegaly (Figure 115.2a).[14,35]

Oxidative hemolytic anemia was identified in a river otter that presented with an acute anemia (PCV = 29%) and red-brown urine containing numerous Hgb casts.[31] More than 90% of the otter's RBCs contained single large Heinz bodies and eccentrocytes were frequently observed on blood smear evaluation.[31] The toxin exposure was determined to be minnows containing methylene blue.[31]

Leukogram Changes

Lymphocytic and myeloid leukemias are rarely observed in ferrets and are poorly characterized. The authors and editor have observed a case of probable myelogenous leukemia in a 6-month-old spayed female ferret that presented for lethargy and weight loss. Hematologic findings include a nonregenerative anemia, thrombocytopenia, and a marked leukocytosis (WBC 79,670/μL). The differential WBC count was comprised primarily segmented neutrophils without toxicity, metamyelocytes, band neutrophils, monocytes, and myelocytes. Small lymphocytes and eosinophils were <1% of the count (Figure 115.2c). A touch impression of the bone marrow was of variable preservation, but myelogenous leukemia was supported by a monomorphic infiltrate of a left-shifted myeloid series (M:E ratio approximately 10:1). The infiltrate consisted predominantly of myelocytes and metamyelocytes with few bands and an apparent maturation arrest at the myelocyte-metamyelocyte stage with a storage pool depletion. Immunophenotypic and cytochemical characterization for leukemia classification in ferrets is needed. Cross-reactive and partially

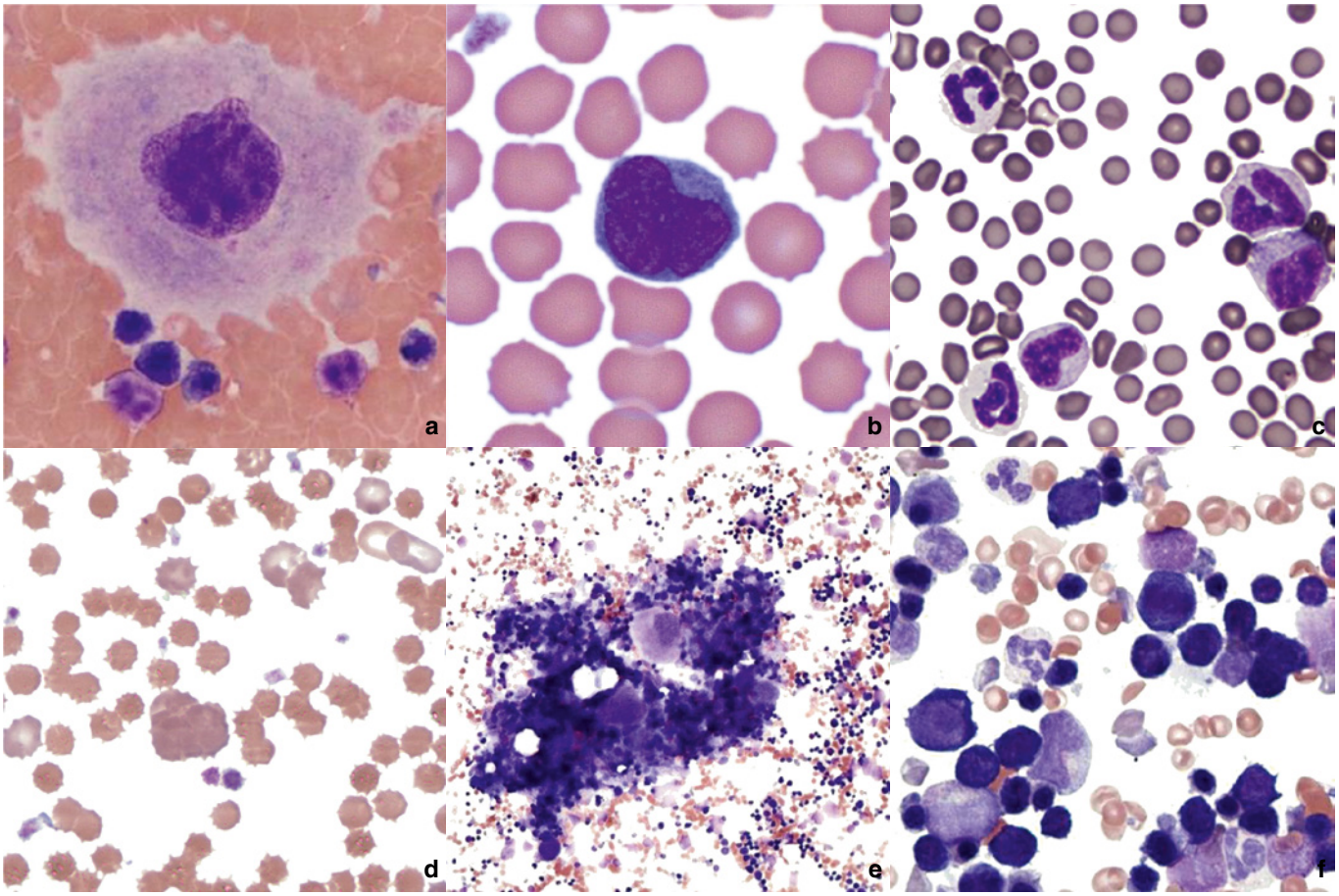


FIGURE 115.2 Abnormalities seen in ferret peripheral blood or spleen. (a) Megakaryocyte indicating extramedullary hematopoiesis, splenic aspirate, 100× objective; (b) Lymphoblast in circulation in apparently healthy ferret; (c) Probable myelogenous leukemia. Neutrophils are left-shifted to myelocyte in image. Myeloblasts were also found (not pictured) 50× objective; (d) Reticulocyte agglutination occasionally found in ferret peripheral blood exhibiting immune-mediated anemia; (e) and (f) Bone marrow aspirate from a ferret with nonregenerative anemia (presumed immune-mediated) exhibiting erythroid hyperplasia and apparent maturation arrest, 20× and 50× objectives. (**Source:** Photos (b)–(f) courtesy of Dr. Kendal Harr.)

validated CD3, CD20, CD14, and CD18 antibodies (Veterinary Diagnostic Laboratory, Michigan State University, Lansing, MI) are available.[28]

The most common clinicopathologic finding in ferrets with lymphoma is anemia. Lymphocytosis and atypical lymphocytes in peripheral blood are relatively uncommonly reported, though occasionally found in lymphoma-afflicted ferrets.[1,11] In young ferrets with lymphoma, a lymphocytosis is more commonly observed than a lymphopenia, which is more commonly noted in older ferrets.[11,14] Atypical circulating lymphocytes are occasionally observed in lymphoblastic lymphoma, but have low sensitivity for disease detection.[4,11] In young ferrets with mediastinal lymphoma, circulating lymphoblasts are a reported feature.[4] In ferrets with lymphoma over 3 years old, atypical lymphocytes are less commonly observed in peripheral blood.[11] Lymphoma diagnosis should include evaluation of lymph node and splenic and/or bone marrow biopsies.

Eosinophilic gastroenteritis in ferrets, though not commonly reported, can result in a peripheral eosinophilia.[14] The exact etiology of eosinophilic gastroenteritis in ferrets is unknown; no parasitic- or allergen-related associations have been identified to date.[14] In a study of ferrets with eosinophilic gastroenteritis, eosinophil concentrations ranged from 1190–3290/μL.[14] Ongoing monitoring of peripheral eosinophil counts can be an adequate method for determining response to treatment and disease progression.

Aleutian Disease Virus

Aleutian disease virus (ADV) is a parvovirus affecting primarily mink, though it rarely can affect ferrets. Ferrets with ADV may have variable clinical findings and hematology test results are nonspecific requiring further diagnostics (PCR) to confirm diagnosis. Experimental ADV infection of mink results in a severe anemia within several months of infection.[29] The proposed mechanism of the anemia is increased RBC destruction, which may be accompanied by decreased or inadequate erythropoiesis. Leukopenia and thrombocytopenia are observed in later stages of infection.[29] Characteristic findings of Aleutian disease in mink include hypergammaglobulinemia and histologic tissue infiltrates of lymphocytes and plasma

cells.[29] Similar lymphoplasmacytic tissue infiltrates were observed in a case report of a pet ferret with spontaneous Aleutian disease; however, interestingly, the hypergammaglobulinemia typically seen in infected mink was not noted.[41]

Hemoparasites

Blood parasites have been reported in mustelids. A *Hemobartonella*-like organism has been reported in a river otter.[17] The affected animal presented with a normocytic normochromic anemia (PCV 12%) with slight polychromasia and anisocytosis, and moderate numbers of parasites were observed on blood smear evaluation as in other species.[17] Microfilariae, most suggestive of *Dirofilaria lutrae*, were identified in approximately 20% of examined river otter blood smears over a 6-year period, though clinical disease was inapparent in those animals.[38] The same study reported lower median MCV of unknown significance in microfilaria-positive animals.[38] *Babesia microti*-like parasites were reported in a North American river otter without any clinical signs.[6] Intraerythrocytic piroplasms were observed in 1–2% of the RBCs; although no hematologic abnormalities were noted, the pathogenic potential of this parasite is unknown.[6] *Babesia sp. NV-1* was detected molecularly in an American mink in Japan.[18]

HEMOSTATIC DISORDERS

Approximately 30 cases of ferret hemorrhagic syndrome (FHS) in 8–24-week-old kits were reported in 2006.[35] They had all recently been shipped from a commercial breeder. Usually, the only clinical signs were acute hemorrhage and death. The disease is now rarely reported; however, the author (CJD) sees about one case a month in recently shipped ferrets from 6–8 weeks of age. The few affected ferrets that had coagulation panels analyzed showed prolonged prothrombin time (PT) and partial thromboplastin time (PTT). Unfortunately, sampling for coagulation panels has not been possible as the ferrets are found dead, having no previous signs. Live animal testing is necessary to further document this disease and elucidate its pathogenesis. Epistaxis, bleeding from mucosal surfaces, and body cavities filled with hemorrhage are found at necropsy. No etiologic agents have been found upon histologic examination of tissues. Once severe hemorrhage has started, it is usually fatal. Ferrets who seem to recover may relapse. Based on this, and the age of the affected ferrets, a genetic component is strongly suspected.[35]

Rodenticide intoxication should be included as a differential diagnosis for any hemostatic disorder. Rodenticide in wild populations is a significant cause of anemia and hemorrhage. Anticoagulant rodenticides reduce the synthesis of vitamin-K-dependent coagulation factors through inhibition of vitamin K epoxide reductase (see Chapter 89).

Mink are one of the species affected by Chédiak–Higashi syndrome (CHS), an inherited platelet disorder. Chédiak–Higashi syndrome is characterized by altered pigmentation, abnormal giant neutrophil granules, and platelet-related bleeding due to abnormal platelet granulation (see Chapters 82 and 146). Anistoroaei et al. found the *LYST* gene to be responsible for the Aleutian (color) phenotype and associated Chédiak–Higashi syndrome.[2] Hematologic findings include markedly prolonged bleeding and prolonged coagulation times.[30] In a study characterizing platelets from mink with CHS, platelets from affected animals have an impaired aggregation response to collagen as well as a lack of serotonin.[30] They have a near absence of the nucleotide storage pool, comprising 38% of ATP and 9.6% of ADP found in normal mink platelets.[30] In CHS-affected mink, the release of Ca^{2+} and Mg^{2+} was 56% and 28% of normal, respectively.

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CHAPTER 116

Hematology of Cavies

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Introduction
Erythron
Leukon
Platelets and Coagulation
Bone Marrow
Reference Intervals
Partitioning Factors
Hematologic Disease

Acronyms and Abbreviations

F, female; KC, Foa-Kurloff cells; M, male; NS, not specified; PLT, platelet; SD, standard deviation; SE, standard error; WG, Wright–Giemsa.

INTRODUCTION

Caviidae is a family of rodents native to South America, which includes domestic and wild guinea pigs (*Cavia sp.*) along with the capybara (*Hydrochoerus hydrochaeris*), mara (*Dolichotis patagonum*), and rock cavy (*Kerodon rupestris*). The vast majority of what is known about cavy family hematology is due to study of the domestic guinea pig (*Cavia porcellus*). Guinea pigs have been used extensively in biomedical research, and additional information for laboratory strains used in research can be found in Chapter 118. This chapter focuses on clinical aspects of hematology for the exotics, zoo, or wildlife practitioner.

Blood collection from guinea pigs can be challenging and is a balancing act of determining the blood volume needed for analysis and selecting a vessel or method that will supply the required quantity without threatening the animal's well-being. Up to 100–400 µL of blood can be obtained from the lateral saphenous or tarsal veins by filling microhematocrit tubes from the clear hub of a sterile hypodermic needle (23–27 gauge), or using gentle aspiration into a 1 cc syringe.[5] Larger volumes (1–2 mL) can be obtained from the jugular or femoral veins, or cranial vena cava (personal communication, Angela Lennox), in sedated or anesthetized animals.[5,28] For research purposes, blood collection methods that yield significantly larger volumes of blood include cardiac, abdominal aorta, or cranial vena cava punctures (generally used as a terminal procedure in anesthetized animals).[29,35] The blood volume of the adult guinea pig is approximately 69–75 mL/kg of body weight, and about 8–10% of the blood volume (0.5–0.7 mL/100 g of body weight) can be safely drawn in a single collection from a healthy guinea pig.[28]

ERYTHRON

Guinea pig RBCs have mild to moderate anisocytosis with a diameter ranging between 7.1 and 8.3 µm (Figure 116.1).[39] The guinea pig RBC is the largest in comparison with other common laboratory rodents, with a mean cell volume (MCV) of approximately 85 fL.[22,24] Best estimates of guinea pig RBC life span range from 60 to 80 days.[39] RBC indices in normal guinea pigs (i.e., RBC count, hemoglobin (Hgb), and PCV) are relatively low when compared with the values observed in other laboratory rodent species, and are more similar to those seen in rabbits.[24,39,41] Historical RBC indices tend to be lower than values observed in specific-pathogen-free (SPF) guinea pigs from more contemporary studies. Along with historically higher mean WBC counts, this suggests that subclinical disease may have contributed to a mild anemia and leukocytosis in previous reports.[24]

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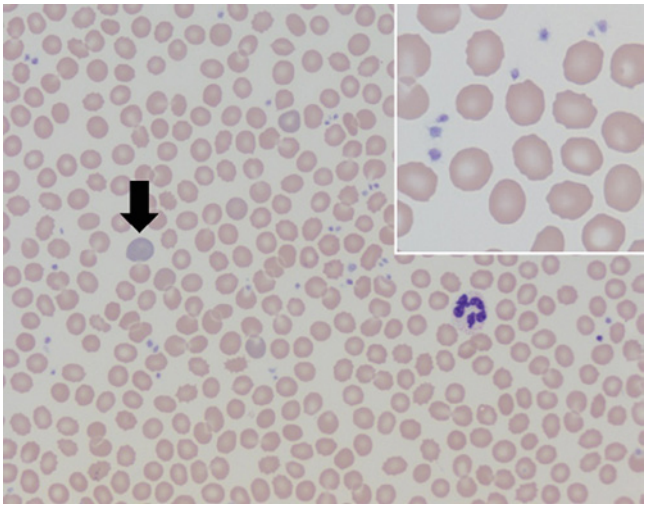


FIGURE 116.1 Moderate-power view of normal guinea pig blood with a heterophil (right) and the occasional polychromatophil (arrow); 500× magnification. Inset: High-power view of normal RBC and platelet polymorphism; 1000× magnification. Modified Wright–Giemsa (WG) stain.

LEUKON

Cavian heterophils are varyingly referred to as neutrophils, depending on the source, and are the functional counterpart of the neutrophil in other species.[42] Heterophils are approximately 10–12 µm in diameter, have a condensed, segmented nucleus (with up to five or more segments), and fine, variably eosinophilic cytoplasmic granules, causing some to refer to them as "pseudoeosinophils" (Figures 116.1, 116.2a, and 116.3a).[27,34,41] As with other mammals, a "drumstick" X chromosome lobe (Barr body) may be present on heterophil nuclei in female guinea pigs.

Lymphocytes are the predominant leukocyte, and small lymphocytes (Figures 116.2b and 116.3c), which are present in greater numbers than large lymphocytes, are not much larger than RBCs. Cavian small lymphocytes have a round, condensed nucleus surrounded by a narrow rim of cytoplasm. Intermediate (approximately 1.5× the size of a RBC) and large lymphocytes (up to double the size of a small lymphocyte), with a less condensed, more oval-shaped nucleus and an expanded amount of pale to moderately basophilic cytoplasm, are also observed.[41] These larger lymphocytes may contain variably sized azurophilic granules (Figure 116.2c).[34] Large granular lymphocytes from the peripheral blood of guinea pigs have been shown to demonstrate cell-mediated cytolytic activity in vitro.[14]

Occasional Foa-Kurloff cells (or Kurloff cells, KC), which are unique to cavian species, may be observed in circulation, accounting for up to 3–4% of the differential leukocyte count in guinea pigs.[23,27] The KC is a specialized mononuclear leukocyte that contains a large (1–8 µm), intracytoplasmic, finely granular to fibrillary, eosinophilic inclusion body consisting of mucopolysaccharide (Figures 116.2g–i and 116.3a).[23,29] The inclusion is membrane-bound, displaces the nucleus, and is metachromatic and periodic acid–Shiff (PAS)-positive.[29] KCs are known to be estradiol-dependent with increased numbers found in the peripheral blood of females compared with males.[30] Among females, their numbers also increase with pregnancy and fluctuate with different stages of the estrous cycle.[3,23] In addition to peripheral blood, they are found in large numbers in the spleen, thymus, bone marrow, and pulmonary capillaries, rarely in lymph nodes, and within vascular channels of the placenta, where they may help prevent maternal rejection of the fetal tissues.[24,30,3] KCs are counted as lymphocytes in the leukocyte differential count in peripheral blood. The exact origin and function of these cells are unknown, although it has been speculated that they may function similarly to natural killer cells.[24,42] These cells have been observed in the peripheral blood and organs of capybara (Figure 116.3a), as well as other species found in the family Caviidae.[19] They are also found in paca (*Cuniculus sp.*), which is in the superfamily, Cavioidea, but separated into the family, Cuniculidae (personal communication, Kendal Harr).

The morphology of cavian monocytes is very similar to that of common domestic species and humans. They are usually larger than the large lymphocytes (about 13–17 µm in diameter), have an oval to reniform or amoeboid nucleus with a dispersed chromatin structure, and have a gray-blue, occasionally vacuolated cytoplasm that is darker than the cytoplasm of large lymphocytes (Figures 116.2d and 116.3d).[35,42]

Cavian eosinophils are easily distinguished from heterophils ("pseudoeosinophils"). They are larger than heterophils (about 10–15 µm in diameter), have a less-segmented nucleus with a less-clumped chromatin, and dense, round, brightly eosinophilic granules that almost completely fill the cytoplasm (Figures 116.2e and 116.3b).[35,42]

As with many mammalian species, basophils are uncommonly found in guinea pig blood except in association with parasitic infection, along with eosinophilia.[37] They are slightly larger than heterophils (about 10–14 µm in diameter) and have a lobulated nucleus. Their cytoplasm is closely packed with round, dark purple granules of varying size (Figure 116.2f).[28,42]

PLATELETS AND COAGULATION

Guinea pig platelets are typically quite small (2–3 µm), with an oval, irregular, or spindle shape and fine purple granulation (Figure 116.1). By electron microscopy, their platelets contain many dark granules (dense bodies) and an open canalicular system.[25] Reference values for circulating platelet counts have been reported to vary from about 250 to 850 × $10^3/\mu L$[24] and tend to be lower than in other laboratory rodents.[35] Coagulation studies demonstrate that the guinea pig has a longer prothrombin time than other domestic species, and a relatively

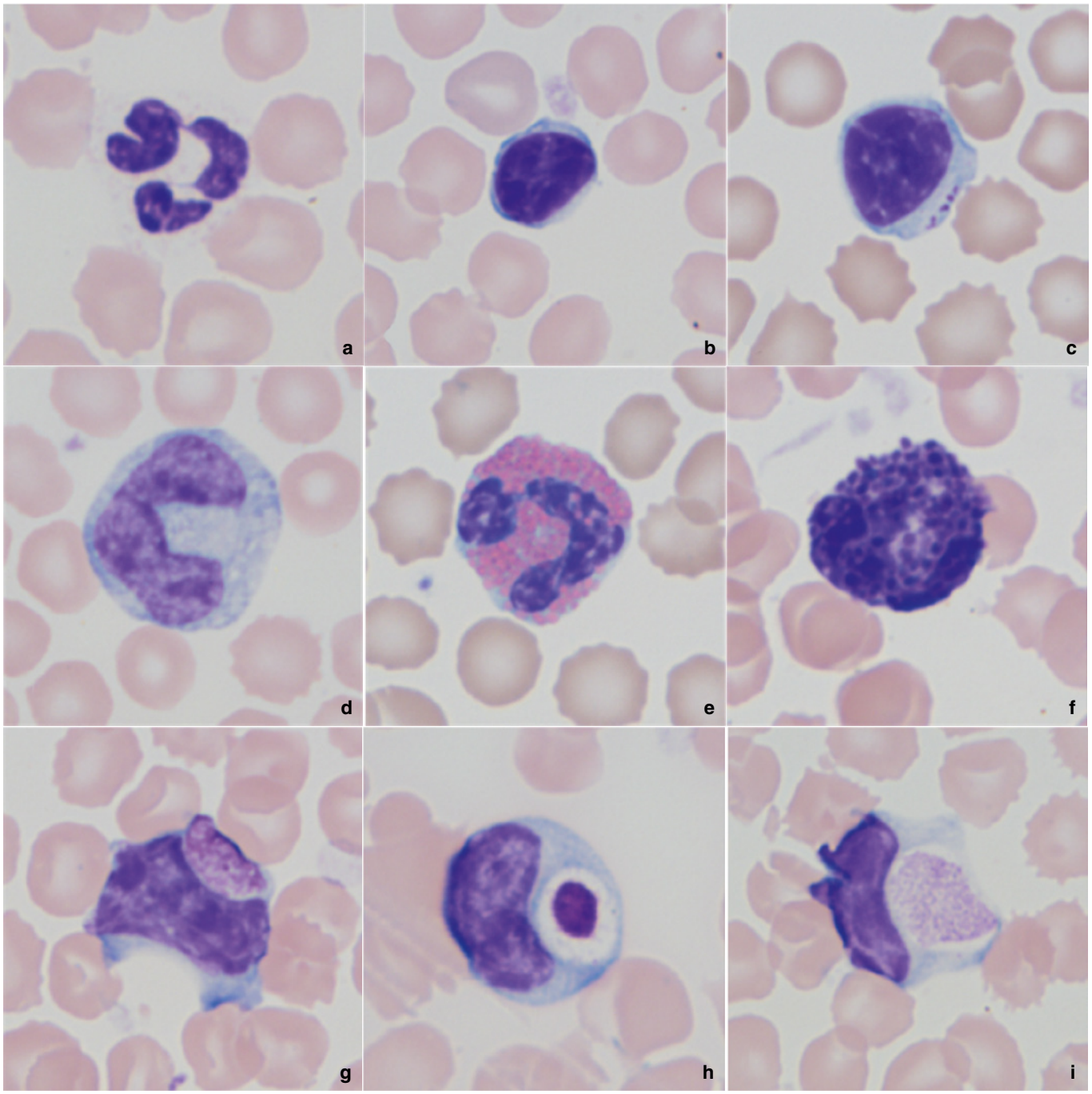


FIGURE 116.2 Normal leukocytes found in guinea pig blood. (a) Heterophil with faint, fine eosinophilic granulation; (b) Small lymphocyte with scant cytoplasm; (c) Intermediate-size lymphocyte with expanded cytoplasm and azurophilic granules; (d) Monocyte with irregular nucleus and foamy basophilic cytoplasm; (e) Eosinophil with numerous small, round, brightly eosinophilic, slightly refractile granules; (f) Basophil with numerous small, round to oval, deeply basophilic granules; (g)–(i) Variable appearance of Foa-Kurloff cells, each with a single, large, eosinophilic, cytoplasmic inclusion. Modified WG stain (1000× magnification).

long partial thromboplastin time.[21,39] Variability in reported coagulation parameters is undoubtedly derived in part from the variety of methods that have been used. Thromboelastography (TEG) has also been reported in guinea pigs which were shown to have a shorter *r* (time to initial clot formation), shorter *k* (time to reach an amplitude of 20 mm), and higher maximum amplitude (MA), indicating that guinea pigs are hypercoagulable and have increased clot stability compared with humans.[21]

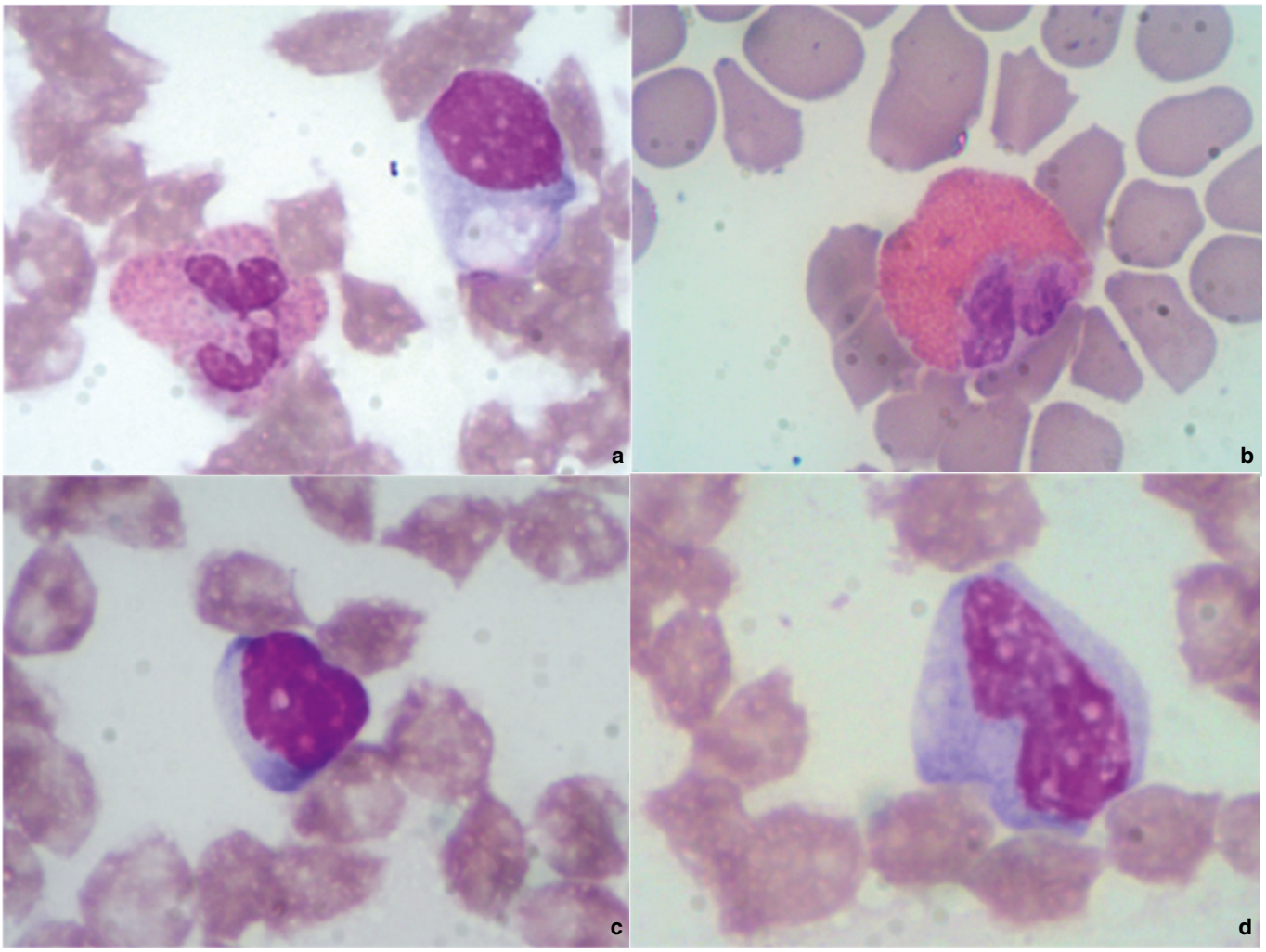


FIGURE 116.3 Normal leukocytes from a 2-year-old captive male capybara. (a) Heterophil (left) and Kurloff cell (right) with a pale-staining Kurloff body; (b) Eosinophil; (c) Small lymphocyte; (d) Monocyte. Wright stain (1000× magnification). Photos courtesy of Juliana Peña Stadlin and Ana María Henao at Fundación Zoológica de Cali, Columbia.

BONE MARROW

Bone marrow can be obtained from the femur, tibia, or iliac crest in live guinea pigs under general anesthesia.[34,35] Collection of a small volume of marrow (3 µL) into a glass capillary is recommended to avoid excess blood contamination, and use of anticoagulant is usually not necessary if slides are made quickly. Postmortem collection can also be obtained by splitting the femur (or another bone) with bone cutters and using a paint brush to spread the marrow across a glass slide[35] (see Chapters 141 and 118).

Bone marrow components in guinea pigs are similar to those observed in other species (Figure 116.4), with the addition of Kurloff cells found as scattered individual cells or small clumps, particularly in the metaphyseal region.[14,30] The M:E ratio has been estimated to be between 1.5 and 1.9 in normal guinea pigs.[9,14,28] As in many mammals, the cellularity of guinea pig bone marrow decreases proportionally with age, and appears to vary slightly based on location (i.e., guinea pig femur and tibia marrow is less cellular than rib, sternum, and vertebrae).[17,39] Indications for bone marrow evaluation in guinea pig patients include cavian leukemia, unexplained peripheral cytopenia(s), or suspicion of other infiltrative bone marrow neoplasia; however, at the author's institution, the latter two are rarely encountered clinically. Although bone marrow evaluation is relatively common in the research setting for a variety of purposes,[39] reports in clinical practice are not widely available.

REFERENCE INTERVALS

It is difficult to find hematologic studies on cavies which fully comply with the guidelines for reference interval generation set forth by the American Society for Veterinary Clinical Pathology (ASVCP).[11] Guinea pig hematology studies were performed in the 1950s and so analytical methods are now significantly different.[2,4,14]

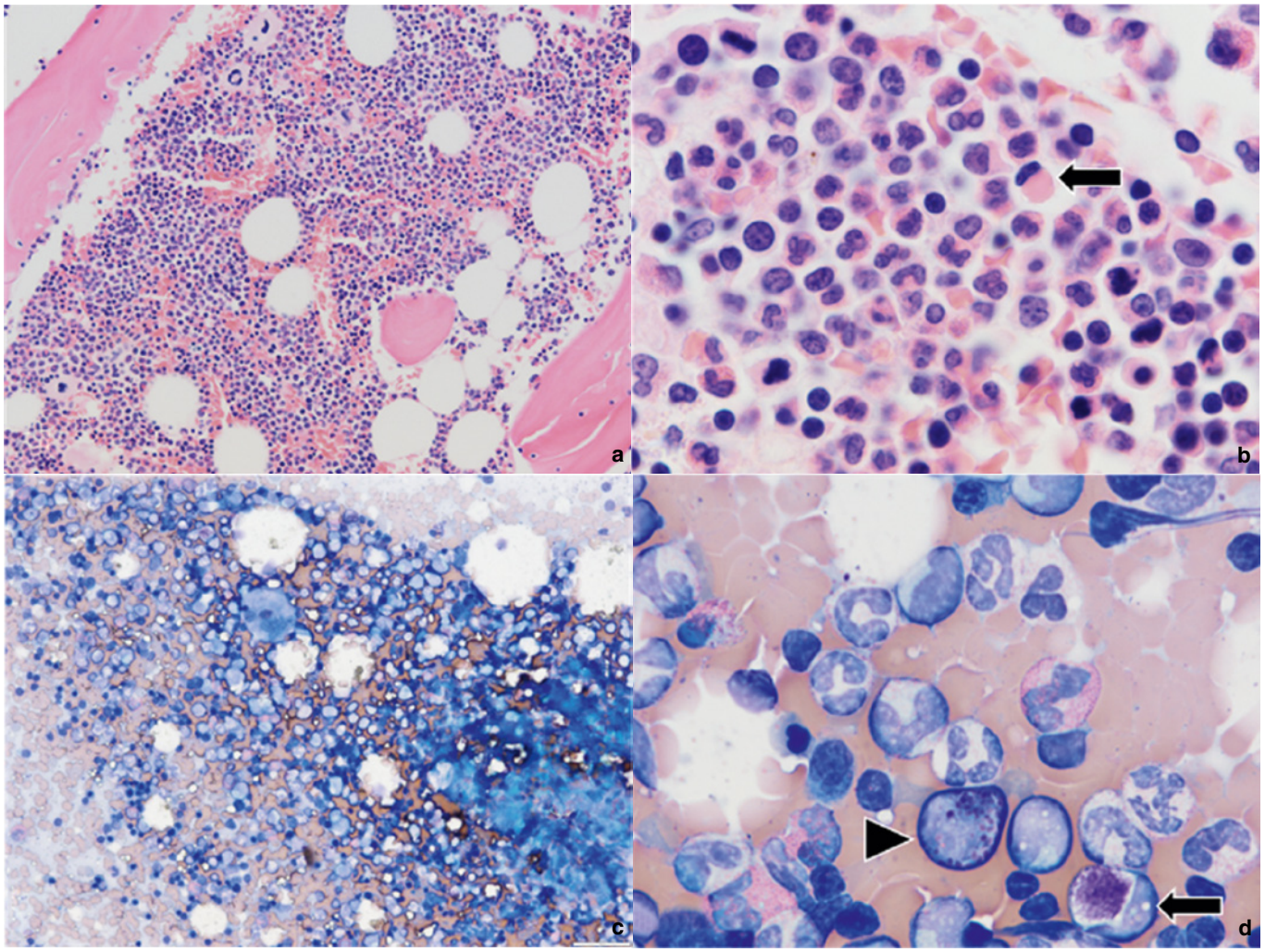


FIGURE 116.4 Normal bone marrow from an adult (7-month-old) female Hartley guinea pig demonstrating a heterogenous combination of megakaryocytic, granulocytic, erythroid, and monocytic lineages; lymphocytes; plasma cells; histiocytes; and Kurloff cells (KC). (a) and (b) Histologic section of the femur. (a) Several megakaryocytes, lipid vacuoles, and bony trabeculae are seen at low magnification; (b) A KC (arrow) admixed with various hematopoietic cells is seen from higher magnification. H&E stain ((a) = 200×; (b) = 1000× magnification). (c) and (d) Cytologic smears. (c) A large megakaryocyte can be seen on the periphery of a cellular bone marrow particle at low magnification; (d) Several heterophil precursors, four eosinophil precursors, one basophil precursor (arrowhead), a KC (arrow), and varying stages of erythroid precursors seen at higher magnification. Modified WG stain ((a) = 200×; (c) = 1000× magnification). Slides courtesy of Dr. Kelly Santangelo and Dr. Mary Afzali at Colorado State University.

Therefore, this chapter aims to represent the best data available for these species with the recognition that the information may be imperfect.

Partitioning Factors

As in many species, age may be a significant partitioning factor. PCV is lower in young (<30-day-old) animals than in adults, except in the first few days postnatal, in which the PCV is reported to be equal to or higher than adult levels.[26,39] Reticulocyte counts are generally higher in immature guinea pigs relative to adults, which have average estimates between 0.9% and 2.3%, depending on the study.[22,26,39] Reference interval studies from client-owned patients are not readily available; descriptive statistics from one study of laboratory animals are presented (Table 116.1).

Data are conflicting with regard to differences between sexes, with some studies reporting a significant difference in some values and others reporting no differences.[22,39,42] Even when sex differences are found, discrepancies exist in the literature as to the direction of difference (e.g., some reports find Hct/PCV is greater in females than males, and others find the opposite).[22,42] Perhaps differences in reproductive status at the time of sampling could explain some of these discrepancies. Nevertheless, sex does not appear to be a consistent partitioning factor of guinea pig hematology.

TABLE 116.1 Hematologic parameters for the guinea pig (Cavia porcellus).[22]

Number of animals (sex)	83 (M) Mean (± SD)	53 (F) Mean (± SD)
RBC (× 10^6/μL)	5.7 (± 0.5)	5.5 (± 0.3)
PCV/Hct (%)	48 (± 4)	47 (± 3)
Hgb (g/dL)	14.6 (± 1.1)	14.5 (± 1)
MCV (fL)	83.5 (± 2)	84.4 (± 1.7)
Mean corpuscular hemoglobin (MCH, pg)	25.4 (± 1.3)	26.3 (± 1.5)
Mean corpuscular hemoglobin concentration (MCHC, g/dL)	30.4 (± 1.8)	30.9 (± 2)
Reticulocytes (%)	1.2 (± 0.9)	0.9 (± 0.8)
WBC (× 103/μL)	6.1 (± 2)	5.6 (± 1.8)
PLT (× 10^3/μL)	427 (± 63)	448 (± 75)

HEMATOLOGIC DISEASE

Guinea pigs are common laboratory animal models, and the myriad of hematologic diseases experimentally induced in this species is beyond the scope of this chapter (see Section 2 and Chapter 118). Primary hematologic disease is uncommon as a presenting concern for guinea pigs in clinical practice.[23] However, the following clinically significant conditions have been reported.

Nonregenerative anemia is a common finding in aged guinea pigs affected by chronic renal disease secondary to advanced nephrosclerosis.[3,24] Flea-bite anemia has been reported secondary to infestation with *Ctenocephalides felis*, the cat and dog flea, and *Nosopsyllus fasciatus*, the northern rat flea.[24,40] Anemia is also associated with vitamin C deficiency.

Guinea pigs appear to be relatively resistant to the effects of steroids in that the numbers of circulating lymphocytes are not markedly reduced by corticosteroid injection.[24,39] Thus, a corticosteroid-induced stress leukogram characterized by lymphopenia is not expected in this species. As with many other species, eosinophilia with or without basophilia have been documented secondary to a number of parasitic infections, including mites, ticks, nematodes, and others.[24,37] Heterophilia, monocytosis, eosinophilia, and basophilia have been reported to rise between two and four times preinfection values in guinea pigs experimentally infected with *Trixacarus caviae* (guinea pig mange).[33] Neutrophilia (heterophilia) and/or lymphocytosis have been reported in response to experimental bacterial and viral infections as well.[13,36]

Lymphocytic leukemia has been well-described in guinea pigs, with leukocyte counts of 200,000/μL or greater.[3,8,16] At the author's institution, cavian leukemia is the most common hematologic disease encountered in pet guinea pig patients. Guinea pigs possess an endogenous type C retrovirus which has been associated with leukemia and exogenous transmission in an inbred strain of guinea pigs,[12,20] but spontaneous viral-induced leukemia has not been well-established in a natural setting. An immunocytochemical assay for immunophenotyping of leukemia and lymphoma (as T-cell or B-cell phenotype) has been developed for this species.[10]

Hypovitaminosis C (also known as scurvy) is a multisystemic disease affecting guinea pigs and other species (primates, bats, and others) that lack the hepatic enzyme l-gulonolactone oxidase to produce ascorbic acid (vitamin C).[24] Vitamin C is an antioxidant and a necessary cofactor in collagen biosynthesis. A spectrum of clinical findings, due to defective collagen and capillary fragility, manifest as subcutaneous, periarticular, and possibly pericardial hemorrhage. These patients may develop both anemia, secondary to blood loss, and coagulopathy, manifested as a high normal to increased prothrombin time.[29,32,43] Somewhat paradoxically, vitamin-C-deficient guinea pigs have also been reported to have normal platelet concentrations up to significant thrombocytosis.[3]

Guinea pigs are important natural reservoirs of *Trypanosoma cruzi* (Chagas disease in humans) in South America,[6] though clinical signs and pathologic findings in naturally infected guinea pigs have not been well characterized. Experimentally, guinea pigs demonstrate infection kinetics and pathologic changes, especially myocarditis, similar to those found in the human disease.[7] Capybara are considered to be asymptomatic reservoirs of *Trypanosoma evansi*,[1] an important pathogen of horses, and have been shown to be seropositive for antibodies against *T. cruzi*.[38] *Leishmania enriettii* is a parasite mainly found in guinea pigs which causes cutaneous leishmaniasis.[27] Although the parasite is thought to be spread hematogenously, detection in the blood has not been reported. Similarly, naturally acquired toxoplasmosis, infection with *Toxoplasma gondii*, has been described in guinea pigs[24] and is potentially hematogenously spread. Experimental infections with many other hemotropic organisms have been performed in guinea pigs, which are beyond the scope of this chapter.

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CHAPTER 117

Hematology of Lagomorphs

FRANCISCO O. CONRADO

Introduction
Blood Collection
Erythron
Leukon
Platelets and Coagulation
Reference Intervals
Partitioning Factors
Bone Marrow Collection and Evaluation
Hematologic Abnormalities
Physiologic
Pathologic
Hemostatic Disease

Acronyms and Abbreviations

APTT, activated partial thromboplastin time; ASVCP, American Society for Veterinary Clinical Pathology; DIC, disseminated intravascular coagulation; H:L, heterophil to lymphocyte; PT, prothrombin time; RHD, rabbit hemorrhagic disease; RHDV2, rabbit hemorrhagic disease virus 2.

INTRODUCTION

European rabbits (*Oryctolagus cuniculus*) are frequently used as an experimental model in human and veterinary research, are raised for meat, and are popular pets. In addition, there are numerous wild hare species which are not covered in this chapter. The effects of clinical diseases on rabbit hematology and diagnostic test application, especially as prognostic indicators, are increasingly being explored. Hematologic assessments are an essential tool for clinicians and researchers alike in the evaluation of health and potential clinical disease.

BLOOD COLLECTION

Rabbit blood hemolyzes easily and clots quickly, which may negatively affect the accuracy and precision of hematology results. Postprandial lipemia may cause artifactual changes in hematologic parameters and it is usually not possible to obtain a truly fasted sample from rabbits due to cecotrope ingestion. Fasting of cecal herbivores should be done with caution in sick animals. Mechanisms of lipemia interference are numerous and depend on the methodology (e.g., turbidity affecting spectrophotometric analyses, volume displacement effect impacting electrolyte measurement).[43] Physicochemical interference on electrophoretic and spectrophotometric methods is the most common and, as lipoproteins absorb light, Hgb and the values calculated from it (MCH, MCHC) may be affected.[64] Interference with the sample's light scatter properties may result in spurious WBC and platelet counts and lipid interference should be suspected when WBC scattergrams demonstrate high numbers of low- to moderate-sized particles.[64] Because lipemia can interfere in numerous tests and methods, the minimum degree of lipemia sufficient to cause interference is variable, and manufacturer guidelines should be followed.[50]

Rabbits can be restrained in a commercial rabbit restrainer, a zippered cat bag, or by securely wrapping the rabbit in a large towel. Common recommended sites for venipuncture in rabbits include the lateral saphenous, cephalic, and jugular veins. Cardiac puncture may be used in research in anesthetized rabbits, typically during a terminal procedure. The ear vessels are not recommended, particularly in small-eared breeds, due to the propensity of hematoma formation, bruising, thrombosis, and skin sloughing.[5] The jugular vein is convenient and allows for a larger volume of blood (up to 6 mL) to be drawn quickly, though obese rabbits with a substantial dewlap may present a challenge.[6] For collection

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from the jugular vein, rabbits are restrained similarly to cats, with the neck gently extended upward and the feet drawn straight down over the edge of the table. The neck should not be fully extended to prevent damage to the spine. The rabbit should have a towel placed over its back and secured on each side to ensure that struggling and kicking, which can damage the spine, are prevented. The jugular furrow may be clipped to improve visualization of the jugular vein and improve sterility, especially if repeated venipuncture is expected. The lateral saphenous vein can be accessed easily for smaller blood volumes (0.5–2.0 mL) with minimal stress. It is away from the animal's head and its use preserves cephalic veins for potential catheterization.[5] Rabbits can be restrained with the head between a restrainer's elbow and body, and the leg extended over the table's edge, held at the stifle to occlude venous return. The lateral saphenous vein is superficial and is prone to hematoma formation, therefore, gentle pressure is recommended after phlebotomy.[6] When collecting large volumes of blood in research, direct cardiac venipuncture may be performed using an 18-gauge or larger needle, 1.5–2 in (3.8–5.1 cm) in length, which is inserted in the third left intercostal space, lateral to the sternum.[35] Cardiac blood collection is reserved for terminal procedures and should not be used for clinical patients because it can result in myocardial damage, hemothorax, pericardial tamponade, and death. Rabbits should be anesthetized before cardiac blood collection is attempted.[32]

The total blood volume in healthy rabbits is estimated to be 4.5–8.1% of total body weight, or approximately 56 (44–70) mL/kg.[11,35] However, there may be inaccuracies in calculating blood volume based on body weight. Therefore, one should consider correlating blood volume with lean body weight or body surface area in obese or emaciated animals.[25] Approximately 6–10% of the blood volume can be collected,[38] and the recommended maximum blood volume that can be safely collected during one sampling is 7.7 mL/kg of body weight.[39]

ERYTHRON

The rabbit RBC is a typical mammalian, anucleated, biconcave disc with a diameter of 5.0–7.8 μm.[45] This variation results in significant anisocytosis appreciated in blood smears from rabbits (Figure 117.1), with microcytes of one-quarter the diameter of normal-sized RBCs sometimes seen in smears from apparently healthy rabbits.[8] Anisocytosis can be more prominent in male rabbits,[45] resulting in higher RDW values than female rabbits. Anisocytosis is also, in part, attributed to the increased size of reticulocytes, and polychromasia is found in up to 2% of RBCs[8] (Figure 117.1). This is likely a reflection of a high RBC turnover and a predicted random destruction rate of 0.5% per day. The average life span of rabbit RBCs is 57 (45–70) days.[25] Poikilocytosis is a normal feature of healthy rabbit RBCs. Most rabbits have mild (<3%) poikilocytes appreciable on blood smear, the majority of which are acanthocytes and echinocytes. Fragmentation morphology (low numbers of schistocytes, microcytes, keratocytes, and spherocytes) is rarely observed in blood smears from healthy rabbits, while diseased rabbits exhibit mild to moderate fragmentation, often associated with septic and inflammatory disorders or malignant neoplasms.[8]

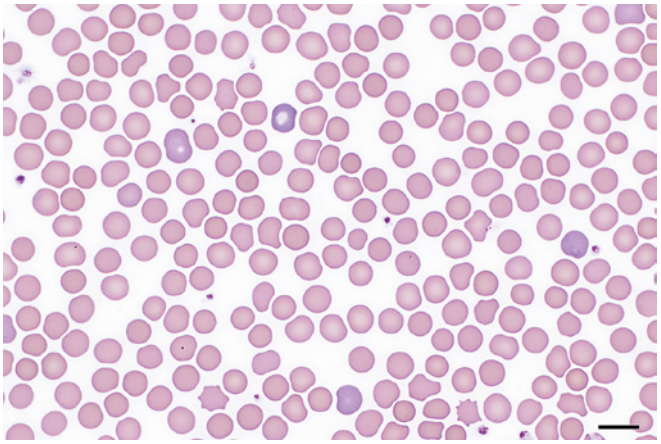


FIGURE 117.1 Photomicrograph of a representative blood smear from a healthy rabbit. Anisocytosis, a few larger, polychromatophilic cells, and rare RBCs containing small, basophilic Howell–Jolly bodies are illustrated. 1000×, aqueous Wright–Giemsa. Bar = 10 μm.

Reticulocytes account for approximately 1–7% (median of 2.7% [168 K/μL], minimum of 0.8% [54 K/μL], and maximum of 7.8% [413 K/μL]) of RBCs in healthy adult rabbits.[10] Automated reticulocyte counts may be higher in samples collected in lithium heparin when compared to EDTA-anticoagulated blood, and there is good correlation between manual reticulocyte counts and quantification by the ADVIA 2120i hematology analyzer.[30] Interestingly, rabbit blood is considered to be an excellent quality control material for flow cytometric reticulocyte enumeration in humans, since the reticulocyte concentration and reticulocyte fluorescence intensity distribution are similar to those of human blood.[48] Nucleated RBCs and RBCs containing Howell–Jolly bodies are found in low numbers in healthy rabbits,[37,38] and do not indicate a regenerative response (Figure 117.2k).

Rabbit Hgb has high oxygen affinity.[7] Neonatal rabbit Hgb has a higher oxygen affinity than that of the dam with the difference gradually disappearing over time.[57] While the general pattern of membrane proteins is similar in all mammalian RBCs, the polypeptide pattern of the rabbit RBC membrane is unique in exhibiting decreased amounts of bands 2.1–2.3 and 6, a band 4.1 which appears splitting into two sub-bands, and higher amounts of band 4.9.[36]

LEUKON

The rabbit heterophil is approximately 10–15 μm in diameter[49] and contains a segmented nucleus that stains variably purple with a darker nuclear membrane. Rabbit

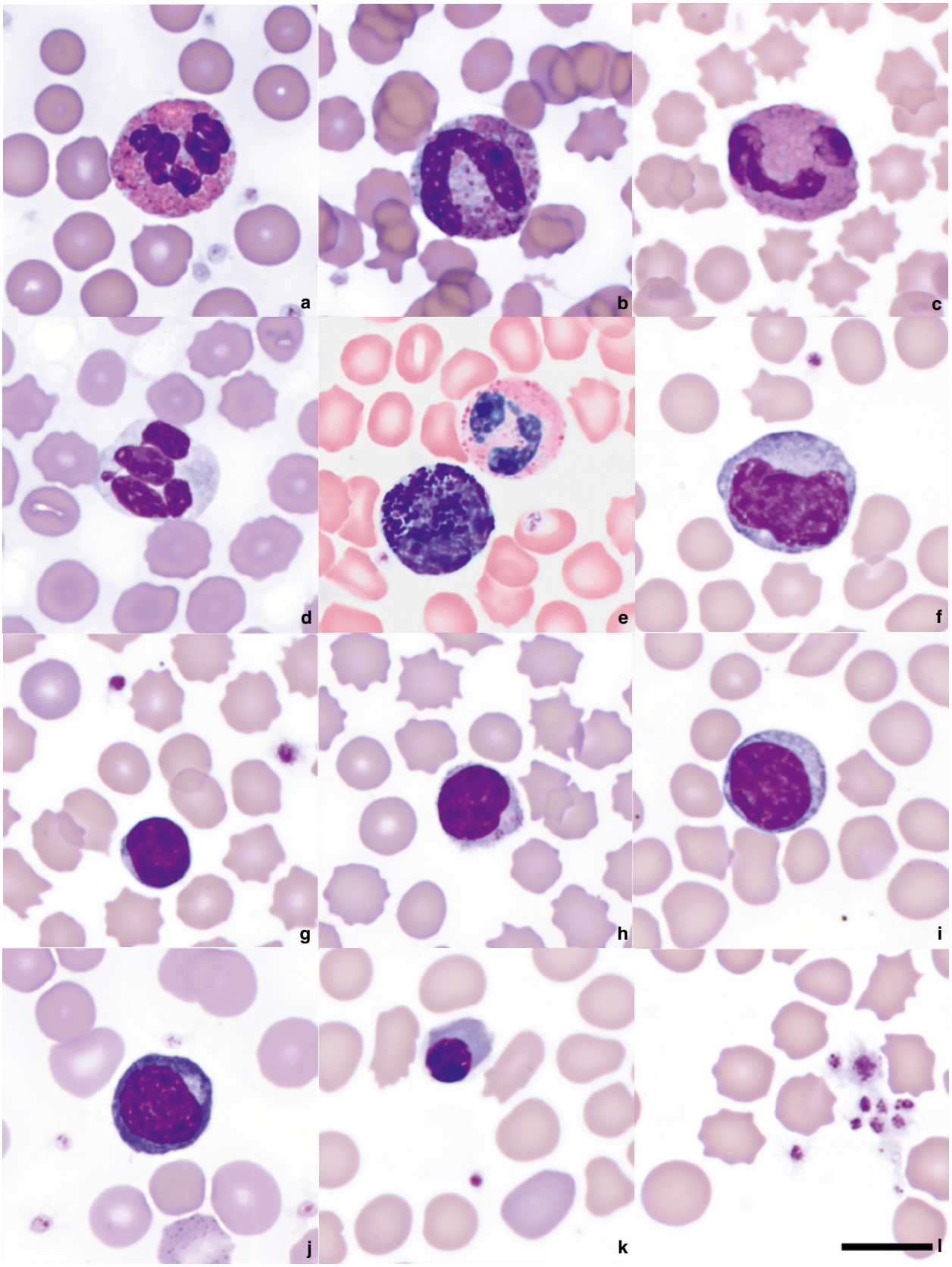


FIGURE 117.2 Photomicrographs of blood elements from pet rabbits (*Oryctolagus cuniculus*). (a) Heterophil; (b) Toxic band heterophil; (c) Eosinophil; (d) Basophil using aqueous Wright-Giemsa stain; (e) Heterophil (upper right) and basophil (lower left) using methanolic Wright-Giemsa stain; (f) Monocyte; (g) Small, well-differentiated lymphocyte; (h) Granular lymphocyte; (i) Intermediate-sized lymphocyte; (j) Reactive-appearing lymphocyte; (k) Polychromatophilic metarubricyte (nucleated RBC); (l) Small platelet clump; 1000×, aqueous Wright–Giemsa. Bar = 10 μm.

heterophils contain acidophilic granules within a colorless to lightly basophilic cytoplasm. Small, azurophilic (primary) granules may give these cells a light purple color, while variable numbers of larger, bright red (secondary, specific) granules are typically present in normal heterophils (Figure 117.2a).

Lymphocytes are the predominant leukocytes in peripheral blood from healthy, unstressed rabbits. The rabbit lymphocyte nucleus is round, condensed, and occasionally indented. The nucleus of the small, well-differentiated lymphocyte (7–10 µm in diameter) is surrounded by a narrow band of pale-blue-staining cytoplasm (Figure 117.2g). Intermediate-sized lymphocytes (10–15 µm in diameter) are commonly found and their slightly more abundant, pale to medium basophilic cytoplasm may occasionally contain a few azurophilic granules[46] (Figure 117.2h,i). Reactive-appearing lymphocytes exhibiting increased cytoplasmic basophilia and a prominent paranuclear (Golgi) clear zone are occasionally identified, and likely reflect antigenic stimulation (Figure 117.2i).

The rabbit monocyte is a large cell (15–18 µm in diameter)[49] with a pleomorphic nucleus (lobulated, horseshoe, or bean-shaped) and diffuse, lightly stained nuclear chromatin. The pale basophilic to light gray cytoplasm is abundant and may contain a few to numerous vacuoles (Figure 117.2f).[46] Morphology is dependent upon response to antigen.

The rabbit eosinophil is slightly larger than the heterophil (12–16 µm in diameter) and has a segmented nucleus that may appear bilobed or horseshoe-shaped.[49] The cytoplasm stains slightly darker blue than the heterophil, and its acidophilic, dull, round pink-red granules are more numerous and larger, up to four times the size of heterophil granules (Figure 117.2c).

Rabbit basophils are generally more numerous than in other vertebrates; while there are lower numbers of tissue mast cells, which are sparsely distributed compared to most other animals and most numerous in the skin, paws, nose, and ears.[22] Basophils measure 10–15 µm in diameter.[49] The nucleus stains light purple and the pale basophilic cytoplasm is packed with deeply purple metachromatic granules.[46] The granules of rabbit basophils typically do not stain with aqueous Romanowsky stains, giving these cells a similar appearance to neutrophils in other species[1] (Figure 117.2d,e). Interestingly, while canine and feline basophils are typically not identified by hematology analyzers, the ADVIA 2120i (Siemens Healthineers AG, Erlangen, Germany) is capable of enumerating rabbit basophils.[34]

PLATELETS AND COAGULATION

Rabbit platelets appear as small clusters of azurophilic granules surrounded by pale blue cytoplasm,[35,46] and measure 1–3 µm in diameter with occasional larger forms seen in blood smears from healthy animals[49] (Figure 117.2l). Previously reported platelet reference intervals varied considerably, from tight to broad ranges, but were not generated in accordance with American Society for Veterinary Clinical Pathology (ASVCP) guidelines (Table 117.1).

TABLE 117.1 Hematologic reference intervals for mixed sex, adult rabbits ($n > 500$). Results generated using Sysmex, Vetscan 5, and manual methods are combined. Outliers are removed and statistics are performed in accordance with ASVCP guidelines. As blood samples from healthy animals were collected in-clinic, a component of stress induced leukograms may be present in the data.

	SI units	Reference interval
WBC	10^9/L	3.4–12.3
RBC	10^{12}/L	4.9–7.5
Hgb	g/L	105–158
Hct	L/L	0.32–0.47
MCV	fL	52–72
MCH	pg	19–24
MCHC	g/L	305–363
Band	10^9/L	0
Heterophil	10^9/L	0.8–8.0
Lymphocyte	10^9/L	0.8–7.6
Monocyte	10^9/L	0–0.8
Eosinophil	10^9/L	0–0.3
Basophil	10^9/L	0–0.7
Platelets	10^9/L	200–1060

Source: Harr, personal communication.

Rabbit hemostasis is, generally, very similar to other mammals and rabbit thromboplastin is often used to construct commercial prothrombin time (PT) assays. Rabbits have a lower factor VII activity and comparatively slow fibrinolytic response compared to humans. Factor VII circulates as a relatively long-lived protein in the healthy rabbit in comparison to the reported shorter half-life for factor VII in the human circulation.[20] Rabbits are known models for venous thrombosis,[59,62] and a study using thromboelastometry identified a comparable maximum clot firmness (MCF) between rabbits and humans, explained by their similar platelet count and fibrinogen levels, supporting the usefulness of rabbits as an animal model for examining platelets.[51] Rabbits have a more active intrinsic system of coagulation and higher clotting factor activities than humans. Rabbit platelets contain 20–25 times the amount of serotonin in human platelets and fail to aggregate in the presence of epinephrine. These and other differing biologic characteristics of rabbit hemostasis warrant caution in extrapolating results of studies in this species to humans.[2,14]

REFERENCE INTERVALS

When assessing rabbit diseases, hematologic reference values are helpful in health status evaluation, providing important information for clinicians. However,

numerous factors such as age, sex, diet, environmental conditions, disorders, stress, pregnancy, and circadian rhythm may influence hematologic values. There are several published rabbit reference intervals which are derived from data collected from homogeneous laboratory animals. These do not reflect the myriad of rabbits housed in laboratory medical facilities, farms, and private homes kept as companion animals.[35] These homogeneous rabbit groups contain animals of the same breed, strain, age, housed in the same environmental conditions, and their reference intervals can be very different from a heterogeneous population of pet rabbits. Additionally, a few texts combine reference intervals from different sources to create ranges too wide to be clinically useful,[38] and/or that do not address the use of different methodologies.[58] Reference intervals generated in accordance with ASVCP guidelines are presented in Table 117.1.

Partitioning Factors

RBC and WBC counts in New Zealand white rabbits undergo significant postnatal change and become comparable with those of adult rabbits by 6 weeks of age. Rabbit RBC counts increase linearly with time after birth.[27] Erythrocyte counts are low in newborn kits, whereas MCV and MCH values are typically higher than in adults.[4,41] New Zealand white rabbits (1–2-month-old) also have reticulocyte counts of 7.4 ± 4.7%, with a 50% reduction in those counts occurring during the third month of life and a subsequent smaller decrease to 3.0 ± 1.3% in adult rabbits.[24] Total WBC counts are lowest in newborn and juvenile rabbits.[27] The absolute lymphocyte concentration sharply increases during the first month of life.[27] In newborns, the initial heterophil:lymphocyte (H:L) ratio (58:31) is reversed in comparison to adults declining to approximately (31:59) by 2 weeks of age. The ratio at 2 months of age is reported as 33:60, increasing to 45:45 after 12 months of age.[25] The increased H:L ratio in neonatal rabbits has been attributed to higher corticosteroid concentrations in neonates.[26] Rabbits delivered by caesarean section on day 29 of gestation showed a comparable H:L ratio to adult rabbits.[4]

The WBC count in rabbits may vary due to age, sex, breed, circadian rhythms (diurnal fluctuations), nutritional status, diet, age, sex, and breed. Additionally, the differential WBC count in a healthy rabbit fluctuates considerably when evaluated over a single month.[39] Total WBC numbers demonstrate diurnal variation, with the lowest counts in the late afternoon and evening. Lymphocyte numbers are typically highest in the early morning and lowest in late afternoon and evening, whereas heterophil numbers are lowest in the early morning and increase to peak numbers in late afternoon and evening.[38]

Progressive alterations in several hematology values of pregnant does occur during gestational development. Platelet concentration increases progressively from organogenesis through the stages of fetal growth.[40] A shortened PT and increased antithrombin III concentration are noted during the late period of pregnancy, and are thought to represent physiologic responses to prevent excessive hemorrhage and/or hypercoagulability at parturition.[40] Progressive decreases in RBC count, Hct, and Hgb concentration are also seen during pregnancy, and are attributed to hemodilution, although a decreased RBC life span during pregnancy has been documented.[31] Dams may also have an initial increase (during organogenesis) followed by a decline in reticulocyte count,[40,60] probably associated with increased demand at this stage. While the WBC count also increases progressively during pregnancy, the changes are typically not statistically significant.[31]

BONE MARROW COLLECTION AND EVALUATION

Bone marrow evaluation is indicated in cases of pancytopenia, nonregenerative anemia, leukemia, and/or gammopathy.[35] Bone marrow aspirates may be obtained from an anesthetized rabbit from the wing of the ilium, the proximal femur, or the proximal humerus. An 18-gauge Rosenthal pediatric biopsy needle can be used for bone marrow collection from the humerus and femur (see Chapter 141).

The morphology of most cells and the maturation sequence in rabbit bone marrow are similar to that in humans and other mammals,[37] and excellent color photomicrographs of rabbit bone marrow are available elsewhere.[49] The M:E ratio of rabbit bone marrow is approximately 1:1.[49] The M:E ratio in rabbits from birth to 5 months of age is reported in older references.[12] Mitotic figures are rarely identified. Lymphocytes compose 3–5% of the nucleated cells in rabbit bone marrow, with occasional plasma cells seen.[49]

Few nonexperimental conditions affecting rabbit bone marrow are reported in the literature. Toxicosis with benzimidazole, a compound used to treat nematode and trematode infections and routinely used in the treatment of *Encephalitozoon cuniculi*, produces histopathologic changes including radiomimetic lesions such as hypoplasia and necrosis.[18] Bone marrow neoplasms are more commonly reported, lymphoma being most prevalent.

HEMATOLOGIC ABNORMALITIES

Physiologic

Rabbits respond to adrenocortical stress or exogenous steroids by mounting a leukocytosis characterized by mature heterophilia and concurrent lymphopenia and eosinopenia.[37] Rabbit lymphocytes are particularly susceptible to the effects of stress. Administration of exogenous epinephrine results in a lymphocyte shift from the spleen and bone marrow to peripheral blood. Epinephrine causes splenic contraction through stimulation of α and β receptors in the splenic capsule.

The mechanism of lymphocyte mobilization from bone marrow is uncertain.[56] Corticosteroid administration results in a redistribution of lymphocytes from the peripheral blood, bone marrow, and spleen to the lymphatic tissue, though the exact mechanism for this process is not fully elucidated.[55] Prolonged stress results, ultimately, in heterophilia and lymphopenia.[58] Transportation or handling stress, if prolonged, can cause an inverse H:L ratio that persists for 24–48 hours.[38]

Pathologic

Rabbits are used as a disease model for drug and vaccine development, in studies of cellular and molecular immune mechanisms underlying many human diseases, including syphilis, tuberculosis, HIV–AIDS, and viral diseases, which is beyond the scope of this chapter[15] (see Chapter 118). Inflammatory and neoplastic diseases are the most common etiologies of anemia in rabbits with severe dental disease, encephalitozoonosis, and neoplasia (commonly lymphoma) diagnosed most frequently.[10] Chronic infections (e.g., otitis, abscesses, endometritis, pyometra) or neoplastic diseases (e.g., uterine adenocarcinoma, lymphoma) may lead to nonregenerative anemias. Rabbits with pneumonic tularemia exhibit leukopenia characterized by monocytopenia and lymphopenia, which are identified on day 4 postexposure. Granulocytes remained largely unchanged. A significant thrombocytopenia was also noted, an interesting finding given that coagulopathies are not reported in humans with this disease.[47] Hematology can be useful in differentiating *E. cuniculi* from *Pasteurella multocida* infection. No known hematologic changes are associated with encephalitozoonosis, while rabbits with pasteurellosis often develop heterophilia and left shift.

Hemorrhage is the second most common cause of anemia in rabbits and is associated with trauma and genitourinary diseases.[10] Erythroid regeneration induced by blood loss will occur after approximately 4–5 days, if not impacted by an inflammatory response. Lead toxicosis is a fairly common known cause of regenerative anemia in rabbits. Reticulocytosis, nucleated RBCs, hypochromasia, poikilocytosis, anisocytosis, and basophilic stippling may occur with lead toxicosis,[29] along with overall anemia and thrombocytopenia.[54]

There are several potential etiologies for leukocytosis, heterophilia, and granulocyte immaturity. Pelger–Huët anomaly, a noninflammatory autosomal dominant disorder characterized by abnormal segmentation of blood granulocytes, was previously documented in rabbits[42] (see Chapter 146). Rabbits affected by infectious disease may present with leukocytosis, but mild inflammatory disease typically results in a shift to a heterophil-predominant differential cell count. Commonly, rabbits with acute infection present with an H:L ratio greater than one. H:L ratio inversion may also occur with transport and handling stress, so this must be interpreted cautiously with clinical signs. Though possible, rabbits generally do not develop severe leukocytosis in response to bacterial infections. Occasionally, rabbits with an infectious process present with leukopenia with toxic change (Figure 117.2b).

The most sensitive and specific indicators of systemic inflammation are acute phase proteins. Normal rabbits typically have <20 mg/L of C-reactive protein which has been described as a biomarker of inflammation in rabbits with suspected encephalitozoonosis.[9] Serum amyloid A is currently being investigated in rabbits and shows promise, though further investigation is necessary.[33] Transferrin and ceruloplasmin are additional candidates.[16]

Lymphocytosis, often associated with viral disease, can also be associated with nonspecific immunologic responses. Paradoxically, some viral infections may induce lymphopenia. Experimental infections of cottontail rabbits (*Sylvilagus fioridanus*) with herpesvirus induce lymphocytosis with proliferation of atypical lymphocytes,[21] while a profound lymphopenia is observed in New Zealand white rabbits experimentally infected with influenza, human immunodeficiency, or Myxoma viruses.[19,28,52] Lymphoproliferative disorders are relatively uncommon in pet rabbits. Multicentric lymphoma is the most common, having been reported in several rabbit breeds. Some rabbits may develop a leukemic phase and circulating neoplastic lymphocytes will cause lymphocytosis in these patients.[23] Lymphoid and myeloid leukemias are rarely reported in rabbits.

Eosinophilia may be caused by parasitic disease such as ascariasis, hepatic coccidial infestation with *Eimeria stiedae* in rabbits housed on the ground, or, most commonly, *E. cuniculi* infestation.[38] Eosinophilia associated with hypersensitivity reactions is less common in pet rabbits than in the laboratory setting where rabbits have been used for cosmetic and drug-safety testing. Cutaneous eosinophilic granulomas have been associated with mite infestation (*Cheyletiella* sp. or *Psoroptes cuniculi*) in the pet rabbit.[61]

There are a few reports of hemoparasites in lagomorphs. Molecular detection of *Babesia* sp. and *Anaplasma phagocytophilum* was reported in black-tailed jackrabbits (*Lepus californicus*), desert cottontail (*Sylvilagus audubonii*) rabbits, and eastern cottontails (*Sylvilagus floridanus*) from the Southern USA. A large piroplasm (3.8 μm × 1.9 μm) was observed in the blood smear from one of the PCR-positive jackrabbits.[63] Parasites morphologically consistent with *Babesia divergens*, the agent of bovine redwater and of European human babesiosis, were identified on blood smears from PCR-positive cottontail rabbits (*S. floridanus*) in Massachusetts.[17] Circulating microfilariae have been rarely reported in rabbits.[13]

Hemostatic Disease

Thrombocytopenia and marked hypofibrinogenemia, along with other indicators of disseminated intravascular coagulation (DIC), including prolonged PT and activated partial thromboplastin time (APTT), are associated with rabbit hemorrhagic disease (RHD) caused by a calicivirus.[3,44] RHD is highly infectious and fatal, resulting in significant economic losses and heavy

mortality among wild and domestic rabbit populations.[53] The mortality is believed to result from circulatory dysfunction associated with DIC and necrotizing hepatitis.[44] Three main RHD virus groups with subtle differences in morbidity and mortality have been characterized based on their genetic, antigenic, and epidemiologic properties at the time of publication. Introduction of rabbit hemorrhagic disease virus 2 (RHDV2) in North America is of significant concern in naïve populations and has resulted in significant mortality across the west coast of the USA.[3]

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CHAPTER 118

Hematology of Laboratory Animals

KARYN E. ENOS and DAVID M. MOORE

Normal Hematology of Nonhuman Primates
Erythron
Leukon
Platelets
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Acronyms and Abbreviations

CO_2, carbon dioxide; EMA, European Medicines Agency; FDA, Food and Drug Administration; ICR, Institute of Cancer Research; NHP, nonhuman primate; O_2, oxygen; PLT, platelet; RANTES, Regulated on Activation, Normal T Cell Expressed and Secreted.

Clinical pathology end points are a standard component in many types of nonclinical (preclinical) studies, e.g., toxicology studies, and are included as deemed applicable in others, e.g., efficacy studies. This chapter reviews recommendations and industry standards for hematologic evaluation in laboratory animals. Regulations regarding preclinical toxicologic safety assessments are discussed in Chapter 10.

NORMAL HEMATOLOGY OF NONHUMAN PRIMATES

In preclinical toxicology and drug development studies, cynomolgus monkeys or crab-eating macaques (*Macaca fascicularis*) are the most commonly evaluated nonhuman primate (NHP) species. Marmosets (Callitrichidae family) are becoming increasingly popular due to their small size. Rhesus macaques (*Macaca mulatta*) are used for xenotransplantation and human immunodeficiency virus research due to their similarity to humans and presence of a naturally occurring immunodeficiency virus (simian immunodeficiency virus). Other species studied in research include chimpanzees (*Pan troglodytes*), baboons (*Papio sp.*), squirrel monkeys (*Saimiri sp.*), owl monkeys (*Aotus sp.*), and tamarins (*Saguinus sp.*).

Blood from NHPs may be collected from a number of venipuncture sites depending on the animal's size, method of restraint, and sample volume required. The femoral vein is the most common site for collection of large volumes (>0.5 mL). The cephalic, saphenous, coccygeal, and jugular veins also can be used. For smaller sample volumes, a sterile lancet may be used to puncture the marginal ear vein, a finger, or a heel. The

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average circulating blood volume of rhesus macaques, cynomolgus macaques, and marmosets have been estimated at 56, 65, and 70 mL/kg, respectively, making them relatively volume-rich versus rodent species, but not without limitations.[9]

ERYTHRON

Erythrocytes (RBCs) from NHPs are anucleate, biconcave disks with central pallor. Some species, such as macaques, have a large area of central pallor. A high degree of variability between NHP species exists for RBC diameter and mean cell volume (MCV). Macaque RBC diameter ranges from 6.5–7.5 μm, and RBC diameters across NHP species range between 3.5 and 8.5 μm.[82] Similar to humans, the circulating life span of macaque RBCs is approximately 100 days, and normal reticulocyte counts are relatively low (0–80,000/μL).

Similar to felines, marmoset RBCs may be more susceptible to Heinz body formation during times of stress and disease. Heinz bodies have been described frequently in blood smears from healthy captive marmosets and occasionally from healthy captive tamarins.[36,37] The clinical significance of these inclusions has been debated. Heinz body hemolytic anemia has caused significant morbidity and mortality in marmoset colonies and has been linked to a frequently fatal disease, Wasting Marmoset Syndrome.[36] Wasting Marmoset Syndrome is considered a form of inflammatory bowel disease, but there is no known etiology.[88] Vitamin E supplementation has been reported to decrease the incidence of anemia.[12]

Low numbers of nucleated RBCs, typically one to two per 100 WBCs, are frequently seen in healthy captive New World monkeys (marmosets, tamarins, owl monkeys, squirrel monkeys) and probably reflect the relatively high RBC turnover rate of these species.[36,37,60] Similarly, slight to mild anisocytosis, polychromasia, and Howell–Jolly bodies are also commonly described in owl monkeys, squirrel monkeys, and tamarins.[14,36,37,60]

Stomatocytosis is a frequent finding in blood smears from normal squirrel and owl monkeys. The cause is not known, and the number of stomatocytes varies. A study performed on a colony of seven captive squirrel monkeys identified 9–89% stomatocytes (average 52%).[23] In addition, this study documented an association between increased numbers of stomatocytes and elevated hematocrits (Hct) that was probably caused by alterations in the normal packing characteristics of RBCs. Unlike many inherited and acquired forms of stomatocytosis in other species, squirrel monkey RBCs do not show increased osmotic fragility or shortened survival time.[23]

LEUKON

In rhesus, cynomolgus, stump-tailed, and pig-tailed macaques, neutrophils and eosinophils measured 10.7–13.3 μm, lymphocytes measured 9.5–12.5 μm, monocytes measured 12.5–15.5 μm, and basophils measured 10.5–13.0 μm.[81] With regard to absolute number, neutrophils generally exceed lymphocytes in the peripheral blood of healthy adult NHPs, including chimpanzees, baboons, cynomolgus monkeys,[17] and cotton-top tamarins.[86] Lymphocytes exceed neutrophils in healthy adult rhesus monkeys[16] and in many New World monkeys including owl monkeys, squirrel monkeys, marmosets, and some species of tamarins.[9] In general, similar to other mammalian species, monocyte, eosinophil, and basophil counts are low in healthy NHPs, typically ranging from 0% to 4% of the total WBC count. The typical NHP neutrophil contains a condensed, twisted, or segmented nucleus that commonly has five to six lobes, but may possess as many as 10–12. A small drumstick-shaped appendage (Barr body) may extend from the neutrophil nucleus of females. The cytoplasm varies from pale to light pink, and fine granulation is a feature of most, if not all, neutrophils from NHPs (Figure 118.1). Lymphocytes from NHPs have scant basophilic cytoplasm and large round nuclei. Lymphocyte morphology is variable and "atypical" or "reactive cells" may be seen. Monocytes in NHPs are larger than lymphocytes and may appear in two forms. The first form has a broader band of cytoplasm than is observed in the lymphocytes, with a mottled gray-blue color with a foamy appearance or with discrete vacuoles. The second type of monocyte, a transitional form, has a large oval nucleus devoid of depressions, and its cytoplasm appears darker adjacent to the nucleus and often has delicate pale reddish granulation (Figure 118.1a).

As compared to other mammalian species, eosinophils and neutrophils have an equivalent size and both may have azurophilic or eosinophilic granules. Eosinophils can be confidently identified using the following criteria: (1) eosinophil granules are almost twice as large and rounder than those of neutrophils; (2) eosinophil granules have a brighter yellow-red color, and may be superimposed on the nucleus; (3) the cytoplasm of eosinophils is slightly more gray-blue; and (4) the eosinophil nucleus is not as condensed or segmented.[82] Marmoset eosinophils contain irregular spherical granules which often have a bluish tinge and create a "muddy" gray-blue cytoplasm that can be confused with basophils. The color of eosinophil granules has been reported to vary from eosinophilic to azurophilic to basophilic in cotton-top tamarins, squirrel monkeys, and chimpanzees, respectively, though type of stain was not always reported[13,14,37] (Figure 118.1e, f, and h).

Idiopathic eosinophilia has been described for specific owl monkey karyotypes. K-I (*Aotus nancymaae*) and K-VI (*Aotus azarae boliviensis*) are associated with low and high levels of eosinophils, respectively. The results indicated that the genetic basis of the eosinophilia in K-VI monkeys was likely a function of heightened eosinophilopoiesis and depressed recruitment kinetics from the peripheral circulatory pool in response to Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES).[2]

Basophils contain dull gray cytoplasm with dull gray rods and additional pale violet to dark purple, round or rod-like granules of varying sizes. The granules typically do not obscure the nucleus, but the morphology and number of the granules may vary significantly between species (Figure 118.1c,g).

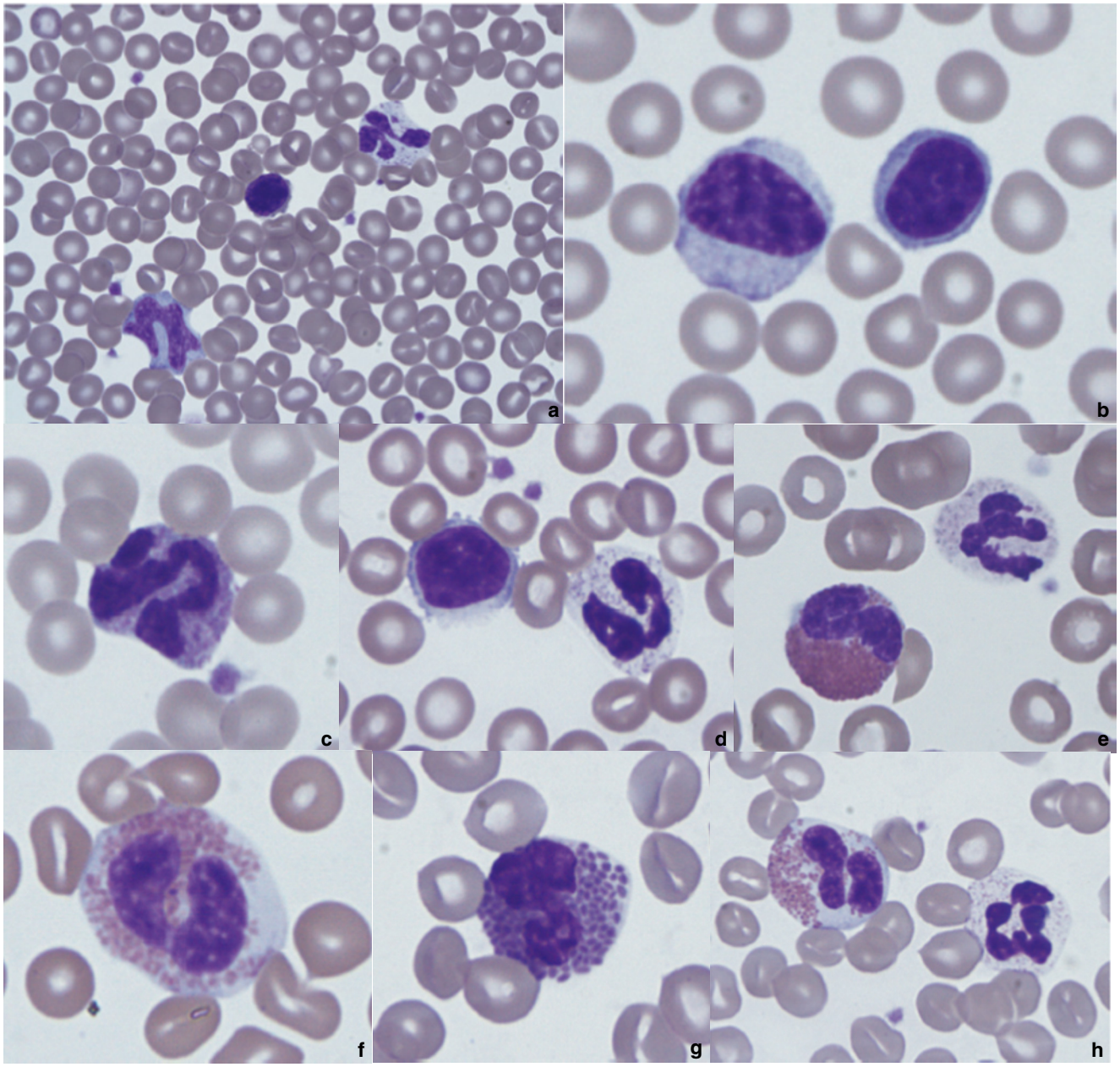


FIGURE 118.1 Peripheral blood cells: (a) Owl monkey (*Aotus nancymae*)—from left to right a monocyte, lymphocyte, and neutrophil; (b) Bolivian squirrel monkey (*Saimiri boliviensis*)—two lymphocytes; (c) Bolivian squirrel monkey (*S. boliviensis*)—basophil; (d) Bolivian squirrel monkey (*S. boliviensis*)—lymphocyte and neutrophil; (e) Chimpanzee (*Pan troglodytes*)—eosinophil and neutrophil; (f) Guyanese squirrel monkey (*Saimiri sciureus sciureus*)—eosinophil; (g) Rhesus macaque (*Macaca mulatta*)—basophil; (h) Rhesus macaque (*M. mulatta*)—eosinophil and neutrophil. Modified Wright–Giemsa stain.

PLATELETS

Platelet morphology in NHP blood smears is similar to platelets from other species. They display an amorphous pale blue ground substance and pronounced violet granulation. Transmission electron micrographs of chimpanzee and monkey platelets show that most of the internal structures are similar to those seen in many mammalian species with the exception of large clumps of glycogen and structures of unknown character circumscribed with fibrous strands.[53] Platelet numbers vary between species, and are generally higher in macaques and lower in chimpanzees among common NHP species.[28] Although few reports on platelet activity in NHPs exist, platelets from baboons and chimpanzees reportedly aggregate with collagen, ristocetin, and ADP. Biphasic aggregation (i.e., aggregation followed quickly by disaggregation) to ADP is common

in both species; however, chimpanzee platelets are not responsive to arachidonic acid.[53] Platelets from rhesus monkeys aggregate in response to collagen, ADP, and arachidonic acid.[45,96] Marmoset platelets aggregate with arachidonate, ristocetin, and collagen at high concentrations, but not with epinephrine. In addition, they have a monophasic response to ADP[55] (see Figure 118.1d and h).

BONE MARROW COLLECTION AND EVALUATION

In larger NHPs, bone marrow can be collected from a number of sites including the iliac crest, the ischial tuberosity, the trochanteric fossa of the femur, the tibial crest, or the proximal humerus.[30] The trochanteric fossa and the proximal humerus are useful for collection from smaller animals. Bone marrow constituents are reportedly similar when sampled from three separate sites: sternum, ribs, and femur[82] (see Chapter 141).

With regard to bone marrow evaluation, the myeloid to erythroid (M:E) ratio ranges from 1.1–1.3 in cynomolgus macaques (mainland China or Vietnam) with no significant differences between sexes.[15] The morphology of hematopoietic cell populations has been described for cynomolgus and rhesus monkeys with strong similarity between the two species.[73,90] A unique feature of bone marrow from rhesus and cynomolgus macaques is the presence of "giant" band neutrophils and metamyelocytes (Figure 118.2g), which is important to recognize as a difference from other species[73,90] (Figure 118.2).

COAGULATION

In general, coagulation values for most species are similar to those reported for humans. Comparisons of the clotting system of rhesus macaques, cynomolgus macaques, and humans concluded that both NHP species are suitable for studying human coagulation defects. Specific differences included higher concentrations and activities of factors II, VII, VIII, and XII in rhesus macaques and lower factor IX activity in both rhesus and cynomolgus macaques as compared to humans.[8,87] Activation of fibrinolysis, intrinsic coagulation, prothrombin consumption, and functional levels of most coagulation factors in cynomolgus macaques were very similar to humans.[8] Clotting times for baboons, chimpanzees, and macaques are typically short, with clots being retracted partially or completely by 4 hours.[30] A recent comparison of 18 analytes that assessed excessive thrombin activation, clotting inhibition, endothelial damage, and fibrinolysis between normal baboons and humans showed a high level of concordance. Exceptions included elevated thrombin/antithrombin complex and fibrinopeptide A and lower plasminogen activity in NHPs.[27]

HEMATOLOGY OF LABORATORY ANIMALS

The Regulatory Affairs Committee (RAC) of the American Society for Veterinary Clinical Pathology (ASVCP) recently published a best practice paper that made clear recommendations on hematology values to assess in toxicology studies[92] (see Chapter 10). Briefly, CBC should include Hct, total and differential WBC counts, Hgb, blood smear collection, RBC, bone marrow smear collection (case-by-case basis for carcinogenicity studies), MCH, MCHC, MCV, RDW, and reticulocyte count. Absolute counts (concentrations) are more relevant than relative counts (percentages). Blood films should be evaluated if cell morphology evaluation, platelet clumping, left shifts to immature cells, presence of nucleated or atypical cells, and confirmation of automated cell counts or other variables are necessary. These criteria need to be defined in standard operating procedures (SOPs). These recommendations apply to all preclinical studies and all species; however, in general less commonly used species are more likely to require manual review of a blood smear. Particularly for mice, even though they are the most often used laboratory animal species, automated counts often do not match manual counts.[65] There may be changes in the list of values to assess depending on anticipated changes related to both the species and the model being studied. Preanalytical factors that can affect hematologic test results are outlined in Table 118.1.

PREANALYTICAL FACTORS AFFECTING HEMATOLOGIC TEST RESULTS

Blood Collection Site

During the course of a study, care must be taken to be aware of body-weight and species-specific requirements (maximum blood volumes) for both single time points and total volume over time with multiple collections. Clinical pathology samples should be collected prior to other study-specific blood samples to more accurately reflect in vivo hematologic values.[72] There are differences in hematologic values and quality of samples for hematologic analyses, as well as effects on animal welfare and behavior, depending on the method and site of blood collection. Standardizing the venipuncture technique for each study is recommended. Venipuncture techniques are discussed in species-specific chapters found in Section IX. In general, the same venipuncture sampling site should be used throughout the study to minimize increased variation in hematologic values due to sampling site whenever possible.

In mice, blood collection sites for in-life assessments include the tail vein, submandibular vein, and sublingual vein (under anesthesia); and for terminal assessments, direct cardiac puncture (Table 118.2).[83] Bleeding from the retrobulbar sinus is no longer recommended in light of tissue trauma, contaminated and clumped samples,

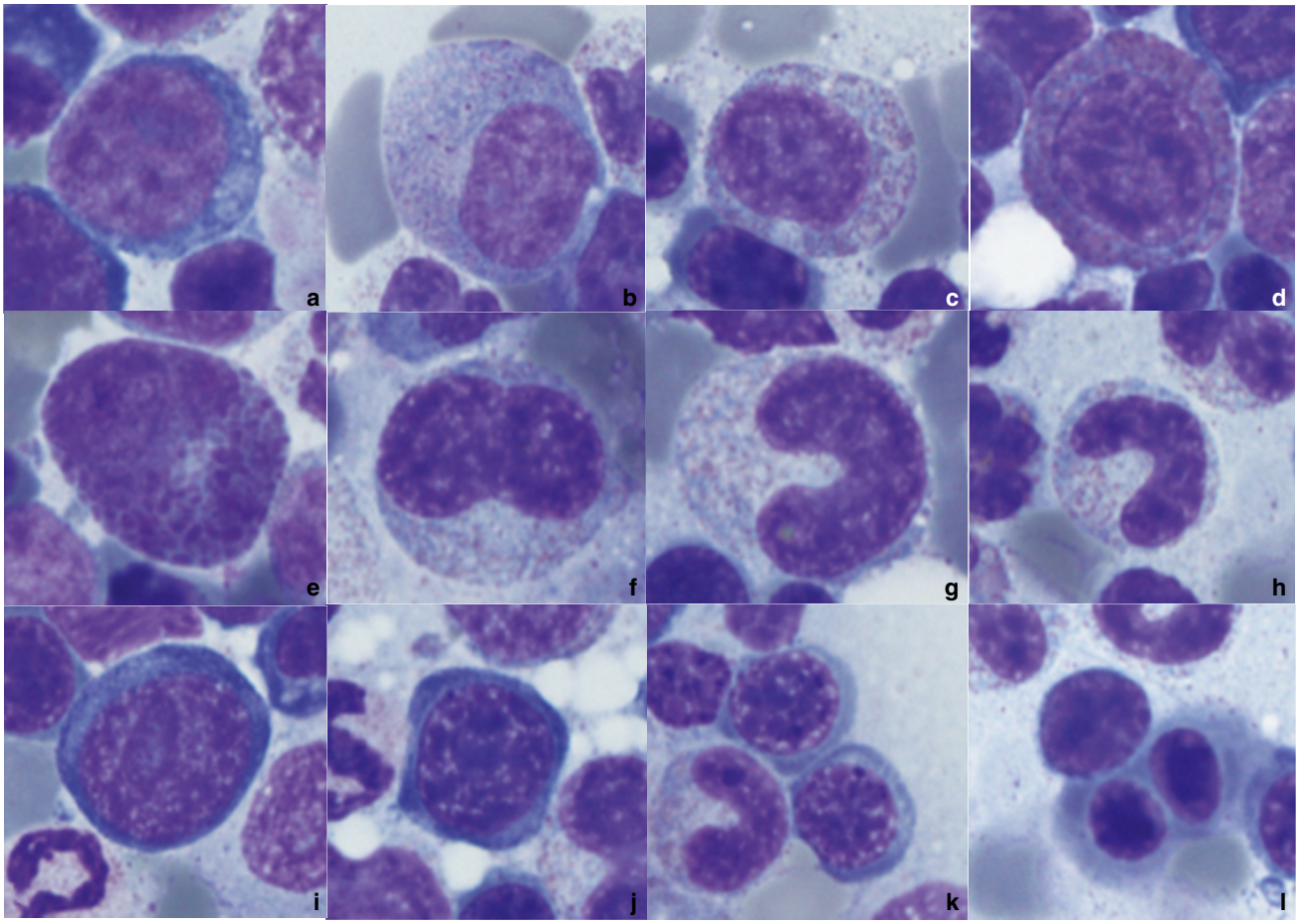


FIGURE 118.2 Rhesus monkey bone marrow aspirate: (a) myeloblast; (b) promyelocyte; (c) neutrophilic myelocyte; (d) eosinophilic myelocyte; (e) basophilic myelocyte; (f) metamyelocyte; (g) giant band neutrophil; (h) band neutrophil; (i) rubriblast; (j) prorubricyte; (k) two rubricytes; (l) two metarubricytes and one lymphocyte. Modified Wright–Giemsa stain.

TABLE 118.1 Preanalytical and analytical factors.

Preanalytical		Analytical	Postanalytical
Blood collection site	Environment	Sample handling	Data presentation
Frequency and time points	Handling/restraint	Analyzer	Data interpretation
Age	Transport	Control groups	
Sex	Circadian rhythms	Reference intervals	
Strain	Anesthesia/analgesia	Historical controls	
Vendor/geographic location	Euthanasia		
Diet			

post collection morbidity, postprocedural complications including blindness and induction of chronic stress, and the need for anesthesia.[25,65,92]

In rats, similar to mice, higher RBC indices (RBC count, Hgb, and Hct), leukocyte, and platelet concentrations are observed in blood collected from more peripheral vessels as compared to more central vessels or the heart.[6,26] Lower basophil counts, total RBC counts, Hgb, Hct, MCH, and MCHC values were observed in rat vena cava blood samples as compared to sublingual samples.[84] In guinea pigs, many collection sites have been used including, most recently, the gingival vein with no notable changes in hematologic values.[79] In rabbits, the auricular vein was the most commonly used, but recently the jugular vein has been used as a practical alternative for repeat sampling, as a subcutaneous sampling port can be placed for blood collection from the jugular vein. No changes in hematologic values have been reported.[63] Site of collection has less of an impact in larger species such as pigs, dogs, and NHPs, though changing the collection site may increase result variation.

TABLE 118.2 Differences as a result of sampling location in mice: comparison to cardiac.

Cardiac	Sublingual/facial	Tail
Baseline	WBCs	WBCs,
	Neutrophils*	Neutrophils
	Lymphocytes	Lymphocytes
	Total RBCs	Total RBCs
	Platelets	Platelets[64]
	Hct	Hct
	Hgb[32]	Hgb[41]

*versus facial.

Frequency and Time Points

Blood collection for hematology in rodent preclinical studies will have an overall study design that takes into account their limited blood volume, larger group size, and decreases variability (see Chapter 10). Large animal (dog and nonhuman primate) preclinical studies, because of their increased variability, smaller group sizes, and larger blood volume, will generally have baseline blood collection(s). These are performed once or twice before dosing to account for day-to-day variability in individual animals. These prestudy baseline samplings also allow for exclusion or inclusion criteria, if they indicate or support other evidence that an animal is not clinically normal and, therefore, not appropriate to use in the study.

Interim blood collections are generally used for studies longer than 7 days duration and can be tailored based on known or anticipated mechanism of action and pharmacology of the test article. Once again, species and blood volume requirements must be considered when determining the most appropriate time points and frequency of blood collection. For many types of preclinical studies, the need for hematology end points must be balanced with the need for blood collection(s) to evaluate toxicokinetics, pharmacokinetics, and pharmacodynamic markers.

The FDA Redbook 2000 states that blood samples obtained in preclinical toxicity studies should be analyzed individually, and not pooled.[11] Pooling results in averaging the variability and eliminating the range and outliers. It dilutes or masks effects that would be observed when analyzing individual specimens and precludes identification and investigation of changes occurring in individual animals.[1]

Age

Age has a significant impact on hematologic values in all species. When setting up RIs for in-house hematologic evaluations, attention should be given to ages of the reference animals to ensure they are representative of anticipated ages of study animals.

In one study of C57BL/6 mice, hematologic RI were determined using facial vein blood collection with diluted blood samples analyzed on the Sysmex XT-2000iV (Sysmex Corp., Kobe, Japan) and indicated an increase in RBC and platelet concentrations from birth to 35 days of age, with a decrease in Hgb, MCH, and MCV between 7 and 14 days of age before rising to adult values. At birth, there were equal proportions of neutrophils and lymphocytes. As they aged, the differential count was dominated by lymphocytes. Basophils were present in low numbers at birth and were rarely seen after 14 days of age; however, no manual cell counts were performed in this study.[101] In C57BL/6 mice, there is a decline in lymphocytes and increasing neutrophil concentrations with age, and a lower Hct due to expanded plasma volume.[65,71]

Published RI for growing and aging male Wistar rats (from 1 to 24 months of age) noted that: Hct progressively increased and then stabilized at 12 months of age. Reticulocyte count declined and then stabilized at 6 months of age. The relative numbers of lymphocytes decreased and neutrophil increased with age.[29]

New Zealand white rabbits (1–2-month-old) were found to have reticulocyte counts of 7.4 ± 4.7%, with a 50% reduction in those counts occurring during the third month of life. A subsequent smaller decrease to 3.0 ± 1.3% in adult rabbits occurred.[43] RBC counts are low in

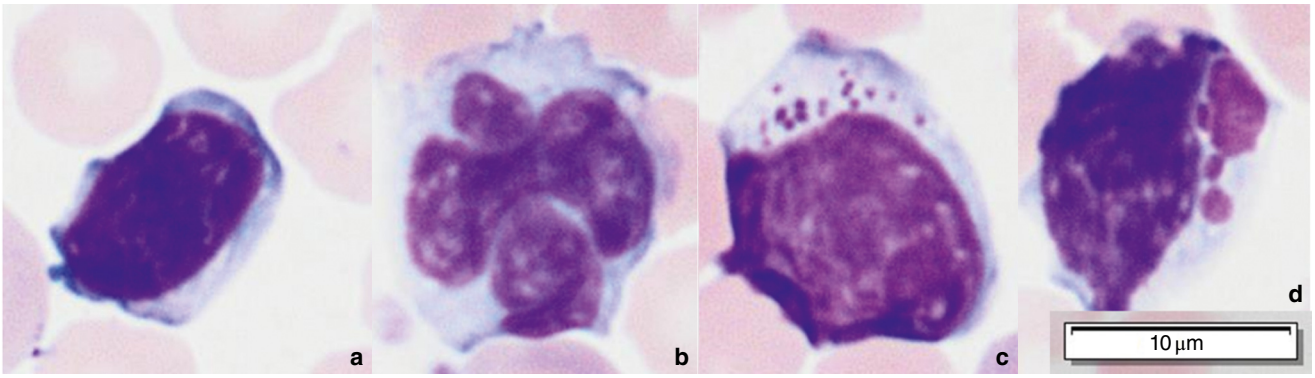


FIGURE 118.3 Hartley guinea pig peripheral blood cells: (a) Small lymphocyte; (b) Lymphocyte with clover-leaf nucleus; (c) Granulated lymphocyte; (d) Lymphocyte with Kurloff body. Wright–Giemsa stain, 100× objective.

newborn rabbits, but attain adult reference intervals by 6 months of age, whereas the MCV and MCH values are higher than in adults.[4] Total WBC counts were lowest in newborn and juvenile rabbits, reaching adult levels after 6–12 months of age (see Chapter 117).

Weiser-Maples guinea pigs had increasing WBC and neutrophil concentrations with decreasing lymphocyte concentration with age as measured on the ADVIA 120 (Siemens Healthineers AG, Erlangen, Germany) with an 18 hour fast and abdominal vein collection under methoxyflurane anesthesia at ages up to 886 days.[50] Possible lymphocyte morphology in Hartley guinea pigs is presented in Figure 118.3.

In a study of Large White Italiana × Duroc × Landrace commercial pig hybrids, where blood was collected via the femoral vein under sevoflurane anesthesia and analyzed on the ADVIA 2120 (Siemens Healthineers AG, Erlangen, Germany), changes from 5 to 30 days of age consisted of increased RBC mass (Hgb, Hct, and RBC) with increased RDW and decreased MCV, as well as decreased platelet concentrations with decreased mean platelet volume.[97] RBC, Hgb, Hct, MCHC, and MCV were also significantly lower in young specific-pathogen free minipigs when compared to adults when collected by cardiac puncture[103] (Figure 118.4).

In laboratory Beagles aged 6–9 months with blood collected from the cephalic vein and analyzed on the T540 Coulter counter, age-related changes included increased RBC, Hct, and Hgb, increased neutrophil concentrations, and decreased lymphocyte concentration. Another study showed similar trends from 9–36 months.[10]

In healthy cynomolgus infants, PCV, MCV, and Hgb concentration dropped significantly from 1–3 months after birth.[89] Changes noted with age in Chinese-origin rhesus macaques aged 3–5 years included increased total WBC, lymphocyte, and platelet concentrations.[16] In cynomolgus macaques under manual restraint with blood collection from the cephalic veins and analyzed on the Sysmex XT-2000iV (Sysmex Corp., Kobe, Japan), decreases in RBC, Hct, and Hgb concentration were observed for females only from 4 to 6 years of age.[102]

Sex

In mice, a sex-associated difference in recovery from blood collection has been observed, with female mice having a more robust recovery from serial blood collections.[74] In general, female mice (and rats) have a lower measured RBC concentration as compared to males,

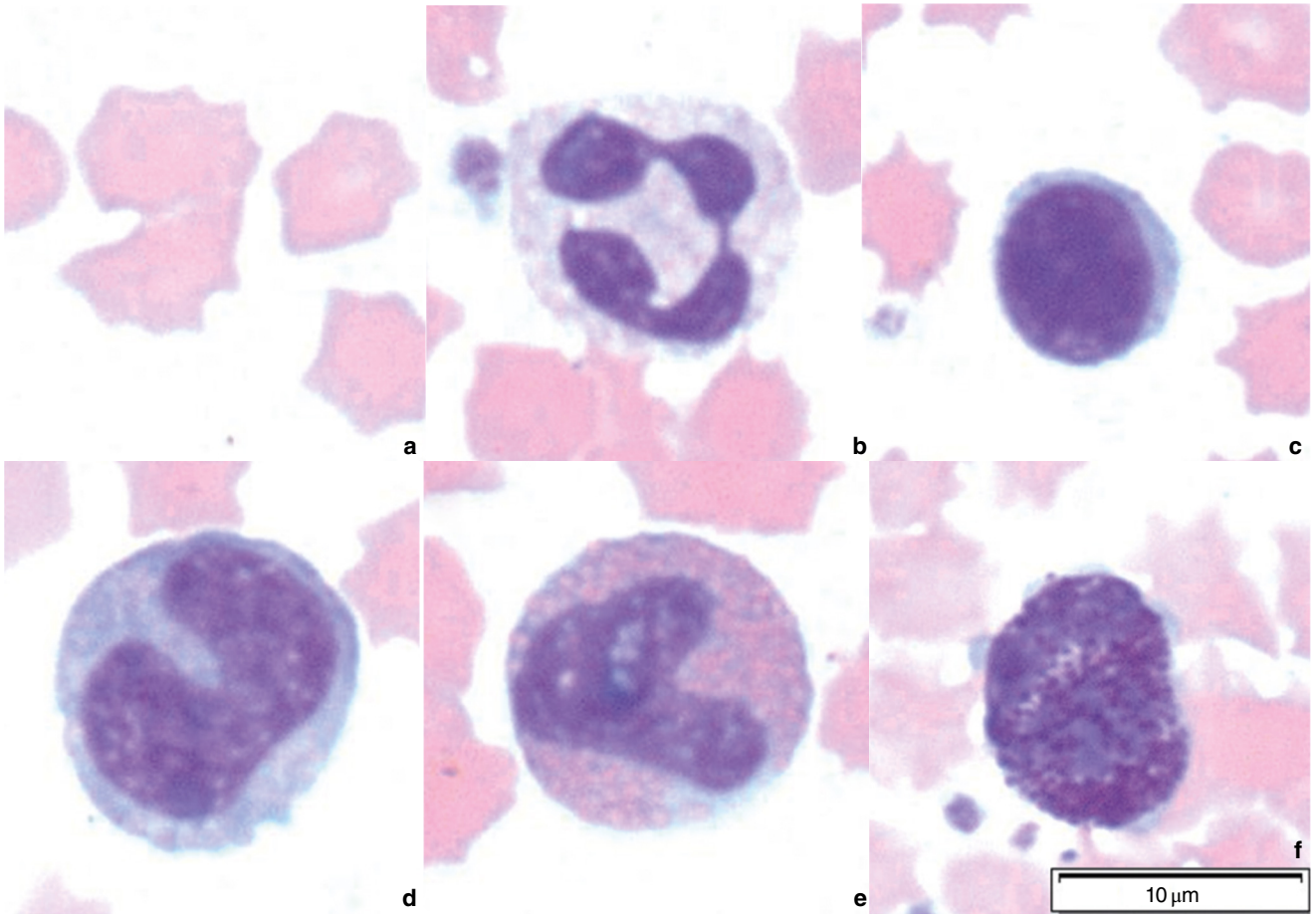


FIGURE 118.4 Yorkshire pig peripheral blood cells: (a) RBCs frequently appear as acanthocytes; (b) Neutrophil; (c) Lymphocyte; (d) Monocyte; (e) Eosinophil; (f) Basophil. Wright–Giemsa stain, 100× objective.

though values may be affected by strain, age, gender, and environment.[71] It should be kept in mind, however, that there are always multiple variables at play.[20]

Male Sprague–Dawley rats have higher Hgb concentration, Hct, RBC concentration, MCV, MCH, WBC, neutrophil, lymphocyte, and monocyte concentrations, while females have higher MCHC, platelet, and eosinophil concentrations when assessed following collection from the abdominal vena cava under sodium pentobarbital anesthesia prior to euthanasia.[38] In contrast to serial blood collections, male Sprague–Dawley rats have a more robust recovery after acute blood collection procedures than did female rats.[61]

In rabbits, RBC count and Hgb concentration are slightly higher in males and reticulocyte counts slightly higher in females.[82] Albino large white (JW-AKT) rabbits with blood collected after 15 hour fast from the auricular vein and analyzed on the Celltac MEK-5258 (Nihon Kohden Corp., Tokyo, Japan) had increased RBC, Hgb, platelet (PLT) in males versus females.

In 5-month-old beagles, PLT concentrations were higher in females.[10] WBC concentrations are reported to increase after 30 days and RBC decrease during the second half of pregnancy.[49]

In studies of adult and juvenile cynomolgus macaques, RBC, Hgb, and Hct were lower in females.[102] In juveniles, reticulocyte concentration was higher in females. In Chinese-origin rhesus macaques under cage restraint, lower extremity veins analyzed on the hematology analyzer XE-2100 (Sysmex Corp., Kobe, Japan) showed that females had lower RBC and Hgb, and higher neutrophil and eosinophil concentrations.[16] In a study of cotton-top tamarins, blood was collected from the femoral vein under anesthesia after an overnight fast, and analyzed on the ADVIA 120 (Siemens Healthineers AG, Erlangen, Germany), PCV, Hct, Hgb, and RBC concentrations were all lower in females than in males and reticulocyte concentrations were higher.[86]

Old World monkeys and great apes have a true menstrual cycle, which can affect RBC indices because a significant amount of blood may be lost during menstruation. Reported changes include decreased RBC mass with increased reticulocyte concentration, MCV, and RDW, as well as increased WBC and platelet concentrations. These changes are most notable with menstruation of >7 days duration.[70] In addition, baboons and chimpanzees display marked turgescence and detumescence of the sex skin in association with the follicular and luteal phases of the menstrual cycle, respectively. These fluid shifts can affect RBC indices causing decreased Hgb, Hct, RBC, and WBC concentration, and increased PLT concentration in the luteal phase.[34]

Pregnancy and Lactation

Hormonal changes through cycling and pregnancy have effects on hematology. RBC counts, Hgb, and Hct values are lower in pregnant Wistar Hannover and Sprague–Dawley rats.[54] Female rats may exhibit leukopenia during estrus.[100] Ovariohysterectomy in female rats results in an increase in the number and percentage of circulating T and B lymphocytes.[71]

In pregnant New Zealand white rabbits, RBC, Hgb, and Hct increased up to day 13, then decreased until day 25 of a 28-day gestation period. Reticulocyte concentrations increased up to day 16, then decreased. Platelets increased up to day 10, and started to decrease on day 13.[99]

In Danish sows at midgestation with blood collection from the jugular vein under manual (snare) restraint and analyzed on the ADVIA 2120i (Siemens Healthineers AG, Erlangen, Germany), changes in laboratory results were consistent with blood volume increases including decreased Hgb concentration and Hct, increased reticulocyte concentration and increased MCV.[7]

Strain

Strain has a major impact on hematologic analyses. It is important that laboratories develop reference intervals that are specific to not only species but also strains of laboratory animals; during studies, appropriate controls must consist of animals of matching strain and vendor/origin. There are notable differences in WBC between BL/6 and BALB/c mice for example.

Differences in circulating mast cells were found between the Th2-prone BALB/c mice and Th1-prone C57BL/6 mice.[18] C57BBL/6 mice have higher RBC counts versus BALB/c or Institute of Cancer Research (ICR); ICR mice have higher platelet concentration than either; C57BL/6 had higher lymphocytes; BALB/c had more granulocytes.[51]

The effects of enrichment are highly variable between strains. A/J and C57BL/6 mice in enriched environments had higher total WBC concentrations than those in nonenriched cages, whereas BALB/c mice had higher WBC concentrations in a nonenriched environment.[95]

Vendor/Geographic Source

Significant differences were found for MCV, MCH, PLT, mean platelet volume, plateletcrit, and WBC, RBC, lymphocyte, monocyte, eosinophil, and reticulocyte concentrations for male and female Wistar rats obtained from two different vendors.[47] Hematologic analysis of eight different outbred stocks and two separate inbred strains of mice from three vendors demonstrated that RBC-dependent values (RBC concentration, Hgb concentration, Hct, MCV, MCH, and MCHC) exhibited less variability than did the WBC concentrations (total WBC, neutrophils, and lymphocytes).[85]

Geographic source is also an important factor in laboratory animal hematology, especially if considering wild-caught animals. One of the most notable examples is the variation of hematologic values between cynomolgus monkeys that originate from Mauritius and those from South-East Asia. Specific differences include higher RBC counts with decreased MCV, MCH, reticulocyte counts, and lower WBC counts in Mauritian monkeys.[21] Differences in cynomolgus monkeys from Cambodia include higher neutrophil, monocyte, and eosinophil concentrations, higher MCHC, and lower lymphocyte concentrations, Hct, and MCV.[17]

Diet

In general, overnight fasting is recommended in all laboratory animal species except mice. Fasted mice do not consume as much water, resulting in hemoconcentration and increased Hct.[24] Fasting of rats can cause increased erythroid indices as well.[100]

Male Wistar rats fasted for 0–48 hours had increased Hgb concentration at 16 hours.[46] Restricted diet provided during evening hours maintains diurnal rhythm, while diet restriction in the morning disrupts the diurnal rhythm of cortisone and adrenocorticotropic hormone.[5] In a 2-year dietary restriction study in Sprague–Dawley rats, decreased total WBC, neutrophil, lymphocyte, and platelet concentrations were identified and were linked to decreased hematopoiesis.[42] Copper-deficient diets provided to male rats resulted in lowered Hgb concentration and MCV as well as increased reticulocyte concentrations. Combined copper and iron deficiencies yielded lowered Hgb concentration and increased reticulocyte, nucleated RBC, and platelet concentrations.[78]

Environment

RBC indices generally increase with environmental heat, and decrease with cold.[100] Warming the whole body or warming the tail is used to induce peripheral vasodilation to facilitate blood sample collection. Warming the tail has been shown to reduce the numbers of WBCs, neutrophils, and lymphocytes observed as compared to nonwarmed tail blood samples.[41] In both warmed and nonwarmed tail blood collection methods, RBC indices and platelet concentration were elevated when compared to both cardiac and retroorbital sinus collection.

Exposure to electromagnetic fields has been studied extensively in mice and rats with variable results consisting of changes in RBC counts, Hgb concentration, Hct, MCV, MCH, and MCHC that depend on exposure frequency, duration, and other parameters.[44] A recent study in C57BL/6NCrl mice showed no changes in hematology or other biologically or clinically relevant values after up to 1 year of exposure.[77]

In rabbits where blood was collected from the ear vein using a commercial restrainer and analyzed on the CELL-DYN 3700 (Abbott Laboratories, Abbot Park, IL, USA), heat stress (hot tropical conditions) caused increased PCV (dehydration) and lymphopenia (stress).[62]

Handling and Restraint

Mouse lymphocyte concentrations decrease with handling and other stressors, while neutrophilia is associated with stress or excitement.[65] To minimize generation of a stress leukogram, handling and restraint for other procedures, electrocardiograms, behavioral testing, physical examinations, and other room disturbances should be conducted after the collection of clinical pathology samples.[26] Blood collection via the submandibular vein in mice has been shown to be more stressful in the absence of anesthesia.[39] Acute stress induces lymphopenia in rodents by changing the distribution from the circulation into peripheral organs such as the skin or lymph nodes and bone marrow.[67] Long-term restraint for infusion studies or nose-only inhalation studies can impact hematologic results, resulting in decreased lymphocyte and eosinophil concentrations, and increased neutrophil concentrations.[68]

Transport

Movement of animals to treatment or procedure rooms, over both short and longer distances, can result in hematologic changes. In BALB/c mice, a decrease in lymphocyte concentrations at 4 hours after transport was observed, consistent with a stress-induced leukogram.[22,67] In F344/N rats that had blood collected for hematologic assessment on the day of shipment receipt versus 12 days later, the newly arrived rats had neutrophilia, eosinopenia, and monocytosis.[19] There were no notable differences in lymphocyte or platelet concentrations. Rhesus macaques had increased platelet counts during the acclimatization period, so 3 months acclimatization was recommended.[35]

Circadian Rhythms

Just as circadian rhythms impact toxicity and treatment effects, they can also affect hematologic results. For example, in rodents, peak numbers of mature circulating WBC and lymphocytes are seen during the light phase, with lowest numbers observed during the dark phase.[105] In mice, WBC, lymphocyte, and eosinophil concentrations peak in morning, and are 3× higher than in the evening. In rats, WBC numbers are lower during periods of activity than during rest periods.[100] Thus, if rodents are maintained on a reverse light cycle and blood is collected during the dark phase, their WBC concentrations will not correspond to RIs from animals on a normal diurnal cycle. To reduce variability in experimental groups when blood sampling takes several hours to complete, it is best to randomize sampling across groups rather than to complete each group one at a time (i.e., random selection of group order and animals within groups, or one animal from mid-dose, control, high dose, low dose, then repeat). Since setting up and initiating blood collection in the animal or procedure room may cause excitement in the first animals sampled, randomization of sampling order by group will reduce this temporal bias associated with excitement.[75]

Although many prosimian NHPs are nocturnal, owl monkeys are the only nocturnal species of simian NHPs and the only nocturnal NHP species commonly used in research. Diurnal variation in both RBC and WBC parameters is due to circadian rhythms and alterations in hydration.[9] These factors should be taken into consideration when sampling blood from owl monkeys.

Anesthesia and Analgesia

Any drug can have effects on hematology values and therefore effects of anesthetic or analgesic agents to be used in laboratory animals should be understood before such treatments can be administered during a study.

One study evaluating the effects of subcutaneous analgesic administration on hematologic values in mice using blood collected by cardiocentesis and analyzed on the Hemavet HV950 (Drew Scientific, Erba Diagnostics, Inc., Transasia Biomedical Ltd, East Mumbai, India) found that after 6 hours, WBC concentrations were higher in mice treated with carprofen and Hct values were lower in mice treated with either buprenorphine or tramadol compared to untreated controls.[40] In albino male mice administered oral aspirin acutely (600 mg/kg 3×/day for 7 days) or chronically (150 mg/kg daily 6 days per week for 25 weeks), blood collected by tail vein was assessed by manual methods. Effects consisted of decreased RBC count, Hgb concentration, and PCV, and increased WBC, neutrophil, eosinophil, and lymphocyte concentrations with acute or chronic doses of aspirin as compared to controls.[57] In this study, ferritin expression in the liver was also measured to assess potential for altered iron uptake from the gastrointestinal tract (as reported in humans), but was similar between dosed and control animals.

In Wistar rats, use of an injectable combination of medetomidine, midazolam, and butorphanol for anesthesia resulted in transient (<24 hours) increases in Hct values and Hgb levels, and transient decreases in total WBC counts, when assessed on the Sysmex XT-2000iV (Sysmex Corp., Kobe, Japan) after jugular vein collection.[66] In Sprague–Dawley rats (Charles River Laboratories), changes in clinical pathology values induced by carbon dioxide (CO_2) inhalation consisted of increased lymphocyte concentration in males following CO_2 inhalation, consistent with an adrenaline response. In this study, blood was collected from the orbital sinus or via cardiocentesis (terminal samples) and evaluated by automatic cell counter with manual slide differentials.[98] In a study using Wistar rats that compared hematologic results associated with three different methods of anesthesia (intramuscular injection with detomidine or ketamine, isoflurane delivered by an anesthetic vaporizer, or isoflurane-soaked cotton ball in a nose cone), blood was collected at euthanasia via the caudal vena cava and analyzed on the ADVIA 120 (Siemens Healthineers AG, Erlangen, Germany). While there was no control group, Hct values were higher than reference intervals for all anesthetic methods, and were lower with vaporizer administration compared to intramuscular and nose cone administration.[52] Increased WBC concentrations were observed in albino rats treated with indomethacin orally when blood was collected by cardiocentesis at euthanasia and analyzed using hemocytometer and manual slide differential.[1]

In pigs, RBC count, Hgb concentration, and Hct measured at 24 hours after experimental femoral fracture repair surgery were increased in the control group (fentanyl only) compared with the treatment group (fentanyl and local bupivacaine).[80]

In a study of beagle dogs, samples collected from the jugular vein and analyzed on Celltac Alpha (Nihon Kohden Corp., Tokyo, Japan) showed that isoflurane anesthesia caused a decrease in RBC and lymphocyte concentration at 2 hours, consistent with blood dilution seen with anesthesia in humans.[91]

In NHPs, the most commonly used anesthetic agent is ketamine, but other agents such as Telazol®, isoflurane, and sevoflurane are frequently used.[48] A physiologic leukocytosis, characterized by lymphocytosis and sometimes a mature neutrophilia, typically occurs when blood is collected from nonanesthetized and untrained macaques. Ketamine anesthesia reportedly reduces lymphocyte concentrations and MCV, but increases the RBC counts, Hgb concentration, and MCHC in macaques of both sexes.[48]

Euthanasia

For most laboratory animal species, blood for hematologic analysis at terminal time points is collected prior to euthanasia. For small rodents, blood is often collected during or immediately following euthanasia in order to maximize the available blood volume. During CO_2-induced euthanasia in mice, when blood was collected by cardiocentesis at the time of euthanasia and analyzed on the Vet ABC Hematology Analyzer (Scil Animal Care Company, Henry Schein, Inc., Melville, NY, USA)-exposure to 100% CO_2 resulted in leukocytosis.[69] Ideally, antemortem blood samples should be collected, prior to euthanasia, as samples collected following death may contain artifacts.

ANALYTICAL CONSIDERATIONS FOR LABORATORY ANIMAL HEMATOLOGY

Automated Hematology Analyzers

The ASVCP Regulatory Affairs Committee recommends all hematology data be generated using automated instruments.[92] If the instrument flags a potential error, a manual smear should be reviewed. When considering acquisition of an automated hematology analyzer, clinicians should assess the abilities and limitations of available commercial units, to ensure that the unit has the capability to recognize and appropriately enumerate cells from the variety of species seen in the preclinical or research laboratory.[11,59]

An industry survey on blood volume requirements for hematology panels using automated analyzers noted that most respondents used sample volumes greater than the minimums recommended by the manufacturer.[72] Issues arise when hematologic analyses are required for small rodents with limited blood volumes. Substantial differences in RBC indices (MCV and MCHC), WBC, and PLT concentrations were observed with 1:5 dilution of rat and mouse blood samples with Cellpack (diluent for Sysmex XT-2000iV, Sysmex Corp., Kobe, Japan) in comparison to nondiluted samples.[59] Different anticoagulants were used in this study, which also induced significant differences, but no other diluents were used. Siemens ADVIA 120 (Siemens Healthineers AG, Erlangen, Germany) analyzer found that several variables were unacceptably altered by dilution of rat blood.[104]

Automated devices used for mouse hematology may have several problems resulting in inaccurate

identification and decreased precision. These are due to the nature of mouse blood components, e.g., small RBC diameter, relatively high numbers of circulating immature and nucleated RBC, variable WBC morphology, platelet clumping, undercounting monocytes, and inability to differentiate atypical lymphocytes, granulocyte types, bands and early myeloid precursors, nRBC, and neoplastic cells.[65] Since accuracy may be compromised, it is recommended to make blood smears for mice for manual microscopic counts (Figure 118.5).

USE OF CONTROL GROUPS, REFERENCE INTERVALS, AND HISTORICAL CONTROLS

Control groups in preclinical studies may include a control article, a vehicle-dosed group, or a sham surgery/procedure group as indicated. It is appropriate to compare test-article-dosed animals to control group animals at the same time points. When possible, generally in dogs and NHPs, individual and group baseline values should be considered and may be used for statistical analyses. Typically, in clinical pathology reports, changes are discussed using descriptive terms such as higher, increased, lower, or decreased as opposed to diagnostic terms (see Chapter 11). Factors such as repeated blood collections can have effects that occur in both dosed and control animals. In this case, having the control animals' data provides weight of evidence that a decreased RBC mass, e.g., is not a test-article-related occurrence. In the case of most rodents, control groups are particularly necessary, since blood volume almost never allows baseline sampling.

At most contract research organizations (CROs), records are kept of control animal values for as many values as is feasible at that given site. This is analogous to a reference interval at a diagnostic laboratory. Few CROs have applied ASVCP's reference intervals guidelines and so the intervals supplied are quite variable. Intervals would be stored with metadata and so ideally could be broken down by age, sex, type of control article, type of study, etc. However, because there can be shifts in values over time, these are not always useful and should never replace real-time, study-specific control animals and baseline values for individual animals where feasible.

Historical control data are useful in some situations. When a dosed individual or group has a value that is different than a control individual or group, historical controls could be used to support that change as being related or not. If the animals' value is out of the controls' range for a particular study, but lacks any other evidence that the change in question is real, having historical control data can be used to help rule it out as a true drug effect if the value is within the historical control data interval.

In general, site-specific reference intervals should be developed for each laboratory and should follow the ASVCP guidelines.[31] Reference intervals should be used

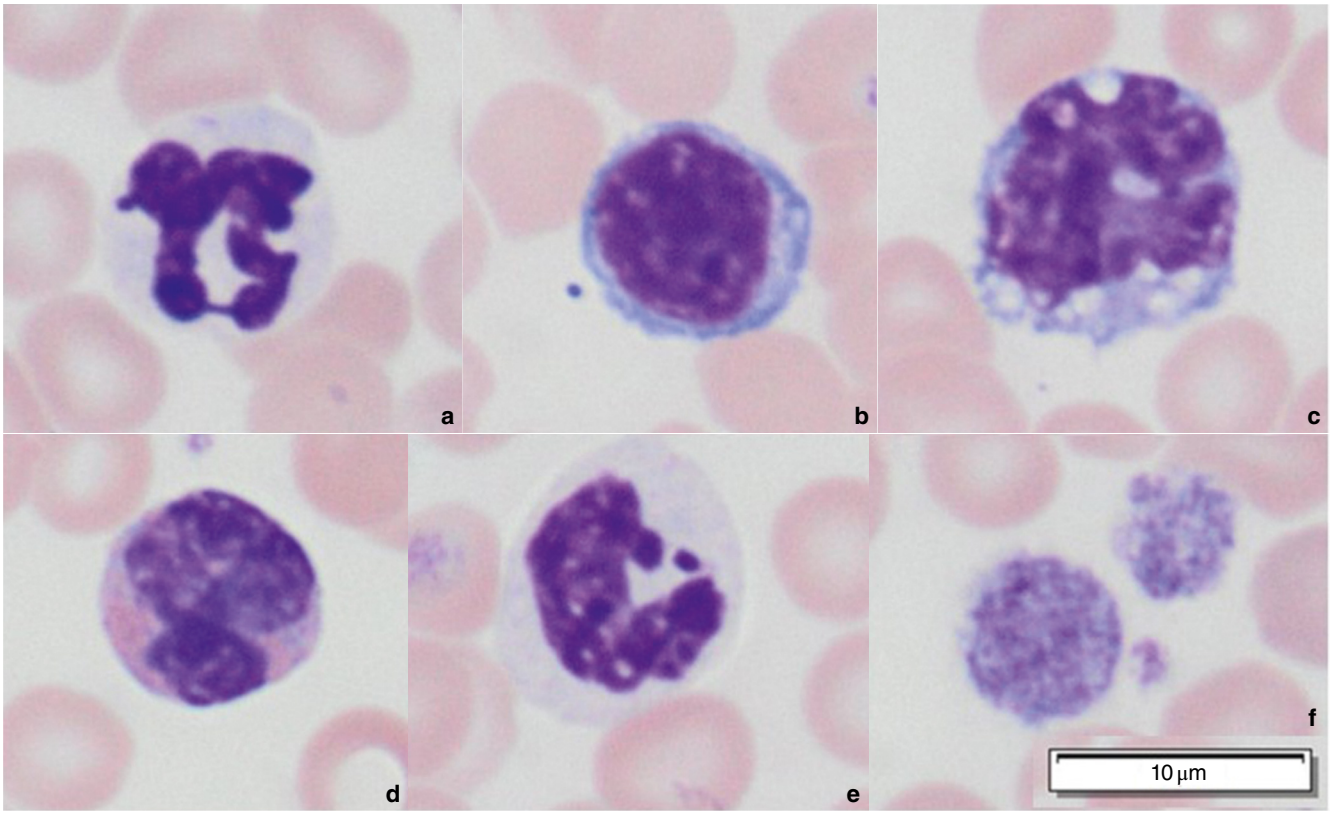


FIGURE 118.5 CBA–CaJ mouse blood peripheral blood cells: (a) Neutrophil with dense chromatin; (b) Lymphocyte; (c) Monocyte; (d) Eosinophil; (e) Neutrophil with open chromatin; (f) Three platelets with variable sizes. Wright–Giemsa stain, 100× objective.

as a tool, but not as the sole guide to determine if values are normal or abnormal, or to determine whether hematologic changes are the result of an experimental procedure. See species-specific chapters in Section IX for available control data.

POSTANALYTICAL CONSIDERATIONS FOR LABORATORY ANIMAL HEMATOLOGY

Data Presentation

Hematologic data can be presented as spreadsheets or graphs depending on personal or institutional preference. Color coding and variations in level of detail shown in tables and reports are also highly variable and often site-specific.[94] Hematologic data are nearly always numerical, and as such represented as either a percent difference or a fold change with a corresponding text modifier.[3,33] Consistent data presentation methods should be maintained throughout a particular report or program.

Data Interpretation

When interpreting hematologic data from laboratory animals, there are some inherent differences from interpreting clinical data. For example, it is important not to overinterpret results in individual animals, as there is often variability in the population. As part of the overall process of hematology data interpretation, comparisons between groups and statistically significant differences need to be critically assessed.[33]

There are also some species-specific hematologic differences that should be considered during study design. Rodent species have more reticulocytes, so will have more polychromasia and anisocytosis on blood smears. Howell–Jolly bodies and nucleated RBC are also commonly observed in blood smears from normal mice.[58] Differential counts vary in different species; mice have relatively more lymphocytes, while dogs and some species of NHPs have more neutrophils. See species-specific chapters in Section IX.

Numerous software packages can aid in streamlining data interpretation and improving consistency and standardization. The simplest data visualization tool may be Excel (Microsoft Corporation, Redmond, Washington, USA) spreadsheets. GraphPad Prism (GraphPad Software, San Diego, California, USA) and Spotfire (TIBCO Spotfire, Somerville, MA) also offer visual displays with color coding and more advanced options that can automate data correlations. Formulas can be created to determine group effects and highlight changes in individual animals.

BONE MARROW

Bone marrow histology is performed as part of any standard preclinical study and includes assessment of H&E-stained slides from all animals. Bone marrow cytology slide preparation is typically performed at necropsy and slides are stained with a Romanowsky-type stain.[76] Smears are only evaluated if indicated by hematology and bone marrow histopathology.[93] Bone marrow cytology is often collected but not evaluated unless there is a specific reason. These situations include a need to differentiate early hematopoietic precursors, investigation of incompletely characterized changes in hematopoietic cellularity on bone marrow histology, determination of whether changes in bone marrow cellularity reflect erythroid or lymphoid cell lineages, and uncharacterized atypical or abnormal cells in the peripheral blood.[76] In the bone marrow, many special stains can be employed including Giemsa to highlight bacteria or erythroid cells, new methylene blue to highlight reticulocytes or Heinz bodies, toluidine blue for mast cells and basophil granules, periodic acid schiff to detect and differentiate erythroid, lymphoid, and myeloid precursors, Masson trichrome for collagen, Prussian blue for ring sideroblasts, and reticulin for collagen III reticulin fibers.[65]

Bone marrow samples from rodents are collected at necropsy for histology as well as cytology (evaluated if indicated). Most frequently, a sagittal section of the sternum is used; a sagittal section of the femur is often also evaluated and sometimes cross-sections of the vertebrae.[76] See species-specific chapters in Section IX for bone marrow evaluation and Chapter 141 for collection techniques. An in vivo evaluation of bone marrow at an interim time point prior to necropsy is required to understand the kinetics of the hematologic response. Core biopsies or fine needle aspirates may be obtained from the iliac crest in dogs and nonhuman primates.[76]

TABLE 118.3 Reference intervals for laboratory beagle dogs.

Age and sex (number of animals)	9–34 months, both sexes (340)[10]	
	Mean	Reference interval
RBC (× 10^6/μL)	7.1	5.7–9.0
Hct (%)	47.1	38–58
Hgb (g/dL)	16.5	12.9–20.4
MCV (fL)	66.9	58–74
MCH (pg)	23.4	20.9–25.8
MCHC (g/dL)	35.1	32.2–37.5
WBC (× 10^3/μL)	9.5	4.9–18.7
Neutrophils (10^3/L)	5.9	2.2–12.2
Lymphocytes (10^3/L)	2.6	1.2–4.5
Monocytes (10^3/L)	0.5	0.1–1.5
Eosinophils (10^3/L)	0.4	0.1–1.5
Basophils (10^3/L)	–	–
PLT (× 10^3/μL)	333	192–538

TABLE 118.4 Reference intervals for cynomolgus monkeys. It is likely that there is a component of a steroid induced leukogram in these values.

Age and sex (number of animals)	19–72 months, both sexes (119)[17]	
Parameter	**Mean**	**Reference interval**
RBC (× 10^6/μL)	5.5	4.6–6.4
Hct (%)	38.0	29–47
Hgb (g/dL)	13.2	11.2–15.2
MCV (fL)	69.6	54.9–84.3
MCH (pg)	24.2	21.3–27.1
MCHC (g/dL)	35.1	27.4–42.8
WBC (× 10^3/μL)	10.3	2.52–18.08
Neutrophils (10^3/μL)	5.3	0.0–11.68
Lymphocytes (10^3/μL)	4.1	0.2–8.0
Monocytes (10^3/μL)	0.7	0–1.5
Eosinophils (10^3/μL)	0.1	0.0–0.4
Basophils (10^3/μL)	0.1	0.0–0.2
PLT (× 10^3/μL)	392	154–630

REFERENCE INTERVALS FOR LABORATORY ANIMALS

The ASVCP has published guidelines for development and use of RIs.[31] It is strongly recommended that institutions develop their own RIs for age- and sex-matched control animals from specific colonies/vendors, to ensure accurate, reliable comparison to hematologic values from test group animals. General reference intervals that are available may be found in the species-specific chapters in Section IX and in Tables 118.3 and 118.4.

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CHAPTER 119

Hematology of Camelids

SUSAN J. TORNQUIST

Erythron
Leukon
Platelets and Coagulation
Reference Intervals
Partitioning Factors
Bone Marrow Collection and Evaluation
Hematologic Disease

Acronyms and Abbreviations

ASVCP, American Society for Veterinary Clinical Pathologists; MPV, mean platelet volume; N:L ratio, neutrophil to lymphocyte ratio; TEG, Thromboelastography.

The biological family Camelidae includes two Old World species, the Bactrian (*Camelus bactrianus* and *C. ferus*) and dromedary camels (*C. dromedarius*), and four New World species, the domestic llama (*Lama glama*), alpaca (*Vicugna pacos*) and the wild guanaco (*Lama guanicoe*) and vicuna (*Vicugna vicugna*). In their respective environments, the camels adapted to severe, dry deserts and the South American camelids adapted to the high-altitude Altiplano.

Llamas and alpacas are sometimes considered to be the same with respect to laboratory reference intervals and interpretation, but there are differences between these species and separate reference intervals should be used.[5]

Venipuncture of the jugular vein is the most common blood collection method. The jugular vein lies deep in the jugular furrow and is not easily visualized in camelids, but with occlusion of the vein and ballottement, a fluid ripple may be seen. The overlying skin is thicker over the anterior neck than it is toward the forelimb, but there is more of a separation between the jugular vein and the carotid artery, so the possibility of arterial puncture is less. Posterior on the neck, the overlying skin is thinner, but a carotid artery stick is more likely. The left jugular vein is very close to the esophagus, so sampling from this side occasionally results in accidental puncture of the esophagus.

ERYTHRON

Camelids' RBCs are elliptical in shape, unlike those of any other mammals.[7,9,13] These elliptical erythrocytes are flat and thin with no central pallor. Because of their shape, they sometimes appear folded on blood smears. Hgb crystals that appear as darkly eosinophilic, dense, rhomboid-shaped structures are frequently seen on blood smears from healthy, nonanemic camelids. The cause and significance of these structures are not known.[34] Cabot rings are also occasionally seen in RBCs from clinically healthy camelids. These are narrow, thread-like structures in the form of rings or figure eights within RBCs. They are thought to be remnants of mitotic spindles and have no known clinical significance in camelid blood.[13]

As in bovids, reticulocytes are present in the normal animal, but typically in lower relative numbers than found in other mammalian species. Normal, nonanemic llamas may have reticulocyte concentrations in the range of 12,000–79,000/μL (0.2–0.6% or 0–0.4%).[9,34] Mean reticulocyte percentages for normal alpacas and vicunas are reported to be 1.4% and 1.2%, respectively. As many as two to three nucleated RBCs per 100 WBCs may be seen in nonanemic llamas.[9]

Several structural and physiologic features of camelid RBCs contribute to their high resistance to osmotic lysis,

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decreased deformability, and high affinity for oxygen. These attributes are adaptive for life in areas of low water availability or lowered oxygen availability. Camelid erythroid membranes contain higher concentrations of the integral membrane protein, band 3, compared to other species.[16] In addition, increased cross-linking of band 3 with other membrane proteins in camelid RBCs probably contributes to their decreased deformability and resistance to hypotonic lysis.[25] Although decreased RBC deformability could be disadvantageous for animals facing a variety of environmental pressures, the disadvantage is minimized by the shape and small size of camelid RBCs that allow them to easily pass through capillaries without having to deform.

The size and shape of camelid RBCs facilitate oxygen diffusion by providing a higher effective surface area for gas exchange.[36] Goat RBCs that are of similar size, but with a smaller surface area, have a much lower oxygen capacity as well as a decreased oxygen affinity in blood.[36] Another adaptation to life at high altitude and low oxygen tension is an oxygen–hemoglobin dissociation curve that is shifted to the left, especially at lower partial pressures of Po_2, facilitating uptake of oxygen by the lung.[36] In addition, the percentage of alkali-resistant (fetal) Hgb with increased oxygen affinity is higher in adult alpacas than in adults of other species.

LEUKON

Camelid leukocytes do not have the unique features of their RBCs and are generally similar to other mammals (Figure 119.1). Neutrophils have a typical segmented nucleus and may have fine pink to purple stippling in normal cells. The neutrophil to lymphocyte (N:L) ratio in adult llamas and camels is about 1.5, which is more similar to humans, dogs, cats, and horses than it is to ruminants.[9,11,34] The N:L ratio was reported to be lower (0.5–2.9) in a group of 29 alpacas, but these were 10–18 months old, so the difference may be based more on age than species.[12] In a study of acute inflammation, the baseline mean N:L ratio in 12 healthy adult alpacas was 4.6.[26] Many published reference intervals do not include an N:L and the relatively small group sizes on which older values are reported suggest that better information is needed to make valid species comparisons. Rare band neutrophils may be seen in peripheral blood from normal animals, but they are more commonly seen in response to inflammation and their morphology is like that of other species.

Camelid lymphocytes vary in size as they do in ruminants. The percentage of granular lymphocytes in circulation in healthy llamas has been reported to be higher than that seen in cattle and horses. In four different studies, the percentage of granular lymphocytes was 7–29%, 1–19%, 0–14%, and 10–30% of blood lymphocytes.[5,11,34] Granules also vary in size.

Low numbers of monocytes are typically found in healthy camelids and have similar morphology to other species including thick band nuclei with coarse chromatin, which may round as the cell matures and reacts to antigen (see Tables 119.1 and 119.2). Cytoplasm is blue to gray and may contain numerous vacuoles (Figure 119.1c,h).

Eosinophil numbers tend to be higher in healthy adult llamas than in other species.[9,13] Eosinophils are usually hyposegmented with band-shaped to bilobed nuclei. The cytoplasm contains numerous fine to small round eosinophilic granules in light blue cytoplasm (Figure 119.1d,i).

Basophils are occasionally found in peripheral blood smears from normal camelids and can be easily identified by their copious blue granules which may obscure the banded nucleus (Figure 119.1e,j).

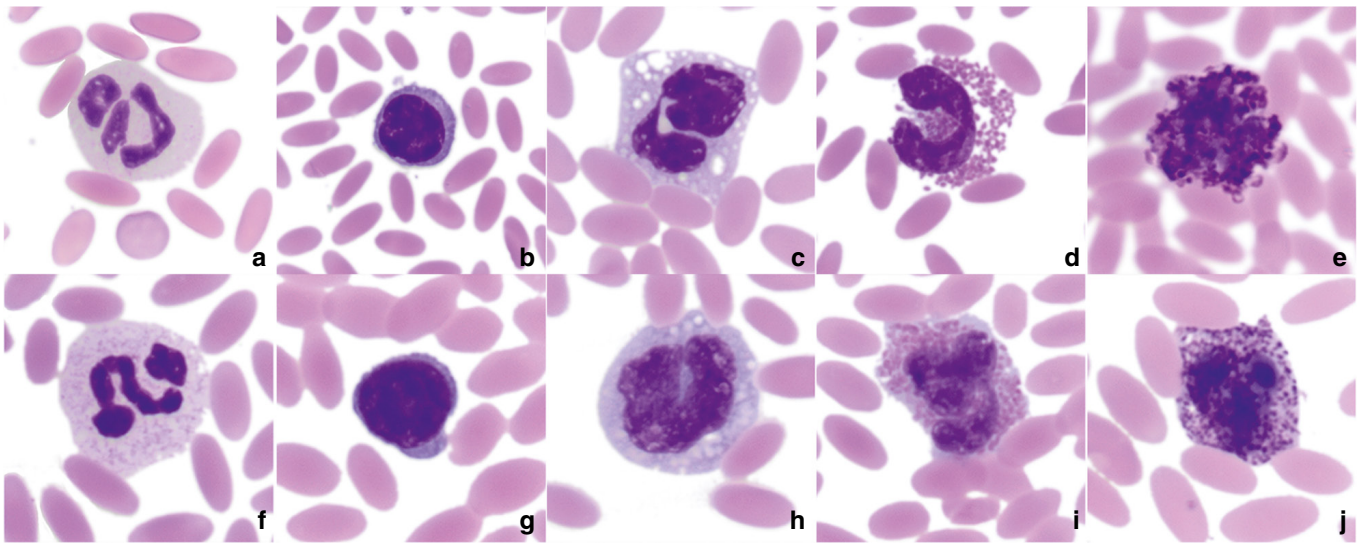


FIGURE 119.1 Camelid leukocytes with normal morphology. Alpaca: (a) Segmented neutrophil; (b) Lymphocyte; (c) Monocyte; (d) Eosinophil; (e) Basophil. Llama: (f) Segmented neutrophil; (g) Lymphocyte; (h) Monocyte; (i) Eosinophil; (j) Basophil. Wright's stain; magnification 1000×.

PLATELETS AND COAGULATION

Platelets from camelids are round to irregular and contain azurophilic granules. Camelid platelets are generally small in volume and platelet counts are higher than in many other species.[5] A study of alpaca platelet indices using the ADVIA 2120 showed that the average mean platelet volume (MPV) in healthy alpacas is lower than that of other species. The same study showed no significant difference between the ADVIA platelet counts and blood-smear-estimated platelet counts suggesting that automated counts are acceptable for this species.

Coagulation testing may be performed in camelids using instruments and reagents that are used in other mammalian species. Thromboelastography (TEG) has been investigated in adult animals and in crias. Mean values of TEG parameters (± standard deviation) for adult alpacas and neonatal crias were, respectively, R = 7.7 (±2.9), 5.6 (±2.2); K = 3.53 (±1.43), 2.55 (±1.18); α = 48.1 (±11.2), 56.2 (±10.4); maximum amplitude (MA) = 55.5 (±8.35) and 59 (±3.21) mm. Mean prothrombin time (PT) and partial thromboplastin time (PTT) were 8.6 (±0.6) and 18.9 seconds (±1.9) in adults versus 9.4 (±0.6) and 18.1 seconds (±1.96) in neonates. A significant difference was not observed between age groups.[2]

REFERENCE INTERVALS

Published reference intervals for New World camelids have been based on relatively small numbers of animals and have been obtained from animals living in a variety of situations including altitude and with different management conditions such as diet and deworming protocols.[7,8,12,34] Values presented in Tables 119.1 and 119.2 have been generated and/or verified using apparently healthy mixed-sex animals by the author in the Pacific northwest region of the United States.

Because camelid Hct and mean cell volume (MCV) cannot be accurately measured with automated hematology analyzers due to the unique RBC shape, reference intervals have most often used data generated with manual methods. Comparisons between manual methods and automated methods are now being made.[5,35] The resulting variability in published intervals highlights the value of both using reference intervals specific to a laboratory/instrument and of having information about the populations from which reference intervals are derived. In using automated hematology analyzers for camelid blood, use of the equine setting with modifications is most often reported.[5,35]

A recent study of 65 healthy adult alpacas showed that automated differential WBC counts correlated well with manual counts for neutrophils, lymphocytes, and eosinophils, and correlated poorly for monocytes and basophils. Comparisons of analyzer-based RBC reference intervals with previous reported intervals for alpacas and llamas showed differences in PCV, Hgb concentration, and RBC counts; but these were somewhat herd-dependent.[5] In a comparison of a

TABLE 119.1 Hematologic reference intervals of alpacas (*Vicugna pacos*). Reference interval is generated from 37 apparently healthy adult, mixed-sex alpacas using an ADVIA 120 on the equine setting. Pcv and differential wbc counts were performed manually.

	Oregon State University Clinical Pathology Lab
PCV (%)	25–37
RBC (× 10^6/μL)	9.5–17.02
Hgb (g/dL)	11.5–16.4
MCV (fL)	19–28
MCH (pg)	8.5–13.9
MCHC (g/dL)	41.9–48.9
WBC (× 10^3/μL)	10.5–23.4
Neutrophils (× 10^3/μL)	2.9–15.3
Bands (× 10^3/μL)	0–0.36
Lymphocytes (× 10^3/μL)	2.8–10.1
Monocytes (× 10^3/μL)	0–1.3
Eosinophils (× 10^3/μL)	0.3–6.3
Basophils (× 10^3/μL)	0–0.7
Platelets (× 10^3/μL)	286–657
Total protein (g/dL)	6–7.5
Fibrinogen (mg/dL) by heat precipitation	100–500

TABLE 119.2 Hematologic reference intervals of llamas (*Lama glama*). Reference interval of 174 adult, mixed-sex llamas verified by the Oregon State University Clinical Pathology Laboratory using an ADVIA 120 on the equine setting.[9]

PCV (%)	27–45
RBC (× 10^6/μL)	10.5–17.2
Hgb (g/dL)	11.9–19.4
MCV (fL)	22.8–29.9
MCH (pg)	39.3–46.8
MCHC (g/dL)	10.1–12.7
nRBC/100 WBCs	0–2
WBC (× 10^3/μL)	8.0–21.4
Neutrophils (× 10^3/μL)	4.7–14.9
Bands (× 10^3/μL)	0–0.15
Lymphocytes (× 10^3/μL)	0.7–4.8
Monocytes (× 10^3/μL)	0–1
Eosinophils (× 10^3/μL)	0.6–4.9
Basophils (× 10^3/μL)	0–0.3
Platelets (× 10^3/μL)	
Total protein g/dL	5.1–7.9
Fibrinogen (mg/dL) by heat precipitation	100–500

point-of-care blood gas analyzer (Element POC, Heska Corp., Loveland, CO) with both a manual PCV method and automated hematology analyzer (ADVIA 120, Siemens Inc., Malvern, PA), in analyzing blood from 104 alpacas and 20 llamas, there were strong correlations between the point-of-care method and both of the two other methods, but constant and proportional biases were present for both comparisons.[35] This indicates reference intervals should be method-specific when optimized.

Reference intervals for adult, mixed-sex, alpacas and llamas for PT and PTT based on mechanical clot detection have been published, but were not generated or verified in accordance with American Society for Veterinary Clinical Pathologists (ASVCP) guidelines.[5,10,14,29] The reported PT reference intervals for llamas were all <13 seconds. There is more variation between the species in the reported PTT reference intervals: 8.8–16 seconds for llamas and 17–22.3, 12.3–21.8, and 8.85–15 seconds for alpacas, again without outlier removal and statistics in accordance with ASVCP guidelines.[5,10,14,29]

Partitioning Factors

Some of the more frequently reported age-related differences in camelids include lower PCV and Hgb concentration, and higher absolute lymphocyte and platelet numbers in juvenile llamas and guanacos compared to adults.[9,13,20] Juvenile alpacas have fewer absolute eosinophil numbers compared to adults in one report.[9]

There are few reported significant differences in camelid reference intervals between males and females. Packed cell volume is reported to be higher in adult male llamas than in adult female llamas.[20] Eosinophil numbers were reported to be significantly higher in females than males in a group of 29 10–18-month-old alpacas.[12] The median MPV of female alpacas is significantly lower than that of males.[5]

The reported effects of altitude on hematologic values in camelids are not clear. For example, Ellis reported a PCV interval of 33–42% for 40 alpacas at the La Raya, Peru, experimental station located at an altitude of 4200 meters, but Tornquist reported a mean PCV of 30.4% for 64 healthy llamas and 30.7% for 171 healthy alpacas at this same station.[7,33] Fowler reported a PCV interval for 116 alpacas at 3900 meters as 24–45%.[9] The PCV interval for 37 healthy Oregon alpacas living at an altitude of approximately 68 meters was 25–37% and for 29 alpacas in Australia (altitude not reported) was 30–42%.[12,33] Hydration status and centrifugation technique may also contribute to the reported differences.

Other environmental factors such as seasonality have not been well-documented. A study of apparently healthy free-ranging (n = 20) versus captive guanacos (n = 17) showed a higher total WBC count and higher PCV interval in the free-ranging population than in the captive population, but factors such as handling and stress could not be standardized.[15]

BONE MARROW COLLECTION AND EVALUATION

Bone marrow is most frequently obtained from the sternum or ribs of camelids with the animals standing or in left lateral recumbency. Intravenous xylazine is often used for sedation.[1,18] A good marrow sample may be obtained from the midline of the sternum at the level of the caudal aspect of the elbow when positioned naturally.[18] The site is aseptically prepared and an 11–16 gauge bone marrow biopsy needle is used to obtain samples. Bone marrow samples are most frequently aspirated into EDTA, but may be collected with no anticoagulant if bone marrow smears are prepared immediately. Slides are air-dried and stained with appropriate stains including Wright or Wright–Giemsa for general evaluation or with Perls iron stain to assess iron stores (see Chapter 141).

Cellularity of normal camelid bone marrow is reported to be similar to or somewhat increased in comparison with other mammals. In studies of seven llamas and 20 dromedary camels, cellularity of 50–85% was noted.[1,24] The morphology and maturation of erythroid cells in bone marrow was similar to that seen in other species in both llamas and camels. Erythroid cells started to change from round to elliptical as they became less polychromatophilic.[1] No dacryocytes, spindle-shaped RBCs, or uneven Hgb distribution were reported in bone marrow samples. No unique features of WBC morphology or maturation have been reported in camelid bone marrow samples. Percentages of bone marrow eosinophils are increased in comparison to other species, which parallels the higher percentages seen in blood.[1] Megakaryocyte numbers and morphology appear to be similar to those of other species. Stainable iron was present in moderate amounts in macrophages in the bone marrow of both llamas and dromedary camels.[1,24] The average M:E ratio in bone marrow of healthy camelids is similar to that of other species. It is reported to average 1.2 in dromedary camels,[24] 0.9–2.9 in llamas,[1,8] and 0.5–1.0 in alpacas.[18]

HEMATOLOGIC DISEASE

Anemia is common in sick camelids and is often hard to classify. The morphologic features of a regenerative response to anemia are less predictable in camelids than they are in many other species. Anisocytosis, polychromasia, reticulocytosis, and nucleated RBCs may be seen in response to anemia, but also may be present in nonanemic animals. In alpacas made acutely and severely anemic by phlebotomy, reticulocyte percentages increased, but only to a maximum of 1.5%, with the peak reticulocyte response occurring at a mean of 10.4 days after induction of anemia.[33] Other morphologic features of camelid RBCs that are occasionally observed in anemia include dacryocytes (tear-drop-shaped cells), spindle-shaped erythrocytes, and uneven

Hgb distribution in the RBCs.[21,22] Although these have been reported in association with iron deficiency anemia, they are seen in moderate to marked anemias of other etiologies in camelids. Anemia due to gastrointestinal and external parasites may be quite severe due to blood loss, inducing an associated iron deficiency.

Anemia of chronic disease is likely to be a factor in many sick camelids with no identifiable cause of anemia. As in other species, the anemia is most likely mild to moderate with little evidence of bone marrow response. The pathogenesis involves the influence of inflammatory mediators, including the acute phase protein hepcidin, on iron transport and erythropoietin responsiveness. In a study of 22 anemic and 22 nonanemic alpacas, no difference in serum hepcidin levels was found.[33]

Hemoparasites have been documented in camelids. *Candidatus Mycoplasma haemolamae*, a hemotropic mycoplasma, may be observed as small, round, or ring-shaped basophilic organisms on the RBC membrane, or in the background, if blood smears are not made promptly after blood collection.[19,27,31] While infection with this organism can certainly be associated with mild to marked anemia, there are many infected animals that are not anemic.[32] The prevalence of this infection in camelids in the USA is around 30% in both llamas and alpacas, with the majority of infections being subclinical.[33] Diagnosis by PCR is the most sensitive method, though some infected animals may have organism numbers that drop below the detection limit at times.[31] *Anaplasma phagocytophilum*, a tick-borne organism that infects granulocytes, has been reported rarely in alpacas, llamas, and one camel, and was associated with mild to moderate anemia.[17,30] In one of those cases, concurrent infection with *M. haemolamae* was present[17] (Figure 119.2f).

Other causes of anemia reported in other species may occur in camelids, but have not been well-documented. These include chronic renal failure, hypothyroidism, and copper deficiency. Hemolytic anemia with Heinz bodies has been reported in two alpacas ingesting red maple leaves.[6] A few reports of bone marrow neoplasia and myelodysplastic syndrome have been associated with severe, nonregenerative anemia in camelids.[3,23,28]

In addition to hemoconcentration due to dehydration as a common cause of relative polycythemia, there is evidence that camelids have a large splenic RBC reserve that may be released into circulation in response to excitement. Miller and Banchero showed that llamas given injections of epinephrine to simulate stress or excitement had increases in PCV of approximately 10% at 3 and 6 minutes postinjection. The PCV typically dropped to resting level in 20 minutes.[20]

Physiologic neutrophilia, a shift of marginated neutrophils to the circulating pool in response to catecholamine release during handling and blood sampling, may account for at least some of the discrepancies reported in N:L in healthy camelid.[7,8]

Hematologic responses to inflammation appear to be similar in camelids to those in other species. In studies where acute inflammation was induced with lipopolysaccharide or inoculation with *Streptococcus zooepidemicus*, neutrophilia and increased band numbers were noted, along with a decrease in lymphocyte counts and an increased N:L.[4,26] In the study using lipopolysaccharide

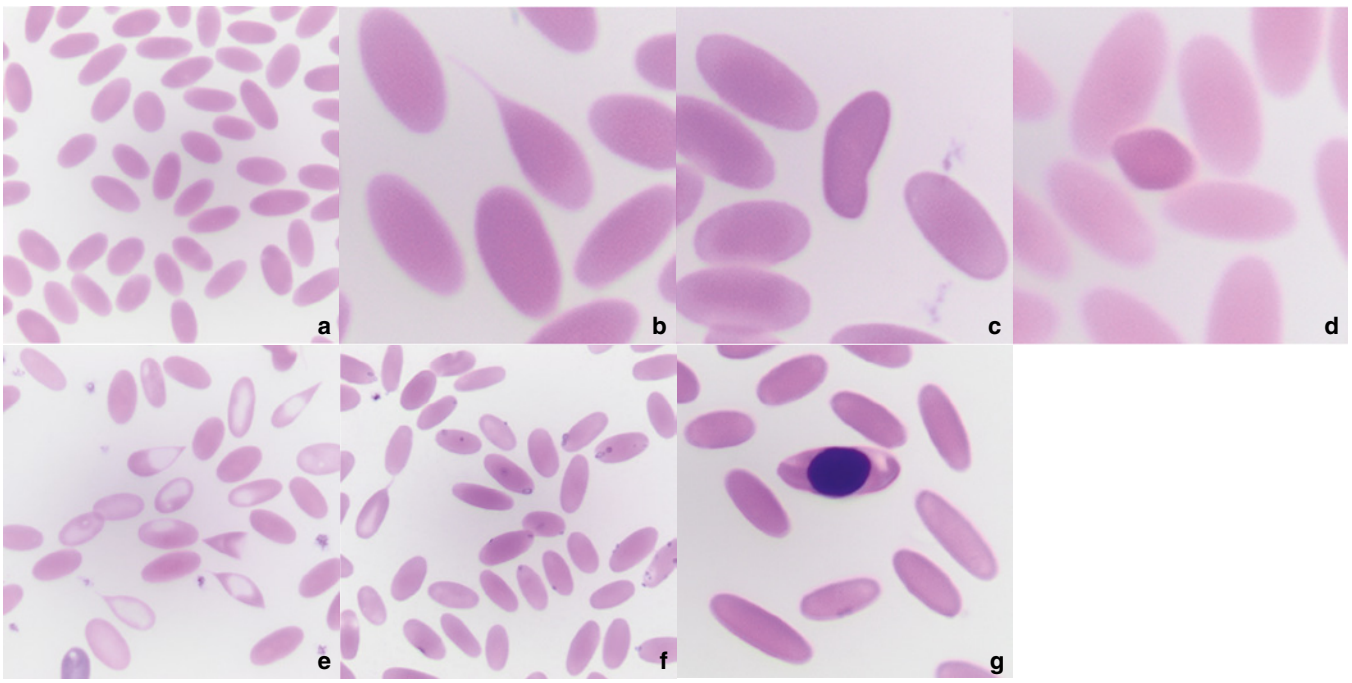


FIGURE 119.2 Normal and abnormal erythrocyte morphology of camelids. Normal: (a) Llama erythrocytes; (b) Alpaca dacryocyte; (c) Folded alpaca erythrocyte; (d) Hemoglobin crystal in a llama. Abnormal: (e) Erythrocyte dyscrasia in an alpaca; (f) *Mycoplasma haemolamae* in an alpaca; (g) Alpaca metarubricyte. Wright's stain, 1000× magnification.

to stimulate acute inflammation, a significantly increased N:L ratio was noted as early as 12 hours.[26] This was accompanied by serum iron levels that were significantly lower than baseline.

Very few cases of leukemia in camelids have been published. They include an acute myeloid leukemia in an alpaca and one case of lymphocytic leukemia.[3,28] A published case of myelodysplasia in an alpaca described no circulating blast cells or leukocytosis, but with atypical erythroid and myeloid cells in the bone marrow (<30% blasts).[23] Considering the lack of publication, it is likely that leukemia occurs uncommonly in these species.

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CHAPTER 120

Hematology of Cervids

BRIDGET C. GARNER

Erythrocytes
Leukocytes
Platelets
Reference Intervals
Hematologic Disease

Acronyms and Abbreviations

ASVCP, American Society for Veterinary Clinical Pathology; LGL, large granular lymphocyte.

The Cervidae family (order Artiodactyla) is a diverse group of animals that has adapted to tropical to arctic climates, and consists of elk, white-tailed deer, mule deer, brocket deer, reindeer, chital, and red deer, among others.[15] Factors including the animal's age, sex, reproductive status, and concurrent disease(s), as well as the methods of capture and blood collection used, can affect cervid hematologic parameters as is seen with other species. As a result, defining normal hematology for such a wide range of species and subspecies can be challenging, but there are noticeable trends.

ERYTHROCYTES

Cervid RBCs are round in vivo and similar in size to, or slightly smaller than, bovine red cells (Table 120.1). More specifically, reindeer RBCs have an average diameter of 5.5 µm, whereas discoid RBCs in white-tailed deer measure 3.5–4.5 µm in diameter.[32,25] Once the blood is collected, RBCs from many cervid species assume a sickle shape, which is described as crescent or holly leaf, and called "drepanocytes" (Figure 120.1a). Other morphology variants include the matchstick (Figure 120.1b) and burr cell.[24,26] The matchstick-appearing cells may transform after initially sickling.[25] The cells are not sickle-shaped in vivo, but change their shape if the blood is alkalinized, oxygenated, or left to stand at 4°C.[23,25,26,45] This peculiarity was first described by Gulliver in 1840, and since that time sickle cells have been observed in a variety of cervids such as barasingha (*Rucervus duvaucelii*), barking deer (*Muntiacus muntjak*), chital (*Axis axis*), fallow deer (*Dama dama*), hog deer (*Axis porcinus*), mule deer (*Odocoileus hemionus*), Père David's deer (*Elaphurus davidianus*), red deer (*Cervus elaphus*), Reeves's muntjac (*Muntiacus reevesi*), rusa deer (*Rusa timorensis russa*), sambar deer (*Rusa unicolor*), sika deer (*Cervus nippon*), and white-tailed deer (*Odocoileus virginianus*).[2,8,9,15–17,33,45] At pH 7.4, most RBCs are sickled, whereas at pH 7.0, few exhibit this shape; thus, the sickling can be reversed by acidifying the blood.[45]

Some Cervidae family members do not exhibit RBC sickling; the RBCs of European reindeer (*Rangifer tarandus*) known as caribou in North America, European elk (*Alces alces*) known as moose in North America, and some North American wapiti (*Cervus canadensis*) do not adopt the sickle shape when exposed to oxygen (Figure 120.1c).[17,45,46] A standard peripheral blood smear from a healthy reindeer shows discoid RBCs with mild anisocytosis, but polychromasia is rare. Central pallor, although present, is often indistinct, and rouleaux formation is uncommon.

Deer RBCs contain several morphologic forms of hemoglobin (Hgb), each of which has different electrophoretic properties, and is associated with a distinct morphologic red cell shape.[25] "Hemoglobin heterogeneity" is not unique to cervids, but the white-tailed deer greatly exceeds the low level of polymorphism reported in other species.[24] An individual deer may contain a

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TABLE 120.1 Hematologic values of cervids.

Animal (study location)	No., Sex[a] (Age)	RBC (× 10^6/μL)	Hgb (g/dL)	Hct or PCV (%)	MCV (fL)	MCHC (g/dL)	Platelet (× 10^3/μL)	WBC (× 10^3/μL)
Red deer[b,d,g] (Spain)[3012]	21 M (5–6.5 yr)	10.41 ± 0.07	16.8 ± 0.11	44.9 ± 0.028	41.2 ± 0.082	34.8 ± 0.21	448.26 ± 8.04	5.52 ± 0.44
Reindeer[e,f,g] (Norway)[4316]	23 F, 9 M (>1.5 y)	10.70 ± 0.17 (9.24–11.96)	17.3 ± 0.258 (14.5–21.1)	47 ± 1 (39–57)	44.4 ± 0.3 (40.3–48.0)	36.6 ± 0.2 (35.1–38.4)	253 ± 24 (74–603)	2.96 ± 0.19 (0.96–5.19)
Roe deer[c,e,g] (Sweden)[4277]	17 M, 18 F (20 >1 y; 15 0–1 y)	11.76 ± 0.14 (9.6–13.8)	18.59 ± 0.190 (15.0–20.4)	52 ± 0.5 (43–59)	44.9 ± 0.44 (39–50)	35.24 ± 0.139 (33.2–37.4)	332.63 ± 18.52 (166–602)	4.91 ± 0.23 (2.8–8.1)

Animal	No., Sex[a] (Age)	Seg neuts (× 10^3/μL)	Band neuts (× 10^3/μL)	Lymphocytes (× 10^3/μL)	Monocytes (× 10^3/μL)	Eosinophils (× 10^3/μL)	Basophil (× 10^3/μL)
	*–	*–	*–	*–	*–	*–	*–
Reindeer[e,f,g] (Norway)[4316]	23 F, 9 M (>1.5 y)	1.91 ± 0.18 (0.56–4.52)	–	0.90 ± 0.06 (0.37–1.69)	0.01 ± 0.01 (0.0–0.03)	0.0 ± 0.0 (0.0–0.01)	0.05 ± 0.01 (0.01–0.13)
Roe deer[c,e,g] (Sweden)[427]	17 M, 18 F (20 >1 y; 15 0–1 y)	2.38 ± 0.21 (0.7–5.6)	0.006 ± 0.004 (0–0.1)	2.40 ± 0.12 (1–4.1)	0.08 ± 0.01 (0–0.3)	0.04 ± 0.01 (0–0.2)	0.05 ± 0.01 (0–0.3)

[a] F, female; M, male; MF, males and females.
[b] Captive animals.
[c] Physically restrained animals.
[d] Serial sampling of the same animals.
[e] Wild animals.
[f] Chemically restrained animals.
[g] Results reported as mean ± standard error (range when available).

mixture of one to six of 21 structurally different Hgb molecules due to variability in the up to three alpha and up to seven beta chains, whereas most other species have less than three different Hgbs.[24,26]

The sickling effect noted in cervid RBCs is due to the formation of spindle-shaped bodies from variant forms of oxygenated Hgb.[25] This phenomenon is an interesting laboratory observation, but it does not appear to have a deleterious effect on the deer or lead to shortened RBC survival.[26,33] Hemoglobin types I and III are the most commonly identified Hgb types in white-tailed deer.[25] These are associated with RBC sickling, or the formation of the crescent or holly leaf shape.[24] Hemoglobin type II, alone or in combination with Hgb types I, III, or IVb, is often associated with the development of a matchstick shape.[24,25] Red cells that contain Hgb type IVa may assume a burr or thorn-apple shape.[24] Sickling does not occur if Hgb type V or VII is present.[24,25]

The average RBC life span varies among cervids. The circulating RBCs of five white-tailed deer persisted for 125 days, one North American wapiti for 149 days, and one sika deer for 159 days.[33] A direct relationship between RBC volume and body size and an inverse relationship between RBC volume and RBC count have been recognized.[18] Such guidelines may be useful for data interpretation when reference intervals are not available.

LEUKOCYTES

Many similarities in cervid WBC morphology are seen upon review of Romanowsky-stained blood smears (Figures 120.2–120.4). The most comprehensive description of cervid leukocyte cytochemical staining and ultrastructural properties has been performed for reindeer (Figures 120.5 and 120.6).[32,19]

Neutrophils, which comprise nearly half of all WBC in reindeer,[4,32] are typically 18–25 μm in diameter with pale eosinophilic cytoplasm.[19] Reindeer neutrophils may occasionally exhibit a low number of basophilic cytoplasmic granules (Figure 120.5). The nuclei resemble those of other species, but they may be more segmented with up to seven lobes.[19]

Lymphocyte diameter ranges from 15 to 35 μm, and these cells may be round, oval, or angular, with variable nuclear to cytoplasmic ratios.[19] The cytoplasm, which is usually lightly basophilic, may contain a few fine azurophilic granules. This cellular morphology is consistent with that of large granular lymphocytes (LGLs). Neutrophil to lymphocyte ratios are typically less than, or equal to one,[1,18,44] but some studies indicate that neutrophils are the most abundant leukocytes in cervids (Table 120.1).[32,43,48]

Monocytes have reniform to oval nuclei with light, blue-gray cytoplasm on peripheral blood smears. Occasionally, there are clear cytoplasmic vacuoles and a small quantity of red-to-pink cytoplasmic granulation.[19]

Eosinophils are slightly larger than neutrophils at 25–30 μm in diameter. The red-orange cytoplasmic granules are typically small and round, with an electron-dense appearance via electron microscopy.[19] The granules frequently group and form aggregates on one or both sides of the nucleus.[19]

Two distinct basophil populations may be seen in some cervids, reindeer in particular (Figure 120.7).[19] The

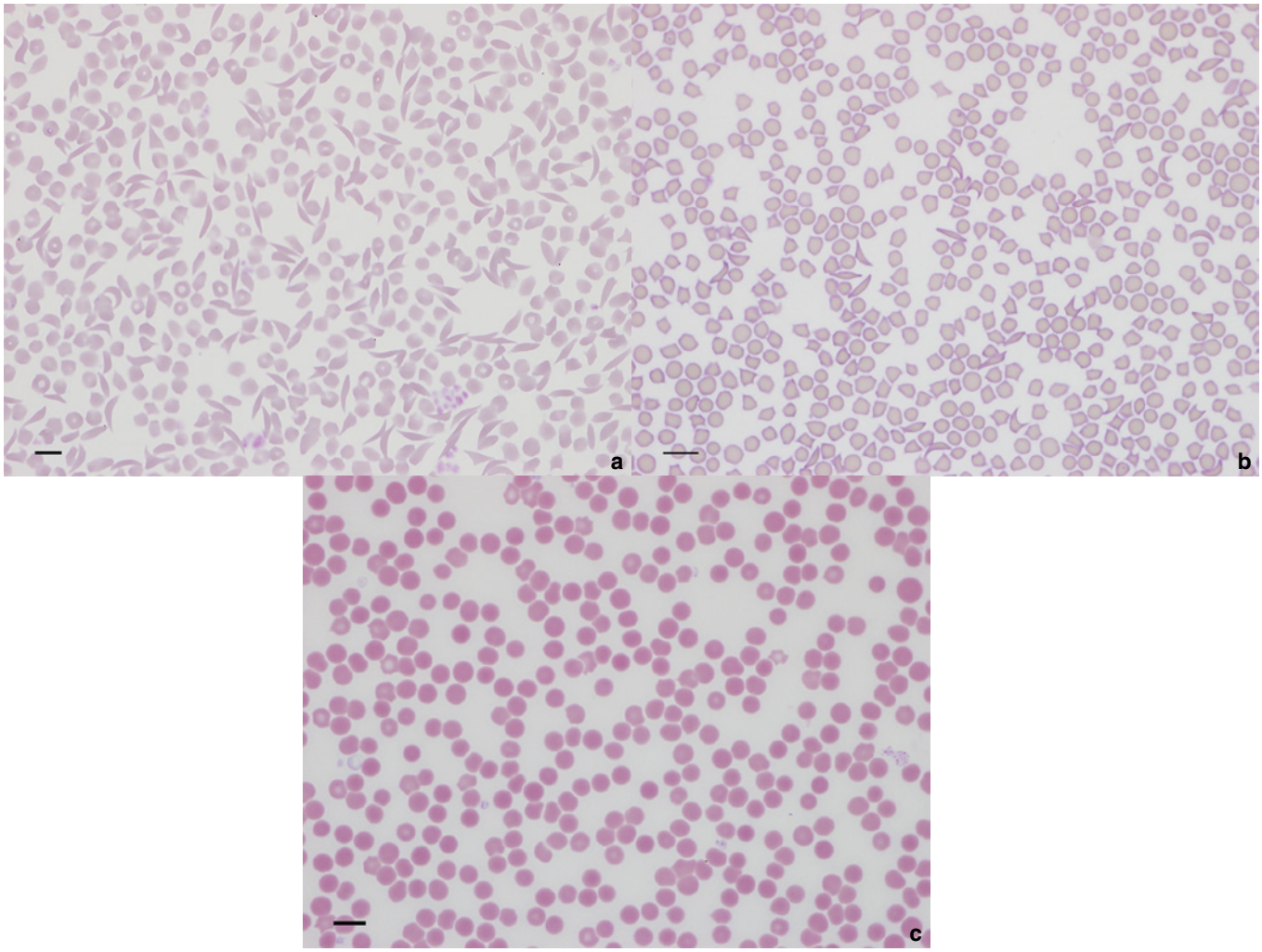


FIGURE 120.1 Normal cervid erythrocytes. Modified Wright stain. Scale bar = 10 µm. (a) Deer, RBCs with normal sickled appearance. Platelets are small and moderately granulated; (b) Deer, RBCs with sickled and matchstick appearances; (c) Reindeer, non-sickled RBCs. Platelets are small and moderately granulated.

first type has the typical appearance noted within most other mammals. These basophils have numerous, pale to dark basophilic granules disseminated throughout the cytoplasm. The second population is cytologically similar to "gray" basophils seen in limited other species. These cells have variable numbers of clear cytoplasmic granules (or vacuoles) that are less than 1.0 µm in diameter interspersed with variable numbers of intensely basophilic, perinuclear granules of similar size. The perinuclear granules may obscure a portion of the nucleus and make it appear ragged or deformed. Both basophil populations can be identified within the same blood smear, and the relative proportions of each can vary between individuals.[19]

PLATELETS

Cervid platelets, including reindeer, are relatively small (Figure 120.1), but the reference intervals for platelet counts are wide with much variability between studies (Table 120.1).[18,30,40] Platelet morphology has not been studied extensively in cervids.

REFERENCE INTERVALS

Complete blood count data are depicted in Table 120.1 that generally followed American Society for Veterinary Clinical Pathology (ASVCP) guidelines for reference interval generation including outlier removal and appropriate statistics. Medical management of cervids should employ species-specific and likely method-specific reference intervals with appropriate outlier removal and statistics. Given the effects of age, sex, reproductive status, season, and the manner in which the blood is collected, there are many variations of reported reference intervals for cervids.

Age has several effects on hematologic values in cervids. There is a direct correlation between age and RBC values. Newborn chital deer have lower RBC counts than juveniles and adults, and newborn reindeer have

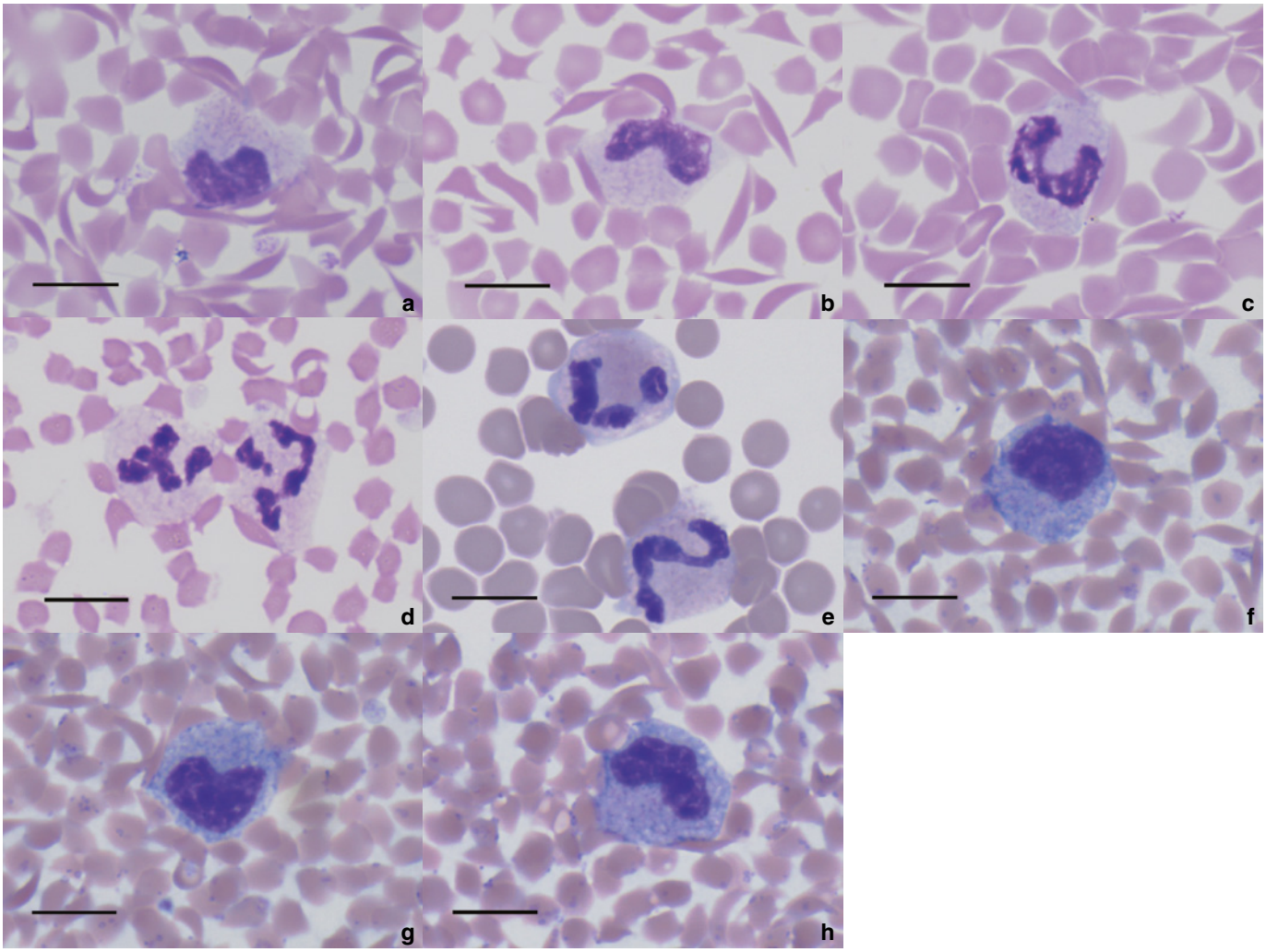


FIGURE 120.2 Normal and toxic circulating cervid neutrophils and neutrophil precursors. Modified Wright stain. Scale bar = 10 µm. (a) Deer, metamyelocyte neutrophil; (b) Deer, early band neutrophil; (c) Deer, late band neutrophil; (d) Deer, segmented neutrophils; (e) Wapiti (elk), segmented neutrophils; (f) Deer, toxic myelocyte neutrophil and *Mycoplasma* infection; (g) Deer, toxic metamyelocyte neutrophil and *Mycoplasma* infection; (h) Deer, toxic band neutrophil and *Mycoplasma* infection.

RBC counts, PCV, Hgb, and mean cell hemoglobin concentration (MCHC).[4,5,43] As white-tailed deer fawns age, their energy requirements increase, as do their RBC counts and their Hgb concentration.[5,36] In chital deer, RBC counts and Hct increase by 40–60% from birth to 10 months of age.[6] Similar findings have been reported for captive reindeer and white-tailed deer.[4,36] In contrast, reticulocyte and platelet counts are higher in newborn reindeer.[4] Reindeer hematologic values stabilize at approximately 5 months of age; therefore, adult reference intervals may be useful when interpreting data from animals 5 months or older if juvenile reference intervals are not available.[32,43]

Anisocytosis, poikilocytosis, basophilic stippling and sickling vary between young animals and adults. Marked anisocytosis and poikilocytosis are found in blood smears from healthy newborn reindeer, but only mild anisocytosis is expected in normal adults. Basophilic stippling and a small number of nucleated RBCs may also be identified in the neonates.[4] Neonatal and juvenile deer RBCs are unlikely to sickle due to the predominance of fetal Hgb (approximately 95% of total Hgb at birth).[22,25] Over time, fetal Hgb is replaced by adult Hgb, and sickling is expected to appear by 8–12 weeks of age if the appropriate adult Hgb forms are present.

There is an unclear connection between age and WBC parameters in cervids, which may be due to species, variation in the measurands, or reference interval generation. Some studies have established a correlation between age and WBC counts, but others have found conflicting results or no correlation at all.[34,36,44] One study that identified an association between age and WBC count determined that neonatal chital deer had significantly greater WBC counts than juveniles or adults.[6] In another study, white-tailed deer WBC counts were highest at 1 month of age.[21]

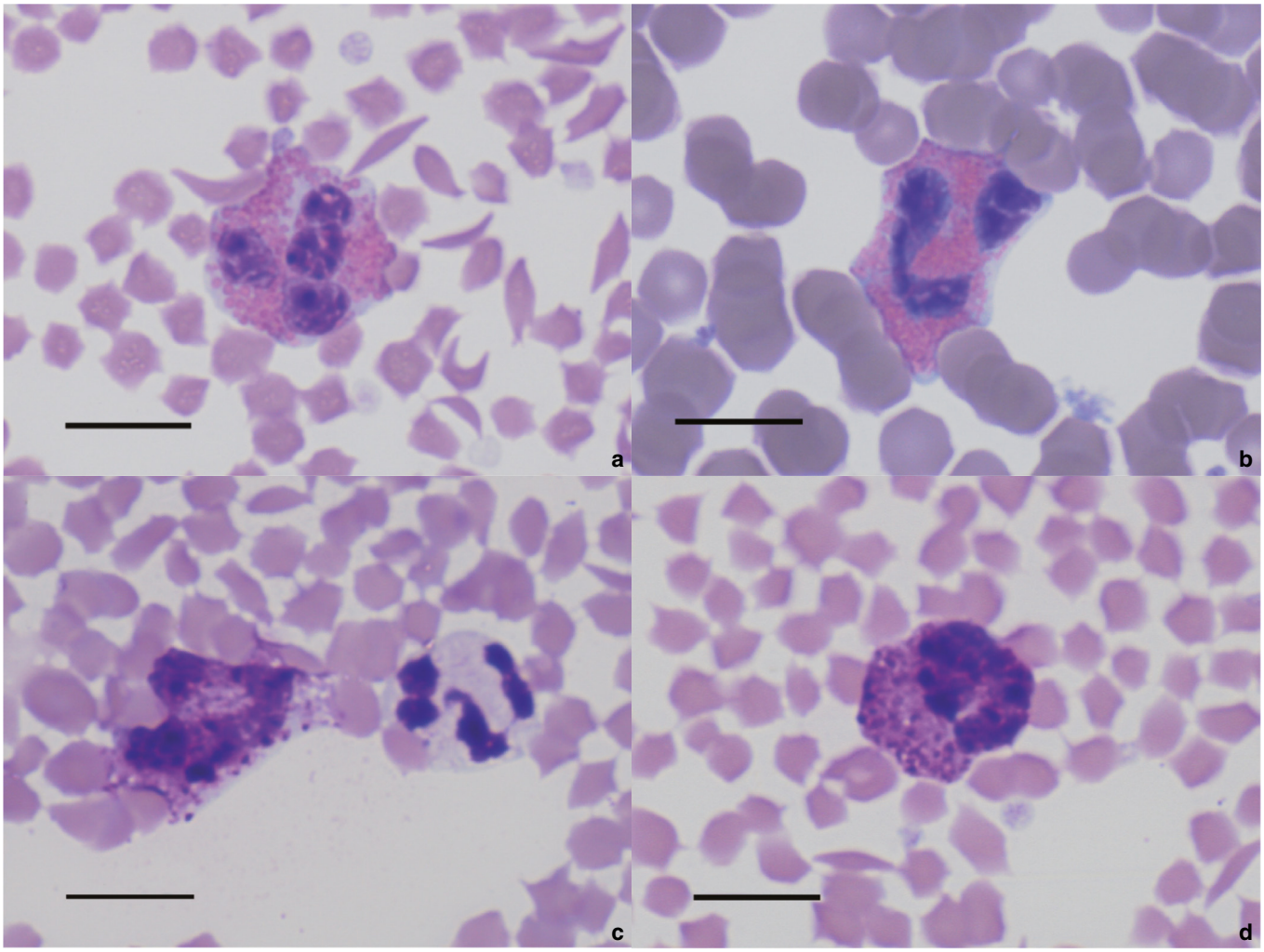


FIGURE 120.3 Normal circulating cervid eosinophils and basophils. Modified Wright stain. Scale bar = 10 µm. (a) Deer, eosinophil; (b) Wapiti (elk), eosinophil; (c) Deer, basophil (left) and segmented neutrophil (right); (d) Deer, basophil.

Meanwhile, WBC counts were typically lower in newborn than juvenile reindeer.[4] The total WBC count is not the only discrepancy found between age groups. In adult chital deer, lymphocytes predominate, but an increased number of neutrophils and a neutrophil:lymphocyte ratio of 2:1 were found in neonates.[6] In rusa deer <90 days old, the neutrophil:lymphocyte ratio was also greater than one, but in older deer, the ratio was less than one.[44]

Sex-related changes in hematologic patterns are most evident in adult cervids.[6] Significant differences between newborn and juvenile male and female reindeer are not typically found, since sex-related variations in hematologic parameters are more significant with sexual maturity and adulthood.[4] RBC count, Hgb concentration, and Hct as well as total WBC count were higher in chital stags than in chital hinds.[5] Similar patterns were noted in male and female rusa deer.[2] However, this observation does not appear to be absolute, since studies did not observe these RBC or WBC variations in mule deer or red deer.[1,30] Adult female reindeer living in zoological parks had higher Hgb, PCV, mean corpuscular volume (MCV), and neutrophil and lymphocyte counts than adult males.[43]

Changes in the erythron due to reproductive status are reported in both male and female cervids. As mating season approaches, RBC, Hct, and Hgb increase to maximum values in male red deer (*C. elaphus*), and decrease thereafter.[12] In female tule elk, pregnancy results in increases in Hgb, RBC count, Hct, and MCV.[40] In female reindeer, PCV, MCV, and Hgb values are higher (and may peak) during the autumn months and then decrease with parturition and the early lactation period.[43] In particular, the Hgb concentration of pregnant reindeer decreases by approximately 3 g/dL or more leading up to and following parturition.[43] The decreases in RBC count, Hgb concentration, and PCV seen in female reindeer in the early stages of lactation were attributed, at least partially, to iron deficiency.[32]

An animal's reproductive status can also affect the WBC and platelet concentrations. During the annual

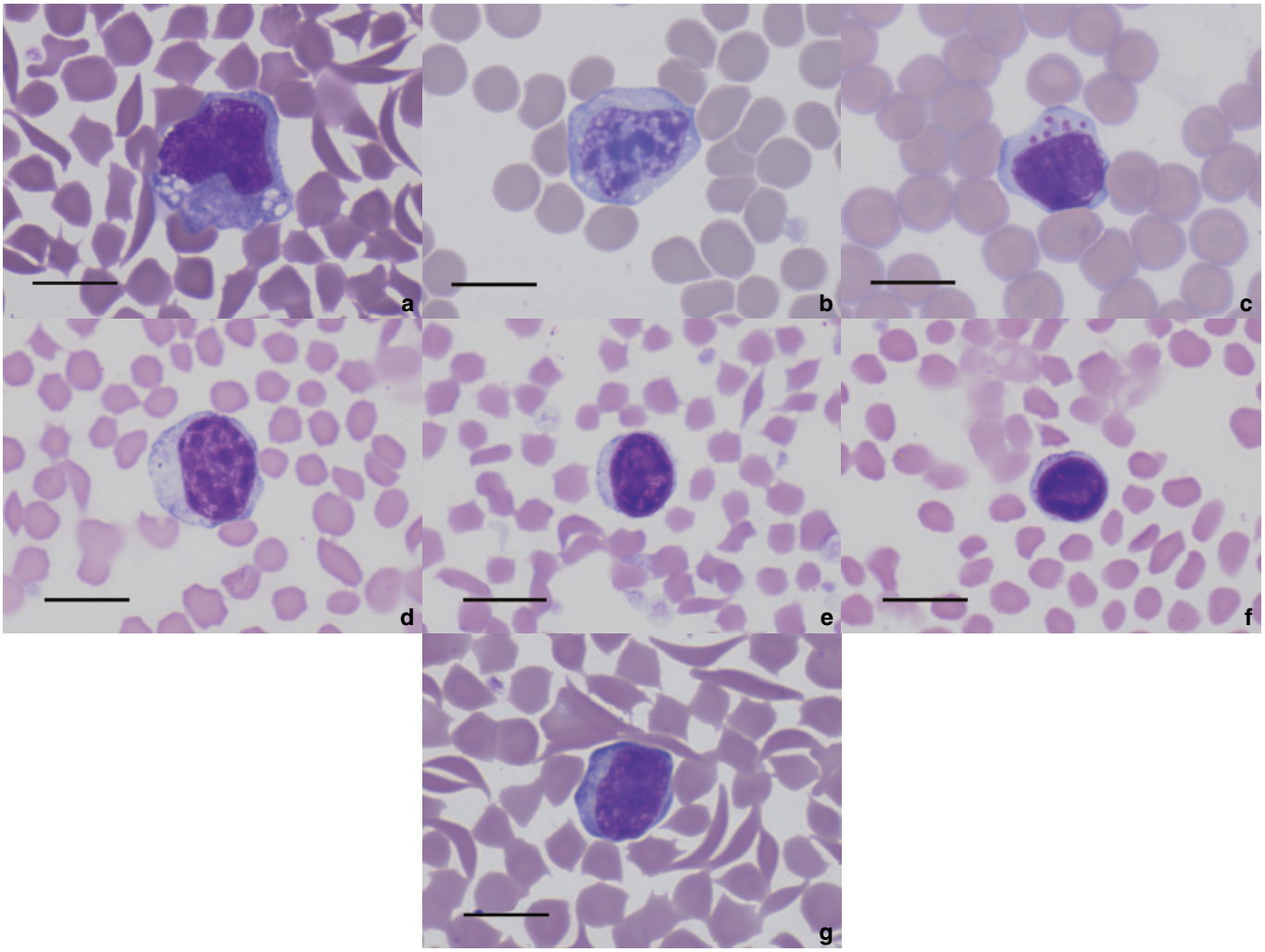


FIGURE 120.4 Normal circulating cervid monocytes and lymphocytes. Modified Wright stain. Scale bar = 10 μm. (a) Deer, monocyte; (b) Wapiti (elk), monocyte; (c) Wapiti (elk), large granular lymphocyte; (d) Deer, large lymphocyte; (e) Deer, medium-sized lymphocyte; (f) Deer, small lymphocyte; (g) Deer, reactive lymphocyte with basophilic cytoplasm.

rutting period, chital deer stags had altered differential leukocyte counts. The WBC count increased in pregnant tule elk due to increased lymphocyte numbers.[40] The mean platelet count in red deer (*C. elaphus*) peaked twice throughout the year.[12] The most significant peak (reaching a mean platelet count of 607.94 ± 54.18 × 10^9/L) occurred in October, during mating season, whereas a smaller peak occurred in the spring.[12]

Cervids are easily excitable, and this is demonstrated by the hematology data. As a result, caution should be used when applying captive animal reference intervals to free-ranging populations and vice versa.[34] Red and white cell values are significantly higher in nonanesthetized deer than in chemically restrained animals.[42] A greater stress response is evidenced by increased plasma cortisol and noradrenaline concentrations. Subsequent splenic contraction contributes to the changes in the awake, physically restrained cervids, but drug-specific effects may be present in sedated animals.[30,28,38,42] A stress response to various handling conditions is repeatedly demonstrated in reindeer.[37,38] Reindeer stressed during venipuncture demonstrate increased WBC counts and decreased lymphocyte numbers, as well as moderate increases in PCV, Hgb, and RBC count.[37]

Changes in the erythron and leukon due to handling and restraint are not consistent across species and sex. Chital does had significantly greater increases in PCV and RBC and WBC counts than chital bucks when both groups were physically restrained for blood collection.[6] Nonanesthetized male marsh deer had higher PCV than females.[42] In contrast, little or no difference in WBC count was seen between excited and resting male sika deer or between farmed male and wild female sika deer.[28]

Intermittent handling of cervids may decrease their stress and subsequently decrease stress-induced hematologic changes. In chital hinds, Hgb, RBC count, and Hct values rose 10–20% during early restraint periods, but declining values suggested that hinds adapted to

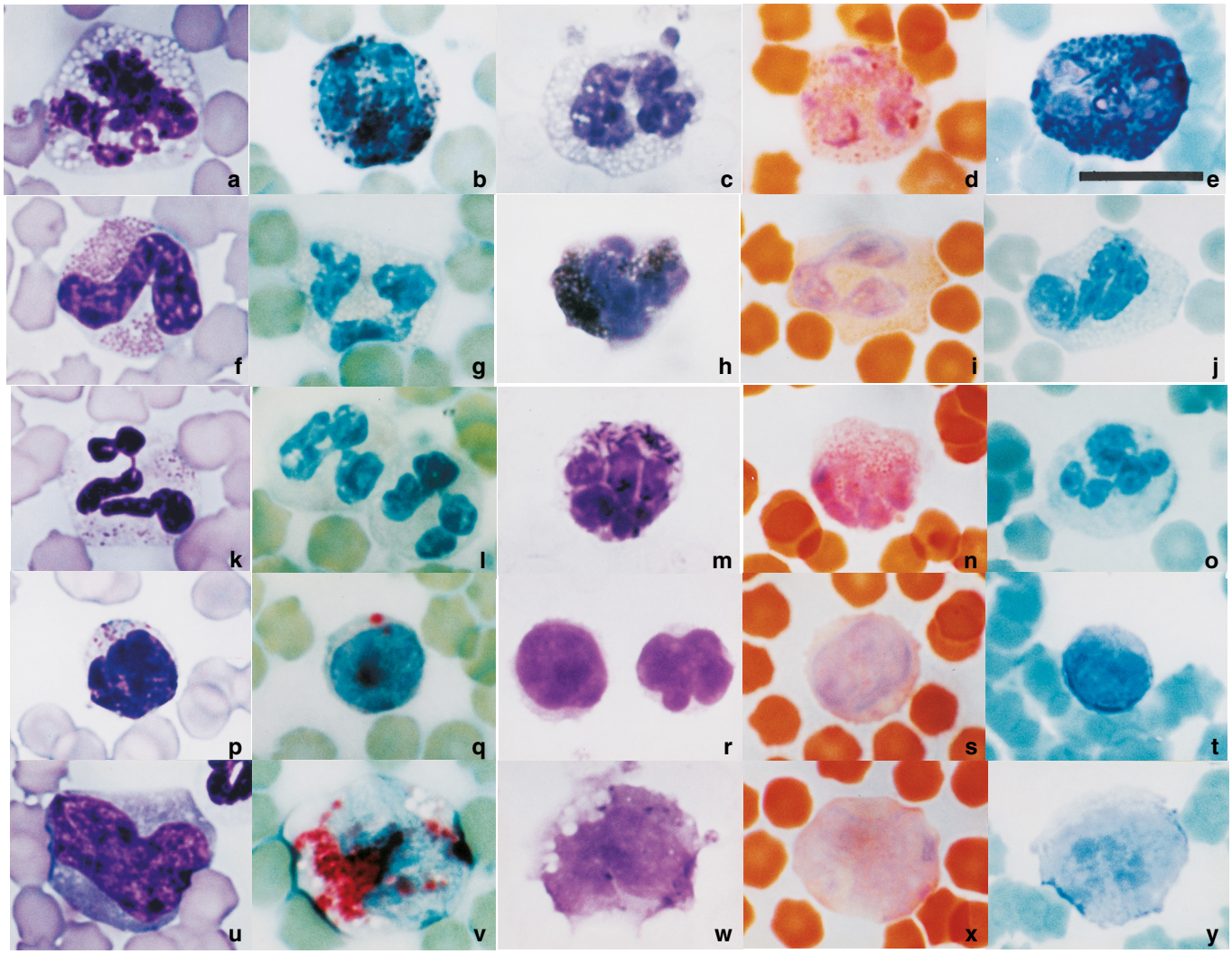


FIGURE 120.5 Cytochemical staining of reindeer leukocytes. Scale bar = 10 μm. (a) Gray basophil, Romanowsky's stain; (b) Basophil, alpha-naphthyl butyrate esterase (α-NBE); (c) Basophil, Sudan black B (SBB); (d) Basophil, chloroacetate esterase (CAE); (e) Basophil, alkaline phosphatase (ALP); (f) Eosinophil, Romanowsky's stain; (g) Eosinophil, α-NBE; (h) Eosinophil, SBB; (i) Eosinophil, CAE; (j) Eosinophil, ALP; (k) Neutrophil, Romanowsky's stain; (l) Neutrophil, α-NBE; (m) Neutrophil, SBB; (n) Neutrophil, CAE; (o) Neutrophil, ALP; (p) Lymphocyte, Romanowsky's stain; (q) Lymphocyte, α-NBE; (r) Lymphocyte, SBB; (s) Lymphocyte, CAE; (t) Lymphocyte, ALP; (u) Monocyte, Romanowsky's stain; (v) Monocyte, α-NBE; (w) Monocyte, SBB; (x) Monocyte, CAE; (y) Monocyte, ALP. (**Source:** Reproduced from Henkel et al.[19] with permission.)

repeated handling within 4 weeks.[5] Stags did not show these distinct and sequential erythron changes with handling, although the relative percentages of WBCs changed after the initial period.[6] The annual male reproductive cycle may have confounded hematologic alterations associated with adaptation to handling stress.[6]

HEMATOLOGIC DISEASE

Infections from epizootic hemorrhagic disease virus, bluetongue virus, and *Yersinia pseudotuberculosis*, among others, contribute to hematologic changes in cervids.[7,20,35] Lymphopenia is commonly identified, and it may be an indicator of decreased survival.[7,35] Acute bacterial infections or other inflammatory conditions contribute to neutrophilia in reindeer similarly to that seen in other species.[4] Two North American elk infected with *Babesia odocoilei* (Figure 120.8a) presented with a normocytic normochromic nonregenerative anemia, but the leukogram abnormalities differed between animals.[10]

Multiple hemoparasites are reported in cervids, but direct connections between these infections and clinical signs are rarely made. A *Mycoplasma ovis*-like organism was initially recognized in a farm-raised white-tailed deer fawn due to the presence of many coccoid, bacillary, and ring-shaped organisms on the blood smear

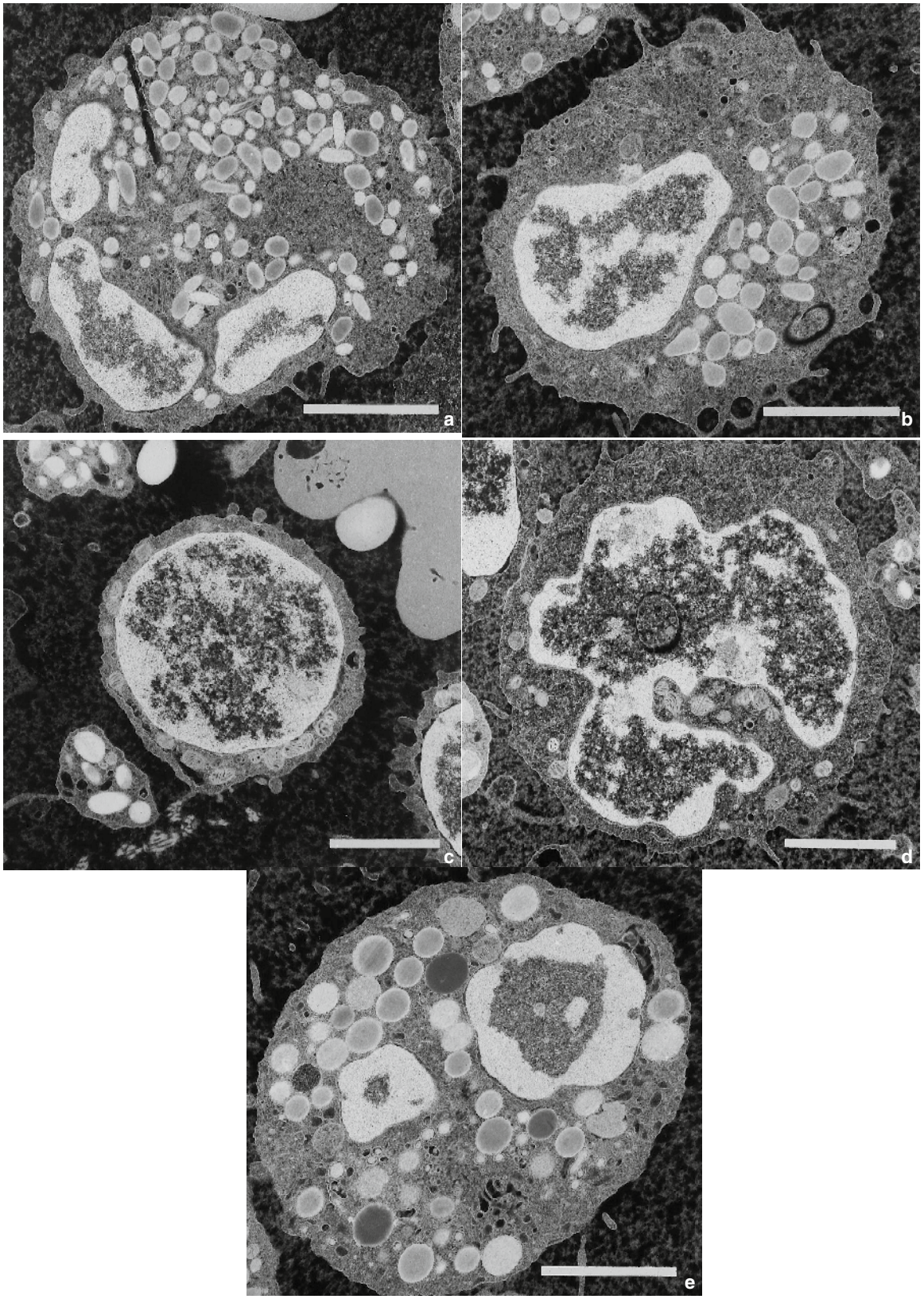


FIGURE 120.6 Electron micrograph, reindeer leukocytes. Scale bar = 2 μm. Left to right top row: (a) Neutrophil; (b) Lymphocyte. Second row left to right: (c) Eosinophil; (d) Monocyte. Third row: (e) Basophil. (**Source:** Reproduced from Henkel et al.[19] with permission.)

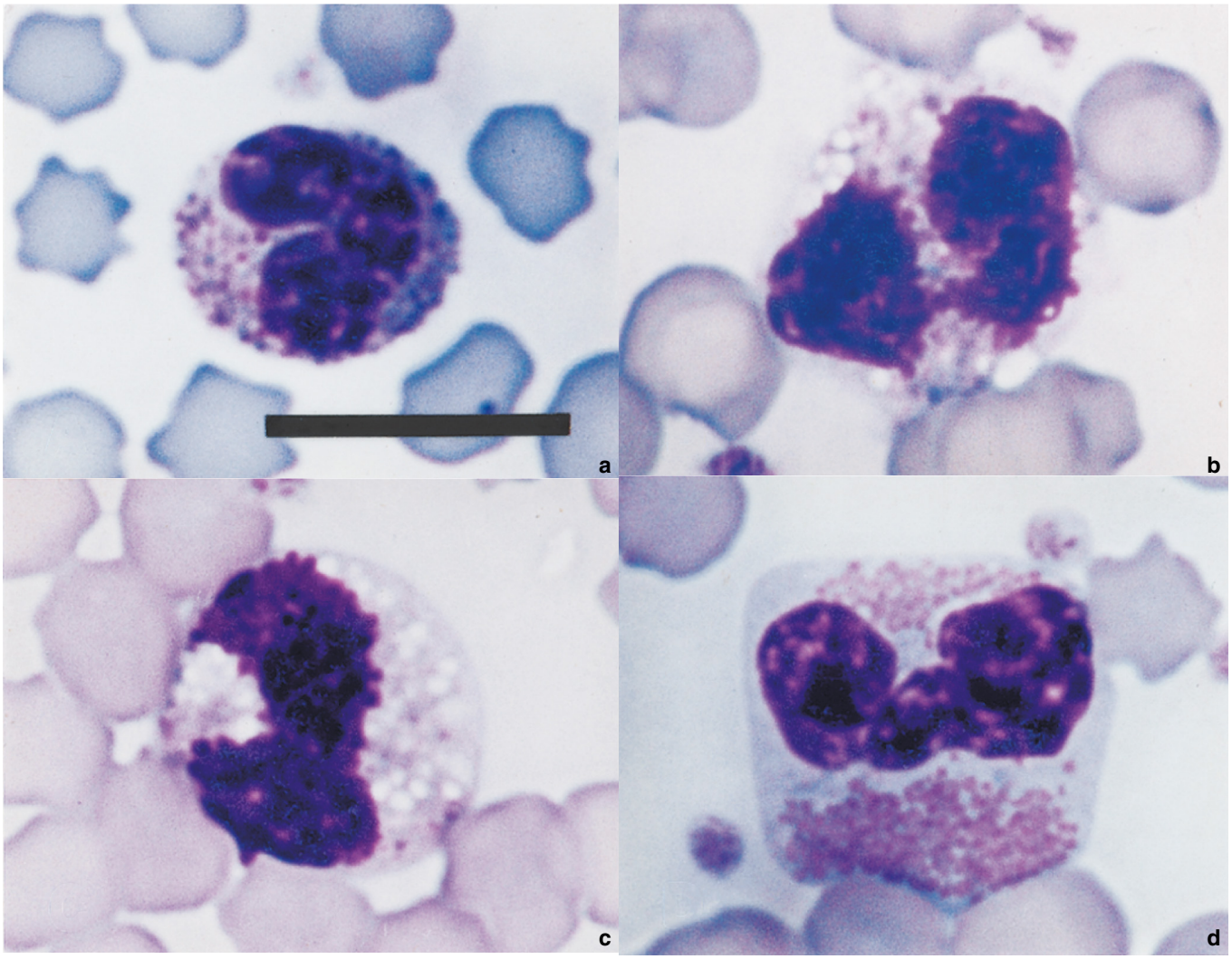


FIGURE 120.7 Reindeer blood. Romanowsky's stain. Scale bar = 10 µm. Comparison of a (a) dark basophil with (b) and (c) gray basophils and (d) an eosinophil. The dark basophil granules are similar to basophil granules in other ruminants. (**Source:** Reproduced from Henkel et al.[19] with permission.)

(Figure 120.8b).[3] Concurrent respiratory and gastrointestinal diseases were confounding factors in the fawn, but multiple affected females from the herd were subclinical.

DNA screening of free-ranging white-tailed deer showed a high prevalence of *Mycoplasma sp*. infections comprised of multiple species.[29] Clinical abnormalities were not detected during PCV measurement or on postmortem analysis. Free-ranging and captive Brazilian deer also had a high prevalence of hemoplasma infections that contained multiple genetically distinct species, but the pathogenic potential was not elucidated.[13,14] A hemomycoplasma species was identified in a herd of reindeer.[41] These animals were often anemic (characterized by either microcytosis or macrocytosis), with abundant poikilocytosis and visible organisms on the blood smear. Concurrent nematode infection may or may not have contributed to the anemia and RBC morphology changes.

Theileriosis was confirmed in a farmed reindeer with anorexia and weight loss.[11] Ring-shaped organisms were found on blood smear review, and PCR confirmed the organism was closely related to, albeit distinct from, *Theileria cervi* (Figure 120.8c). Both *Trypanosoma cervi* (Figure 120.8d) and *Theileria cervi* were seen on a blood smear from a white-tailed deer fawn.[47] Lesions associated with systemic trypanosomiasis and theileriosis were not found on necropsy.

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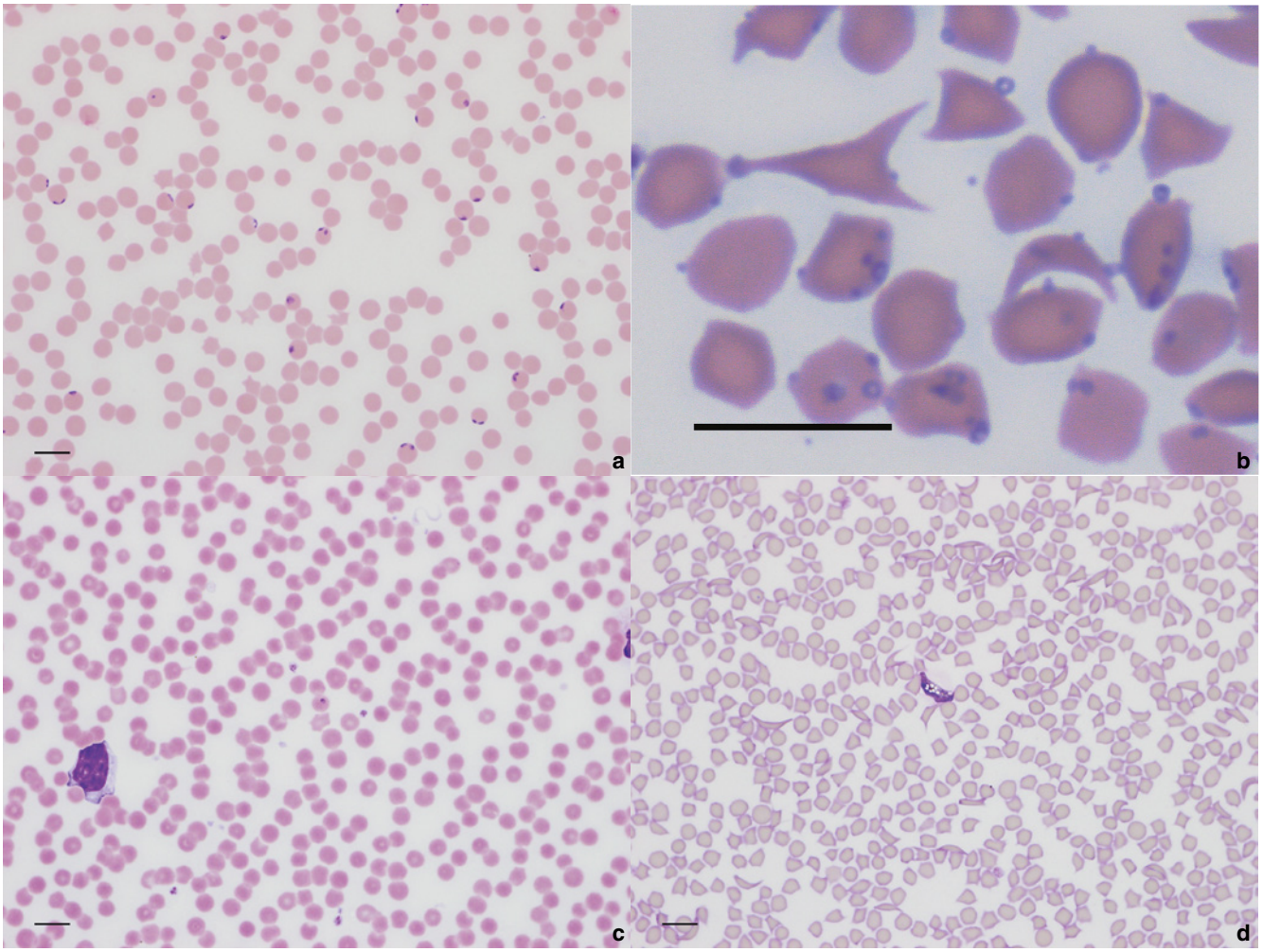


FIGURE 120.8 Hemoparasites in cervid blood. Modified Wright stain. Scale bar = 10 µm. (a) Reindeer, *Babesia odocoilei*; (b) Deer, *Mycoplasma ovis*-like organisms; (c) Reindeer, Theileriosis; (d) Deer, *Trypanosoma cervi*.

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CHAPTER 121

Hematology of Paenungulata: Elephants, Sirenians, and Hyraxes

EMMA H. HOOIJBERG

Elephantidae
- Erythron
- Leukon
- Platelets and Coagulation
- Reference Intervals
  - Partitioning Factors
- Blood and Bone Marrow Collection
- Hematologic Disease

Sirenia
- Erythron
- Leukon
- Platelets and Coagulation
- Reference Intervals
- Blood and Bone Marrow Collection
- Hematologic Disease

Hyracoidea
- Erythron
- Leukon
- Hematologic Disease

Acronyms and Abbreviations

ANAE, alpha-naphthyl acetate esterase; ANBE, alpha-naphthyl butyrate esterase; APTT, activated partial thromboplastin time; ASVCP, American Society for Veterinary Clinical Pathology; AT, antithrombin; CAE, chloroacetate esterase; CSS, cold stress syndrome; DIC, disseminated intravascular coagulation; EEHV, elephant endotheliotropic herpesvirus; HAB, harmful algal bloom; LAP, leukocyte alkaline phosphatase; MA, maximum amplitude; PAS, periodic acid–Schiff; PT, prothrombin time; SBB, Sudan Black B; SAA, serum amyloid A; TEG, thromboelastography.

The Paenungulata clade contains the extant mammal orders of Sirenia (manatees and dugongs), Hyracoidea (hyraxes), and Proboscidea (containing the family Elephantidae, or elephants). This clade is distantly related to the horse. As well as sharing genomic and morphological similarities[52], some members of this clade display similar and unusual hematologic characteristics: very large RBCs, the presence of heterophils instead of neutrophils, and the existence of a unique bilobed monocyte.

ELEPHANTIDAE

The Asian elephant, *Elephas maximus*, is found naturally in south-east Asia and the Indian subcontinent. Asian elephants do occur in the wild, but have also been commonly used for work and ceremonial purposes. The African elephant, *Loxodonta africana*, does not have a close history of association with humans.

Erythron

With recently reported mean cell volume (MCV) ranging from 104–138 fL, and an average cell diameter of 9 µm, the elephant RBC is the largest among terrestrial mammals.[1,25,30,51,65] The hematocrit (Hct) or PCV ranges from 27% to 47%, with an average of 35%.[1,25,30] Because of the large RBC size, the RBC count tends to be lower than in other mammals, with reported ranges of 1.4–6.6 × 10^{12}/L.[1,25,30,65] RBC, PCV, hemoglobin concentration (Hgb), and RBC indices appear to be fairly similar when comparing African and Asian elephant data, taking different analytical methodologies and reference populations into account.

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Anisocytosis is mild and occasional schistocytes and acanthocytes may be present. Reticulocytes and nucleated red blood cells (nRBCs) are generally absent from the blood of healthy elephants.[1,30,12] Elephant RBCs exhibit strong rouleaux formation, similar to that seen in horses, and the RBC sedimentation rate is high.[51,53] Both Asian and African elephant RBCs display greater osmotic resistance compared to those of humans and domestic animals, probably due to the high surface area to volume ratio of the elephant RBC.[55,65]

Leukon

Leukocytes have similar morphology and cytochemical staining characteristics in both elephant species (Figure 121.1). A unique feature of the elephant leukon is the bilobed monocyte, which has been described in both African and Asian species. In addition, like the rest of the Paenungulata clade, the elephant "neutrophil" contains many pink cytoplasmic granules, and has inconsistently been called a heterophil by some authors and will be referred to as such here.[25,53]

Elephant heterophils are 12–16 μm in diameter, with many fine, round to oval, variably sized reddish granules and a nucleus containing three to five lobes.[25,30,51] The heterophil of the Asian elephant shows variably positive staining for peroxidase, strong positivity for Sudan Black B (SBB), moderate positivity for leukocyte alkaline phosphatase (LAP) and chloroacetate esterase (CAE), and negative staining for alpha-naphthyl acetate esterase (ANAE), alpha-naphthyl butyrate esterase (ANBE), and periodic acid–Schiff (PAS).[25,51] When viewed with electron microscopy, these cells are round with short microvilli and small pores, and contain larger electrolucent primary granules and smaller secondary granules, which can be either round or oblong.[25,30]

Lymphocytes have a similar appearance to those of domestic livestock, and small, medium, and large lymphocytes are seen on blood smears, with the latter two being most common. The nucleocytoplasmic ratio is high, with a round to oval to slightly indented nucleus and a thin rim of pale blue cytoplasm.[25] Purple granules may rarely be present. Elephant lymphocytes stain variably positive with ANAE and ANBE and negative with SBB, peroxidase, CAE, and LAP.[1,25,51]

Monocytes in both elephant species have variable morphologies, ranging from a large cell similar to that seen typically in domestic animals with a polymorphic nucleus and vacuolated blue cytoplasm, to a bilobed cell, to a monocyte with a round to oval nucleus. The bilobed monocyte measures 16–18 μm in diameter, with a pale blue, occasionally vacuolated cytoplasm. The nucleus is horseshoe-shaped with two lobes; trilobed forms have been described.[25,51,53] Monocytes may also display a round to oval nucleus when spread out on a smear, and these forms have been confused with lymphocytes. Discriminating features include the presence of cytoplasmic vacuoles and a coarser, more clumped chromatin pattern in the monocyte.[25] Both of these unusual leukocytes have been conclusively shown to be monocytes in both African and Asian elephants, using cytochemical staining and electron microscopy. They stain weakly or focally positive for peroxidase, indicating a phagocytic function.[1,25,53] Further cytochemical staining characteristics include variable positivity for ANAE, SBB, and CAE and negativity for LAP and ANBE.[1,25] When viewed with the electron microscope, both bilobed and round nucleus monocytes contain a low number of small round variable electron dense granules (100–300 nm in diameter), numerous mitochondria, and variable numbers of tubulovesicular structures.[25]

Eosinophils are slightly larger than heterophils with a 14–18 μm diameter and have a pale blue cytoplasm filled with small but distinct, round, red-orange, refractile granules. Nuclear shape ranges from band to bilobed to trilobed, with coarsely clumped chromatin.[25,51] They stain strongly positive for peroxidase, ANBE, and ANAE and moderately positive for SBB; while being negative for alkaline phosphatase and CAE.[25,51] Ultrastructurally, granules are round to oval to rod-shaped.[25,51]

Basophils have a band-shaped to bilobed nucleus and contain numerous small lavender to magenta granules. They stain moderately positive with ANAE and negative with SBB and peroxidase.[25,51]

Disparities in elephant differential cell counts are present in the literature. Old publications tend to count the bilobed monocytes as lymphocytes, or as a separate population of cells, resulting in highly variable results.[1,16,45,65] Even when reviewing only studies from the last 20 years, where the bilobed monocyte has been counted as a monocyte, or as a separate population, there are discrepancies. This is partially due to the difficulty in differentiating reactive monocytes with round to oval nuclei from lymphocytes; however, the correct identification of these cells with round nuclei as monocytes has been confirmed with special stains. For example, in one study the lymphocyte (all cells with round nuclei, likely incorrectly categorized) was found to be the dominant cell type in both captive (not immobilized) and free-ranging (immobilized) Asian elephants from Sri Lanka, making up an average of 40–44% of the differential, with heterophils and monocytes (including bilobed cells) each around 25–30%. Percentages of eosinophils were roughly 5% and basophils were generally less than 1%.[53,54] This is different to results from a small group of captive elephants in Thailand, where bilobed monocytes (counted separately) had a mean differential percentage of 37%, with lymphocytes at 27%, heterophils at 29%, and "normal" monocytes at 1%.[51] This is in turn completely different to another recent study from Thailand in which hematology data from 149 male and female Asian elephants in tourist camps in Thailand were analyzed; bilobed monocytes were not identified at all.[30] Samples were measured on an automated hematology analyzer with manual differential counting; the authors attempted to count bilobed monocytes as a separate population but did not find any. (These data are not presented in this chapter.) Leukocyte counts using cytochemical staining on blood smears from a

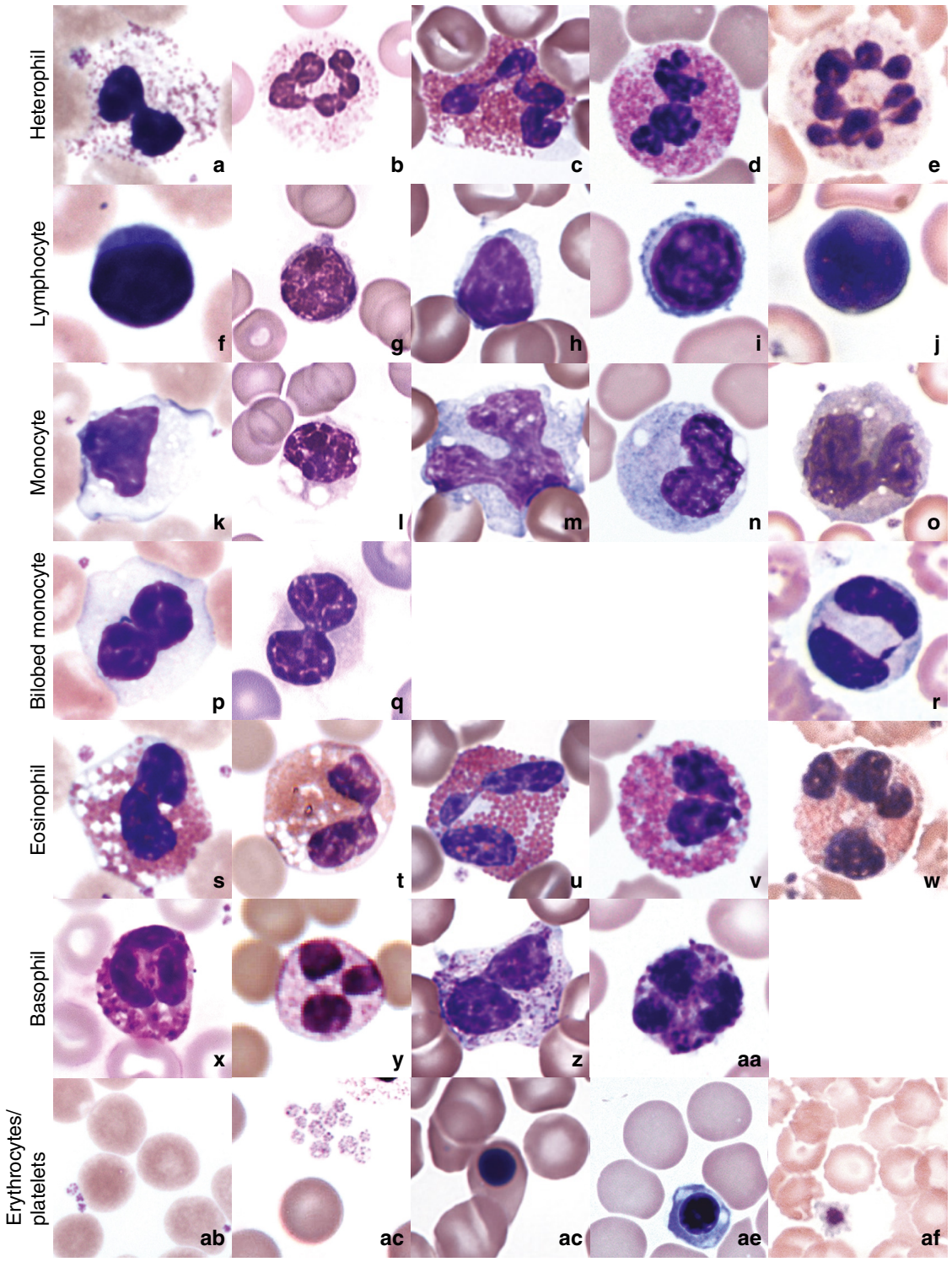


FIGURE 121.1 Representative images of Wright-Giemsa stained normal WBCs, RBCs, and platelets from members of the Paenungulata clade. African elephant (*Loxodonta africana*): (a) Segmented heterophil; (f) Small lymphocyte; (k) Monocyte with vacuolation; (p) Bilobed monocyte; (s) Eosinophil with occasional clear granulation; (x) Basophil; (ab) Platelets in comparison to the large elephant RBC. Asian elephant (*Elephas maximus*): (b) Segmented heterophil; (g) Small lymphocyte; (l) Reactive monocyte with round nucleus; (q) Bilobed monocyte; (t) Eosinophil with similar granulation to the African elephant; (y) Basophil; (ac) RBC with platelets. Florida manatee (*Tritrechus manatus latirostris*): (c) Heterophil; (h) Lymphocyte; (m) Monocyte; (u) Eosinophil; (z) Basophil; (ad) nucleated RBC which are rarely found in healthy manatee. Bilobed monocytes are not pictured as they have not been documented in manatee at this time. Dugong (*Dugong dugon*): (d) Heterophil; (i) Lymphocyte; (n) Monocyte; (v) Eosinophil; (aa) Basophil; (ae) Polychromatophilic metarubricyte. Bilobed monocytes are not pictured as they have not been documented in dugong at this time. Rock hyrax (*Procavia capensis*): (e) Heterophil; (j) Lymphocyte; (o) Monocyte; (r) Bilobed monocyte illustrating the filamentous nuclear attachment frequently observed; (w) Eosinophil; (af) Platelet with the smaller hyracoid RBC. Basophils are rarely found in rock hyrax and are not pictured. (**Source:** Asian elephant photos courtesy of Dr Kendal Harr. Manatee photos courtesy of Dr. John Harvey and Dr. Kendal Harr. Dugong photos courtesy of Dr. Lucy Woolford. Hyrax photos courtesy of Dr. Itamar Aroch. African elephant basophil courtesy of the San Diego Zoo.)

group of captive Asian elephants in the USA, where all cells described here as monocytes (bilobed and round nuclei) were counted as such, revealed that monocytes are the dominant cell type, followed by heterophils, lymphocytes, eosinophils, and basophils[25] (Table 121.1). Current automated techniques cannot be expected to correctly identify elephant leukocytes and manual differential cell counts are always required. It is vital that monocytes are identified correctly, as described here, and that appropriate reference intervals are used.

TABLE 121.1 Hematology reference data for Asian elephants (*Elephas maximus*) and African elephants (*Loxodonta africana*). Note that in the first case, sample size was small and therefore the minimum–maximum range is reported. The Asian elephant reference population comprised 25 adult, predominantly female animals, from zoological collections in the USA.[1,25,58] The free-ranging African elephant population comprised 48, mostly male adult animals, from the Kruger National Park in South Africa.[58] Only 23 samples from the automated hematology analysis were finally included after applying the QA screening process.

Measurand (units)	Method	Sample size	Mean	SD	Min–max range	RI
Asian elephant (*Elephas maximus*)[25]						
PCV (L/L)	3	20	0.357	0.038	0.27–0.44	
Hgb (g/L)	1	20	123	13	96–153	
RBC (× 10^{12}/L)	1	20	2.9	0.4	2.1–3.6	
MCV (fL)	1	20	125	7	111–138	
MCHC (g/L)	1	20	347	6	334–357	
RDW (%)	1	18	16.8	1.1	14.9–18.7	
PLT (× 10^{12}/L)	1	18	513	98	342–719	
WBC (× 10^9/L)	1	20	13.6	2.7	8.6–18.2	
Bands (× 10^9/L)	2	20	0.0	0.0	0.0	
Heterophils (× 10^9/L)	2	20	3.2	0.8	1.8–4.3	
Lymphocytes (× 10^9/L)	2	20	2.7	1.5	1.0–5.0	
Monocytes (× 10^9/L) (includes bilobed cells)	2	20	7.1	2.8	1.6–12.6	
Eosinophils (× 10^9/L)	2	20	0.2	0.2	0–0.6	
Basophils (× 10^9/L)	2	20	0.1	0.1	0–0.2	
Free-ranging African elephant (*Loxodonta africana*)[58]						
Hct (L/L)	3	48	0.41	0.04	0.33–0.49	0.34–0.49
Hgb (g/L)	4	23	141	13	116–163	114–167
RBC (× 10^{12}/L)	4	23	3.44	0.3	2.8–4.0	2.8–4.1
MCV (fL)	5	23	121	6	112–134	109–133
MCHC (g/L)	5	23	338	15	314–364	306–370
PLT (× 10^{12}/L)	4	23	284	54	182–386	171–398
WBC (× 10^9/L)	4	23	11.2	2.2	7.5–15.2	6.6–15.7
Bands (× 10^9/L)	2	23	0.1	0.1	0.0–0.2	0.0–0.6
Heterophils (× 10^9/L)	2	23	2.8	0.7	1.5–4.0	1.4–4.3
Lymphocytes (× 10^9/L)	2	23	2.7	1.3	1.1–5.5	0.7–7.0
Regular monocytes (× 10^9/L)	2	23	3.8	0.9	2.7–6.1	2.5–6.9
Bilobed monocytes (× 10^9/L)	2	23	1.1	0.5	0.3–2.3	0.3–2.6
Round monocytes (× 10^9/L)	2	23	0.3	0.3	0.0–0.9	0.0–1.7
Eosinophils (× 10^9/L)	2	23	0.4	0.2	0.0–0.9	0.1–0.9
Basophils (× 10^9/L)	2	23	0.0	0.0	0.0–0.1	0.0–01

Method 1: Cell-Dyn 3500 (Abbott Diagnostics Inc., Abbott Park, IL, USA).
Method 2: Manual differential using Wright–Giemsa-stained blood smears.
Method 3: Manual microhematocrit (packed cell volume).
Method 4: Combined from scil Vet abc and Horiba ABX Micro ESV60.
Method 5: Calculated from Hct, RBC, Hgb.
PLT, platelet count, SD, standard deviation; RDW, red cell distribution width.

Platelets and Coagulation

Platelet counts in 22 wild caught African elephants ranged from 229–622 × 10^9/L and in captive Asian elephants from 106–719 × 10^9/L.[25,30,17] Ultrastructural examination, using transmission and scanning electron microscopy, has shown that platelets from both species do not have a surface open canalicular system.[14,17] They do contain α-granules, dense bodies, mitochondria, Golgi bodies, glycogen, and filamentous structures of unknown function.[14,17] Elephant platelets show reversible aggregation in response to a low dose of an agonist such as platelet-activating factor or adenosine diphosphate.[14] Activated platelets demonstrate numerous cytoplasmic protrusions, rather than pseudopodia, and there is no interaction between adjacent platelets.[14] As has been observed in bovines, gaps form in the cell membrane during activation, which may provide the route of granule extrusion in the absence of a canalicular system.[14]

Activity of Asian elephant coagulation factors VII, IX, X, and XI and antithrombin (AT) was found to be similar to that of humans. Activity of factor VIII was about twice that of humans. Commercially available reagents (in particular recombinant tissue factor) can be used for coagulation testing in Asian elephants, but results will differ between different manufacturers. For seven elephants, prothrombin time (PT) ranged from 8.8–12.3 seconds and activated partial thromboplastin time (APTT) from 52.1–83.5 seconds. Although factor XII activity was higher than in humans, APTT was up to two times longer than in humans, using the same analytical system. The reason for this is not clear.[20]

Asian elephants have hypercoagulable tracings using thromboelastography (TEG), in particular high maximum amplitude (MA) and α-angle with minimal clot lysis, compared to domestic species, although similar to manatees.[46]

An Asian elephant originating in Malaysia and housed in an Australian zoo was found to have persistently elevated PT. Factor VII deficiency caused by a homozygous missense mutation in the gamma-carboxyglutamic-acid-rich domain of the factor VII gene, resulting in arginine for glycine substitution, was identified. This elephant did not show any clinical signs of a hemostatic disorder, and the mutation was not found in the rest of the Australian zoo elephant population.[35]

Reference Intervals

Multiple publications presenting hematology data from healthy elephants of both species exist.[1,16,25,30,46,53,54,58,65] Very few studies, however, have followed accepted guidelines, e.g., those from the American Society for Veterinary Clinical Pathology (ASVCP), for reference interval generation.[18] Additionally, all of the older papers and some recent ones use manual and not automated methods, which are subject to a high degree of analytical imprecision. In studies where automated hematology systems were used, there is usually no information about analytical performance or validation of the method, which is likely to be important in these animals with very large RBCs as well as very different WBC morphologies. As discussed in the earlier text, there are also huge discrepancies in monocyte identification, leading to significant variation in monocyte and lymphocyte reference intervals.

The hematology data presented in Table 121.1 for 20 captive Asian elephants were generated using an automated hematology analyzer, with manual differentials using modified Wright–Giemsa staining and cross-checked using cytochemically stained slides. Bilobed and round nucleus monocytes were counted as monocytes. A recent study generated clinical pathology reference intervals according to ASVCP guidelines for free-ranging apparently healthy African elephants from the Kruger National Park, South Africa.[58] After a strict outlier elimination procedure, hematology reference intervals were derived from 24 EDTA whole blood samples analysed on the scil Vet abc (Scil Animal Care Company, Henry Schein, Inc., Melville, NY, USA) or Horiba ABX Micro ESV60 (Horiba Ltd., Kyoto, Japan) automated analyzers. Reference intervals were also generated for manual PCV from 48 samples. Other studies on African elephants used samples from culled animals and manual counting methods and are not reported here.[16,65] Hemostasis reference intervals for TEG, clotting times, and fibrinogen were generated according to ASVCP guidelines for up to 26 captive Asian elephants.[46] Data and methods used are presented in Table 121.2.

Partitioning Factors

Hematologic values appear to be similar for African and Asian elephants, apart from the WBC differential cell count, which is highly method-dependent. Various studies have shown few differences between subgroups of elephants. There was no difference in results between a group of captive, nonimmobilized Asian elephants and a group of free-ranging, immobilized individuals of the same species.[54] MCV and mean cell hemoglobin concentration (MCHC) were higher in female versus male Asian elephants in one study, but not in others.[30,53,54] No age or sex differences in hemostatic measurands have been identified; however, studied partitioned subgroups were small and the statistical testing lacked power.[46]

RBC counts and PCV have been reported to be slightly lower in pregnant versus nonpregnant Asian elephants, due to volume expansion associated with pregnancy.[45] Higher values for RBC, Hgb, MCHC, and lower values for MCV were found in free-ranging African elephants in the dry season compared to the wet season, probably due to dehydration.[65] Both young Asian and African elephants have higher WBC counts than adults.[12,65]

Biological variation of hematology or hemostasis measurands in elephants is minimally reported. One study concluded that there were no remarkable seasonal changes in hematology results collected from four captive Asian elephants every two weeks over a 12-month

TABLE 121.2 Hemostasis reference data for Asian elephants(*Elephas maximus*). The reference population comprised 8 male and 30 female captive animals from zoological collections in the USA and United Kingdom.[46]

Measurand (units)	Method	Sample size	Mean	SD	Min–max range	RI
TEG R (min)	1	38	4.9	1.4	2.2–7.5	2.0–7.8
TEG K (min)	1	38	1.2		0.8–2.3	0.7–2.2
TEG α (°)	1	38	74.6	3.7	64.0–81.0	65.3–80.7
TEG MA (mm)	1	38	80.9	3.2	73.3–85.8	73.2–86.4
G (d/cm^2)	1	38	21.8	4.1	15.2–30.2	14.3–31.0
APTT (s)	2	23	11.2		8.2–21.3	7.6–25.1
PT (s)	2	23	11.0		9.7–14.9	
Fibrinogen (g/L)	2	23	3.62	0.6	2.66–4.91	2.57–5.10

Method 1: Kaolin-activated whole blood TEG, 60 minutes postcollection, TEG 5000 Analyzer (Haemonetics, MA, USA).
Method 2: Thermo Scientific Pacific Hemostasis Reagents (Thermo Fisher Scientific, MA, USA) on a STart 4 Hemostasis Analyzer (Diagnostica Stago, NJ, USA).
RI = Reference intervals: 95% RIs generated according to ASVCP guidelines.

period, but did not evaluate the data statistically.[44] The author of this chapter calculated intraindividual variation in a group of four captive female African elephants using data measured on a CELL-DYN 3500 (Abbott Laboratories, Abbot Park, IL, USA) hematology analyzer and found intraindividual coefficients of variation of 10.3% for WBC, 9.4% for RBC, 7.3% for Hgb, 7.4% for PCV, 3.3% for MCV, and 2.1% for MCHC. These values are similar to those found for dogs.[27]

Blood and Bone Marrow Collection

Many captive or working elephants are trained to allow blood collection without restraint or sedation. Blood is collected from the auricular veins on the medial surface of the ear, in a standing or laterally recumbent elephant. Vasoconstriction may occur in cold weather, making these veins difficult to visualize. A hair dryer may be used to warm up the area to promote vasodilation. The lateral auricular veins, cephalic vein (medial proximal forelimb), or saphenous vein (distal hindlimb) may also be used for collection. If these sites are chosen, a tourniquet (e.g., bungee cord) will be needed to raise the vein. A large bore needle is advised to prevent damage to the very large elephant RBCs.

Elephant long bones do not have open marrow cavities and are filled with dense trabecular bone.[43] Hematopoiesis may take place in the vertebral bodies; further research is needed.

Hematologic Disease

Elephants with inflammatory disease may not show leukocytosis, heterophilia, lymphocytosis, and/or monocytosis. More common and specific changes include left shifted heterophilia, heterophil toxicity, reactive lymphocytes, plasmacytosis, and marked monocytic activation.[56] A left shift may include the presence of giant toxic heterophils and precursors as early as myelocytes. Toxic heterophils exhibit Döhle bodies and cytoplasmic basophilia. Serial blood smear examination in elephants with inflammatory disease is important, as resolution of the abovementioned morphological changes is associated with clinical improvement, while increasing heterophil toxicity indicates an increasing severity of disease.[56]

Elephant endotheliotropic herpesviruses (EEHV) belong in the Proboscivirus genus of the beta herpesvirus subfamily. Multiple EEHV strains have been recognized in both Asian and African elephants; and the strains appear to be species-specific with large intrastrain genetic variation. African elephants are naturally infected with EEHV2, EEHV3A, EEHV3B, EEHV6, EEHV7A, and EEHV7B, which are usually nonpathogenic or of low pathogenicity in this species.[34] EEHV1A, EEHV1B, EEHV4, EEHV5A, and EEHV5B naturally infect Asian elephants globally and infections are generally not clinically significant in most elephants.[34] Captive Asian elephant calves between the ages of 1 and 4 years can, however, develop a severe hemorrhagic disease, which is often fatal. Up to 20% of captive calves may be affected.[48] The majority of cases have been caused by EEHV1A, followed by EEHV1B, EEHV3A, EEHV4, and EEHV5A. The disease is rare in African elephant calves in similar husbandry situations, although two lethal cases of EEHV2, one mild case of EEHV3B, and one of EEHV6 have been reported.[48] EEHV1 infection in Asian elephants is not a crossover infection from African elephants as previously thought, but has been a natural infection of Asian elephants at least since evolutionary divergence between the two species.[34] Affected elephants present with lethargy and anorexia, edema of head, neck, forelimbs, and trunk, and cyanosis that begins at the tongue tip and moves caudally.[47] Characteristic pathologic lesions include herpetic inclusion bodies within endothelial cells, particularly of tongue, myocardium, and liver; extensive microhemorrhage in the heart and tongue muscle leading to microscopic and macroscopic edema, including pericardial effusion and ascites, macroscopic cardiac hemorrhage, and petechiation of visceral and parietal serous membranes.[47,48] Endothelial cell

necrosis and fibrinoid degeneration of blood vessel walls leads to thrombosis, edema, and hemorrhage. These lesions may also be found in renal, brain, and retinal tissue.[19] Hematologic changes may include lymphopenia, thrombocytopenia, and anemia.[48] One African elephant calf that survived an infection with EEHV3B showed an initial leukopenia with a left shift, lymphopenia, monocytopenia, and mild anemia. Over the course of the 14 days of viremia, lymphocytosis, monocytosis, and neutrophilia developed, the left shift was absent by day 11 and the leukogram was normal by day 30. There was an initial drop in platelets over the first five days of infection followed quickly by return to normal.[11] Serum amyloid A (SAA), although not disease-specific, is increased in Asian elephants with nonpathogenic EEHV viremia and decreasing viral titres are associated with return of SAA to normal levels.[57] SAA concentrations in the elephant calf described in the earlier text increased to 12-fold of the elephant's baseline values by day 12 and returned to baseline by day 14.[11] In a suspected case, blood and trunk wash fluid should be submitted for PCR examination based on Association of Zoos and Aquariums protocols. More information can be found on eehvinfo.org.[22]

Mycobacterium tuberculosis has been isolated from captive and working Asian and African elephants across the globe.[39,40,49,62] The prevalence of *M. tuberculosis* in the captive North American elephant population has been estimated to be between 3 and 4%.[39] Clinically apparent disease is not seen in most infected elephants. When present, clinical signs include weight loss, anorexia, exercise intolerance, dyspnea, and coughing.[39] Pathology occurs primarily in the lungs and draining thoracic lymph nodes, and occasionally in extrathoracic locations. Lesions range from granulomatous, caseous, and/or mucopurulent solitary nodules to coalescing areas in affected locations.[40] Captive Asian elephants with tuberculosis may have a moderate normocytic, normochromic anemia and hypoferremia, and have also been found to have lower MCHC and higher band neutrophil and eosinophil concentrations than tuberculosis-negative elephants, as well as a lower albumin to globulin ratio.[24,51] Serological tests are available for screening but do not indicate clinical disease, which must be confirmed by interferon-γ release assay, trunk wash culture, and PCR according to United States Department of Agriculture protocols when in the United States.[40,42] Transmission is probably from humans to elephants, but this disease can be zoonotic.[39] Cases of mycobacteriosis caused by *Mycobacterium avium* and nontuberculous mycobacteria have also been reported.[33,67]

Microfilaria, in most cases *Dipetalonema sp.*, have been identified in blood smears from both African and Asian elephants.[7,12,36] *Dipetalonema gossi* was found to be associated with intrahepatic vasculitis and hepatitis in free-ranging elephants in South Africa.[7,12] *Trypanosoma evansi* (surra) has been reported in Asian elephants, and causes fever, progressive anemia, edema of dependent parts, and petechiation of mucous membranes.[29] Parasites may be seen in blood smears of affected animals, but this method is insensitive. *Trypanosoma congolense* and *Trypanosoma brucei* have been identified in African elephants.[61]

SIRENIA

This order contains four species in two families: *Dugong dugon* (dugong), *Trichechus manatus* (West Indian manatee), *Trichechus senegalensis* (African manatee), and *Trichechus inunguis* (Amazonian manatee). There are two subspecies of the West Indian manatee: *Trichechus manatus latirostris* (Florida manatee) and *Trichechus manatus manatus* (Antillean or Caribbean manatee). The sirenians are large aquatic herbivorous mammals that inhabit various fresh water and marine habitats.

Erythron

Like elephants, sirenians have similar Hgb and PCV values to domestic species, but a higher MCV and lower RBC (Table 121.3). MCVs of 105–140 fL and 116–167 fL have been reported in Florida manatees and dugongs, respectively, using different automated methods.[26,66]

Typical blood smear findings for Florida manatees include mild polychromasia, mild to moderate rouleaux (less than cats and horses), stomatocytosis, low numbers of schistocytes, echinocytes, nRBCs (in 40% of animals), Howell–Jolly bodies, and <15% Heinz bodies.[26] A manatee nRBC is shown in Figure 121.1. The presence of schistocytes and Heinz bodies may suggest a degree of inefficient removal of abnormal RBCs by the spleen. Unlike elephants, reticulocytes are present in the blood of healthy Florida manatees, with mean relative reticulocyte count of 2%.[26] Reticulocytes were not detected in healthy Antillean manatees in one study, although low numbers of nRBCs may be present.[37] Extramedullary hematopoiesis occurs normally in the sirenian spleen, liver, kidney, and vertebrae, which may explain the presence of circulating nRBCs in healthy animals.[8] Erythron findings in dugongs are similar, with mild anisocytosis, rare schistocytes, low numbers of Howell–Jolly bodies, and low numbers of nRBCs in around 70% of animals, especially pregnant females.[66]

Leukon

Sirenian WBC counts are slightly lower than most domestic mammals, although similar to other marine mammals.[26] Dugongs have lower WBC concentrations than manatees.[66] The heterophil to lymphocyte ratio in manatees is around 1.0–1.6.[26,37] In a hematologic evaluation of dugongs, lymphocytes were predominant in one half of animals, heterophils in one third of animals, with equal proportions of the two cell types in the remainder.[66] This variation may have been due to stress during capture. Free-ranging dugongs have higher eosinophil counts than manatees, possibly due to the presence of internal parasites.[66] Ultrastructural images of dugong blood cells are available in the literature.[66]

TABLE 121.3 Hematology and hemostasis reference data for Florida manatees (*Trichechus manatus latirostris*) and dugongs (*Dugong dugon*). The reference populations of Florida manatees were: hematology, 52 free-ranging animals and 62 captive animals from institutions in the USA; coagulation, 21 male and 19 female free-ranging animals; TEG, 17 male and 12 female free-ranging animals. The dugong reference population consisted of 92 free-ranging animals from Australia.[5,6,26,66]

Measurand (units)	Method	Sample size (n)	Mean	SD	Min–max range	RI
Florida manatee (*Trichechus manatus latirostris*)[5,6,26]						
Hct (L/L)	1	52	0.35		0.30–0.44	
		62	0.35		0.28–0.46	
Hgb (g/L)	1	52	112		94–135	
		62	112		86–149	
RBC ($\times 10^{12}$/L)	1	52	2.8		2.2–3.4	
		62	2.8		2.3–3.5	
MCV (fL)	1	52	128		114–140	
		62	124		105–140	
MCH (pg)	1	52	41.0		37.0–45.0	
		62	40.0		33.0–44.0	
MCHC (g/L)	1	52	320		280–354	
		62	320		294–336	
RDW (%)	1	52	16.2		13.9–22.8	
		62	16.6		13.5–21.4	
PLT ($\times 10^{12}$/L)	1	52	242		111–424	
		62	274		137–507	
MPV (fL)	1	52	6.44		5.02–8.49	
		62	6.40		5.19–8.80	
WBC ($\times 10^9$/L)	1	52	6.0		2.8–13.5	
		62	6.6		2.9–14.2	
Bands	2	52	0.0		0.0–0.2	
		62	0.0		0.0–0.2	
Heterophils ($\times 10^9$/L)	2	52	2.3		0.8–6.5	
		62	3.3		1.4–8.5	
Lymphocytes ($\times 10^9$/L)	2	52	2.9		1.0–7.2	
		62	2.7		0.8–8.5	
Monocytes ($\times 10^9$/L)	2	52	0.5		0.1–1.7	
		62	0.6		0.1–2.8	
Eosinophils ($\times 10^9$/L)	2	52	0.2		0.0–1.2	
		62	0.1		0.0–0.4	
Basophils ($\times 10^9$/L)	2	52	0.0		0.0–0.3	
		62	0.0		0.0–0.1	
nRBCs ($\times 10^9$/L)	2	52	0.0		0.0–0.2	
		62	0.0		0.0–0.3	
Reticulocytes ($\times 10^9$/L)	3	17	51.0		16.0–96.0	
		15	74.0		0–127	
Reticulocytes ($\times 10^9$/L)	4	10	17.0		7.0–45.0	
		9	21.0		3.0–39.0	
APTT (s)	5	40	9.5	1.5	5.8–12.5	
PT (s)	5	40	10.7	0.5	10.4–12.0	

(*Continued*)

TABLE 121.3 (Continued)

Measurand (units)	Method	Sample size (n)	Mean	SD	Min–max range	RI
Fibrinogen (g/L)	5	40	3.69	0.79	1.72–6.10	
ATIII (%)	6	40	131.5	11.0	112–161	
D-dimer (μg/L)	7	40	142	122	0–588	
TEG R (min)	8	29	2.0	0.5	1.4–4.4	
TEG K (min)	8	29	0.8	0.0	0.8–0.8	
TEG α (°)	8	29	83.1	2.0	78.9–85.9	
TEG MA (mm)	8	29	77.0	3.4	56.1–83.6	
TEG LY30 (%)	8	29	0.3	0.4	0.0–2.5	
Dugongs (*Dugong dugon*)[66]						
Hct (L/L)	9,10	91	0.37	0.03	0.3–0.45	0.32–0.44
	9	27	0.36	0.03	0.30–0.42	
	10	64	0.38	0.03	0.32–0.45	0.32–0.44
Hgb (g/L)	9,10	91	114	9.0	89–135	92–132
	9	27	111	9.4	93–129	
	10	64	116	8.3	89–135	90–134
RBC ($\times 10^{12}$/L)	9, 10	91	2.7	0.2	2.1–3.4	2.3–3.2
	9	27	2.8	0.2	2.3–3.4	
	10	64	2.7	0.2	2.1–3.2	2.2–3.1
MCV (fL)	9, 10	91	138	11.0	116–167	119–161
	9	27	128	6.0	116–138	
	10	64	143	10.0	122–167	123–164
MCH (pg)	9, 10	91	42.0	3.0	35.0–50.0	36.0–49.0
	9	27	40.0	2.0	35.0–44.0	
	10	64	43.0	3.0	36.0–50.0	36.0–50.0
MCHC (g/L)	9, 10	91	305	11	278–330	284–327
	9	27	309	10	290–330	
	10	64	304	11	278–329	282–325
PLT ($\times 10^{12}$/L)	9, 10	91	329	97	103–666	170–152
WBC ($\times 10^{9}$/L)	9, 10	92	5.6	1.6	2.1–9.6	2.6–9.3
		56 adults	5.1	1.4	2.1–9.2	2.3–8.9
		36 subadults	6.3	1.6	4.0–9.6	
Heterophils ($\times 10^{9}$/L)	2, 9,10	92	1.8	0.6	0.5–3.3	0.7–2.9
Lymphocytes ($\times 10^{9}$/L)	2, 9, 10	92	2.3	1.1	0.4–4.9	0.7–4.6
Monocytes ($\times 10^{9}$/L)	2, 9, 10	92	0.3	0.3	0.0–2.0	0.0–0.8
Eosinophils ($\times 10^{9}$/L)	2, 9, 10	92	1.2	0.5	0.4–2.7	0.4–2.5
Basophils ($\times 10^{9}$/L)	2, 9, 10	92	0.1	0.1	0.0–0.4	0.0–0.3
nRBCs ($\times 10^{9}$/L)	2	79	0.2	0.3	0.0–1.5	0.0–1.4

Method 1: Elephant setting on CELL-DYN 3500R (Abbott, IL, USA).
Method 2: Manual 200-cell differential using Wright–Giemsa-stained blood smears.
Method 3: Manual count on new-methylene-blue-stained blood smears.
Method 4: Human setting on ADVIA 120 (Siemens Healthcare Diagnostics Inc., NY, USA).
Method 5: STA Compact (Diagnostica Stago, NJ, USA).
Method 6: Chromogenic, Stachrom ATIII (Diagnostica Stago, NJ, USA).
Method 7: Immunoturbidimetric, HemosIL (Instrumentation Laboratory, MA, USA).
Method 8: Kaolin-activated TEG, TEG 5000 Analyzer (Haemonetics, MA, USA).
Method 9: Cell-DYN 3700 (Abbott, IL, USA).
Method 10: Sysmex XT-2000iV (Sysmex Corp., Hyogo, Japan).
MCH, mean cell hemoglobin; MPV, mean platelet volume; RDW, red cell distribution width; SD, standard deviation; RI = reference intervals: 95% RIs generated according to ASVCP guidelines.

Like the elephant, manatees and dugongs have heterophils rather than neutrophils. The heterophil of the Florida manatee and dugong measures 9–12 μm in diameter, contains numerous pink to red-staining round to oval to rod-shaped granules in a pale blue cytoplasm, and has a nucleus with three to five lobes (Figure 121.1).[26,66] These cells stain strongly positive for PAS and myeloperoxidase (like neutrophils of domestic animals, but unlike heterophils of rabbits and birds) and show weak staining with CAE; but are still called heterophils due to their appearance.[31,66]

In all species, lymphocytes are small to medium in size (diameter 7–10 μm), occasionally with magenta cytoplasmic granules (Figure 121.1).[26,37,66] In approximately half of dugongs, lymphocytes are the predominant cell type. While most lymphocytes exhibited negative staining, a subset of dugong lymphocytes had mild to moderate granular focal cytoplasmic reactivity with ANBE and PAS consistent with a T-cell subset.

Sirenian monocytes are similar to other mammals with a large diameter of 16–18 μm, polymorphic nucleus, and occasionally vacuolated blue cytoplasm. Unusual monocyte morphology, in particular the bilobed monocyte of elephants and hyraxes, has not been reported in sirenians. Cytochemical staining of dugong monocytes revealed moderate patchy cytoplasmic staining with ANBE, scant staining with alkaline phosphatase, and diffuse pale granular cytoplasmic staining with PAS. Monocytes did not stain with ANAE, peroxidase, SBB, and napthol AS D chloroacetate esterase.

The eosinophil has a reported diameter of 9–13 μm with a more basophilic cytoplasm and less lobulated nucleus, as shown in Figure 121.1. [26,37,66] Granules are bright pink or dark red, larger, and more uniform than those of the heterophil. Dugong eosinophils all stained positive with ANBE, some stained positive with peroxidase, PAS, and SBB, similar to elephants and domestic ungulates.[66]

Basophils (Figure 121.1) range in diameter from 10–11 μm and tend to have a band-shaped nucleus and purple granulation, which is minimal in manatees but abundant in dugongs.[26,66] Basophils are uncommonly observed in sirenian blood and have not been identified in cytochemical stains to date.[66]

Platelets and Coagulation

Platelet counts are similar to those in domestic mammals; platelets are small (2–3 μm), ovoid to irregular in shape, with light blue to pink granules.[26,66] Platelet clumping is often observed on sirenian blood smears.

Several studies have evaluated hemostasis in healthy and debilitated Florida manatees.[5,6,21,37] APTT is fast compared to other species, and like the elephant, manatees have comparatively high concentrations of factors VIII and XII, compared to humans.[5,37] TEG studies in clinically normal manatees have demonstrated shorter R and K and greater α and MA compared to other species, indicating a relative hypercoagulability.[6,21] The adaptive advantage of this relative hypercoagulability is not known; however, it has been speculated that these animals may be naturally predisposed to thromboembolic disease.[6]

Reference Intervals

Recent publications have focused on Florida manatees with a single study reporting hematology results for dugongs.[5,6,21,26,66] Most studies were either published prior to, or did not explicitly follow ASVCP guidelines. Hematology data generated for 52 free-ranging and 62 captive healthy Florida manatees are presented in Table 121.3.[26] This study compared results between free-ranging and captive animals, as well as between different life stages, but did not generate reference intervals. Free-ranging manatees had significantly higher MCV than captive animals and calves had lower MCV than adults. Heterophil numbers were lower in free-ranging versus captive, with higher values in calves compared to adults, possibly due to a longer, stressful restraint period before blood sampling in captured animals. Eosinophils were lower in captive animals. Automated reticulocyte count was higher in free-ranging manatees. Calves tended to have more reticulocytes and metarubricytes than adults.

Studies presenting hemostasis data included between 29 and 40 Florida manatees.[5,6,21] Due to the low number of animals, recommended statistical methods for reference interval generation were not always possible and results are generally reported as mean, standard deviation, and minimum–maximum range. Data for coagulation profiles generated from 40 free-living healthy Florida manatees are presented in Table 121.3.[5] Reference intervals, generated by the robust method for PT, APTT, fibrinogen, and D-dimers from up to 29 rehabilitated Florida manatees, are available but not presented in the table, as the authors neglected to report any methods used to produce the results.[21] Results for all clotting profile measurands apart from D-dimers appear to comparable in both studies. Kaolin-activated TEG was studied in 29 free-living healthy Florida manatees (Table 121.3).[6] Resting times between blood collection and TEG analysis ranged from 3 to 12 hours. No differences were found for sex or size class in any of these hemostasis studies.

Hematology data were collected from 82 free-living Antillean manatees, caught off the coast of Belize.[59] The reference sample group included adults, subadults, and calves of both sexes. The analytical methods and outlier detection used are not described; the sample size differs between measurands and data presented are not always plausible, e.g., MCV minimum–maximum range starts at zero. If these data are accessed, they should not be used for medical diagnosis.[59]

Reference intervals have been generated, according to ASVCP guidelines, for a population of free-living dugongs in southern Queensland, Australia (Table 121.3).[66] The reference population consisted of 42 males and 50 females (11 pregnant), 56 adults and 36 subadults. WBC and lymphocyte counts were higher in

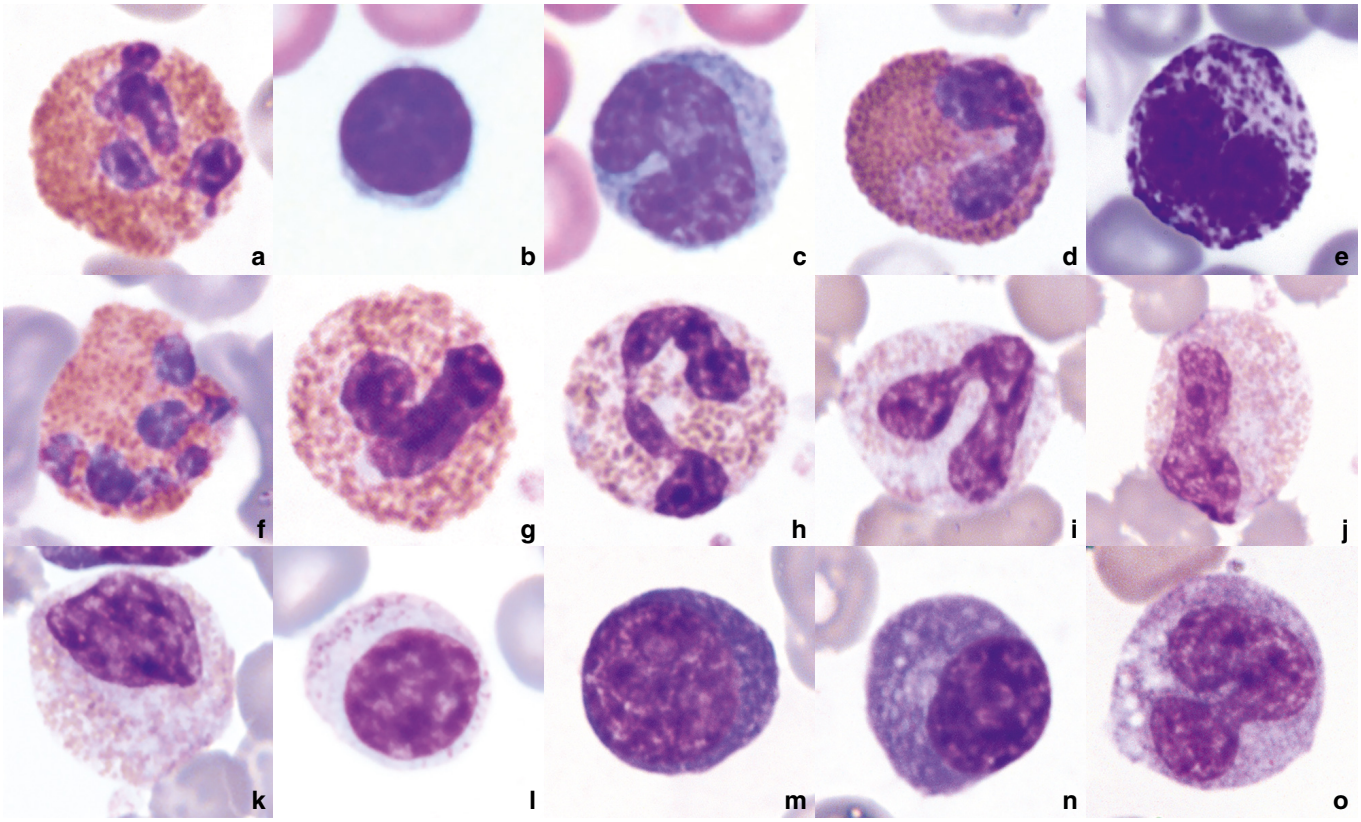


FIGURE 121.2 Normal and abnormal leukocytes of the West Indian manatee (*Trichechus manatus latirostris*). (a) Segmented heterophil; (b) Small lymphocyte; (c) Monocyte; (d) Eosinophil; (e) Basophil; (f) Hypersegmented heterophil; (g) Band heterophil; (h) Mildly toxic (1+) heterophil; (i) Mildly to moderately toxic (1–2+) heterophil; (j) Moderately toxic (2+) metamyelocyte; (k) Moderately toxic myelocyte; (l) Granular lymphocyte; (m) Reactive lymphocyte; (n) Plasmacytoid lymphocyte/plasma cell; (o) Reactive monocyte. (**Source:** Photos courtesy of Dr. Nicole Stacy.)

subadults versus adults. PCV, MCV, and nRBC were higher and platelet count lower in pregnant females. Results were generated from two hematology analyzers, and differences were found between analyzers for some measurands (Table 121.3).[66]

Blood and Bone Marrow Collection

Manatees and dugongs are docile, gentle animals and can be captured without sedation using nets or stretchers.[59,66] Blood is collected in a nonsedated animal from the brachial plexus between the radius and ulna from either pectoral flipper, using a 18–22 gauge needle and a butterfly extension, or wing-tipped blood collection set.[5] There is a vascular bundle here formed by axillary and brachial arteries.

This order has amedullary pachyosteosclerotic long bones which contain loose connective tissue rather than sites of hematopoiesis. Hematopoiesis occurs primarily in the inner, cancellous bone portion of the vertebral bodies. Myeloid, erythroid, and megakaryocytic precursors have been identified. No lymphoid precursors were observed.[8]

Hematologic Disease

nRBCs increase in Florida manatees with inflammatory disease and a mild leukocytosis may also be present. Both changes are specific, but poorly sensitive and other inflammatory markers such as SAA or plasma albumin:globulin ratio have better diagnostic accuracy.[23] Band heterophils and toxic change are indications of active immune response (Figure 121.2).

Cold stress syndrome (CSS) is one of the most common causes of fatalities in Florida manatees and results after prolonged (>72 hours) exposure to water with a temperature of <20 °C. Manatee with CSS suffer from metabolic and nutritional disorders, systemic inflammation, infectious disease, and hemostatic derangements, specifically thromboembolic disease.[23] Animals commonly present with epidermal lesions ranging from skin bleaching to ulceration and orthokeratotic hyperkeratosis.[23] These lesions frequently occur at the extremities, probably due to small vessel vasoconstriction combined with localized inflammation and activation of coagulation leading to endothelial dysfunction.[6] Using TEG, manatees with CSS were found to have longer R and shorter K times, and increased fibrinolysis at LY30, suggesting a procoagulant excess with activation of the fibrinolytic pathways. MA and α-angle were not different, indicating that final clot strength is not affected in CSS.[6] The use of TEG as a prognostic tool has not been examined. APTT and PT are extended in cold-stressed manatees. Seven out of 10 cold-stressed manatee samples did not clot by 90 seconds, while the normal

TABLE 121.4 Hematology reference data for 25 adult, mixed-sex rock hyrax (*Procavia capensis*) using an Abacus (Diatron, Vienna, Austria). Manual 400-cell differential cell counts were performed using modified Wright-stained blood smears. Statistical outlier removal was not performed.[2]

Measurand (units)	Mean	SD
Hct (L/L)	0.31	0.03
Hgb (g/L)	83.0	14.0
RBC ($\times 10^{12}$/L)	4.7	0.7
MCV (fL)	70.0	5.0
MCH (pg)	18.0	2.0
MCHC (g/L)	262	28.0
RDW (%)	19.7	0.7
PLT ($\times 10^{12}$/L)	270	98.0
MPV (fL)	7.1	0.4
WBC ($\times 10^{9}$/L)	16.0	5.0
Neutrophils ($\times 10^{9}$/L)	12.1	4.2
Lymphocytes ($\times 10^{9}$/L)	2.6	1.5
Monocytes ($\times 10^{9}$/L)	0.6	0.5
Eosinophils ($\times 10^{9}$/L)	0.7	0.7
Basophils ($\times 10^{9}$/L)	0.0	0.2

MCH, mean cell hemoglobin; MPV, mean platelet volume; RDW, red cell distribution width.

manatee PT reference interval is <12 seconds.[6] Mean D-dimer concentrations of cold-stressed manatees were around seven times higher than healthy animals. D-dimer values exceeding 1500 µg/L or increasing values over the course of rehabilitation were indicative of a poor prognosis.[6,21] ATIII was lower in CSS animals, suggesting a consumptive coagulopathy. Three CSS animals had ATIII <70% with PT and APTT prolonged beyond clot end points, indicating disseminated intravascular coagulation (DIC). Factor XI activity was increased in CSS manatees; this finding has been associated with an increased thrombotic risk in humans. Although the dataset was small, these findings all suggested that CSS is associated with hemostatic derangements.[6] Lymphocyte proliferation was decreased in manatees with CSS, indicating that immune dysfunction is a component of this syndrome, predisposing these animals to opportunistic infectious diseases such as bronchopneumonia, enterocolitis, and dermatitis.[64]

Brevetoxicosis or a harmful algal bloom (a.k.a. HAB) is another leading cause of death in Florida manatees, caused by a neurotoxin produced by a plankton, *Karenia brevis*, during algal blooms. Congestion, hemorrhage, and inflammation at multiple sites are major pathological findings.[9] Submucosal infiltration of lymphocytes, plasma cells, and macrophages, containing brevetoxin antigen, was present in the respiratory tract and leptomeninges. Hemosiderosis was seen in hepatic, splenic, and central nervous system tissues suggesting a chronic hemolytic process. The pulmonary congestion and hemorrhage suggest acute cardiovascular collapse from shock. The central nervous system changes could explain neurological signs seen clinically. It was theorized that brevetoxin-containing cells undergo apoptosis with a concurrent cytokine storm that leads to shock and death. Ongoing apoptosis could also lead to chronic immunosuppression. In addition to apoptosis, lymphocytes from animals with brevetoxicosis, exposed in vitro to brevetoxin, show decreased proliferative responses compared to lymphocytes from healthy animals.[63,64]

A DIC scoring system was developed for Florida manatees, based on platelet count (1 point 75–150 $\times 10^{9}$/L, 2 points < 75 $\times 10^{9}$/L), D-dimer (1 point 150–500 µg/L, 2 points > 500 µg/L), PT (1 point > 12 seconds), APTT (1 point < 14 seconds), and fibrinogen (1 point > 4.50 g/L, 2 points ≤ 3.00 g/L). DIC was present with a combined score of ≥4. DIC was diagnosed, based on the scoring system, in 56% of manatee afflicted with CSS and 51% with trauma, but was not diagnosed in manatee with brevetoxicosis. The presence or absence of DIC in CSS did not affect long term survival.[3] Manatee that had an increasing DIC score over the course of rehabilitation did not survive.[3]

HYRACOIDEA

The hyrax is a small, approximately 10-pound (4–5 kg), terrestrial herbivorous mammal which occurs naturally over most of Africa, the Middle East, and western part of the Arabian peninsula. There are five species within this order: *Procavia capensis* (rock hyrax or dassie), *Dendrohyrax validus* (eastern tree hyrax), *Dendrohyrax arboreus* (southern tree hyrax), *Dendrohyrax dorsalis* (western tree hyrax), and *Heterohyrax brucei* (bush hyrax). *P. capensis*, the rock hyrax, has the widest distribution and is commonly found in zoological collections worldwide.[13]

Erythron

Published information on hyrax hematology is scarce. One study obtained hematology data from 25 free-ranging clinically normal rock hyraxes, from the eastern Mediterranean region.[2] The reference population consisted of 18 female and 9 male, 17 subadult and 8 adult animals (Table 121.4). Males generally had higher values than females, apart from MCV, monocyte, eosinophil, and basophil counts, which were not different between the sexes. RBCs are similar in size and volume to the dog (MCV = 59–81 fL), and while not as big as those of elephants and sirenians, are large for a mammal of its size. PCV and Hgb concentration appear to be lower than other members of the Paenungulata clade. This may be linked to low iron intake from their natural diet, as this species is susceptible to iron overload in captivity. Blood smears from healthy rock hyraxes may exhibit mild hypochromasia, anisocytosis, schistocytosis, polychromasia, microcytosis, macrocytosis, and a variable number of codocytes. nRBCs are rare.[2]

Leukon

Neutrophils in hyraxes do show a variable amount of pink granulation similar to manatee and elephants, but have not been described as heterophils in the few reports available (Figure 121.1). Lymphocytes were mostly small. The prevalence of large granular lymphocytes ranged from 0.5–20% of the lymphocyte population. Eosinophils in this species contain many small, round brick red granules.

A low percentage (1–3%) of bilobed WBCs with a light to medium blue cytoplasm are found in blood smears. The nuclear lobes are connected by a thin filament of chromatin. The morphology of these cells is similar to the bilobed monocytes described for elephants, and were categorized as monocytes in the study, although no cytochemical staining was performed to confirm this.

Hematologic Disease

WBC and neutrophil counts were fairly high in some animals in one study, which may reflect an adrenaline and/or glucocorticoid response.[2]

Hemosiderosis is a common postmortem finding in captive rock hyrax, indicating iron overload. This is most likely due to suboptimum nutrition with excessive bioavailable iron in captivity, leading to excessive iron absorption.[32] No clinicopathological information is available.

Cases of tuberculosis due to the dassie bacillus (*Mycobacterium tuberculosis complex sp.*) have been reported in rock hyraxes living in or imported from South Africa and the United Arab Emirates.[15] This is the genetic clade of Mycobacterium which causes tuberculosis in people and elephants. Typical granulomatous inflammation caused by the bacteria was present in the lungs as well as other organs on postmortem. No hematology findings have been reported.

Hyraxes are reservoirs for leishmaniasis. The presence of and seropositivity for *Leishmania tropica* and *Leishmania aethiopica* has been demonstrated in rock hyraxes in Israel and Saudi Arabia, respectively.[41,58] Both organisms cause cutaneous leishmaniasis in humans.

Rare reports of hemoparasites exist in hyrax. *Babesia thomasi* has been reported to occur in free-ranging hyraxes in sub-Saharan Africa.[28]

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CHAPTER 122

Hematology of Marine Mammals

NICOLE I. STACY and HENDRIK H. NOLLENS

Blood Sampling
Erythrogram
Leukogram
Platelets and Coagulation
Bone Marrow Collection and Evaluation
Reference Intervals
  Partitioning Factors
Hematologic Manifestation of Disease

Acronyms and Abbreviations

AID, anemia of inflammatory disease; aPTT, activated partial thromboplastin time; CD, cluster of differentiation.

BLOOD SAMPLING

Hematology is a well-established and essential diagnostic tool in marine mammal medicine and is used in a variety of research and clinical settings, from individual animal monitoring in managed care to stranding situations and population-level health assessments. The bottlenose dolphin (*Tursiops truncatus*) is the best studied marine mammal species regarding hematology, with studies published as early as 1964.[20]

Given the unique anatomic features of marine mammals, a physical examination is more limited in comparison to terrestrial mammals. However, blood sampling is often feasible and easily obtainable, providing an opportunity to pursue a suite of diagnostics, of which hematology is considered minimum baseline data. General principles of hemogram interpretation across mammalian species apply to marine mammals. The hemogram is useful for detection of systemic inflammation, guidance for additional diagnostic testing, and patient monitoring during treatment.

Blood collection in marine mammals can be challenging and trained behaviors have become essential tools. Venipuncture sites of marine mammals are well described in the literature. Common blood sampling sites include ventral fluke vasculature, the ventral peduncle where the fluke veins converge, and the central vein of the dorsal fin in cetaceans (dolphins and whales);[25] the caudal gluteal vasculature and jugular vein in otariidae (true sea lions); the plantar veins and the extradural sinus in phocidae (seals); the extradural sinus and interdigital hind flipper veins in odobenidae (walruses);[9,21] and jugular and sublingual veins in polar bears (*Ursus maritimus*).[3] Anticoagulants include EDTA or lithium heparin which may be used for blood collection for CBC, with EDTA preferred. Sodium citrate is used for fibrinogen and hemostasis testing. Given the incomplete intrinsic coagulation pathway in cetaceans, thrombin-activator blood sampling tubes are recommended when serum is to be collected, since blood samples without thrombin additive may clot slowly, resulting in potential artifactual changes and variability in test results.[27] In situations in which only a small blood volume can be acquired (e.g., logistical challenge, difficult restraint), a whole blood sample of minimum 1.25 mL can be collected in sodium citrate. Considering the dilution factor of 1.1, this sample can be used for CBC, excluding erythrocyte sedimentation rate, and then centrifuged to obtain plasma for fibrinogen and serum chemistry excluding calcium, sodium, and total carbon dioxide.[27]

ERYTHROGRAM

Marine mammals have the largest mammalian erythrocytes (RBC) compared to terrestrial mammals, with only elephant RBCs ranging similarly in size to pilot whales (*Globicephala sp.*).[34] RBC sizes range from a minimum mean cell volume (MCV) of 106 fL in bottlenose dolphins

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up to MCV 183 fL in beluga whales (*Delphinapterus leucas*) in the toothed whales (Figure 122.1a–c). No information is available to date in the baleen whales. Compared to other marine mammal species, northern elephant seals (*Mirounga angustirostris*) have higher MCV, mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), and lower RBC counts, which is presumptively associated with their physiologic adaptation to deep diving requiring increased oxygen storage, a need that develops at time of weaning.[15] Polychromasia, assessed by blood film evaluation, appears to be mild in clinically normal pinnipeds, sea otters, and walrus, but is absent to minimal in clinically normal cetaceans. Erythrocyte morphology in health may include up to 50% codocytes in walrus[27] and occasional Howell–Jolly bodies and nucleated RBC (nRBC) in healthy cetaceans. Heinz bodies are uncommon in healthy marine mammal species other than the manatee (see Chapter 121).

Erythrocyte morphology in health at different life stages versus changes in disease and clinical significance have yet to be determined in marine mammal species. For example, prominent metarubricytosis in two captive-born neonatal bottlenose dolphin calves that died from different causes was associated with regenerative anemia of undetermined etiology; these two animals also showed increased erythropoiesis in bone marrow, liver, and spleen, and erythrophagocytosis, but it was unknown whether the erythroid regenerative response was normal for the life stage or could be attributed to disease.[4]

The erythrocyte sedimentation rate is readily available, cost-effective, and routinely included in marine mammal CBCs at in-house laboratories, as its clinical value in the diagnosis and monitoring of inflammatory disease in marine mammals has been anecdotally documented.[25,27] Erythrocyte sedimentation rate is reportedly higher in pilot whales and false killer whales compared to other cetaceans;[27] therefore, it is essential to consider species-specific ranges as for other diagnostic tests when interpreting results.

LEUKOGRAM

Leukocyte (WBC) morphology in marine mammals is generally comparable to other mammals, though there is some variability. Neutrophil morphology is similar across marine mammals and all have segmented nuclei. While pinnipeds, walruses, and polar bears have clear cytoplasm, bottlenose dolphins and other cetaceans may exhibit variable numbers of fine dust-like pink or orange-colored secondary granules similar to other ungulate species, e.g., giraffe, equine foals (Figures 122.2 and 122.3). The conventional band versus mature neutrophil definition of no segmentation being <66% of the width of the neutrophil nucleus is an established method of defining bands in mammalian hematology.[13] A stricter method that identifies a band neutrophil if any chromatin broader than a thin filament spanning between lobules has been established in marine mammals, particularly cetaceans[27] (Figure 122.4). Furthermore, a segmented neutrophil may be defined as having at least two lobes separated by filament(s)[25] (Figures 122.2–122.4). Given that more band neutrophils are identified by this method, clinically healthy cetaceans commonly have 1–3% band neutrophils, which may vary up to 5%.[27] This method of quantifying bands is believed to be more sensitive in detecting early inflammation and in monitoring cetaceans during treatment by marine mammal clinicians.

Small lymphocytes are similar in size and morphology to other mammalian species with clumped chromatin and scant pale basophilic cytoplasm. Atypical or reactive lymphocytes and plasma cells are infrequently found in healthy marine mammals (Figure 122.5).

Monocytes typically exhibit a reniform, bi or multilobed nucleus with smooth chromatin and pale to medium basophilic cytoplasm (Figures 122.2 and 122.3). Some monocytes may appear reactive or activated, i.e., have a variable number of cytoplasmic vacuoles with bluer cytoplasm, in apparently healthy marine mammals.

Eosinophils in marine mammals vary in size, number, and color. The cytoplasm may be slightly basophilic in bottlenose dolphins and Hawaiian monk seals which may be confused with toxic neutrophils (Figures 122.2d and 122.3d). Basophils are typically present in very low numbers or possibly absent in marine mammals. They vary significantly in granule number, size, and color intensity, but can be easily differentiated from neutrophils and eosinophils (Figures 122.2 and 122.3).

PLATELETS AND COAGULATION

Platelet concentrations in marine mammals are interpreted similarly to their terrestrial counterparts. Significant differences in platelet physiology are likely adaptations to diving (see Section VI). For example, although killer whales produce concentrations of thromboxane similar to other mammals, the low response of their platelets to thromboxane is presumed to be a protective mechanism for deep diving marine mammals that prevents clotting.[27] Pressure changes associated with diving do not result in platelet activation or changes in platelet numbers in killer whales.[26]

The predominant coagulation pathway in cetaceans is the extrinsic pathway through tissue factor activation, since most cetaceans are reportedly deficient or lacking factor XII (Hageman factor), thus resulting in a nonfunctional intrinsic pathway.[29,32] Consequently, activated partial thromboplastin time (aPTT) and activated coagulation time are comparatively prolonged when compared to other mammalian species, and both tests are considered inaccurate.

BONE MARROW COLLECTION AND EVALUATION

Due to the difficulty in accessing active representative bone marrow (especially in adult animals) and the

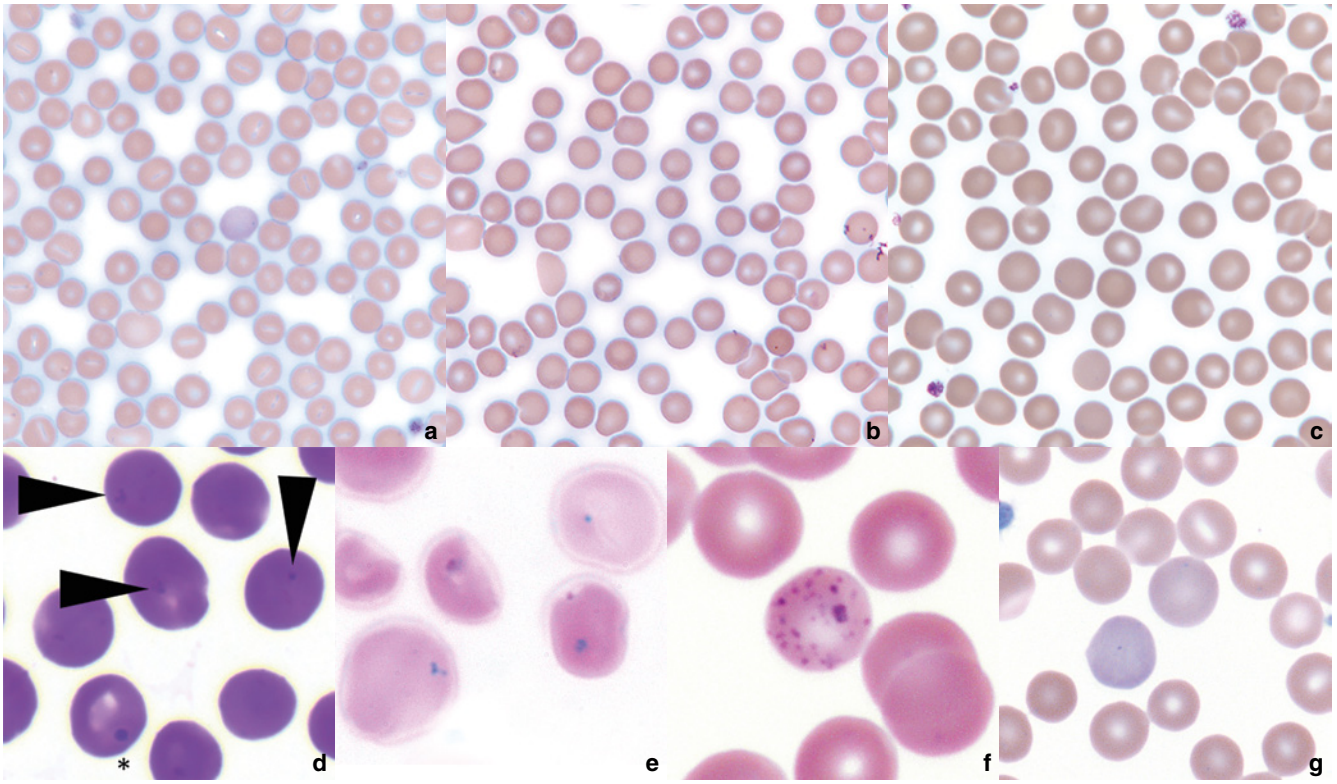


FIGURE 122.1 Erythrocytes from marine mammal species. (a) Bottlenose dolphin (*Tursiops truncatus*) RBCs; (b) Killer whale (*Orcinus orca*) RBCs; (c) Beluga whale (*Delphinapterus leucas*) RBCs. Bottlenose dolphin (*T. truncatus*): (d) Focal fine granular basophilic aggregates (arrowheads) and a Howell–Jolly body (asterisk) suggestive of siderocytes; (e) Turquoise-colored positive-staining aggregates consistent with siderotic inclusions using Prussian blue stain in the same patient sample; (f) Basophilic stippling; (g) Anisocytosis and polychromasia in a regenerative anemia. 1000×, modified Wright–Giemsa stain (unless otherwise indicated).

potential to cause harm to the marine mammal patient, bone marrow sampling should be performed only if considered clinically useful, under anesthesia, and in species in which bone marrow is more readily accessible, such as pinnipeds. Indications for bone marrow sampling are uncommon and include persistent cytopenias, leukocytosis with the suspicion of neoplasia, or presence of circulating cells with abnormal morphology as in other mammals. The underlying causes for such conditions may often be explained using clinical and medication history, and other clinicopathological changes. Sampling in live animals has the potential to cause harm to the patient and is invasive at the anatomic sites, including ribs and vertebra, and has only been used in pinnipeds under anesthesia.

Bone marrow sampling can be performed postmortem. Autolytic changes in carcasses >1 day old, bone marrow sample preparation technique, and the animal's age have to be considered when interpreting the specimen's quality for use in diagnosis. The proximal appendicular or axial skeleton is a common collection site in any mammalian species throughout life. Cetacean bone marrow can be challenging to obtain, since active bone marrow is mainly located in the axial skeleton, and vertebral bodies are difficult to access due to their dense internal structure. Bone saws are useful to access the bone marrow. Imprints for cytology and tissue sections for histopathologic evaluation may provide useful diagnostic information (Figure 122.6).

REFERENCE INTERVALS

Although there is abundant literature on hematology data from various marine mammal species in managed care and wild populations, few publications report hematologic reference intervals established in accordance with American Society of Veterinary Clinical Pathology guidelines.[10,24,30] Blood analyte databases with summarized data from various institutions with animal populations of the same species but exposed to different extrinsic factors, e.g., husbandry, nutrition, environment, etc., and likely using different analytical methodologies should not be used for medical interpretation. As in other species, any hematology data of an individual marine mammal needs to be interpreted in the context of history, administration of recent or current medications, and clinical examination findings. Many extrinsic and intrinsic factors in published hematology data have to be considered when applying data from published studies to an individual, such as analytical methodology and biological

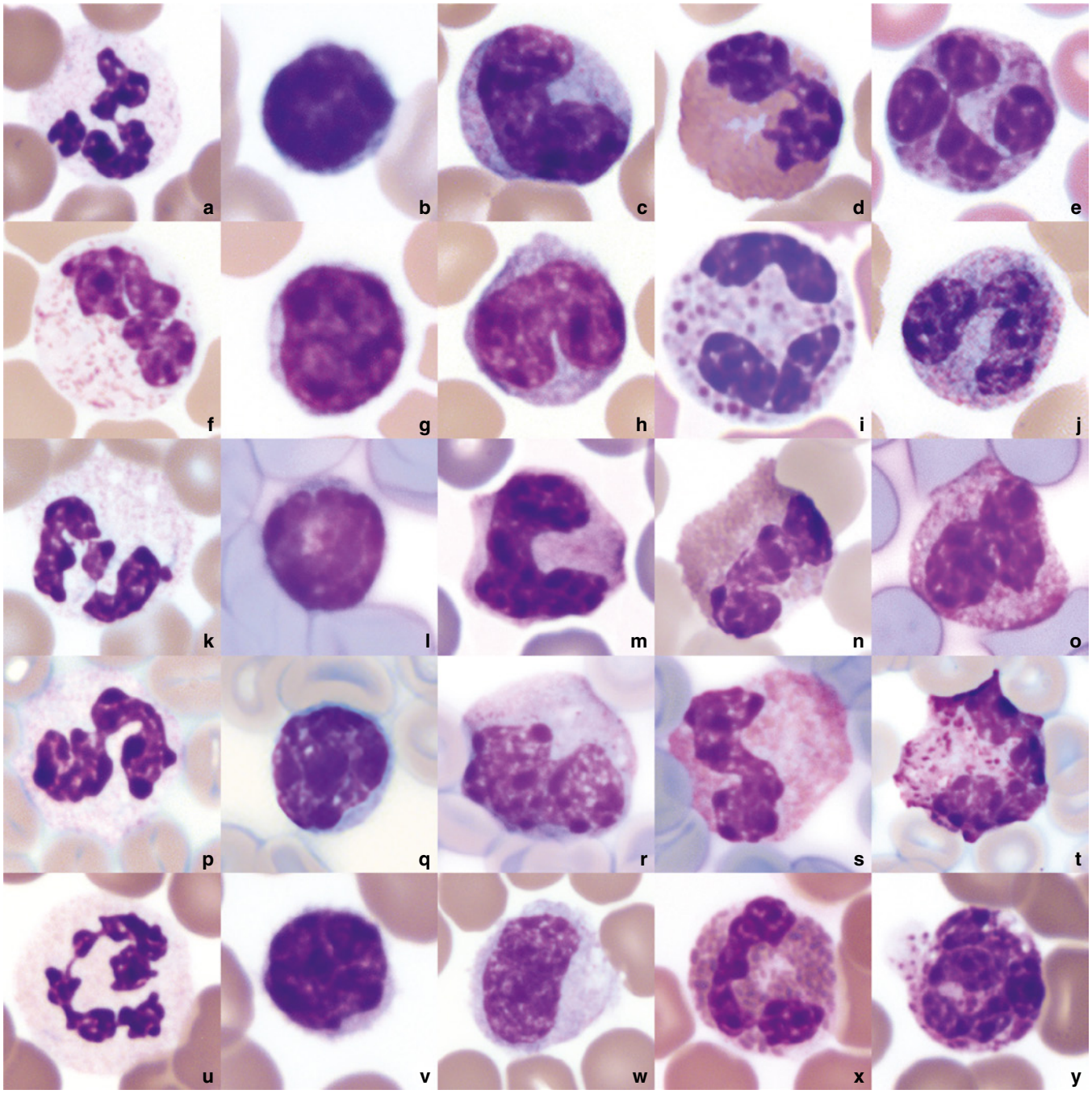


FIGURE 122.2 Normal cetacean leukocytes. Bottlenose dolphin (*Tursiops truncatus*): (a) Segmented neutrophil; (b) Small lymphocyte; (c) Monocyte; (d) Eosinophil; (e) Basophil. Atlantic spotted dolphin (*Stenella frontalis*): (f) Segmented neutrophil; (g) Small lymphocyte; (h) Monocyte; (i) eosinophil; (j) Basophil. Beluga whale (*Delphinapterus leucas*): (k) Segmented neutrophil; (l) Small lymphocyte; (m) Monocyte; (n) Eosinophil; (o) Basophil. Killer whale (*Orcinus orca*): (p) Segmented neutrophil; (q) Small lymphocyte; (r) Monocyte; (s) Eosinophil; (t) Basophil. Long-finned pilot whale (*Globicephala melas*): (u) Segmented neutrophil; (v) Small lymphocyte; (w) Monocyte; (x) Eosinophil; (y) Basophil. 1000×, modified Wright–Giemsa stain.

variables,[11] as well as inclusion criteria of animals in a study. Various partitioning factors have been documented in marine mammals, with the clinically relevant factors including age, sex, season, and habitat,[11,24] and physiological factors, e.g., fasting in northern elephant seals.[39]

Consistent methodology allows for the generation of population- and subject-based reference intervals, and the trends found over continuous routine wellness examinations can be evaluated. The use of subject-based reference intervals offers the best opportunity for close monitoring of an individual, observation of

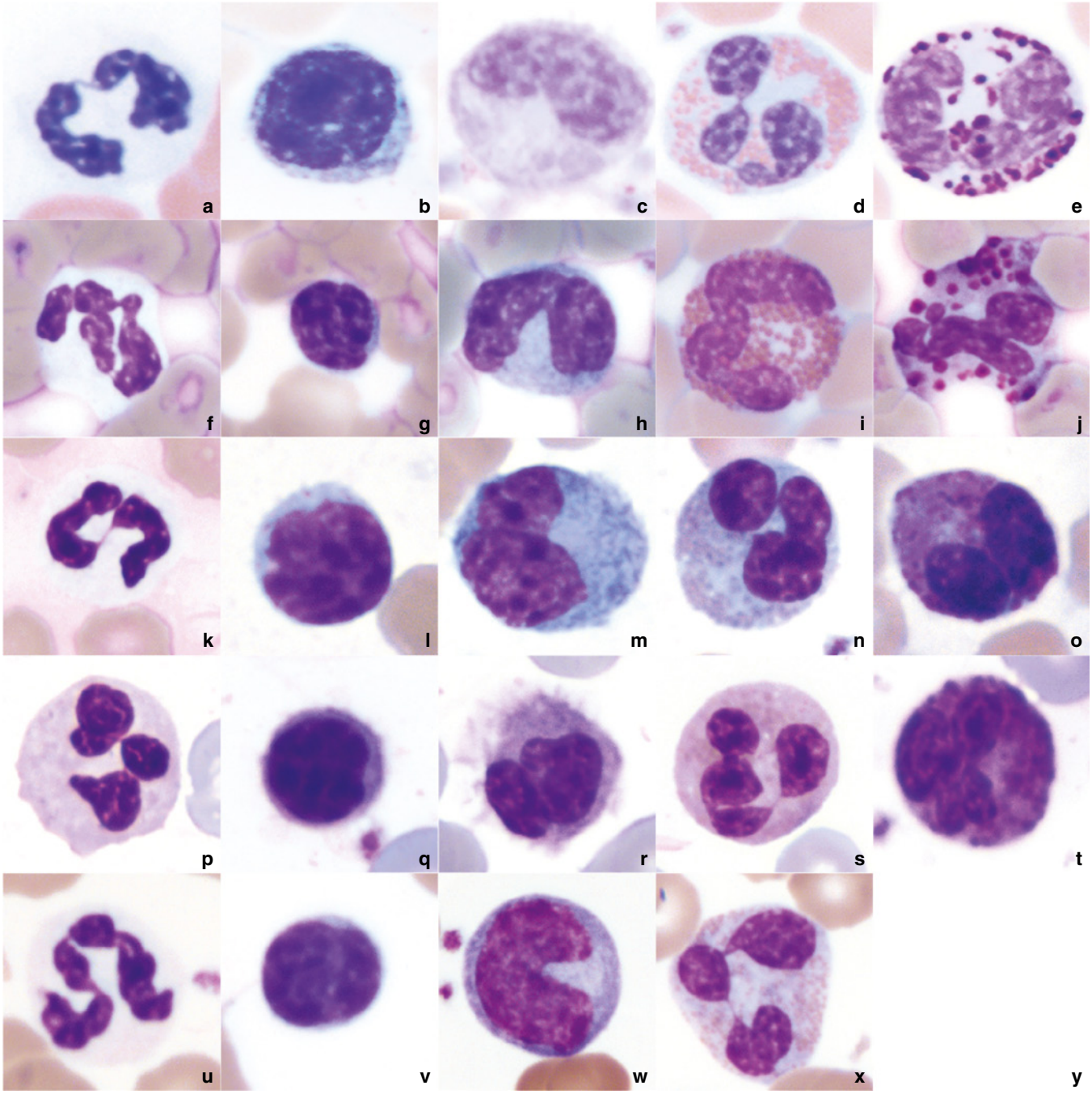


FIGURE 122.3 Normal pinniped leukocytes. Monk seal (*Neomonachus schauinslandi*): (a) Segmented neutrophil; (b) Small lymphocyte; (c) Monocyte; (d) Eosinophil; (e) Basophil. Harbor seal (*Phoca vitulina*): (f) Segmented neutrophil; (g) Small lymphocyte; (h) Monocyte; (i) Eosinophil; (j) Basophil. Northern fur seal (*Callorhinus ursinus*): (k) Segmented neutrophil; (l) Small lymphocyte; (m) Monocyte; (n) Eosinophil; (o) Basophil. Guadalupe fur seal (*Arctocephalus townsendi*): (p) Segmented neutrophil; (q) Small lymphocyte; (r) Monocyte; (s) Eosinophil; (t) Basophil. Steller sea lion (*Eumetopias jubatus*): (u) Segmented neutrophil; (v) Small lymphocyte; (w) Monocyte; (x) Eosinophil; (y) image not available. 1000×, modified Wright–Giemsa stain.

individual variability, and early detection of hematologic abnormalities during suspected illness. However, reference intervals created from a single animal's data may result in increased false positives (type 1 error), i.e., diagnosis of disease which is not present. Broader population-based reference intervals can be applied in the initial assessment of stranded animals, and then trends of the individual observed, with consideration of analytical error, e.g., allowable total error (see Chapters 138 and 140).

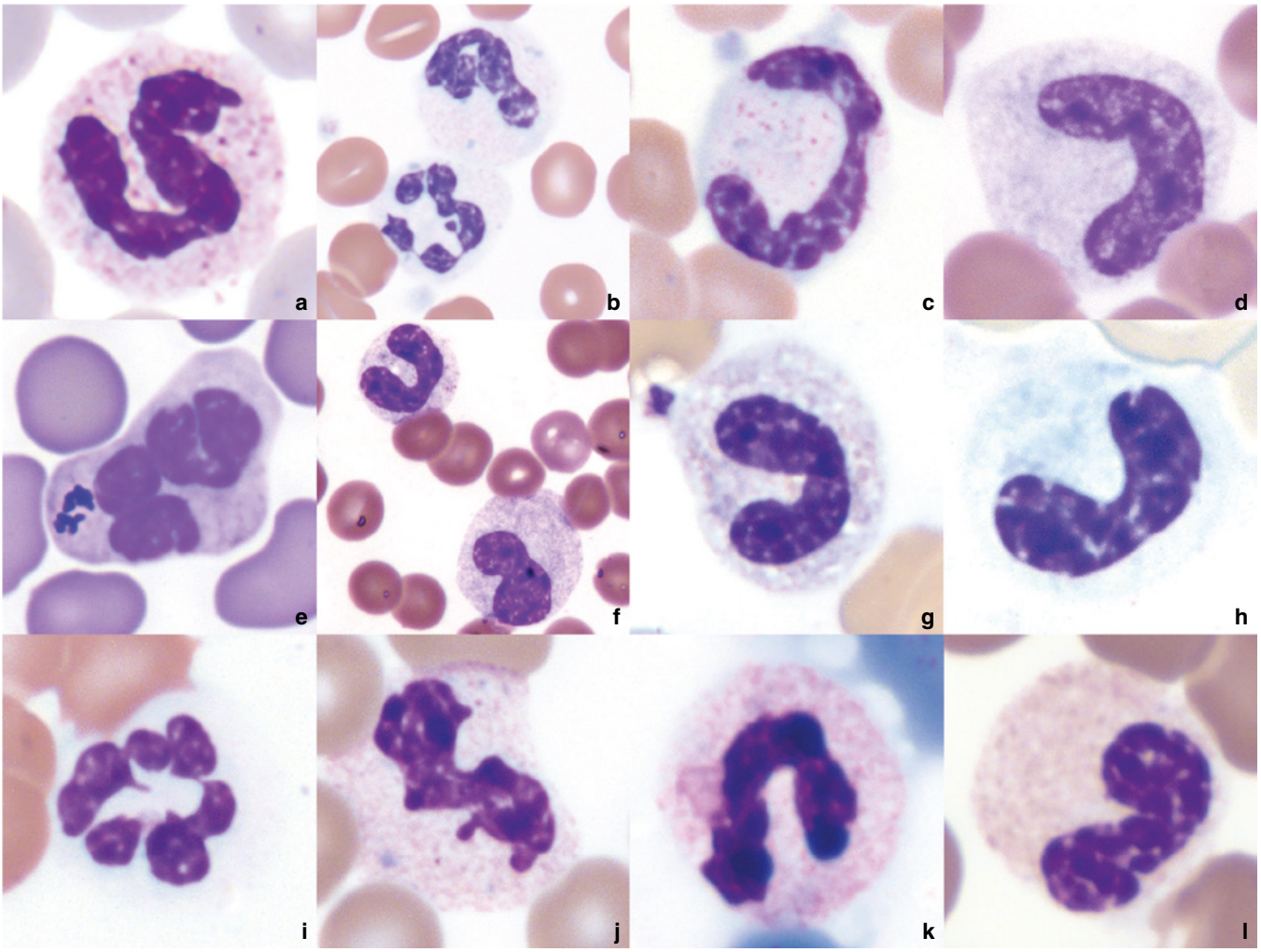


FIGURE 122.4 Abnormal neutrophil morphology in cetacean and pinniped blood. Bottlenose dolphin (*Tursiops truncatus*): (a) Band neutrophil; (b) Mildly toxic (1+) neutrophils, lower magnification to show comparison; (c) Moderately toxic (2+) band neutrophil; (d) Markedly toxic (3+) band neutrophil; (e) Neutrophil with phagocytized small cocci, blood culture identified *Peptostreptococcus sp.*; (f) markedly toxic (3+) band (top) and metamyelocyte (bottom), lower magnification to show comparison; (g) Guadalupe fur seal (*Arctocephalus townsendi*): markedly toxic (3+) band neutrophil; (h) Monk seal (*Neomonachus schauinslandi*): moderately toxic (2+) band neutrophil; (i) Steller sea lion (*Eumetopias jubatus*): hypersegmented neutrophil; (j) Beluga whale (*Delphinapterus leucas*): mildly toxic (1+) band neutrophil; (k) Blainville's beaked whale (*Mesoplodon densirostris*): band neutrophil; (l) Long-finned pilot whale (*Globicephala melas*): band neutrophil. 1000×, modified Wright–Giemsa stain, unless otherwise specified.

Partitioning Factors

The growing embryo and fetus induce significant hematologic changes in the mother. In killer whales (*Orcinus orca*), decreasing Hct, Hgb, and RBC concentration during gestation were linked to plasma volume expansion prior to parturition, which resolved postnatally.[28] Physiologic leukocytosis around parturition has been attributed to increased neutrophil concentration in killer whales, with a sharp drop after parturition.[28] In bottlenose dolphins, while WBC counts remain stable, lymphocytes and platelets reportedly decrease during the 2nd and 3rd semester, and evidence of hemoconcentration may be observed including increased RBC count, Hgb, and Hct.[1] These nonpathologic hemogram changes need to be considered when interpreting blood data from pregnant killer whales and bottlenose dolphins.

HEMATOLOGIC MANIFESTATION OF DISEASE

Mild anemia of chronic disease associated with inflammation is seen in marine mammals. Underlying mechanisms and causes for anemia are presumptively similar to those of other mammals and interpretation is based on similar principles (see Section III). Anemia of inflammatory disease (AID) is typically nonregenerative; however, mild increases in anisocytosis, polychromasia, nRBC, and mild basophilic stippling reflect slight

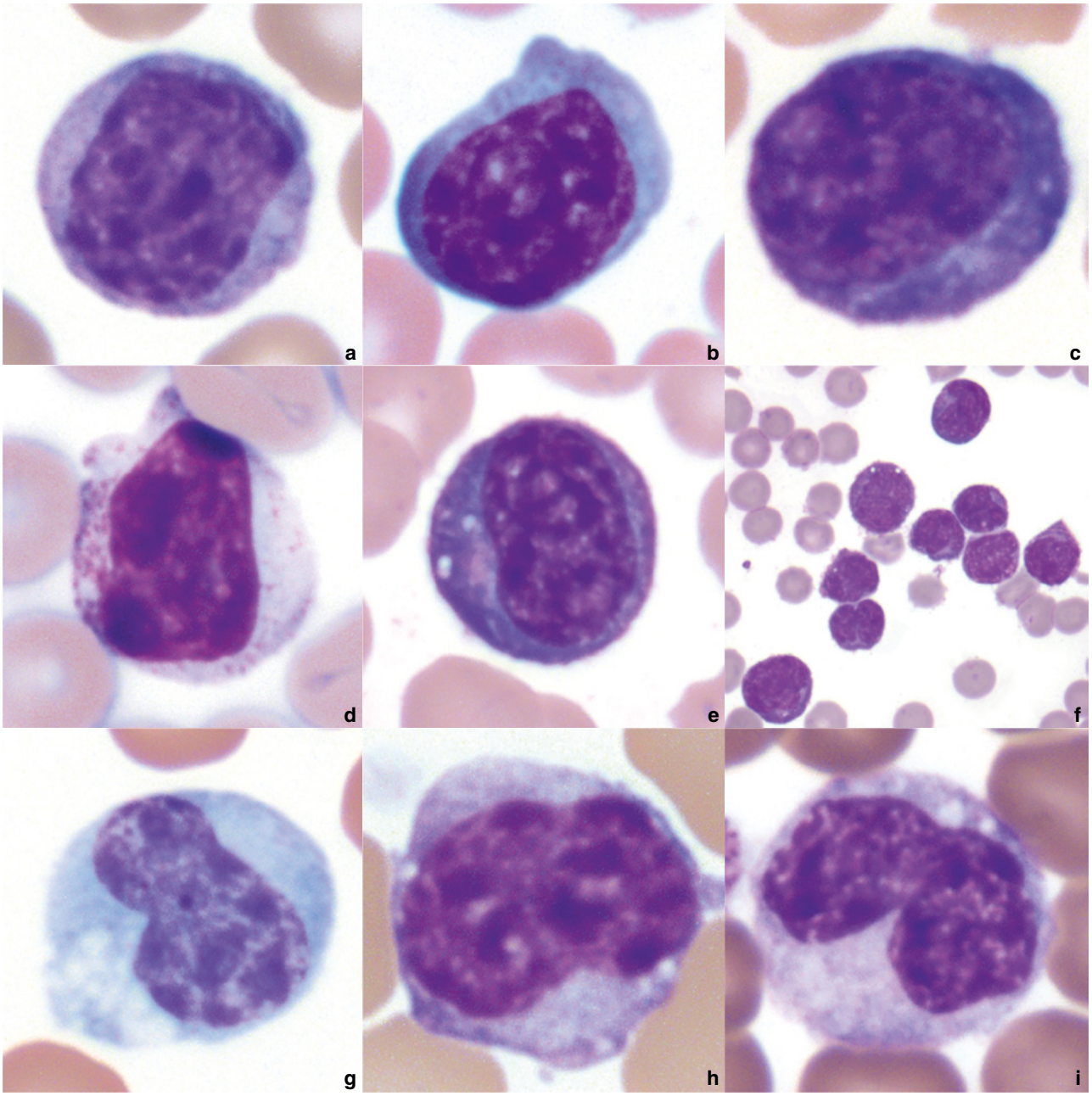


FIGURE 122.5 Abnormal lymphocyte and monocyte morphology in cetacean and pinniped blood. Bottlenose dolphin (*Tursiops truncatus*): (a) Reactive lymphocyte; (b) Atypical lymphocyte; (c) Atlantic spotted dolphin (*Stenella frontalis*): reactive lymphocyte; (d) Killer whale (*Orcinus orca*): granular lymphocyte; (e) California sea lion (*Zalophus californianus*): reactive lymphocyte; (f) California sea lion (*Z. californianus*): circulating neoplastic lymphocytes in patient with confirmed intestinal lymphoma, 20× objective; (g) Bottlenose dolphin (*T. truncatus*): reactive monocyte; (h) Atlantic spotted dolphin (*S. frontalis*): reactive monocyte; (i) Long-finned pilot whale (*Globicephala melas*): reactive monocyte. 1000×, modified Wright–Giemsa stain.

increases in reticulocyte counts, which may become evident during recovery from anemia of inflammatory disease in the cetacean patient (Figure 122.1f,g). Since speed, degree, and confounding factors of erythroid regenerative responses are not well understood in marine mammals and difficult to study given the lack of controlled research settings, trends of the individual patient may be followed over time during treatment. Blood loss anemia is a rather rare condition in marine mammal medicine, but may result in prominent reticulocytosis after several days of blood loss, similar to other mammalian species, as observed by the authors in bottlenose dolphins. Bone marrow depression is diagnosed based on exclusion of other etiologies of anemia. Bone marrow aspirate and/or biopsy is possible in pinnipeds, but is often not feasible in other taxa (see Chapter 141).

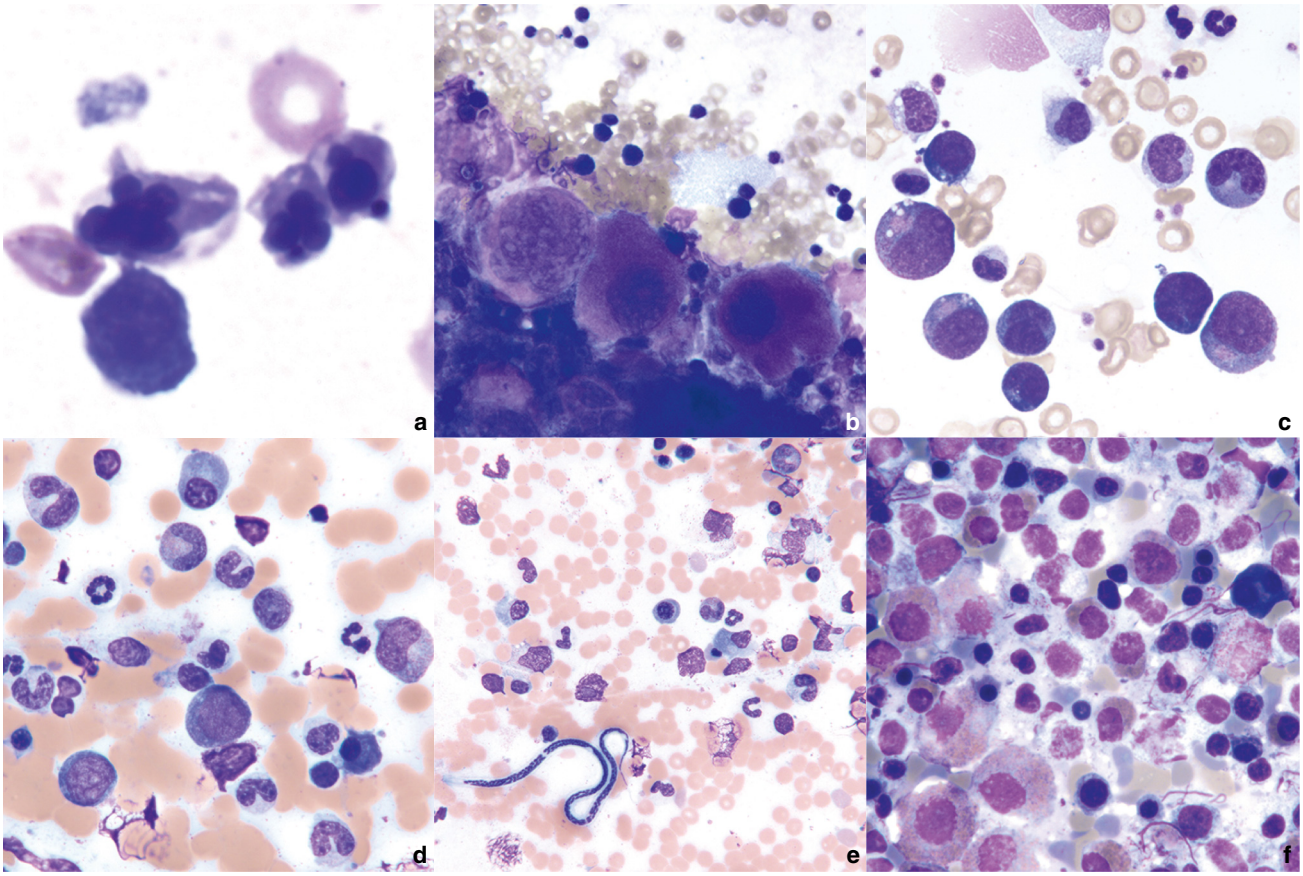


FIGURE 122.6 Bone marrow cytology. Guadalupe fur seal (*Arctocephalus townsendi*): (a) Dysplastic changes in erythroid precursors, possibly from antimicrobial effects and/or nutritional deficiencies; (b) Megakaryocytic hyperplasia in patient with inflammatory leukogram and peripheral thrombocytosis; (c) and (d) Myeloid hyperplasia; (e) California sea lion (*Zalophus californianus*): microfilaria likely from hemodilution; (f) Bottlenose dolphin (*Tursiops truncatus*) with confirmed brevetoxicosis: postmortem collected bone marrow imprint showing cell lysis from autolysis, yet overt eosinophilic hyperplasia. Modified Wright–Giemsa stain.

Increased numbers of Heinz bodies in two bottlenose dolphins were presumptively attributed to oxidative damage from liquid amber tree toxicity and/or hydrogen peroxide administration by the authors. Bottlenose dolphins in managed care can develop iron storage disorder, which is not believed to be hereditary and associated with diet, among other factors.[18] The authors have observed low numbers of siderocytes, confirmed by Prussian blue, in blood smears from captive dolphins with clinically suspected or confirmed iron storage disorder (Figure 122.1d,e). The presence of siderocytes should prompt evaluation of serum iron indices.

The leukogram is a key CBC component in marine mammals, as it assesses inflammation from infectious and noninfectious causes. Common infectious causes of inflammation include secondary bacterial infections, pneumonia, and gastrointestinal disorders in marine mammal species.[25,35] Considerations for noninfectious causes include urogenital carcinoma in California sea lions, oral carcinomas in bottlenose dolphins, or other noninfectious causes of tissue necrosis. In response to inflammatory disease, marine mammal leukocyte morphologic changes may include neutrophil left-shifting, toxicity, monocytic reactivity, atypical or reactive lymphocytes, and plasmacytoid cells.

Given the importance of early diagnosis of inflammation in clinical settings, inflammatory markers are routinely included during wellness examinations and treatment monitoring. The most important inflammatory markers include all leukogram analytes, especially WBC count, polymorphonuclear bands, toxic change, platelets (e.g., inflammatory thrombocytosis), Hgb, PCV, fibrinogen, erythrocyte sedimentation rate, serum alkaline phosphatase, acute phase proteins, and serum iron.[25,28] Serum amyloid A (SAA) has been proven clinically useful in the diagnosis of systemic inflammation in northern elephant seals.[33] The clinical utility of SAA and other acute phase proteins to detect systemic inflammation in cetaceans is still under investigation.[6] Toxic change in neutrophils is frequently observed with inflammatory disease or other causes of accelerated neutrophil activation or production and is evaluated as for other mammals. Harvey (2012) proposed a scale of

1+–3+ for the evaluation of toxic changes, which appears to introduce less interobserver variability than other grading scales published in the literature (e.g., 1+–4+)[13] (Figure 122.4).

Inflammatory disease in marine mammals often results in leukocytosis. However, cetaceans with clinically suspected low-grade inflammation frequently only display mild left-shifting and minimal toxicity without neutrophilia. Identification of these mild changes can be important. For example, low-grade pneumonia in cetaceans can be life-threatening.[25] Anecdotally, leukopenia in cetaceans can be a poor prognostic indicator when observed as a trend in an individual, reflecting acute, overwhelming inflammation and possible sepsis. Drug reaction should be considered in patients with anemia of undetermined cause, neutropenia, and bone marrow dyscrasia as documented in other mammals.[36] Neutropenia has been observed in cetaceans after sulfadiazine administration; similarly to dogs in which aplastic anemia has been documented.[37]

Algal toxin exposure has resulted in increased eosinophil concentrations with domoic acid exposure in sea lions.[9] Eosinophilic hyperplasia has been seen in the bone marrow of a bottlenose dolphin with confirmed exposure to brevetoxin (Figure 122.6f). Hemoparasitic agents described in the following text should also be ruled out in cases presenting with eosinophilia.

Increasing trends in platelet concentrations and large platelets are often observed with inflammatory disease, similar to terrestrial mammals in which one of the most common causes of thrombocytosis is cytokine production in inflammatory disease[23] (Figure 122.6b). Thrombocytopenia is uncommon in marine mammals. Thrombocytopenia may be artifactual resulting from platelet clumping, and blood film evaluation should be performed before considering differential diagnoses. It has been observed by the authors in a few northern elephant seals with disseminated intravascular coagulation from *Otostrongylus circumlitis* infestation, in cetaceans reacting to sulfadiazine,[37] and as a sequelae to bone marrow suppression in various species.

Clinically significant infectious agents may be observed upon blood film review. Filaroid parasites in seals, specifically *Acanthocheilonema (Dipetalonema) spirocauda*, the so-called seal heartworm, can cause clinical disease and mortality. *Acanthocheilonema odendhali* is found in sea lions (*Otariidae*), but considered an incidental finding, similar to canine heartworm infection, which is rarely observed in phocidae and otariidae under managed care[19] (Figure 122.6e). Additionally, the authors observed an intracytoplasmic focal aggregate of cocci in a neutrophil from a bottlenose dolphin that became suddenly anorexic with a WBC count change from 6.2 to 14.9 × 10^9/L within 24 hours (Figure 122.4e). This observation prompted blood culture from which *Peptostreptococcus sp*. was isolated. After adjustment of antimicrobials based on the sensitivity panel, the dolphin recovered rapidly. The definitive cause of the septicemia was not determined, but was presumably associated with chronic pulmonary disease.

Few case reports of leukemia in marine mammals exist. Lymphoma has been documented in cetaceans and pinnipeds, with a description of leukemic manifestation in two harbor seals; herpesvirus-associated B-cell lymphoma in California sea lions; eosinophilic leukemia in a Pacific white-sided dolphin (*Lagenorhynchus obliquidens*); and myelogenous leukemia in a walrus (*Odobenus rosmarus*) and a bottlenose dolphin (*T. truncatus*).[23,36] Reactive lymphocytes, which exhibit increased size, dark cytoplasmic basophilia, smooth chromatin, rare to occasional nucleoli, and a heterogenous appearance, are frequently observed in marine mammals with inflammatory disease (Figure 122.5). When atypical lymphocytes with similar morphologic features are observed during blood film evaluation, the challenge is similar to other mammalian species. The differentiation of reactive (heterogenous lymphoid population) versus neoplastic (monomorphic) lymphocytes is challenging at best with light microscopy alone and any diagnosis must be based on morphologic features, absolute lymphocyte counts, and other laboratory, clinical, and imaging findings (see Section V). Currently, there is no published literature on the use of cluster of differentiation (CD) markers in the diagnosis of leukemia; although, in the authors' experience, CD markers that are functional in dogs are often cross-reactive in pinnipeds. Markers have been used to assess immune functions in cetacean species and could potentially be used in the diagnosis of leukemia.[8,30]

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CHAPTER 123

Hematology of Galliformes

JULIE PICCIONE and JESSICA HOKAMP

Hematopoiesis
Blood Collection
Erythron
Leukon
Thrombon
Reference Intervals and Partitioning Factors
  Chicken
  Turkey
  Quail
  Pheasant
  Guinea Fowl and Peafowl
Bone Marrow Collection and Evaluation
Hematologic Disease
  Anemia
  Inflammation
  Neoplasia
  Coagulopathy
  Hemoparasites

Acronyms and Abbreviations

AGP, α_1 acid glycoprotein; ALV, avian leukosis virus; ASVCP, American Society for Veterinary Clinical Pathology; CV, coefficient of variation; H:L ratio, heterophil:lymphocyte ratio; HSC, hematopoietic stem cell; IL, interleukins; SAA, serum amyloid A.

Members of the order Galliformes are heavy-bodied, ground-feeding birds, referred to as gallinaceous birds. There are hundreds of birds in this order including chicken (*Gallus gallus*), turkey (*Meleagris gallopavo*), quail (*Coturnix sp.*), pheasant (subfamily Phasianinae), grouse (subfamily Tetraoninae), peafowl (*Pavo sp.*), ptarmigan (*Lagopus sp.*), partridge (subfamily Perdicinae), and guinea fowl (family Numididae). They are often raised for egg and meat production or used for recreational hunting. Backyard poultry flocks are increasing in the USA and are typically maintained for food production, show, or as companion animals. These backyard operations can be a source of zoonotic infectious diseases and may have detrimental effects on neighboring food animal operations, as biosecurity protocols may be inadequate.[20] Hematologic evaluation of these animals is a relatively inexpensive method to evaluate health.

HEMATOPOIESIS

The majority of research on avian hematopoiesis has been performed in gallinaceous birds, mostly chicken and quail-chick chimeras. In the embryo, hematopoiesis begins in the yolk sac with a single wave of hematopoietic stem cells (HSC) that will then colonize developing organs, including, in approximate chronologic order, the intra-aortic clusters, para-aortic foci, spleen, liver, bursa of Fabricius, thymus, and bone marrow.[57] Even though the bone marrow is the last organ colonized by hematopoietic precursor cells in the embryonic period, it contains multiple cell lineages by day 12 of embryogenesis. Embryogenesis lasts approximately 21 days in the chicken. Definitive HSC that maintain hematopoiesis during the bird's life will develop within the chicken embryo (versus yolk sac, as previously thought).[16]

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In the first 2–5 days of embryogenesis, HSC are originally derived from the yolk sac.[57] During this early stage, these HSC are committed primarily to erythropoiesis.[57,67] However, the yolk sac will also contain primitive lymphoid cells, granulocytic and macrophagic precursor cells, and likely thrombocytes within 10 days of embryogenesis.[16,67] These cells will eventually enter circulation and colonize other organs and locations (e.g., bone marrow, spleen, liver).

Within the chicken embryo, hematopoietic cells are first confidently identified in the para-aortic foci and intra-aortic clusters around day 3 of embryogenesis.[57,67] Para-aortic foci are groups of cells located in loose mesenchymal tissue ventral to the aorta.[16] These para-aortic foci provide cells for the first wave of thymic colonization and for bone marrow colonization. Interestingly, definitive para-aortic foci have not been identified in mammals.[16] Intra-aortic clusters are areas of hematopoietic cells protruding into the aortic lumen. These cells are CD45- and CD41-positive (pan-leukocytic antigen and HSC marker, respectively).[16] They appear to be predominantly erythroid precursors; however, myeloid and thrombocyte precursors are also present.[57,67]

Between days 8 and 15 of embryogenesis, a rudimentary spleen is colonized by stems cells from the yolk sac, intraembryonic para-aortic foci, and intra-aortic clusters. The spleen then begins to support erythropoiesis, granulopoiesis, and lymphopoiesis.[57,67] After hatching, the spleen will become one of the major secondary lymphoid organs. During this same time period, epithelial cells in the bursa of Fabricius (primary lymphoid organ) attract stem cells from the yolk sac, spleen, liver, bone marrow, and blood.[57,67] The bursa is responsible for the amplification and differentiation of B-lymphocytes via bursin, which is the major differentiating hormone of B cells.[16] After sexual maturity, the bursa will involute and B-cell amplification and differentiation will primarily occur in the spleen and intestinal germinal centers.

The thymus, the other primary lymphoid organ in birds, is responsible for T-lymphocyte differentiation. T-cell progenitors colonize the thymus in three waves. The first wave occurs at day 6 of embryogenesis, with T cells traveling from the para-aortic foci to the thymus.[16] The bone marrow supplies the progenitors for the second and third waves of thymic colonization, which occur at days 12 and 18 of embryogenesis, respectively.[16] T-cell differentiation is regulated by thymopoietin, which is synthesized by thymic epithelial and dendritic cells.[16] Certain T-cell lymphocyte populations can also be found in the spleen and intestinal epithelium during embryogenesis.[16,57]

The bone marrow contains multiple cell lineages by 12 days of embryogenesis, and after hatching it is the primary site of hematopoiesis. Bone marrow contains specialized niches for different areas of hematopoiesis, likely based on cell–cytokine interactions.[57] The bone marrow primarily produces erythroid cells; however, granulocyte and lymphocyte development also is initiated and may occur here.[67] Unlike in mammals, the liver plays only a minor role in embryonal hematopoiesis, supporting erythropoiesis and granulopoiesis until shortly after hatching.[57] However, the liver does serve as an important organ for extramedullary hematopoiesis in post-hatched gallinaceous birds.

Cytokines are small proteins that are secreted by one cell and cause an effect on another cell. Cytokines involved in hematopoiesis are better defined in mammalian species than in birds. Colony-stimulating factors (CSF) have been shown to act on pluripotent HSC and initiate the development of myeloid cells in chicken. Similar to mammals, there are three identified CSFs in chicken: granulocyte CSF, macrophage CSF, and granulocyte–macrophage CSF.[32] Interleukins (IL), specifically IL-2, IL-15, and IL-21, regulate T-cell proliferation.[32] IL-18 stimulates proliferation of CD4+ T cells.[22] Other cytokine function is poorly defined or varies between manuscripts. A detailed review of cytokines and chemokines is provided elsewhere.[32,57]

In mature gallinaceous birds, erythropoiesis occurs in the vascular sinuses of the bone marrow (medullary sinus lumen), primarily in the vertebrae and long bones.[67] Erythropoiesis is stimulated by avian erythropoietin, which is structurally distinct from mammalian erythropoietin. Similar to other birds, RBCs develop from rubriblasts to prorubricytes to rubricytes to polychromatophils to mature RBCs (metarubricytes). Rubricytes may be separated into basophilic rubricytes followed by early and late polychromatophilic rubricytes.

As discussed in the earlier text, avian bone marrow is primarily dedicated to erythropoiesis and the number of reserve mature granulocytes is very low compared to most mammals. Granulocytes develop in the extravascular space of bone marrow. Due to limited granulocyte reserves, left shifts in the heterophil line occur frequently with inflammation. In adults, the spleen is presumed to be one of the primary locations for lymphopoiesis and granulopoiesis; however, extramedullary hematopoiesis can also occur in the liver and kidney. The role of bone marrow in avian lymphopoiesis is unclear, but there may be small stem cell populations originally from the thymus and bursa that are capable of self-renewal.[67] Granulocyte maturation appears to be similar in gallinaceous birds as in other birds with the following stages: myeloblasts, progranulocytes, myelocytes, metamyelocytes, band granulocytes, and mature granulocytes. Monocytopoiesis is not well described in gallinaceous birds and monocyte precursors are likely similar in appearance to granulocytic precursors.

There is limited literature on thrombocyte proliferation and maturation in gallinaceous or other birds. Thrombocytes are presumed to mature in the vascular sinus of bone marrow and are derived from thromboblasts, which are difficult to differentiate from other blast cells with light microscopy alone. In general, thrombocytes are most commonly found in areas of hemodilution in bone marrow samples. Further study is needed.[67]

BLOOD COLLECTION

The brachial (wing) vein is the most commonly used vessel for blood collection in chicken and turkeys. The wing vein is superficial and easily visible in animals over 2 weeks of age. A quick reference for assisted or unassisted venipuncture in poultry can be found on the Food and Agriculture Organization of the United Nations' website (http://www.fao.org/docrep/005/ac802e/ac802e0a.htm). The maximum blood volume that should be collected is 1% of the body weight (e.g., absolute maximum of 23 mL in 5 lb/2.3 kg chicken). Blood smears should be prepared at the time of collection by well-trained staff. Both wedge (a push preparation using two glass slides) and coverslip (a pull preparation using two square glass coverslips aligned catty-corner) techniques are appropriate for gallinaceous birds (see Chapter 137).

The use of anticoagulants is necessary for hematologic evaluation. In general, EDTA and lithium heparin are the most common options in veterinary medicine, and both are considered appropriate for gallinaceous species, as no significant lysis has been documented to date. PCV and plasma protein can be lower and lymphocyte numbers and platelet clumping can be higher in lithium heparin versus EDTA samples.[25] Therefore, the same anticoagulant should be used to decrease variation.[25] Cellular morphology and staining are adequate with both EDTA and lithium heparin, though EDTA may be preferred to avoid heparin artifact.[25]

ERYTHRON

In general, RBC mass in gallinaceous birds can be evaluated through PCV, hemoglobin (Hgb), or with RBC counts. Packed cell volume is determined by centrifugation of microhematocrit tubes. Hemoglobin can be measured by standard instrumentation after cell lysis and removal of nuclei. Manual RBC counts can be performed with standard erythrocyte Unopette® methods using a hemocytometer. When using automated methods, both WBCs and RBCs will be counted; however, WBC numbers are small in comparison and will likely not skew RBC counts.[67] Mean cell volume (MCV) and mean cell hemoglobin concentration (MCHC) can be calculated from the PCV/RBC count and Hgb/RBC count, respectively. Mean cell hemoglobin (MCH) does not provide diagnostic information.

Mature Galliform RBCs are oval to elliptical with smooth eosinophilic cytoplasm and a centrally located, oval nucleus with clumped to condensed chromatin (Figure 123.1). Chicken RBCs have a more rounded nucleus compared to other gallinaceous birds. The vast majority of RBCs should be mature and relatively uniform in appearance. Minimal anisocytosis (variation in RBC size) is expected and clinically insignificant. Mature chicken RBCs average 12x6 μm.[67] RBCs in white-breed and bronze-breed turkeys average 14x8.5 μm.[6] In Japanese quail and adult peafowl, RBCs average 11x7 μm and 14x7 μm, respectively.[51,65] Most papers cite an older reference reporting that chicken RBC life span has a maximum of 35 days. It does not appear that this has been confirmed experimentally in recent times.[64]

Polychromasia is common on blood smears due to the shorter half-life of avian RBCs. Blood smears from normal gallinaceous birds typically contain 1–5% polychromatophils.[67] With standard Romanowsky stains, polychromatophils are slightly larger and more oval than mature RBCs with increased cytoplasmic basophilia and a round to slightly oval, lighter staining nucleus with less-condensed chromatin. In addition, due to intravascular and intrasinusoidal erythropoiesis, rubricytes are seen in low numbers on peripheral blood smears.[67] Circulating rubricytes also appear to be more common in chicken and turkeys than in other birds, though are uncommon in blood smears from the normal animal and increase with erythroid regeneration.[67] Rubricytes are round and contain basophilic to grayish cytoplasm with a centrally located round nucleus with clumped chromatin.

LEUKON

Nonmammalian RBCs and thrombocytes are nucleated, limiting the use of automated analyzers. Although some studies have shown good agreement between manual and automated granulocyte counts, lymphocyte and monocyte counts are considered inaccurate.[36] Therefore, manual WBC-counting methods (hemocytometer) are primarily used in the diagnostic setting.[12] Natt–Herrick solution can be used to provide a direct WBC count.[11] The eosinophil Unopette® method can provide an indirect WBC count when used in conjunction with WBC differential counts. WBC estimates can also be calculated from the monolayer during blood smear examination. WBC estimates should not be performed by novice microscopists. Estimates should only be used when there is even WBC distribution in the blood smear.

The avian WBC count can be determined directly with a Neubauer-ruled hemocytometer, pipettes, and Natt–Herrick solution. The advantage of this technique is that all WBCs are visualized and enumerated. The disadvantages are that this WBC counting technique is labor intensive, care must be taken to differentiate thrombocytes from small lymphocytes, and WBCs may be swollen and distorted if analysis of the diluted specimen is delayed. This method works well for an in-house laboratory, but may prove difficult with samples that are mailed to a diagnostic laboratory due to increased lysis over time.

The WBC count can be quantitated indirectly with the Unopette® system. In this technique, heterophils and eosinophils are selectively stained by phloxine dye and counted in a hemocytometer. Based on the WBC differential cell count of a stained blood smear, the combined heterophil and eosinophil hemocytometer count is mathematically corrected to account for basophils and

mononuclear cells (e.g., lymphocytes and monocytes) that were not counted. Thus, the total WBC count per microliter of blood has been determined indirectly. Advantages of this system are that heterophils and eosinophils are directly identified and counted in a hemocytometer; thus, there is no confusion between lymphocytes and thrombocytes because they are not counted with the hemocytometer. However, hemocytometer counts are labor-intensive, a greater potential for mathematical errors exists, and WBC distribution on stained blood smears may not be random.

One can estimate WBC counts from the stained blood smear by multiplying the average number of WBCs per field of view by a given factor; the factor is usually approximately the square of the objective's magnification, e.g., 2500 for the 50× oil-immersion objective. This factor can vary with the optics of different microscopes. Advantages include the stability of the blood film and the labor-efficient process of WBC count estimation. The major disadvantage is that the WBC count is estimated instead of quantitated. Thus, poor smear technique resulting in cell lysis and maldistribution may influence the estimate, and the coefficient of variation (CV) may be significantly higher. Coefficient of variation for hemocytometer-derived WBC counts (eosinophil Unopette® method) is approximately 13% as opposed to 30% for estimated WBC counts from stained blood smears.[35] The variation reported with these techniques may vary with some labs reporting CV that are significantly higher. However, the latter technique may be useful when the lability or specimen volume would otherwise preclude hematologic study. Ultimately, the future of avian hematology depends on the development of semi-automated or automated techniques to determine avian WBC counts in a rapid, accurate, economical, and labor-efficient manner.

Heterophils follow lymphocytes in abundance and an H:L ratio <1 is expected in most gallinaceous birds (except guinea fowl). Heterophils should have colorless cytoplasm with numerous eosinophilic, slightly muddy-appearing to refractile elliptical granules. Nuclei are less lobulated than expected in mammalian specimens; mature heterophils have two to three lobes and coarse chromatin[65,67] (Figure 123.1). Mature heterophils are approximately 13 μm in diameter in chicken and approximately 10 μm in diameter in turkeys, quail, and peafowl.[6,51,65,67]

Lymphocytes are the predominant leukocyte in most gallinaceous birds, which is in contrast to other avian species.[30,52,54,67] Guinea fowl do appear to be heterophil predominant, though a steroid induced leukogram should be ruled out.[46,47] Both small and medium lymphocytes may be found in blood smears from normal animals.[65,67] In adult quail, small lymphocytes and medium lymphocytes measure approximately 5 and 8 μm in diameter, respectively.[65] Small lymphocytes have a scant amount of medium basophilic cytoplasm, a round nucleus with condensed chromatin, and a high nuclear to cytoplasmic ratio (Figure 123.1). In contrast to small lymphocytes, medium-sized lymphocytes have increased amounts of pale to light basophilic cytoplasm and a round to slightly indented nucleus with coarse to condensed chromatin. No clinical significance is typically ascribed to the proportion of medium to small lymphocytes. However, it is important to ensure that small lymphocytes are not classified as thrombocytes and that medium lymphocytes are not classified as monocytes. Rare reactive lymphocytes, with dark blue cytoplasm and clear Golgi zones, are also expected in circulation in normal birds, especially after vaccination.

Monocytes are the largest cells in circulation. They contain a modest amount of light to medium basophilic to blue–gray, often vacuolated cytoplasm and a round to kidney-shaped to pleomorphic nucleus with clumped or smooth chromatin (Figure 123.1). Fine azurophilic cytoplasmic granules may be seen.[67] In chicken and quail, monocytes are 13–14 μm in diameter, while peafowl and turkey monocytes are smaller averaging 11 μm in diameter.[6,51,65,67]

Eosinophils in gallinaceous birds are similar to other mammalian and avian species in that they have pale blue cytoplasm with numerous eosinophilic round granules and a lobulated nucleus with condensed chromatin (Figure 123.1). Eosinophil granules stain very pale with Wright–Giemsa in some gallinaceous birds, such as certain turkeys, pheasants, and grouse. Variation with types of Romanowsky stain may also occur. Eosinophils in gallinaceous birds are approximately 10–12 μm in diameter.[6,51,65,67] Eosinophils can be differentiated from heterophils by their distinct granules, lack of refractile appearance, and typically darker-staining or more visible nucleus.

Basophils contain numerous dark basophilic granules, which often obscure the nucleus (Figure 123.1). Basophils are more commonly observed in blood smears from avian species than mammalian species and may be morphologically similar to mast cells. Basophils in gallinaceous birds are typically smaller than other WBC, measuring 9–12 μm in diameter.[6,51,65,67]

When needed, cytochemical stains can be used to distinguish WBCs in chicken and likely other gallinaceous birds. Acid phosphatase, peroxidase, Sudan Black B, and Luna methods may be used to differentiate chicken eosinophils from heterophils. Periodic acid–Schiff and Grimelius stains differentiate chicken thrombocytes from small lymphocytes, as thrombocytes stain positively with periodic acid–Schiff and Grimelius, while lymphocytes do not.[63] The ultrastructural features of blood cells in select gallinaceous birds have been discussed extensively in other sources.[37–40]

Studies using conventional flow cytometry with fluorescent markers to perform WBC counts and differentials in chicken and quail have generally shown good correlation with blood smear evaluation and manual differential counts in research settings.[8,41,56] In one study, there was poor correlation between monocyte counts obtained with flow cytometry versus manual differentials.[8] Use of fluorescent markers is not common in diagnostic and clinical settings, likely due to cost and long-term instability of cell markers during shipping and transit.

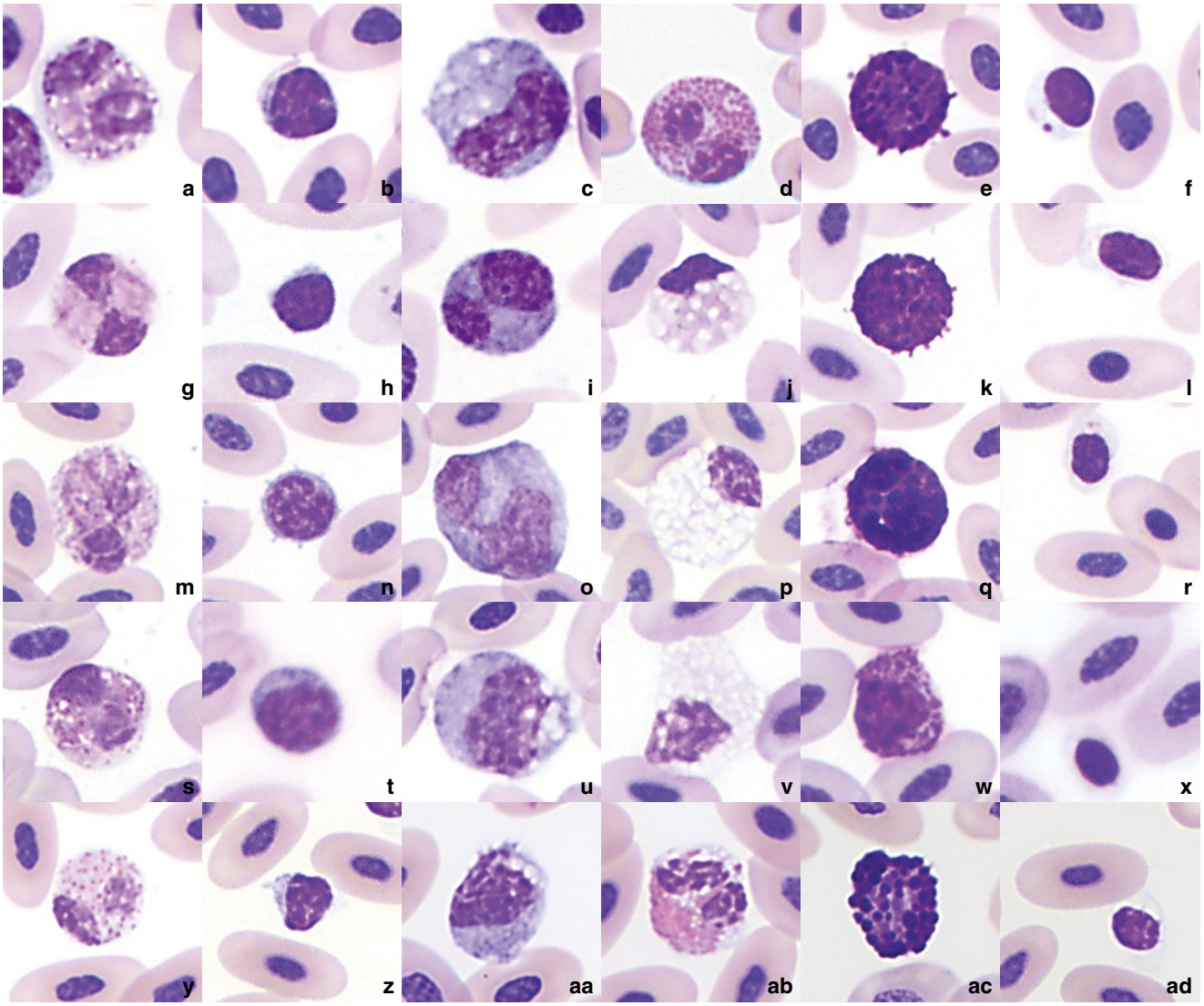


FIGURE 123.1 Peripheral blood of healthy, adult gallinaceous birds. Wright–Giemsa. Chicken (*Gallus gallus domesticus*): (a) Heterophil; (b) Lymphocyte; (c) Monocyte; (d) Eosinophil; (e) Basophil; (f) Erythrocyte and thrombocyte. Ocellated turkey (*Meleagris ocellata*): (g) Heterophil; (h) Lymphocyte; (i) Monocyte; (j) Eosinophil; (k) Basophil; (l) Erythrocyte and thrombocyte. Ring-necked pheasant (*Phasianus colchicus*): (m) Heterophil; (n) Lymphocyte; (o) Monocyte; (p) Eosinophil (with non-staining granules); (q) Basophil; (r) Erythrocyte and thrombocyte (Source: Sample provided by Gladys Porter Zoo). GrouseAttwater's prairie chicken (*Tympanuchus cupido attwateri*): (s) Heterophil; (t) Lymphocyte; (u) Monocyte; (v) Eosinophil; (w) Basophil; (x) Erythrocyte and thrombocyte. (Source: Sample provided by the Caldwell Zoo). Peacock (*Pavo cristatus*): (y) Heterophil; (z) Lymphocyte; (aa) Monocyte; (ab) Eosinophil; (ac) Basophil; (ad) Erythrocyte and thrombocyte. (**Source:** Sample provided by the Fort Worth Zoo)

THROMBON

Thrombocytes are smaller and rounder than RBCs and contain a small amount of clear to pale blue cytoplasm with occasional small clear vacuoles or pink granulation surrounding a small round, condensed nucleus (Figure 123.1). Thrombocyte counts in gallinaceous species appear to be consistent with other avian species at approximately 30,000 cells/μL.[24,33] During blood smear examination, it is important to view the feathered edge for large thrombocyte clumps which falsely decrease the thrombocyte estimate. Thrombocytes are reported to be approximately 4 μm in diameter in quail.[65]

REFERENCE INTERVALS AND PARTITIONING FACTORS

The American Society for Veterinary Clinical Pathology (ASVCP) has published guidelines for the determination of de novo reference intervals in veterinary species.[19] Deviations from the published recommendations

may occur due to study publication prior to ASVCP guidelines, inclusion of low numbers of target species, difficulty in proving health in avian species, inappropriate statistical analysis, inclusion of outliers, etc. Baseline blood profiles from individual animals in times of health may aid in interpretation of future blood profiles. This would likely only be practical in well-managed, small backyard flocks or zoo settings.

Variations in hematologic values can be seen with many partitioning factors (species, breed, sex, age, environmental conditions, reproductive status, etc.). It should be noted that many studies evaluating reference intervals and partitioning factors were performed in different areas of the globe and published in various journals. A detailed review of this literature is beyond the scope of this chapter; however, some guidelines are provided in the following text.

Chicken

There are several CBC changes that are expected during growth of chicken. From 1–7 weeks of age, Hgb, PCV, and MCV increase and WBC count decreases with increasing age.[23] Following this, PCV and Hgb appear clinically similar in Polbar chicken (Poland) across three time points: 8, 12, and 18 weeks of age.[24] By contrast, RBC counts, PCV, and Hgb concentration increased substantially between 10 and 35 weeks of age in clinically normal breeding cocks.[34] In the Polbar chicken, WBC counts decreased from 37 × 10^3/μL to 27 × 10^3/μL between 8 and 18 weeks of age due to decreases in absolute heterophil and lymphocyte counts.[24] A steroid-induced leukogram is possible. In the breeding cocks, WBC counts also decreased substantially, mostly due to decreases in lymphocyte counts.[34]

Significant differences in CBC values between male and female chicken have been found. Hgb concentration and PCV are higher in male than female chicken for several breeds.[7,49,58] Studies evaluating total RBC counts, MCV, MCH, and MCHC in males versus females offer no clear consensus.[24,49] The differences between these values may not be significant or clear between breeds, testing modalities, and environments. In general, total WBC counts are often statistically higher in male versus female chicken.[7,49] However, clinically significant differences may only be apparent in some breeds (e.g., American Red Cornish and Vanaraja breeds).[7,49] Multiple studies have shown that absolute or relative lymphocyte numbers are higher in female chicken than in male chicken.[24,58] In the peak egg-laying period, RBC counts, PCV, Hgb, and WBC counts have been shown to decrease in hens.[62]

Hemoglobin concentration and PCV have been shown to be statistically and clinically different between different breeds (as well as geographic locations) of chicken.[7,48] For example, average Hgb in Aseel (Indian) chicken was 12.9 g/dL compared with 8.7 g/dL in Rhode Island Red (English) chicken with both sites using a comparable cyanmethemoglobin method.[48]

Turkey

One reference interval study published prior to ASVCP guidelines had no mention of outlier removal, and the reported intervals should not be used for medical diagnosis.[9] Although sex has been shown to be a major partitioning factor in CBC findings of chicken, clinically significant differences have not been found between male and female turkeys.[21,54] When both male and female turkeys aged from 19–23 weeks to 30–32 weeks, heterophil numbers increased, lymphocyte counts decreased, and thus the H:L ratio increased.[54] PCV may decrease during egg production/laying and a PCV greater than 45% may be an indicator of poor egg production.[50] PCV in turkeys can drop substantially in the summer (41% to 35% in male turkeys between winter and summer, respectively).[21]

Quail

In some studies evaluating presumably healthy quail, there are substantial discrepancies in total WBC count, with means ranging 2.6–16.8 cells × 10^3/μL.[4,13,43] MCV decreased dramatically at 2 weeks of age, followed by a steady increase until at least 6 weeks of age in Japanese quail (*Coturnix japonica*), similar to a pattern seen in chicken.[4,23] Also similar to chicken, PCV is higher in adult male than female quail.[3,18] PCV differences between sexes are also clinically significant with males averaging 42–52% and females averaging 33–46%.[3,18]

Pheasant

Selected reference intervals for 6-week-old farm-raised Chinese ring-necked pheasants (*Phasianus colchicus*) are provided in Table 123.1.[15] While this study followed guidelines recommended by the ASVCP, some limitations to consider include: cardiac collection, administration of euthanasia solution, and unspecified anticoagulant (presumed EDTA).[19] In addition, while all animals were considered clinically healthy and gross and histopathologic evaluation of many tissues was unremarkable, some animals had substantial abnormalities in serum chemistry results.[15] It is possible that the low reference values represent a mild anemia due to chronic disease.

RBC count, PCV, and WBC count increase as pheasants age from around 30–360 days, which contrasts somewhat with other gallinaceous birds.[24,33,34,53] These changes can be clinically significant with average PCV increasing from 28% to 36% and 34% to 44%, depending on the study.[33,53] However, WBC counts may substantially decrease within time points between 30 and 360 days of age.[26,33,52] Interestingly, one study showed markedly increased WBC counts in both males and females in November (averaging 26 × 10^3/μL) versus March (averaging 9 × 10^3/μL).[26] However, these animals were also aging from approximately 180 to 300 days during this comparison, so the variables were not isolated.[26]

Table 123.1 Selected Galliformes hematology reference intervals. RBC counts were determined using hemocytometer, PCV by centrifugation of microhematocrit tube, Hgb using the cyanmethemoglobin method, and MCV was calculated. Reference interval generation was in compliance with ASVCP guidelines.

	Japanese quail[a] (*Coturnix japonica*)	Chinese ring-necked pheasants[b] (*Phasianus colchicus*)
RBC (× 10^6/μL)		2–3.6
PCV (%)	34–55	28–40
Hgb (g/dL)	9.6–18.4	
MCV (fL)		91–164
Activated coagulation time (sec)	<180	
Prothrombin time (sec)	10–14	

[a] 12 male and 12 female (n = 24), 22-week-old, clinically healthy Japanese quail were housed in British Columbia, Canada and had unremarkable postmortem histopathology. Blood was collected from unsedated birds' jugular veins and the sample was placed in heparinized tubes or tubes with no anticoagulant.[68]
[b] 84 or 107 (depending on analyte), 6-week-old, male and female Chinese ring-necked pheasants were housed in Minnesota, USA and had clinically healthy and unremarkable postmortem histopathology. Blood was collected from the heart after sedation with euthanasia solution given IM and was placed in tubes with unknown anticoagulant (presumed EDTA).[15]

Similar to chicken, male pheasants have higher RBC counts, PCV, Hgb, and MCHC than females.[26] Significant decreases in PCV, RBC counts, and Hgb and increases in total WBC, lymphocyte, and basophil counts can be seen during peak egg laying.[30,55] These changes are likely clinically significant; in one study, average RBC count decreased from 3.5 × 10^{12}/L to 2.6 × 10^{12}/L).[30] The decrease in RBC values during peak egg-laying has also been observed in chicken; however, in contrast to pheasants, chicken appear to have decreased WBC counts during peak egg-laying.[62]

Guinea Fowl and Peafowl

In contrast to other gallinaceous birds, guinea fowl appear to be heterophil-predominant, not lymphocyte-predominant.[46,47] Additionally, WBC counts of guinea fowl appear to be lower than other birds with two studies demonstrating average WBC counts of 1.4 and 3.7 × 10^3/μL in apparently healthy birds of different ages.[46,47] As guinea fowl age from 8 to 24 weeks, Hgb and WBC count increase significantly.[46] Unlike chicken, there are no consistent differences in RBC count, PCV, Hgb, or total WBC counts between male and female guinea fowl.[1,46,47] One study of guinea fowl from two different countries (Belgium and Ghana) showed statistically significant differences in RBC counts and PCV.[46] Another study showed significant differences between all hematologic values between five different varieties of guinea fowl in Nigeria.[1] Different guinea fowl production systems also appeared to cause variations in WBC counts as well as heterophil and lymphocyte proportions.[47] One study in peafowl, published before ASVCP guidelines, has no mention of outlier removal and the reported intervals should not be used in medical diagnosis.[51]

BONE MARROW COLLECTION AND EVALUATION

Bone marrow evaluation can provide valuable information in cases of nonregenerative anemia, heteropenia, persistent thrombocytopenia, pancytopenia, and dysplastic changes. The proximal portion of the tibiotarsal bone is most commonly used for antemortem bone marrow aspiration in gallinaceous birds. The femur and sternum may also be used, but may have variable quantities of fat and there is the concern for perforation of the air sacs at the sternum/keel. Fresh smears should be prepared at the time of collection by well-trained staff. When utilizing a diagnostic laboratory, the following samples should be submitted to provide the most accurate results: freshly prepared bone marrow smears, recent CBC data (within 24 hours) or whole blood for CBC, and bone marrow in EDTA (large birds) (see Chapter 141).

There are limited studies on the cytologic evaluation of bone marrow aspirates in the chicken; however, the composition of chicken bone marrow has been extensively evaluated.[10] In healthy adult chicken, the bone marrow cellularity is high (>70% cells to fat in marrow particles) (Figure 123.2a). The M:E ratio in healthy adult chicken is approximately 1:1 (Figure 123.2b). Mature cells should outnumber immature cells in all cell lines (Figure 123.2c). Certain cell types are difficult to definitively identify in bird bone marrow, such as early thrombocyte and monocyte precursors. Aggregates of variably sized lymphocytes are more commonly identified in birds than in some mammals.[16,44] Other cells that may be visualized in bone marrow aspirate samples include osteoblasts, osteoclasts, plasma cells, and macrophages. Cell distribution can vary with disease; examples are provided in the following text (Figure 123.2d).

Bone marrow cytologic evaluation has also been evaluated in clinically healthy Japanese quail and partridge with no overt hematologic abnormalities.[44,66] The mean M:E ratio is 0.4 in Japanese quail and 1.3 in partridge.[44,66] Erythroid cells predominate (76%) in Japanese quail, while slightly more than half of the cells (52%) are myeloid in the partridges studied.[44,66] The cellularity of samples is typically high, ranging from 60–90% cells to fat.[44,66] In Japanese quail, bone marrow findings can vary by age, with increasing RBC and thrombocyte precursor cells with advanced age.[44] There are no significant

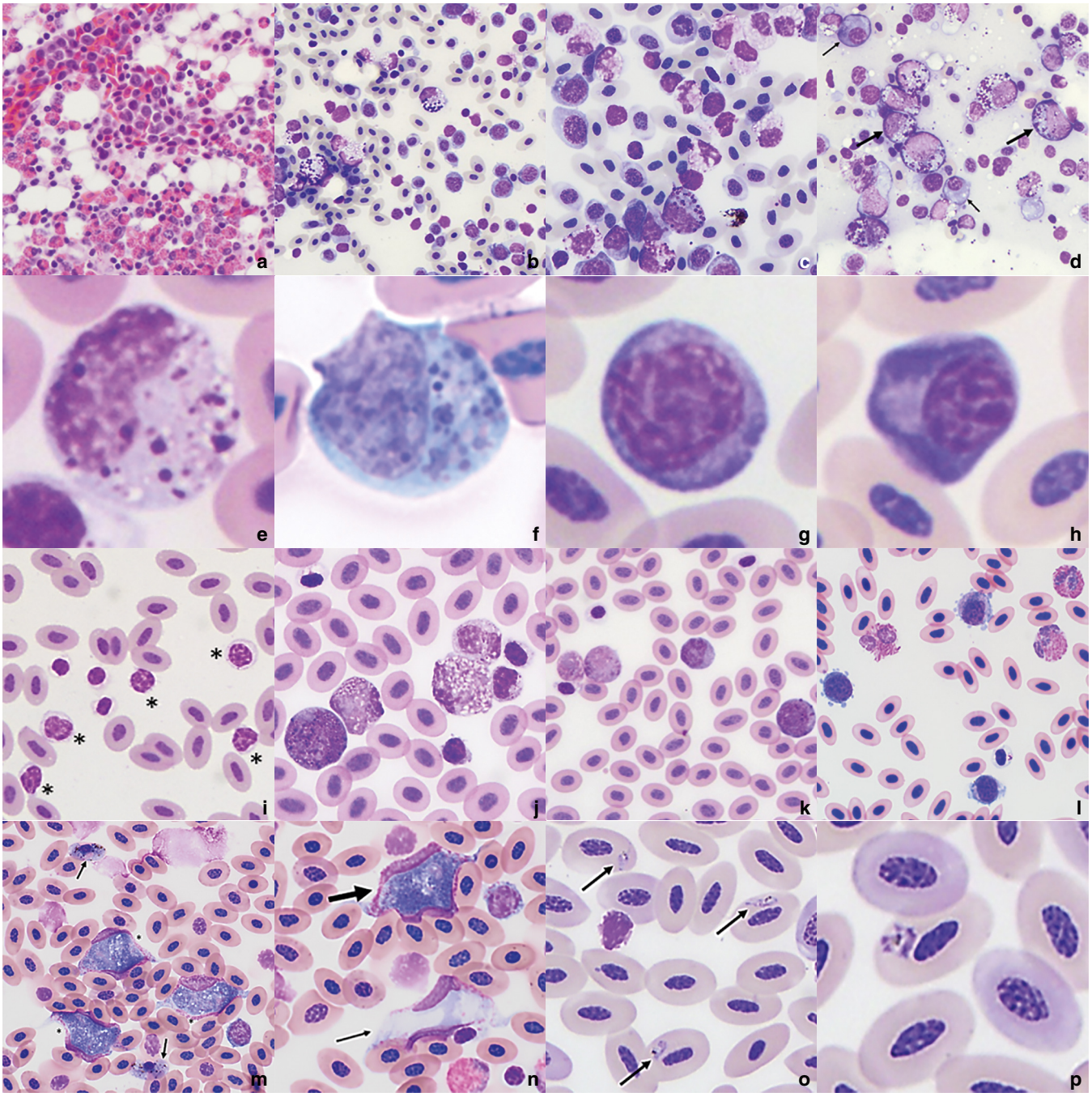


FIGURE 123.2 Bone marrow and abnormal cytomorphology from Galliformes. Samples are stained with Wright–Giemsa unless otherwise noted. Chicken (*Gallus gallus domesticus*): (a) Normal bone marrow, core biopsy, H&E; (b) and (c) Normal bone marrow impression; note M:E ratio is approximately 1–1.5 (Source: Samples provided by Dr. Erin Edwards); (d) Myeloid hyperplasia and plasmacytosis secondary to *Staphylococcus sp*. synovitis and osteomyelitis, bone marrow. Myeloid precursors (large arrows) and plasma cells (small arrows) are increased (Source: Sample provided by Dr. Erin Edwards); (e) Toxic heterophil metamyelocyte with increased basophilic cytoplasm and dark purple granules; (f) Toxic heterophil myelocyte (Sources: images provided by Drs. Nina Zitzer and Gregory Having). Golden pheasant (*Chrysolophus pictus*): (g) Reactive lymphocyte; (h) Plasma cell (Source: Sample provided by Gladys Porter Zoo). Chicken (*Gallus gallus domesticus*): (i) Lymphoid leukosis in an ALV-positive chicken; note increased numbers of small to intermediate lymphocytes (*) and mild to moderate anemia (Source: Slide provided by Dr. Holly Minard); (j) and (k) Myelocytic myeloid leukosis; note immature myeloid precursors and dysplastic change in the heterophils; (l) T-cell leukemic lymphoma in a chicken with confirmed Marek's disease; note immature lymphocytes and anemia (Source: Image provided by Dr. Gary Lee). Turkey (*Meleagris ocellata*): (m) *Leukocytozoon* gametocytes (*) and *Hemoproteus* (arrows); (n) *Leukocytozoon* macrogametocyte (thick arrow) and *Leukocytozoon* microgametocyte (thin arrow) (Source: Images provided by Dr. Francisco O. Conrado). Attwater's prairie chicken (*Tympanuchus cupido attwateri*); (o) Trophozoites and gametocytes consistent with *Plasmodium pedioecetae*; (p) *Plasmodium pedioecetae* meront (**Source:** Sample provided by Drs. Kristin J. Bondo and Blake Grisham).

differences between bone marrow findings in male and female Japanese quail or partridge.[44,66]

HEMATOLOGIC DISEASE

Anemia

When PCV and/or Hgb concentration fall below an accurate reference interval, the anemia should then be classified as regenerative or nonregenerative by quantifying immature RBCs. Immature RBCs can be seen on standard blood smears, as polychromatophils and polychromasia can be documented. When a more accurate indicator of regeneration is needed, reticulocyte counts can be performed with new methylene blue preparations (see Chapter 23). With new methylene blue, reticulocytes show a distinct cytoplasmic ring of granular basophilic material (aggregates of RNA) surrounding the nucleus.[65] Rubricytes may also be enumerated either in percent or per 100 WBC. A nonregenerative anemia is presumed when gallinaceous birds have <5% polychromatophils, <3% reticulocytes, or <3 rubricytes per 100 WBC. It can take up to 3 days for a regenerative response to be detected in peripheral blood, so the duration of anemia should be considered prior to classification. In strongly regenerative anemias or certain hematopoietic disorders, rubriblasts, prorubricytes, and rarely mitotic figures may also be seen in circulation.

In general, regenerative anemias are seen with blood loss and hemolysis. Blood loss can be caused by trauma, internal and external parasitism, intestinal disease, and toxins (e.g., brodifacoum rodenticide).[68] Common internal parasites that may cause anemia in gallinaceous birds include capillariasis, severe ascaridiasis, cecal threadworms (*Trichostrongylus tenuis*), and chronic coccidiosis (*Eimeria tenella*, *Eimeria necatrix*, or *Eimeria brunetti*).[29] External bloodsucking parasites of poultry are numerous and include various species of fleas (e.g., *Echidnophaga gallinacea*), ticks (e.g., *Argas persicus*), mites (e.g., *Dermanyssus gallinae*, *Ornithonyssus sylviarum*), mosquitoes, biting midges (e.g., *Culicoides sp*.), certain flies (e.g., *Simulium spp*.), and bedbugs (*Cimex lectularius*).[29] Causes of hemolysis include *Plasmodium sp*. infestation, bacterial sepsis, and toxins (lead, zinc, etc.). Nonregenerative anemias can be seen with anemia of chronic inflammation, certain bacterial diseases (salmonellosis, chlamydiosis, aspergillosis, avian tuberculosis, *Mycoplasma gallisepticum*), iron deficiency due to chronic blood loss, viral disease (chicken anemia virus, reticuloendotheliosis virus, avian leukosis virus (ALV), Marek's disease), and leukemia.[27,42] Polycythemia, or increased PCV above an accurate reference interval, is rarely reported in gallinaceous birds. Mildly increased RBC counts and PCV can be seen with excitement, stress, or dehydration and are nonspecific clinical changes seen with many etiologies, including poor management. Significant erythroid regeneration in the face of a normal to mildly increased PCV may be observed as a sequela of significant respiratory disease.

Inflammation

Inflammation can cause several changes in peripheral blood such as leukocytosis or leukopenia, altered WBC differential (including heteropenia, heterophilia, lymphocytosis, monocytosis), left shift of heterophils, toxic change, and reactive lymphocytes. In general, band heterophils should be very rare in healthy adult gallinaceous birds. Increased H:L ratio in gallinaceous birds can be seen with inflammation, stress, egg-laying, or in certain age groups and should not be used as the sole indicator of inflammation.[4,5,30,49,50] H:L ratios are also reported to vary between different chicken breeds when determined by conventional flow cytometry using the fluorescent markers anti-CD45-PE, anti-CD4-Alexa 700, and anti-macrophage-fluorescein isothiocyanate (KUL01).[8] Studies using automated analysis should be replicated prior to interpretation.

Increased band heterophils (left shift) can be seen with severe, and often acute, inflammatory diseases of gallinaceous birds. Band heterophils have a more bilobed or horseshoe shaped nucleus with mild cytoplasmic basophilia and possibly decreased cytoplasmic granules. In addition, primary granules (round, purple granules) may be seen in the cytoplasm of band heterophils or earlier precursors such as myelocytes (Figure 123.2e,f). Quail under 1 week of age, and possibly other young birds, may have bilobed nuclei in their heterophils.[65] Toxic change in heterophils can occur with significant inflammation and increased peripheral demand for heterophils. In brief, toxic changes in heterophils can include increased cytoplasmic basophilia, decreased cytoplasmic granulation, indistinct vacuolation of the periphery of the cell, and primary basophilic rounded granules (Figure 123.2f).

Heterophilia is seen with a variety of etiologies including, but not limited to, stress, bacterial pneumonia or sepsis, chlamydiosis, systemic fungal infections, necrosis, and parasitic infestations (e.g., *T. tenuis*).[35] Severe heterophilia (>70 × 10^3/μL) may be associated with pneumonia, chlamydiosis, coelomitis, *Mycobacterium* infections, systemic aspergillosis, and, less commonly, leukemia.[27] Heteropenia is seen with acute, severe, or chronic infections (e.g., bacterial sepsis), certain medications (e.g., cyclophosphamide) or with bone marrow failure due to chicken anemia virus, leukosis, etc.[17,35,42]

Considerations for lymphocytosis include chronic fungal or parasitic disease, chronic viral infections, or lymphoid neoplasms. Increased numbers of reactive lymphocytes and plasma cells can be seen with antigenic stimulation from a variety of etiologies (e.g., infection, inflammation, and vaccination). Reactive lymphocytes are slightly larger and have a small amount of deeply basophilic cytoplasm. The nuclear chromatin of reactive lymphocytes remains mature (coarse to condensed) and there are no visible nucleoli (Figure 123.2g). In comparison, plasma cells have a small nucleus with clumped chromatin and a moderate amount of deep blue cytoplasm with a perinuclear clear zone or Golgi zone; and may also be counted in a differential cell count

(Figure 123.2h). Lymphopenia can be observed in gallinaceous birds with severe stress or with certain medications such as cyclophosphamide and corticosteroids.[17]

Monocytosis in gallinaceous birds is observed with chronic inflammatory processes. *Mycobacterium avium* infections, chlamydiosis, aspergillosis, and necrosis may produce severe increases in monocyte counts.[27,35] Eosinophilia is observed with parasitic infestations, foreign bodies, fungal disease, and chronic antigenic stimulation.[35] Basophilia is seen with parasitic infestations and with acute inflammation from a variety of etiologies.[35] Monocytopenia, eosinopenia, and basopenia are considered clinically insignificant.

The principal acute phase proteins that have been used to identify the acute phase response in chicken, and thus the innate immune response, are serum amyloid A (SAA), α_1 acid glycoprotein (AGP), and ovotransferrin.[45] Ovotransferrin and α_1 acid glycoprotein have long been established using radial immunodiffusion (Mancini method) or ELISA. The majority of investigations have studied the acute phase protein response in experimental models of infection or in response to bacterial endotoxin. For instance, Adler used *Salmonella typhimurium* lipopolysaccharide to demonstrate a response in AGP levels in chicken, which increased fourfold within 24 hours post treatment.[2] More recent investigations have shown that SAA has the greatest and most rapid increase in chicken with systemic inflammation of several etiologies, including to natural red mite infestation.[31] The ELISA assay for chicken AGP and SAA can be obtained from Life Diagnostics Inc (Westchester, USA). Validation according to the ASVCP guidelines is recommended (see Chapter 139).

Neoplasia

Leukemia in gallinaceous birds is often induced by viruses and referred to as leukosis. Common viral causes include ALV and Marek's disease virus. CBC findings vary dramatically depending on the virus, disease stage, species, patient age, etc. Additional diagnostic tests are typically needed to confirm which virus caused the neoplastic transformation. Although case reports of natural, spontaneous leukemia (nonviral induced) are lacking in gallinaceous birds, arguably they could occur.

The leukosis/sarcoma group encompasses a variety of retroviruses that induce neoplasms in chicken.[42] ALV is the most common virus in this group and can cause significant losses in poultry flocks. The most common neoplasm observed is lymphoid leukosis, followed by myeloid and erythroid leukosis. There are six subgroups based on differences in viral envelope glycoproteins (A, B, C, D, E, and J). Lymphoid leukosis is a neoplasm of B-cell (bursa) lymphocytes, which can be small or large lymphocytes (Figure 123.2i). These neoplasms typically do not occur before 14 weeks of age, which can help differentiate an ALV etiology from Marek's disease. Mortality usually occurs closer to 24–40 weeks of age in commercial operations.[42] However, ALV and related leukosis can also been seen in older animals (2–5 years of age) in backyard flocks (K. Harr, personal communication). Rubriblasts are commonly observed in circulation with erythroid leukosis (erythroblastosis).[42] However, it can be difficult to differentiate rubriblasts, lymphoblasts, and myeloblasts on standard blood smears alone. Severe anemia and thrombocytosis can also be observed with erythroid leukosis.[42] Myeloblastic myeloid leukosis (myeloblastosis) and myelocytic myeloid leukosis (myelocytomatosis) are induced by ALV subgroup J. Immature myeloblasts are the predominant cell type in circulation with myeloblastic myeloid leukosis. These large cells contain a small amount of basophilic cytoplasm that lacks cytoplasmic granules, immature chromatin, and variably prominent nucleoli. Promyelocytes, myelocytes, metamyelocytes, and mature heterophils will be present, but likely in smaller proportions. Anemia and thrombocytopenia are often present.[42] In myelocytic myeloid leukosis, granulated myelocytes predominate; however, cellular atypia and immature cells are also observed (Figure 123.2j,k).[28,42]

Marek's disease is caused by an alphaherpesvirus and is common worldwide in chicken. However, natural infections have been documented in quail and turkeys as well.[42] Marek's disease induces lymphoproliferative neoplasms. Lymphoma is far more common (e.g., lymphoid tumors in nervous system, spleen, liver, eyes, or skin); however, large T-cell leukemia may also be seen (Figure 123.2l).[42] Unlike lymphoid leukosis, Marek's disease may occur as early as 4 weeks and the mortality peaks in chicks from 10–20 weeks of age.[42] Anemia may also be observed with Marek's disease, whether leukemic cells are present or not. Marek's disease may be differentiated from leukosis due to a characteristic leg paresis, atherosclerosis, and persistent neurologic disease.

Coagulopathy

There is a paucity of literature on clinical coagulation disorders, thrombocytopenia, and thrombocytosis in gallinaceous birds. Many of the original avian coagulation studies used chicken and were reviewed in the "Avian Hematology" chapter in the 6th edition. Thrombocytosis is thought to occur secondary to inflammatory disorders (*Mycobacterium* infections, etc.) or neoplastic disorders (erythroid leukosis, etc.).[27,42] In gallinaceous birds, thrombocytopenia may be seen with increased thrombocyte destruction or consumption (sepsis, etc.) or with bone marrow disease (myeloid leukosis, chicken anemia virus, cyclophosphamide administration, etc.)[17,42] (see Chapter 95).

Hemoparasites

In general, hemoparasites are uncommon in well-maintained commercial flocks of gallinaceous birds. However, hemoparasites may be observed in backyard flocks, wild animals, and companion animals. The protozoan parasites commonly seen in avian blood smears include *Plasmodium*, *Hemoproteus*, and *Leukocytozoon sp*. These agents share a similar life cycle, with sporogony (asexual spore formation) in the midguts of *Simulium*

flies or *Culicoides* midges. In infected birds, merogony/schizogony (asexual reproduction) occurs in the tissue and sexual differentiation occurs in peripheral blood.[69] *Hemoproteus* and *Leukocytozoon* organisms can be observed in RBCs from apparently healthy wild and companion gallinaceous birds. Infection with multiple agents is common in wild birds.

Hemoproteus gametocytes are occasionally observed in RBCs of gallinaceous birds (Figure 123.2m). Gametocytes are basophilic, oval to elongated, with occasional yellow-brown iron pigment. Large organisms will mold around the RBC nucleus without displacing it. Most commonly, the presence of *Hemoproteus* gametocytes is considered an incidental finding; however, increased numbers may be observed with concurrent systemic illness. In one study of wild grouse and ptarmigan in Alaska, prevalence of *Hemoproteus sp*. detected by PCR was 21%.[59] In another study of wild sage-grouse from Colorado, *Hemoproteus sp*. were detected by blood smear examination in 9% of birds.[60]

Leukocytozoon sp. infestations also occur in gallinaceous birds. Leukocytozoon gametocytes dramatically alter the appearance of the nucleated cells they infect, replacing normal cytoplasm and displacing the nucleus peripherally (Figure 123.2m,n). A recent study in chicken used cell-type-specific antibodies to identify thrombocytes as the primary cell infected by *Leukocytozoon sabrazesi*.[69] Prevalence of *Leukocytozoon* in wild sage-grouse, as determined by blood smear examination, varies from approximately 10% to 45%, with some variation between state and breeding season.[14,60] In one study of wild grouse and ptarmigan in Alaska, prevalence of *Leukocytozoon* detected by PCR was 53%.[60] The true prevalence of *Leukocytozoon* likely varies between species, locations, and environments. Leukocytozoonosis can cause economic loss, especially with concurrent illness.[14,69] In chicken, *L. caulleryi* has been reported to have tropism for the reproductive tract, causing inflammation, edema, and decreased egg production.[14] In sage-grouse, *L. lovati* may cause decreased productivity even though only low numbers of organisms were detected.[14] *L. lovati* in wild grouse appears to be more prevalent during the brood-rearing season (July–August) than in the breeding season (March–May).[14]

Plasmodium sp. can be clinically insignificant or cause mortality. In one study of wild grouse in Alaska, prevalence of *Plasmodium sp*. detected by PCR was 17%.[59] Prevalence of *Plasmodium sp*. is <1% in wild sage-grouse from Nevada, Oregon, and Colorado, when determined by blood smear examination.[14,60] *Plasmodium pedioecetii* has been identified in several species of gallinaceous birds including sage-grouse, greater prairie chicken, and gray partridge.[14,61] *P. pedioecetii* is also seen in Attwater's prairie chicken in Texas (Figure 123.2o,p). *P. pedioecetii* appears to be incidental with no overt clinical signs in the gallinaceous birds studied. *Plasmodium* and *Hemoproteus* gametocytes can be difficult to differentiate (see Chapter 126).

Other hemoparasites that may be observed in gallinaceous birds include *Trypanosoma sp*., microfilaria, *Isospora sp*., *Hepatozoon sp*., *Aegyptianella sp*., and spirochetes (*Borrelia anserina*). These organisms have similar appearances in other animals (see Chapter 125).

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CHAPTER 124

Hematology of Psittacines

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Blood Collection and Preanalytical Factors
Laboratory Evaluation
Erythrocyte Morphology
Leukocyte Morphology
Thrombocytes and Hemostasis
Bone Marrow Collection and Evaluation
Reference Intervals
Partitioning Factors
Hematologic Disease
Anemia
Leukocyte Changes in Disease
Hemic Neoplasia

Acronyms and Abbreviations

K-EDTA, potassium-ethylenediaminetetraacetic acid; G:E ratio, granulocytic to erythroid ratio.

The order Psittaciformes includes four families: the Psittacidae (African and New World parrots and parakeets), the Cacatuidae (cockatoos), the Strigopidae (New Zealand parrots), and the Psittaculidae (other Old World parrots and parakeets). Most psittacine birds are colorful birds that have a strong beak for cracking nuts, seeds, and woody foodstuffs and are commonly kept as pets.

BLOOD COLLECTION AND PREANALYTICAL FACTORS

Most parrots are prone to stress during manual restraint and large parrots may be difficult to safely restrain for blood sampling. In general, blood collection should be done as early as possible, even before a complete physical examination is performed. This limits stress-induced changes on the hemogram and biochemical profile. General anesthesia induces various hematologic changes and intramuscular injection of sedatives may lead to acute local inflammation and biochemical changes lasting several days. Therefore, reversible intranasal sedation is a safe and reliable alternative to either manual restraint or general anesthesia prior to venipuncture. An intranasal combination of midazolam and butorphanol induces safe and reliable conscious sedation in healthy parrots.[14,35]

Blood is typically collected from the right jugular vein in most Psittaciformes regardless of their size. This site is the easiest to sample from while the bird is manually restrained. A large apterium (featherless tract) is present in all parrots and this avian order, being tropical, generally has a large jugular apterium with less adjacent down feathers than birds from temperate ecozones. The skin is particularly thin and the vein is readily visible. Obesity is common in pet birds, especially in Amazon parrots (*Amazona sp*.), and may result in subcutaneous fat deposition around the neck that may complicate venipuncture. Alternatively, the left jugular vein may be used, but is smaller than the right and sometimes too small to sample. The ulnar vein is prone to hematoma formation and is only recommended for venipuncture in anesthetized parrots. Additionally, it is more challenging to restrain parrots for venipuncture at this site than other birds, such as raptors. The medial metatarsal vein is generally too small in most parrots to obtain a large enough volume except in larger parrots such as macaws. A volume of 1% of body weight can safely be collected in most psittacine birds.

A 25–22-gauge needle, depending on the animal size, is typically used with a 1–3 mL syringe. Preheparinization of the needle and syringes is not recommended, as it will result in overheparinization of the sample, cytologic artifacts, dilution artifacts, and is unnecessary when proper techniques are employed. However, blood sampling is

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dependent upon the venipuncturist's experience level and needle heparinization will usually result in adequate, though not optimal, samples. The needle should be removed from the syringe prior to transferring the blood to the collection tube to limit hemolysis. Hemolysis is rarely encountered during blood collection performed by the authors. As avian blood clots quickly, it needs to be transferred as soon as possible into the collection tube. If a hematoma develops during collection, the blood will clot faster in the syringe. Toenail clipping is not recommended, as it leads to bacterial contamination and samples of suboptimal quality.

There are several preanalytical factors that may affect hematologic results. Basic factors associated with psittacine biologic variability such as season, age, sex, comorbid chronic stable diseases (such as feather-damaging behavior, common in Psittaciformes), and stress (induced by transport, restraint, degree of socialization) need to be considered when interpreting the hemogram. Proper collection techniques and anticoagulant choice are also important. K-EDTA is the anticoagulant of choice in most psittacine birds and has not been shown to lead to hemolysis within 12 hours of sampling in any psittacine species unlike in some other avian orders (notably some Passeriformes, Gruiformes, and Struthioniformes). It is superior to heparin for hematologic analysis as it inhibits thrombocyte and WBC aggregation, has slightly better preservation of cellular elements in stored samples, and superior cytologic staining. In a study on anticoagulants in macaws (*Ara sp.*), K-EDTA and heparin resulted in similar hemograms with significant cell lysis at 24 hours in both anticoagulants, rendering blood smear assessment more challenging. Citrate resulted in profound hematologic changes (dilution effect and hemolysis) and is not recommended for hematology in psittacine birds. However, the main drawback of this study was the collection method with all syringes being preheparinized, thereby confounding the results comparing the anticoagulants.[25] In another study in Hispaniolan Amazon parrots (*Amazona ventralis*), PCV was significantly lower and lymphocyte concentrations were significantly higher (although still within expected reference intervals) in lithium heparin samples compared with K-EDTA, although there was similar preservation of cell morphology and staining for both heparinized and K-EDTA samples.[21] Therefore, in small psittacine birds, it is still acceptable to use heparin when performing both hematology and biochemistry from the same collection tube to maximize small sample volume. Insufficient or delayed mixing in blood collection tubes with anticoagulant may cause small clots, which will affect blood cell counts. Prolonged storage of psittacine whole blood is also not recommended, as significant hemolysis and WBC lysis were seen after 12 hours in macaws.[25]

LABORATORY EVALUATION

All WBC counting techniques in birds are manual, which results in increased analytic variability, decreased accuracy, and a decreased ability to transfer reference intervals established within one laboratory to another. Because RBCs greatly outnumber WBCs and thrombocytes, general automated techniques can be used for RBC counts.

Psittacine blood cells tend to be more fragile compared to other avian species such as raptors and poultry. Consequently, blood smears may have more artifacts, especially in the small psittacines, possibly due to substandard preanalytical techniques. Experience may be the largest factor in producing quality blood smears. Specifically, the wedge technique (push preparation) may result in excessive cell lysis in psittacine birds. Other techniques to minimize cell lysis, such as the pull preparation and cover slip techniques, can be used to improve blood smear quality.

Blood cell counting is performed either through hemocytometer-based techniques or by performing an estimate from a blood smear. While estimation from the smear is the least precise and accurate technique, it is relatively quick and easy. Thus, it is still widely performed by practitioners in-house and by commercial laboratories. Hemocytometer counts are performed using a stain such as phloxine B, eosin, Natt–Herrick solution, or Rees–Ecker solution. It is noteworthy that some Psittaciformes, such as cockatoos, have deeply basophilic granules in their eosinophils using Wright's stain, which may not stain positively with eosin or phloxine B. The agreement between different counting methods is generally poor in birds, which has been demonstrated in various avian species including macaws. In particular, a significant disparity has been reported between hemocytometer total cell counts and WBC estimates of blood smears in cases of marked leukocytosis. While hemocytometer cell counts are typically considered more accurate than blood smear estimates, phloxine-B staining protocols may be less accurate when there are increased numbers of monocytes and/or lymphocytes, and in cases where heterophil degranulation and eosinophilia may interfere with phloxine stain uptake.[45]

Early attempts at automation of total WBC and differential cell counts used techniques such as high-throughput digital image cytometry in parrots and antibody-independent flow cytometry protocols in Galliformes[2] (see Chapter 123). None of these techniques are currently available for diagnostic use in psittacines. While flow cytometry has been used successfully in chicken for differential cell counts, it is unlikely that the technique will be efficacious in Psittaciformes because of the large number of species seen in practice in which most antibodies may not cross-react.

ERYTHROCYTE MORPHOLOGY

Psittacine RBCs are large elliptical cells that have abundant orange-red cytoplasm and condensed ovoid nuclei with dense homogenous chromatin when stained with Romanowsky-type stains. Mild anisocytosis and slight poikilocytosis are found in blood smears from healthy birds. Additionally, mild variations in nuclear morphology can be normal; e.g., symmetrically centrally constricted nucleus, nuclear microlobulation, and, less

commonly, binucleation (Figure 124.3d). The presence of these nuclear abnormalities does not correlate with PCV.[7]

As in other avian species, psittacine RBCs compared to mammalian RBCs have a shorter circulating half-life, ranging from 25–45 days in different species, thus have a higher percentage of circulating polychromatophilic RBCs in health.[38] Polychromatophilic RBCs are large elliptical cells and contain a large volume of purple-red cytoplasm compared to the orange-red of mature RBCs. Nuclear morphology is like that of mature RBCs, although the chromatin may be less densely clumped (Figure 124.3c). The use of supravital stains (e.g., new methylene blue) to identify visible reticulum has demonstrated that a high percentage (>90%) of erythrocytes contain some amount of visible reticulum; thus, only cells with a complete ring of reticulum that encircles the nucleus (i.e., ring form) should be classified as reticulocytes in avian species. The recommended staining protocol for avian reticulocyte assessment is a dilution of 1:3 parts blood to new methylene blue, with an incubation time of 10 minutes. The same procedure may be used to identify Heinz bodies and denatured organelles caused by oxidative damage.[24] This mixture can be stored refrigerated for up to 24 hours.[28] Supravital stains require incubation with whole blood, and secondary staining of already prepared blood smears is of low quality and often nondiagnostic. The number of polychromatophilic RBCs observed on a Romanowsky-stained blood smear typically corresponds with the number of ring-form reticulocytes on a new methylene-blue-stained preparation. Variations in staining techniques and counting protocols for avian reticulocytes have resulted in a high degree of variation in published reticulocyte percentages in birds with reports ranging 0.4–6.8% in healthy psittacine species.[38] Additionally, absolute reticulocyte concentrations are superior to reticulocyte percentages, which can be influenced by the PCV. Reference intervals generated using 44 healthy psittacine birds of varying species for absolute polychromatophil count were 5,670–227,640 cells/μL (mean: 131,204 cells/μL).[28] More immature erythroid cells (e.g., early and late polychromatophilic rubricytes) may be seen in low numbers in healthy birds and in increased numbers as part of a regenerative response in anemic birds. Early and late polychromatophilic rubricytes are ovoid to round with a smaller volume of blue-gray to pale purple cytoplasm. These cells can be distinguished from mature and polychromatophilic RBCs by their small round nuclei and more open chromatin pattern. Rubricytes may also be counted, in lieu of reticulocyte counts, to assess regeneration on a Romanowsky preparation (Figure 124.3c,d).

Blood group antigens have not been described in psittacine species, although studies in poultry have identified as many as 28 different blood group antigens.[27] Preformed antigens to blood groups have not been described in birds, and homologous major crossmatches in a small group (n = 15) of untransfused cockatiels (*Nymphicus hollandicus*) were all compatible, as were heterologous crossmatches with blue-fronted Amazon parrots (*Amazona aestiva*).[13] That being said, care must be used when considering blood transfusions between different avian species (i.e., heterologous transfusions), which may result in agglutination and/or hemolysis.[13,27] There are no published reports of transfusion reactions in psittacines, although the clinical features of transfusion reactions in birds have not been well-defined. The half-life of homologous transfused RBCs was much longer (11–17 days) compared with the heterologous transfused RBC (0–3 days) using fluorescent-labeled RBCs. Additionally, nearly all the donor–recipient pairs in the heterologous groups of cockatiels became crossmatch-incompatible after one transfusion.[13] Based on these findings, homologous transfusions are recommended, although single heterologous transfusions may be clinically tolerated in psittacines.[27] Heterologous transfusions are not recommended, as the circulating half-life of the transfused RBC is relatively short and the metabolic effects resulting from clearance of transfused RBC in already debilitated birds can have a negative clinical impact. Only fresh whole blood transfusions are available for psittacines, as high potassium concentrations develop in avian blood products maintained with currently available storage media.[38] Cryopreservation techniques for avian packed RBCs have shown some promise in in vitro pilot studies. However, some protocols result in poor post thaw RBC viability and in vivo studies are lacking, limiting the practical use of these techniques currently.[18] Avian erythropoietin is structurally unique compared to mammalian erythropoietin, and there is no in vivo cross-reactivity when mam-malian erythropoietin is administered to birds[38] (see Chapter 103).

LEUKOCYTE MORPHOLOGY

As in other avian species, psittacine WBCs consist of granulocytes (i.e., heterophils, eosinophils, and basophils) and mononuclear cells (i.e., lymphocytes, monocytes) (Figure 124.1). Heterophils are the most numerous WBC in most parrots (said to be heterophilic predominant), and are largely analogous to mammalian neutrophils. Heterophils have abundant clear cytoplasm filled with numerous secondary granules which are typically rod-shaped to round and a dull reddish-orange, sometimes with a faint refractile quality, when stained with Romanowsky-type stains. Nuclei are segmented to lobulated with dense, condensed dark chromatin and inapparent nucleoli. Assessment of nuclear morphology in mature avian granulocytes can be challenging with the high degree of cytoplasmic granulation. Additionally, some species may have relatively hyposegmented nuclei in health, which can also make determination of a left shift challenging. Evidence of toxic change can help provide support for a suspected left shift. Increased cytoplasmic basophilia and vacuolation should be present, but may be difficult to appreciate in mild cases of toxic change due to cytoplasmic granulation. Other toxic changes reported in avian heterophils include the presence of primary granules (round, metachromatic to magenta cytoplasmic granules), degranulation, and/or consolidation of heterophil granules, although care must

be taken to avoid misinterpreting smear artifact[38] (Figure 124.4a,b). Although functionally like mammalian neutrophils, avian heterophils have decreased activity of some enzymes including myeloperoxidase, alkaline phosphatase, and catalase.[15] It has been suggested that the differences in antimicrobial enzyme composition between avian heterophils and mammalian neutrophils account for the development of caseous rather than liquid necrosis in response to heterophilic inflammation in avian tissues.[38]

Lymphocytes are often the second most numerous of all leukocytes, following heterophils, in most psittacine species. Some psittacine species are lymphocytic predominant, with lymphocytes being the most common WBC type; e.g., some Amazon parrots (*Amazona sp.*), some Pionus parrots (*Pionus sp.*), and Quaker parrots (*Myiopsitta monachus*). Avian lymphocytes are morphologically comparable to those described in mammals. Small mature lymphocytes should predominate with scant pale blue cytoplasm, clumped chromatin, and inapparent nucleoli. Granular lymphocytes with azurophilic cytoplasmic granules may be present in varying, typically low, numbers. Reactive lymphocytes with increased amounts of deeply basophilic

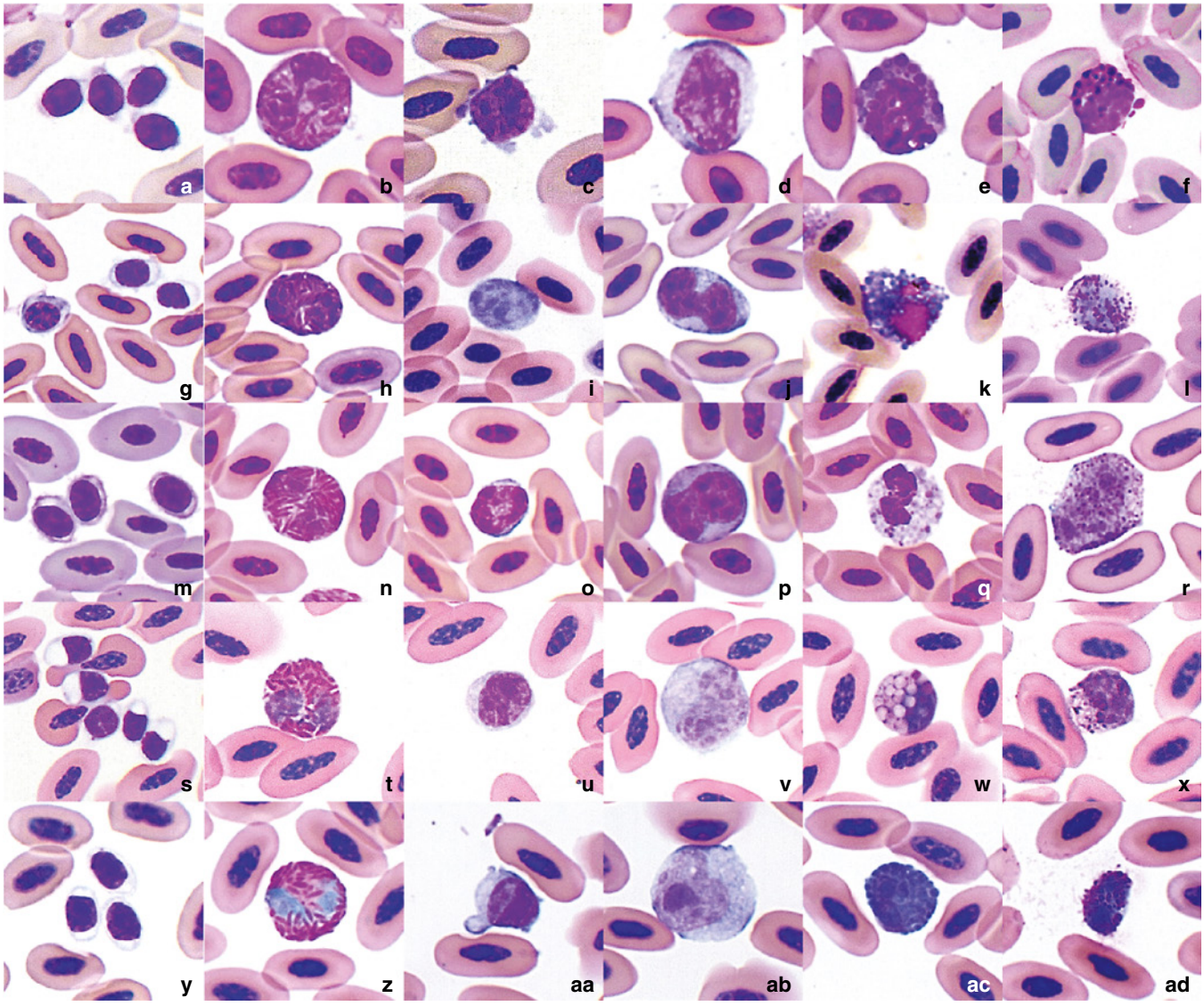


FIGURE 124.1 Comparative WBCs, RBCs, and thrombocyte morphology of selected psittacines. 1000× using modified Wright's stain unless indicated. African grey parrot (*Psittacus erithacus*): (a) Thrombocyte; (b) Heterophil; (c) Lymphocyte with classic cytoplasmic blebbing; (d) Monocyte; (e) Eosinophil; (f) Basophil. Cockatiel (*Nymphicus hollandicus*) stained using Diff-Quik stain: (g) Thrombocyte and a polychromatophil; (h) Heterophil; (i) Large lymphocyte which can be periodically found in normal birds; note the scant tag of basophilic cytoplasm; (j) Monocyte which is larger than surrounding RBCs; (k) Blue eosinophil which raises questions regarding granule pH; (l) Basophil. Eclectus parrot (*Eclectus roratus*): (m) Three thrombocytes; (n) Heterophil; (o) Intermediate-sized lymphocyte; (p) Monocyte; (q) Eosinophil with gray and occasional pink and purple round granules; (r) Basophil. Macaw (*Ara sp.*): (s) Five thrombocytes with variable spreading on the glass slide; (t) Heterophil; (u) Intermediate-sized lymphocyte; (v) Monocyte; (w) Eosinophil; (x) Basophil. Amazon parrot (*Amazona sp.*): (y) Three thrombocytes; (z) Heterophil; (aa) Lymphocyte with cytoplasmic blebbing; (ab) Monocyte; (ac) Blue-gray eosinophil; (ad) Basophil. (**Source:** All photos contributed by Dr. Kendal Harr.)

cytoplasm may also be observed under circumstances of antigenic stimulation and must be distinguished from neoplastic lymphocytes based on other hematologic abnormalities, clinical history, and other diagnostic findings. Finally, avian monocytes are morphologically and functionally comparable to mammalian monocytes (Figure 124.1).

Eosinophils are much less numerous than heterophils and contain abundant pale blue cytoplasm with numerous round cytoplasmic granules. Eosinophil granule morphology varies in different psittacine species. Psittacine eosinophils may have gray to bright blue to pale purple granules, while others have the more typical reddish-orange-colored granules (Figure 124.1). Nuclei are segmented to lobulated, though less so than heterophil nuclei, with dense dark chromatin and inapparent nucleoli. Similar challenges assessing eosinophil nuclear morphology exist, as described for heterophils. The eosinophil granule content of different avian species has not been thoroughly explored with cytochemical stains and ultrastructure, and functionally the exact role of eosinophils is poorly understood. Avian eosinophils are less responsive to parasite antigens compared with mammalian eosinophils, and may be a more general component of inflammation in these species.[38]

Basophils may be smaller than heterophils and eosinophils with a large amount of clear to pale blue cytoplasm and many small, dark purple cytoplasmic granules. Basophil nuclei are like eosinophil nuclei, although may be even less-lobulated. Granules may dissolve or coalesce with the alcohol-based stains routinely used for hematology, such as Romanowsky-type stains. Alcohol-based stains can stain quite differently than aqueous-based stains (see Chapter 117). Some of the basophil granule constituents are similar to those in mammals (e.g., histamine), and, while the function of avian basophils is not known, it is thought to be similar to that of mammalian basophils.[38]

THROMBOCYTES AND HEMOSTASIS

Thrombocytes are analogous to mammalian platelets and morphology can vary from species to species. In general, thrombocytes are small, round to oval cells with clear to light blue cytoplasm, sometimes with a few pink cytoplasmic granules and clear vacuoles (glycogen) (Figure 124.1). When activated, as during collection, storage, and inflammation, they can undergo a shape change resulting in the development of elongated cytoplasmic processes or a more spindloid appearance. Nuclei are round to oval with dense smudged, homogenous chromatin and inapparent nucleoli. Distinguishing thrombocytes from lymphocytes can be challenging but, in general, lymphocytes have a larger more complex nucleus and darker blue cytoplasm compared to the condensed nucleus and clear cytoplasm of thrombocytes. It can be helpful to initially assess thrombocyte morphology near the feathered edge of a blood smear where thrombocyte clumps allow the viewer to easily identify thrombocytes and become familiar with their morphology.

Absolute thrombocyte counts in psittacines are rarely determined due to the tendency of thrombocytes to clump. A direct thrombocyte count can be performed using a hemocytometer and Natt–Herrick solution; however, visual assessment from a blood smear may be sufficient for diagnostic purposes. In general, 10–15 thrombocytes per 1000 RBCs are considered adequate. Quantitative thrombocyte estimation from a blood smear is prone to error due to uneven distribution and thrombocyte clumps in most smears.

Commercially available reagents for hemostasis assessment in humans and other mammals interact with avian coagulation factors, but do not bind with the same affinity; thus, self-made, avian-specific reagents are often required. This limits the availability and practicality of running coagulation assays in a commercial diagnostic laboratory. Additionally, the intrinsic coagulation pathway may be less significant in birds which lack factor XII, although activated clotting time can be activated with diatomaceous earth, resulting in shorter times to clot. The need for avian specific reagents and methods is supported by coagulation times exceeding the upper limit of the reaction time (500 seconds) observed for partial thromboplastin time in many species of birds, including macaws (*Ara sp.*) and African grey parrots (*Psittacus erithacus*), when plasma was analyzed using nonspecific commercially available reagents.[19] The optimum anticoagulant for coagulation analysis in birds has not been determined, as only trisodium citrate has been evaluated. As stated in the earlier text, trisodium citrate causes increased hemolysis in psittacine blood within hours of sampling (see Chapter 95).

Thromboelastography is a more holistic tool for coagulation assessment in mammals, as it incorporates coagulation factors and cellular elements, as well as assessment of fibrinolysis. This assay has been successfully modified for use in avian species, although normal tracings vary in different species.[32,44] As no current reports of thromboelastography performance in birds with coagulopathies exist, the diagnostic value of this assay in psittacines is still unknown.

BONE MARROW COLLECTION AND EVALUATION

Bone marrow collection in psittacines is like that in other avian species. Bone marrow can be collected from any nonpneumatized long bone. The most common site for collection of clinical bone marrow samples is the tibiotarsus proximate to the cnemial crest. Bone marrow samples should be collected into an anticoagulant to prevent clotting, and 1% EDTA has been demonstrated to be superior to heparin in the authors' experience. Preparation of bone marrow smears is also like those described for mammalian bone marrow aspirates, and techniques to minimize excessive hemodilution (e.g., petri dish technique) should be used to maximize the diagnostic yield of the sample. Routine Romanowsky stains are recommended for air-dried bone marrow aspirate smears (see Chapter 141).

Hematopoietic cells of granulocytic and erythroid lineages at all stages of development (Figure 124.2) can be identified in psittacine bone marrow aspirate smears, although cells of the thrombocyte lineage cannot be reliably identified by morphology alone. As in other species, the distribution of hematopoietic precursors favors many more mature than immature forms. Maturation stages in normal psittacine erythropoiesis are largely like those in mammals with the obvious distinction of nuclear retention in mature forms. The six recognized stages of erythroid development in birds are rubriblasts, prorubricytes, basophilic rubricytes, early polychromatophilic rubricytes, late polychromatophilic rubricytes, polychromatophilic RBCs, and mature avian RBCs (metarubricytes). Late-stage rubricytes can be distinguished from polychromatophilic RBCs by the nuclear shape, round in rubricytes and ovoid in RBCs. By convention, mature RBCs are not included in bone marrow cell differentials. Late-stage erythroid precursors (early and late polychromatophilic RBCs) are the predominant cell type observed in psittacine bone marrow aspirates.

Psittacine granulopoiesis is also largely like that observed in mammals with a gradual decrease in cell size and cytoplasmic basophilia as cells mature, and a gradual decrease in nuclear size with development of nuclear segmentation and lobulation in mature granulocytes. The stages of granulocytic development are also similar and include myeloblasts, progranulocytes, myelocytes, metamyelocytes, bands, and mature segmented granulocytes. Unique to avian progranulocytes are multiple different granule types, while mammalian progranulocytes contain only primary granules. Avian progranulocytes contain granules ranging in color from orange to basophilic to metachromatic, and in shape from spherical to ring forms.[5] It has been speculated that these different granule morphologies may represent a unique organelle or potentially different intermediary stages of granulocyte development.

Avian thrombocytes are derived from a line of mononuclear precursors in the bone marrow (see Chapter 123). The thrombocyte series includes early-, mid-, and late-immature thrombocytes. While thrombocyte precursor morphology has been described in psittacines,[5] a recent assessment of bone marrow morphology in Hispaniolan Amazon parrots (*A. ventralis*) revealed discordance in thrombocyte precursor classification between experts in avian hematology. It was concluded that morphologic assessment alone is insufficient to distinguish thrombocyte precursors from later-stage erythroid precursors. Regardless, in the previous studies where thrombocyte precursors were identified, they accounted for <3% of all nucleated cells, and thus omission of this cell lineage is unlikely to change clinical assessment of bone marrow aspirates, unless there is a peripheral thrombocytopenia.

Like other avian species, low numbers of lymphocytes (<2%), plasma cells (<1%), macrophages (<1%), and osteoclasts may be observed in bone marrow aspirates from psittacines. Iron has not been observed in Romanowsky stained bone marrow aspirates from psittacines. The mean G:E ratio derived from 17 clinically healthy, adult Hispaniolan Amazon parrots (*A. ventralis*) was 0.4 ± 0.2. It is possible that the unique histologic organization of avian bone marrow, with erythropoiesis occurring in the medullary sinus lumen and granulopoiesis within hematopoietic chords in the extravascular space, may predispose avian bone marrow samples to biased sampling of erythropoiesis over granulopoiesis (data submitted by authors).

REFERENCE INTERVALS

Partitioning Factors

Reports on the effect of various physiologic, analytic, and environmental factors on hematologic results in psittacines are highly variable largely due to the small numbers of individuals from which these observations were made, with resultant wide confidence intervals around reported reference limits. Additionally, WBC differentials in psittacines have been historically reported as percentages rather than absolute concentrations, which limits the ability to extrapolate this data without concurrent total WBC counts. Current guidelines put forth by the American Society for Veterinary Clinical Pathology require minimum numbers of individuals within partitioned reference intervals, which are largely absent from the current published data in psittacines. Furthermore, statistically significant differences between partitioned groups of individuals may not be clinically relevant.

Preanalytical sampling factors and analytical methodology can influence hematologic results in psittacines. PCV was lower in jugular samples collected from anesthetized Carnaby's cockatoos (*Calyptorhynchus latirostris*) compared to samples collected with manual restraint from the metatarsal vein.[34] Circulatory alterations associated with gas anesthesia are assumed to occur in psittacines. Additionally, many published reference interval studies in psittacines utilize different methodology to generate the hematologic data, and given the high degree of imprecision inherent to manual techniques used for avian CBCs, results obtained using different techniques should not be considered equivalent.

Partitioning factors are typically important biological variability factors, although partitioned reference intervals are not routinely used for diagnostic assessment of psittacines. With these caveats in mind, the following observations have been reported. In general, erythroid parameters tend to increase with age from the neonate stage, and this finding has been reported in both captive and wild psittacines.[1,6,23,31,47] The PCV is often lower in neonates and fledglings than in adults.[5,9,10] There are progressive increases in MCV and hemoglobin (Hgb) concentration in some species.[23,47] In general, WBC counts decrease with age, which is also reported in both wild and captive psittacines.[14,31,47] In some species (African grey parrots, Amazon parrots, and macaws),

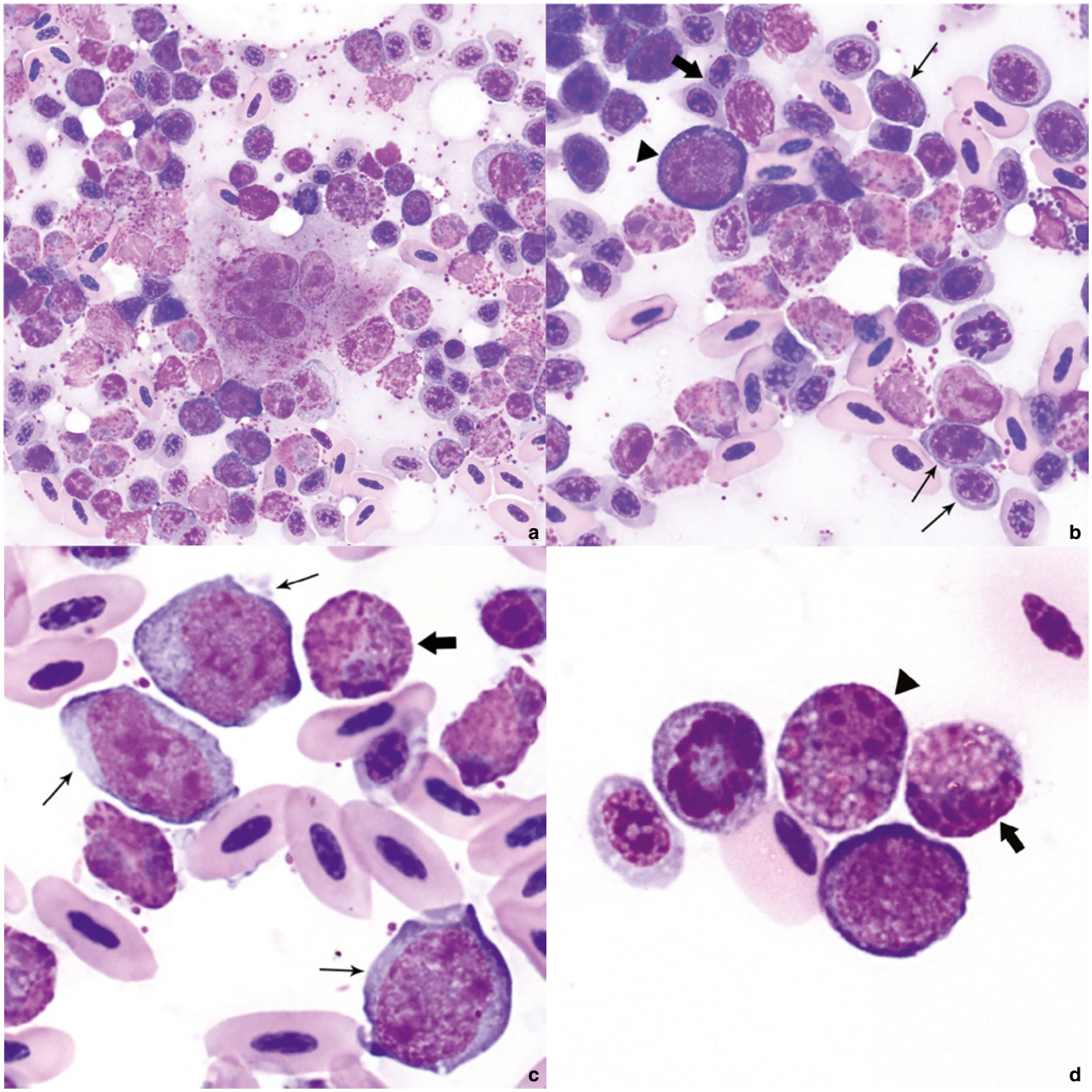


FIGURE 124.2 Erythroid and granulocytic precursor morphology in bone marrow from a Hispaniolan Amazon parrot (*Amazona ventralis*). (a) Large osteoclast surrounded by many proliferative- and maturative-stage granulocytic and erythroid precursors; (b) Representative stages of erythrocyte development including a rubriblast (arrow head), basophilic rubricytes (thin arrows), and early polychromatophilic rubricytes (thick arrow); (c) Representative stages of granulocytic development including myeloblasts (thin arrows) and a heterophil myelocyte (thick arrow); (d) Heterophil myelocyte (thick arrow) and a heterophil progranulocyte with round reddish-orange secondary granules and round magenta primary granules (arrow head) a mitotic figure, polychromatophil, and blastic precursor. 1000× Modified Wright–Giemsa stain.

WBC counts transition to expected adult values at 2–3 months of age.[5,9,10,14] There are inconsistent and often contradictory reports of WBC differential variation with age in psittacines. Lymphocyte concentrations were higher in a population of juvenile, captive Spix's macaws (*Cyanopsitta spixii*) compared to adults, although in other species (cockatoo, Eclectus parrot, macaw) lymphocytes are reported to increase with age and, in some species, may be the predominant leukocyte in adult individuals.[14]

Few reports regarding the effect of sex on hematologic values in psittacines exist, in part due to the challenges in distinguishing individuals of different sexes, as these species often lack significant sexual dimorphism. Higher

RBC, PCV, and Hgb concentrations have been reported in male budgerigars compared with females. In a population of fledging wild hyacinth macaws (*Anodorhynchus hyacinthinus*), females had higher total WBC and heterophil concentrations compared to males.[1] Higher WBC counts due to increased lymphocyte concentrations were observed in female orange-winged Amazon parrots (*Amazona amazonica*) compared with males.[48] Other variables such as stress should also be considered in these studies.

Environment can also influence hematologic data. PCV was higher in winter months relative to autumn in a population of captive Spix's macaws.[14] Species that form large flocks had lower total WBC counts compared to solitary species. Higher total WBC counts due to higher heterophil and eosinophil concentrations were observed in a population of wild blue-fronted Amazon parrots (*Amazona aestiva*) compared to captive individuals of the same species, although these findings were attributed to an epinephrine response as a result of capture and sampling. Lymphocyte and monocyte concentrations were higher in the captive blue-fronted Amazon parrots in this study.[12] Wild nestling burrowing parrots (*Cyanoliseus patagonus*) with higher body condition scores had higher WBC counts due to increased heterophil concentrations, compared to those with low body condition scores[36] (see Table 124.1).

HEMATOLOGIC DISEASE

The most common hematologic abnormality reported from a population of ill pet psittacines presenting to a tertiary referral center was leukocytosis, which was observed in 28% of individuals, followed by anemia, which was present in 26% of individuals. Noteworthy is that over 50% of sick psittacines in this study had no hematologic abnormalities.[4] That being said, a number of important and relevant hematologic abnormalities may be observed in ill psittacines.

Anemia

As in other species, anemia in psittacines should be assessed for evidence of regeneration, which can help in prioritizing potential underlying etiologies. Nonregenerative anemia is a nonspecific finding and can develop more quickly in birds compared to mammals due to the shorter circulating RBC life span in birds. Nonregenerative anemia has been most commonly reported in association with a wide range of chronic infectious diseases, such as chlamydiosis, aspergillosis, and mycobacteriosis. Other diseases associated with nonregenerative anemia in psittacines include egg yolk peritonitis, chronic air sacculitis, chronic liver or renal disease, hypothyroidism, hyperestrogenism, iron deficiency, starvation, self-mutilation, and neoplasia.[6] Mild nonregenerative anemia is frequently observed in African grey parrots (*P. erithacus*) with Psittaciform 1 bornavirus, the putative causative agent of proventricular dilation disease.[26] Severe nonregenerative anemia was reported in an adult hyacinth macaw (*A. hyacinthinus*) secondary to a nutritional vitamin-B12 deficiency. Occasional folded dacryocyte-like RBCs were observed and the anemia gradually resolved with vitamin-B12 supplementation and transition to a balanced commercial pelleted diet.[20] Severe nonregenerative anemia, as a component of pancytopenia, is commonly seen in juvenile old world parrots infected by psittacine circovirus. Hypochromasia and/or microcytosis has been reported in poultry secondary to iron

TABLE 124.1 Reference intervals for psittacine birds, in accordance with ASVCP guidelines for sample size and statistical analysis.

Species	Captive orange-winged Amazon parrot ($n = 29$)[48]	African grey parrot	Amazon parrot	Budgerigar	Cockatiel	Cockatoo	Conure	Macaw
RBC ($\times 10^{12}$/L)	2.4–3.7	3.0–4.0	2.0–4.5	–	2.5–5.0	–	–	2.2–3.9
PCV (L/L)	0.42–0.60	0.40–0.53	0.39–0.54	0.40–0.57	0.40–0.60	0.38–0.52	0.40–0.55	0.38–0.54
Hgb (g/L)	–	142–186	130–160	–	–	–	125–190	129–193
MCV (fL)	138–193	–	–	–	–	–	–	–
WBC ($\times 10^9$/L)	0.7–16.4	3–15	5–15	2.5–8	3.5–11	4–14	3.5–11	5–20
Heterophils ($\times 10^9$/L)	0.7–7.2	1–12	1.5–11	0.9–5	1–9	1.5–10	1–6	1.1–15.0
Lymphocytes ($\times 10^9$/L)	0–10.8	0.6–7	1–10	0.9–4.5	0.4–4	1–7	1–5.5	0.7–8.7
Monocytes ($\times 10^9$/L)	0.1–0.9	0–0.8	0–0.6	0–0.6	0–0.9	0–0.7	0–0.7	0–0.8
Eosinophils ($\times 10^9$/L)	–	0–0.2	0–0.3	0–0.1	0–0.2	0–0.2	0–1.0	0–0.1
Basophils ($\times 10^9$/L)	–	0–0.5	0–0.4	0–0.4	0–0.6	0–0.3	0–0.4	0–0.4

All values except those from the orange-winged Amazon parrot were generated from >500 adult, mixed-sex birds, retrospectively deemed healthy based on clinical history. WBC counts determined via hemocytometer or direct smear estimate, 200 cell WBC differential cell counts. Hgb by cyanmethemoglobin without centrifuging to remove erythrocyte nuclei.

deficiency and presumably could develop in psittacines to do chronic external blood loss, although primary reports are lacking (see Chapter 123).

Other morphologic abnormalities in the erythroid line in psittacines are occasionally striking. An alkylating chemotherapeutic agent (Mitomycin-C) was shown to experimentally induce RBC nuclear abnormalities in conures (*Aratinga canicularis*), resulting in a dose-dependent increase in RBC numbers with micronuclei and/or nuclear budding. A time-dependent increase in numbers of polychromatophilic RBCs was also noted.[17] Therefore, prominent or progressive erythrocyte nuclear atypia should warrant further investigation for potential drug or toxin exposure in psittacines. Marked erythroid dysplasia resulting from apparent loss of nuclear anchoring and orientation has been observed by the editor in lovebirds (*Agapornis sp.*) and cockatiels (*N. hollandicus*), though other bird species may be affected (Figure 124.3).

Hemorrhage is the most common cause of regenerative anemia in psittacines and is most often the result of trauma, ulcerative internal lesions such as gastrointestinal ulcers, or bleeding neoplasms. Less commonly, blood-sucking parasites or coagulopathies (secondary to toxins, bacterial or viral infections, neoplasia, inherited factor deficiencies, or severe hepatic disease) may result in hemorrhage and a regenerative anemia.[6] Conure bleeding syndrome is an uncommon and poorly characterized disease typified by variably acute to chronic recurrent episodes of hemorrhage from the upper respiratory tract, gastrointestinal tract, and body cavities of several species of conures and other small psittacines. The clinically appreciable external hemorrhage induces a marked regenerative response (see Chapter 95).

Presumed immune-mediated hemolytic anemia has been reported infrequently in captive psittacines.[29,30] Anemia in these cases was moderate to severe (PCV of 28% and 17%, respectively) with variable changes in RBC morphology including moderate numbers of anucleated RBCs (erythroplastids) and marked macroscopic agglutination, as well as a subtle rounding to erythrocyte

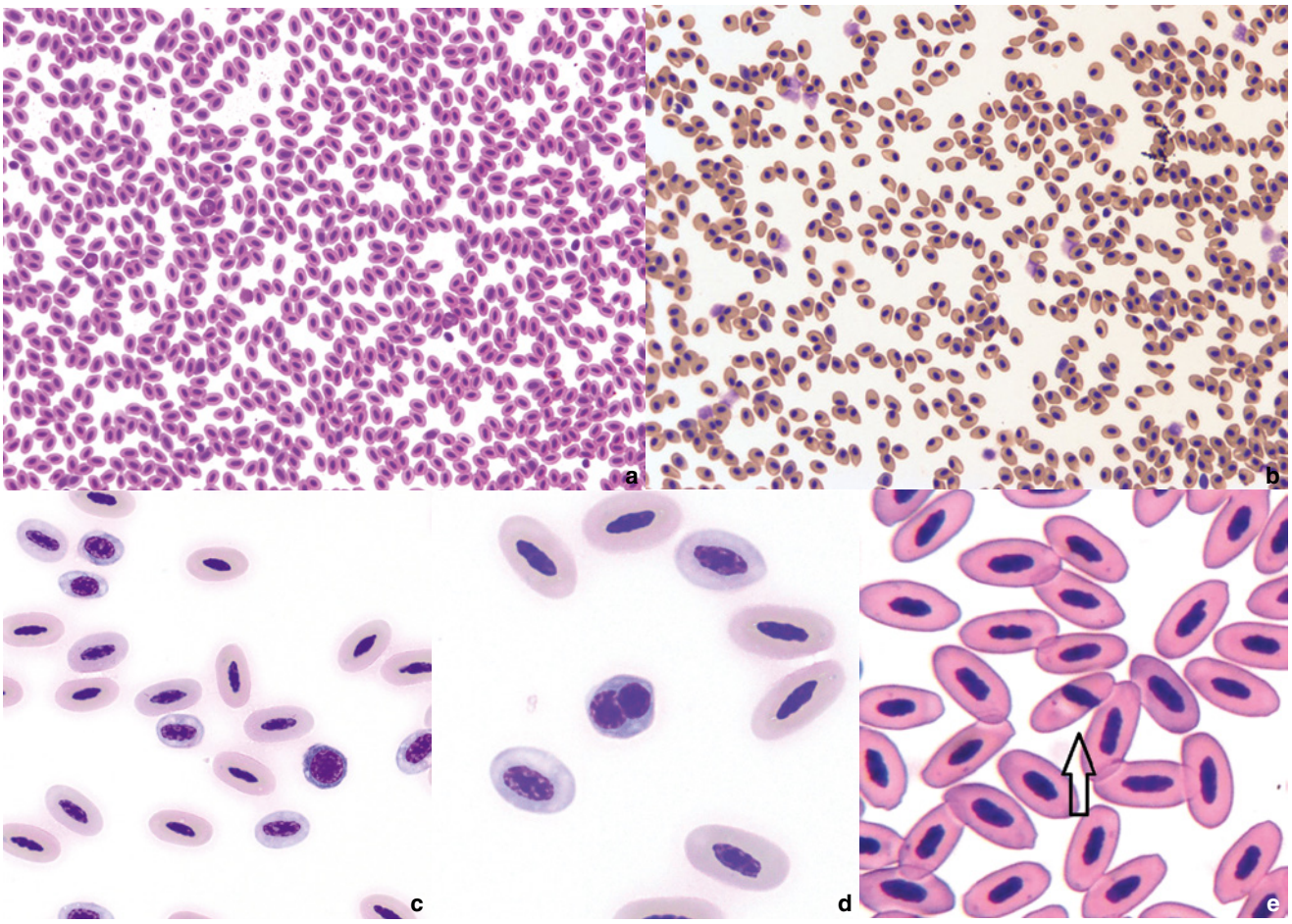


FIGURE 124.3 Erythroid morphologies found in psittacines. (a) A normal blood smear from a lovebird (*Agapornis sp.*) for comparison, 10× objective; (b) Erythroid dysplasia with loss of nuclear anchoring and orientation in a blood smear from a flockmate of the normal bird pictured in (a), 10× objective; (c) Regenerative anemia with increased numbers of polychromatophilic RBCs, late polychromatophilic rubricytes, and one basophilic rubricyte in an Amazon parrot (*Amazona sp.*); (d) Binucleated early polychromatophilic rubricyte in an Amazon parrot. 1000× (e) High magnification view of an RBC nucleus that has inappropriate orientation and is almost perpendicular to the cell (open arrow). (**Source:** Photos courtesy of Dr. Kendal Harr. Modified Wright–Giemsa stain unless otherwise specified.)

cell margins, suggestive of spherocytes.[29,30] Leukocyte findings were inconsistent. Both birds had normal total WBC counts on presentation; although one developed a mild heterophilia,[29] while the other had a heteropenia with a marked left shift.[30] Additionally, bone marrow findings in these birds ranged from unremarkable to erythroid hyperplasia with left shift. Anemia improved in one of the birds following corticosteroid administration, but this individual ultimately relapsed and died of suspected aspiration pneumonia.[30] Intravascular hemolysis with hemoglobinuria has been reported secondary to lead toxicity in Amazon parrots (*Amazona sp*.) and African grey parrots (*P. erithacus*).[41]

As in other species, absolute erythrocytosis due to increased circulating RBC mass must be distinguished from relative erythrocytosis due to hemoconcentration. This can typically be accomplished via physical examination and evaluation of biochemical values that support hemoconcentration, such as increased total protein concentration. Primary absolute erythrocytosis due to polycythemia vera, an idiopathic myeloproliferative disease, has not been reported in psittacines at this time. Secondary erythrocytosis in these species is most often attributed to chronic respiratory disease, especially aspergillosis, pulmonary neoplasia (primary or metastatic), and cardiovascular disease. Erythroid regeneration may be seen in the face of normal to increased PCV in birds with respiratory disease. PCVs ranging from 55–74% have been reported in older Amazon parrots (*Amazona sp*.) with idiopathic chronic pulmonary interstitial fibrosis. Affected individuals also had higher MCV and mean cell hemoglobin concentration compared with unaffected control birds.[49] Chronic obstructive pulmonary disease, also called macaw asthma, is associated with a marked erythrocytosis and has been reported in macaws (*Ara sp*.) housed in poorly ventilated aviaries and exposed to feather dust from cockatoos and African grey parrots.[46] Inappropriate secondary erythrocytosis due to erythropoietin-producing neoplasms has not been reported in psittacines.

Leukocyte Changes in Disease

Physiologic leukocytosis must be distinguished from pathologic leukocytosis in psittacines as in other species, and hematologic manifestations of epinephrine and corticosterone release associated with transport, physical restraint, blood sampling, and secondary to excess endogenous or exogenous corticosteroids are largely similar to those describe in other avian and mammalian species.[37,38,43]

Inflammation is the most common cause of leukocytosis in psittacines. Inflammatory leukocytosis in psittacines is typified by heterophilia, which may also be accompanied by a left shift and toxic change. The magnitude of the heterophilia can vary, typically in the range of 20–50,000 cells/μL,[29,30,41] but may be pronounced, especially in association with systemic mycobacteriosis, which can incite total WBC counts exceeding 100,000 cells/μL.[3,11,16,40,45] Monocytosis, lymphopenia, and reactive lymphocytes may also be observed in association with inflammatory disease in these species.[9,11,40,45] Granulocytic hyperplasia and left shift (increased numbers of myeloblasts and progranulocytes) were reported at necropsy in the bone marrow of a mature female lesser sulfur-crested cockatoo (*Cacatua sulphurea*) with an acute severe heterophilia and left shift with marked toxic change secondary to esophageal perforation from gavage feeding.[3] Infectious agents that have been reported to result in inflammatory leukograms in psittacines include bacterial (avian chlamydiosis,[40] avian mycobacteriosis, salmonellosis,[45] avian coxiellosis, sepsis), fungal (aspergillosis),[45] and protozoal (*Toxoplasma gondii*)[11] diseases. Mild leukocytosis may be observed associated with viral (Pacheco's disease) infections as well.[16] Non-infectious inflammatory diseases have also been associated with leukocytosis and include neoplasia, metabolic disease, intestinal foreign bodies, orthopedic disease, lead or zinc toxicities, and trauma.[6,8]

Lymphocytosis in psittacines typically reflects antigenic stimulation, which may be secondary to viral infections (e.g., herpesvirus, psittacine circovirus), although this may reflect a temporal response, as lymphopenia has also been reported in birds with these viral infections.[38,43] Chronic antigenic stimulation and vaccinal response should also be considered. Eosinophilia and basophilia are less common in birds and have been reported secondary to tissue necrosis, trauma, respiratory disease, intestinal parasites, and presumed primary immune mediated hemolytic anemia.[6]

Leukopenia is less commonly reported in psittacines. Leukopenia with or without concurrent nonregenerative anemia and thrombocytopenia is common in parrots with psittacine circovirus infections, the causative agent of psittacine beak and feather disease. Viral antigen was recovered from the bone marrow and circulating WBCs in a group of affected African grey parrots (*P. erithacus*) indicating a direct viral cytotoxic effect.[43] Leukopenia secondary to bone marrow toxicity from drugs (e.g., fenbendazole, chlorambucil) is a well-established finding in other species of birds and presumably in psittacines as well. Leukopenia due to overwhelming tissue demand for WBCs, as observed in cases of endotoxemia, also occurs in psittacines and is typically characterized by concurrent toxic change and left shift.[6] Finally, care should be taken to assess blood smears with apparent leukopenia for evidence of WBC rupture, as poorly prepared blood smears may be associated with prominent cell lysis and artifactual leukopenia.

Thrombocytosis has been reported concurrent with a marked regenerative response to anemia.[6] Thrombocytopenia, as a component of pancytopenia due to decreased bone marrow production, is observed with viral infections (psittacine circovirus and polyomavirus), lymphoid leukemia, and drug/toxin administration (e.g., chlorambucil).[22,38,39] Thrombocytopenia may also be secondary to peripheral consumption/sequestration,

as a sequela of disseminated intravascular coagulation, or endotoxemia.[6,38]

Hemic Neoplasia

Expected hematologic findings in psittacines with hemic neoplasia have been poorly characterized. Individual case reports indicate that a wide range of hematologic abnormalities may be observed, including lack of any hematologic abnormalities (Figure 124.4d,e). As in other species, leukemia and lymphoma with a leukemic phase can be challenging to distinguish in some cases; however, attempts to determine the primary site of disease burden, blood for leukemia and tissue for lymphoma, may be helpful in classifying the underlying disease process in many cases. Psittacines lack organized lymph nodes found in mammals and so lymphoma is often derived from scattered lymphoid aggregates in organs.

Marked leukocytosis due to a mature lymphocytosis with few slightly larger more immature-appearing lymphocytes, and no other hematologic abnormalities, has been reported in a double yellow-headed Amazon parrot (*Amazona oratrix*) and a green-winged macaw (*Ara chloropterus*) with chronic T-cell lymphocytic leukemia. Marked bone marrow infiltration by small mature lymphocytes was noted in both cases with concurrent marked peripheral lymphocytosis.[21,38,39] The leukemic phase of lymphoma is common in many avian species, and has been reported secondary to cutaneous, nonepitheliotropic B cell lymphoma in an umbrella cockatoo (*Cacatua alba*).[42] This patient had a moderate heterophilia and mild eosinophilia at the time of diagnosis. Heterophilia and a left shift were also reported in a yellow-collared macaw (*Primolius auricollis*) with retrobulbar and multicentric B cell lymphoma. No leukemic phase of lymphoma, i.e., no atypical circulating cells,

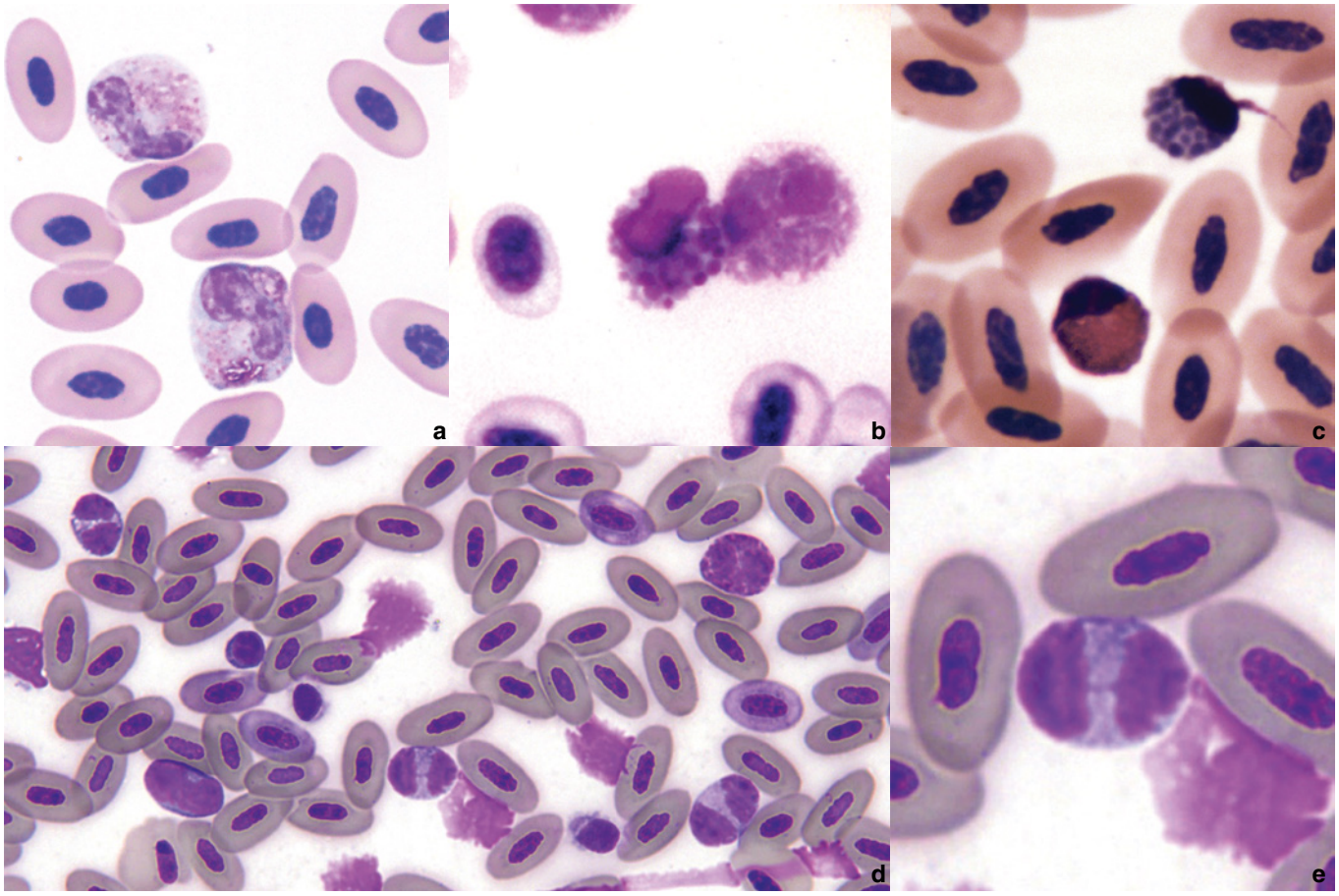


FIGURE 124.4 Leukocyte morphology found in sick psittacines. Wright–Giemsa or modified Wright's stain. RBCs may be used as internal size markers. (a) Toxic band heterophils with increased cytoplasmic basophilia and degranulation from a cockatoo; (b) Metamyelocyte from an African grey parrot (*P. erithacus*) showing distinct round, purple, primary granules next to a heterophil with mild degranulation. The total WBC count was estimated to be 100,000 cells/μL and the bird died within 48 hours of sampling due to intestinal perforation; (c) A heterophilic myelocyte in comparison to an eosinophil in a lilac-crowned Amazon (*Amazona finschi*) with ulcerated fungal dermatitis; (d) and (e) Probable lymphocytic leukemia in a blue-fronted Amazon parrot (*Amazona aestiva*) that presented repeatedly for CBCs with approximately 50,000 lymphocytes/μL for 2 years. The neoplastic cell morphology, which almost appears binucleate with a strand of nuclear material, was a consistent finding.

was noted in this individual despite the observation of 5–20% neoplastic lymphocytes in a patchy distribution throughout the bone marrow at necropsy.[33] In this case, erythroid and granulocytic lineages were admixed with the neoplastic cells and showed normal maturation.

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CHAPTER 125

Hematology of Anseriformes

JESSICA HOKAMP and JULIE PICCIONE

Blood Collection
Erythron
Leukon
Thrombon and Coagulation
Reference Intervals and Influencing Factors
Bone Marrow Collection and Evaluation
Hematologic Disease
Heavy Metal Toxicity
Crude Oil Toxicity
Inflammatory Leukogram
Leukemia
Hemoparasites

Acronyms and Abbreviations

ASVCP, American Society for Veterinary Clinical Pathology; H:L ratio, heterophil:lymphocyte ratio.

The Anseriforme order of birds is composed of at least 180 waterfowl species within three families. These include the largest family, Anatidae, which houses ducks, geese, and swans, and two smaller families, Anhimidae (screamers) and Anseranatidae (of which the only living species is the magpie goose, *Anseranas semipalmata*). These birds are all adapted to an aquatic existence. Members of Anatidae are commonly used for meat production and hunted for sport, but are also companion animals and housed at zoo facilities. Hematology is a rapid, inexpensive, and relatively minimally invasive method to evaluate some aspects of health in these species.

BLOOD COLLECTION

When collecting blood from large waterfowl, use of a 3 mL syringe with a 20–25-gauge needle is recommended. Visualization of the jugular vein can be difficult in these species due to lack of the apterium (featherless tract over the jugular vein); therefore, the basilic vein (medial aspect of the elbow) and the medial metatarsal vein (medial aspect of the hock) are recommended as more accessible sites. Accessing the basilic vein can be difficult and stressful; of course, this is dependent on user experience. The medial metatarsal vein may be easier to access and less stressful for the animal, especially if a light towel or cloth is used to cover the face and eyes, without blocking the airways, to reduce visual sensation. The medial metatarsal vein is more resistant to hematoma formation than the basilic vein. A wrap may be used to ensure hemostasis.[10]

ERYTHRON

The erythron is assessed by the same means as other birds, using total RBC count, PCV, hemoglobin concentration (Hgb), mean cell volume (MCV), mean cell hemoglobin concentration (MCHC), and evaluation of RBC morphology (see Chapter 123). Normal Anseriforme RBCs are oval with eosinophilic cytoplasm and a centrally located oval nucleus with densely clumped basophilic chromatin (Figure 125.1). Mallard (*Anas platyrhynchos*), canvasback (*Aythya valisineria*), and lesser scaup (*Aythya affinis*) RBCs are oval measuring 12–14 µm in length and 6–8 µm in width with nuclei that vary from 5–6 µm in length and 2–3 µm in width.[9,23] These are larger than RBCs of domestic mammals, with an average calculated MCV of 192 fL noted for Indian runner ducks (*Anas platyrhynchos domesticus*), 164 fL for wood ducks (*Chenonetta jubata*), and 145 fL for black ducks (*Anas superciliosa*).[6,29,30]

Normal RBCs of healthy waterfowl display minimal to mild anisocytosis and anisokaryosis. Erythrocyte life span is estimated at a maximum of 42 days in

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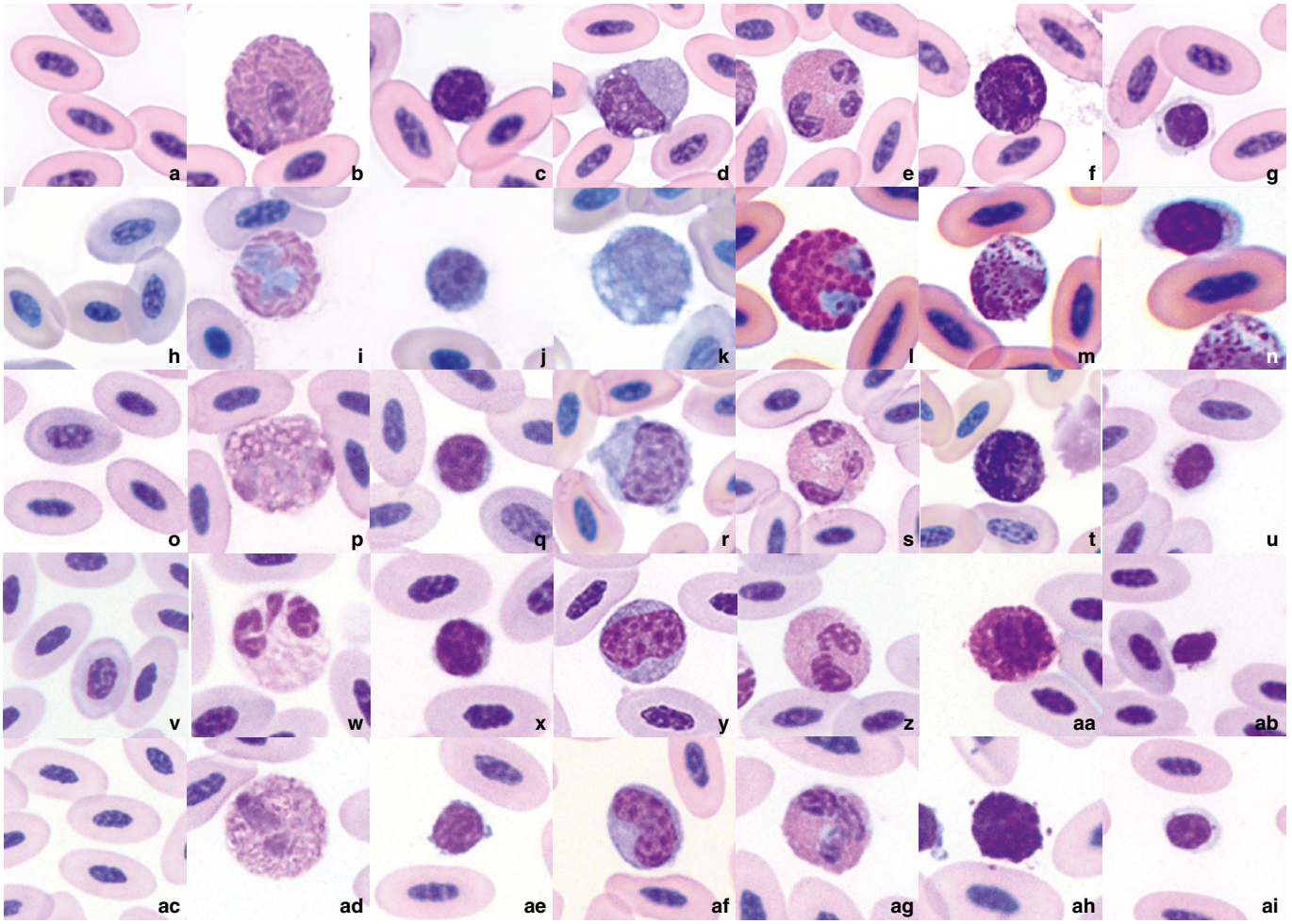


FIGURE 125.1 Normal peripheral blood cells from five species of healthy, adult Anseriformes. Mallard (*Anas platyrhynchos*): (a) Mature erythrocytes; (b) Heterophil; (c) Lymphocyte; (d) Monocyte; (e) Eosinophil; (f) Basophil; (g) Thrombocyte. Pekin Duck (*Anas platyrhynchos domesticus*): (h) Mature erythrocytes and polychromatophils; (i) Heterophil; (j) Lymphocyte; (k) Monocyte; (l) Eosinophil; (m) Basophil; (n) Thrombocyte. Baer's pochard (*Aythya baeri*): (o) Mature erythrocytes and a polychromatophil; (p) Heterophil; (q) Lymphocyte; (r) Monocyte; (s) Eosinophil; (t) Basophil; (u) Thrombocyte. Ringed teal (*Callonetta leucophrys*): (v) Mature erythrocytes and a polychromatophil; (w) Heterophil; (x) Lymphocyte; (y) Monocyte; (z) Eosinophil; (aa) Basophil; (ab) Thrombocyte. Black swan (*Cygnus atratus*): (ac) Mature erythrocytes; (ad) Heterophil; (ae) Lymphocyte; (af) Monocyte; (ag) Eosinophil; (ah) Basophil; (ai) Thrombocyte. Wright–Giemsa, 1000×. (**Source:** Samples and images provided by The Ohio State University, Moody Gardens, the Gladys Porter Zoo, and Dr. Kendal Harr.)

healthy white Pekin ducks (*A. platyrhynchos*). Polychromatophils are more rounded and slightly larger than mature RBCs with increased cytoplasmic basophilia (Figure 125.1). In most healthy ducks of a variety of species, the average PCV is approximately 45% which is similar to tundra swan (*Cygnus columbianus*).[2,28,34] Indian runner ducks have a reported average RBC count of $2.5 \times 10^6/\mu L$ and average Hgb of 13.8 g/dL.[6] The clinician should be aware that calculated hematocrit as determined by the i-STAT 1 (Abbott Point of Care Inc., Abbott Park, Illinois, USA) is consistently lower than PCV determined by microhematocrit tube centrifugation. This discrepancy was demonstrated in mottled ducks (*Anas fulvigula*)[34] and is similar to findings in other avian species. The authors of the study do not recommend direct comparison of i-STAT determined Hgb and hematocrit with other analyzers as these are likely falsely decreased in ducks and other avian species.

LEUKON

The leukon is assessed by the same means as other birds and includes a total WBC count, differential WBC counts, and morphologic evaluation (see Chapter 123). Average total WBC count has been reported between $6.3–20.9 \times 10^9/\mu L$ in ducks.[6,34] Circulating Anseriforme WBCs are composed of the same cell types as other avians and include heterophils, lymphocytes, monocytes, eosinophils, and basophils. Most studies of healthy Anseriformes have found lymphocytes are the predominant leukocyte type and the heterophil to lymphocyte (H:L) ratio is less than 1.[11,15,29,30,40] However, this was inverted in ferruginous ducks (*Aythya nyroca*), mottled ducks, and tundra swans, which had H:L ratios greater than 1 (e.g., H:L was approximately 1.3 in mottled ducks).[2,28,34] Importantly, this H:L inversion may be due to species differences, capture stress, or undiagnosed inflammatory conditions in the population.

Heterophils are round to oval with clear cytoplasm that is filled with elongated to fusiform eosinophilic granules that are slightly refractile in appearance. They contain a bi to multilobed nucleus with condensed chromatin (Figures 125.1 and 125.2). In ducks, heterophils were described as approximately 10 μm in diameter.[36] Rod-shaped heterophil granules can be distinguished from eosinophils by their lack of distinct granule borders and refractile, slightly muddied appearance.

Lymphocytes are predominantly small, ranging from 4–8 μm in diameter with scant amounts of light blue cytoplasm visible on one side of a round to slightly cleaved nucleus with condensed chromatin (Figures 125.1 and 125.2). Low numbers of intermediate-sized lymphocytes with a slightly increased amount of cytoplasm are commonly seen in Anseriforme blood smears as well. Additionally, a few reactive lymphocytes might be seen as part of an antigenic response with deeper blue cytoplasm, pseudopods, and purple cytoplasmic granules.

Monocytes, the largest WBC in circulation, are similar in appearance to other birds and mammals. They are round to irregular in shape with a moderate amount of basophilic to gray cytoplasm that is often vacuolated. They contain a round to lobed to irregular nucleus with variably condensed chromatin (Figures 125.1 and 125.2).

Eosinophils are round with pale blue to clear cytoplasm and many distinct eosinophilic, fine, round to oval granules. They contain a bi to trilobed nucleus with condensed chromatin (Figure 125.1). Eosinophils are reported to average 9.7 μm in diameter in ducks.[36] Duck eosinophils have been reported to have rod-shaped granules, but this has not been confirmed with ultrastructure or cytochemical stains at this time.

Basophils are round to slightly irregular in shape with numerous basophilic round granules that often obscure the nucleus. These average 6.4 μm in diameter in ducks[36] (Figure 125.1).

THROMBON AND COAGULATION

There is a paucity of information regarding thrombocytes and coagulation in Anseriformes. Thrombocytes are nucleated round to oval cells with a small amount of clear cytoplasm that surrounds the ovoid nucleus. The cytoplasm frequently contains one to several round azurophilic granules at one pole (Figure 125.1). Transmission electron microscopy has demonstrated that thrombocytes in ducks are 4.5–6 μm in diameter with large vacuoles.[4] Thrombocyte counts were low in 5-day-old mallards (fewer than five per 10 high-powered fields) and increased to a plateau of approximately 20 thrombocytes per 10 high-powered fields at 18 days old, which is consistent with adult thrombocyte counts.[11] Thrombocytes are also commonly seen in large clumps, mostly on the feathered edge as well as in the body of the smear. Thrombocyte clumping is typically interpreted to indicate an adequate thrombon mass.

REFERENCE INTERVALS AND INFLUENCING FACTORS

Only one study of ferruginous ducks in Israel adheres to the American Society for Veterinary Clinical Pathology (ASVCP) guidelines for correctly establishing and reporting de novo reference intervals in veterinary species.[2,13] The authors of this study reported that guidelines for establishing reference intervals were followed for PCV (38–54%), total WBC count ($0.5–9.2 \times 10^3/mL$), and WBC differentials. However, this study only reported relative values for quantifying polychromasia, and total WBC concentration was estimated. Furthermore, the low reference values for all WBC types (heterophils, lymphocytes, monocytes, eosinophils, and basophils) were $0.0 \times 10^3/mL$; therefore, these data were not included in this text. Articles that did not adhere to reference interval guidelines are cited.[28,34] Deviations from the guidelines included publication of the study

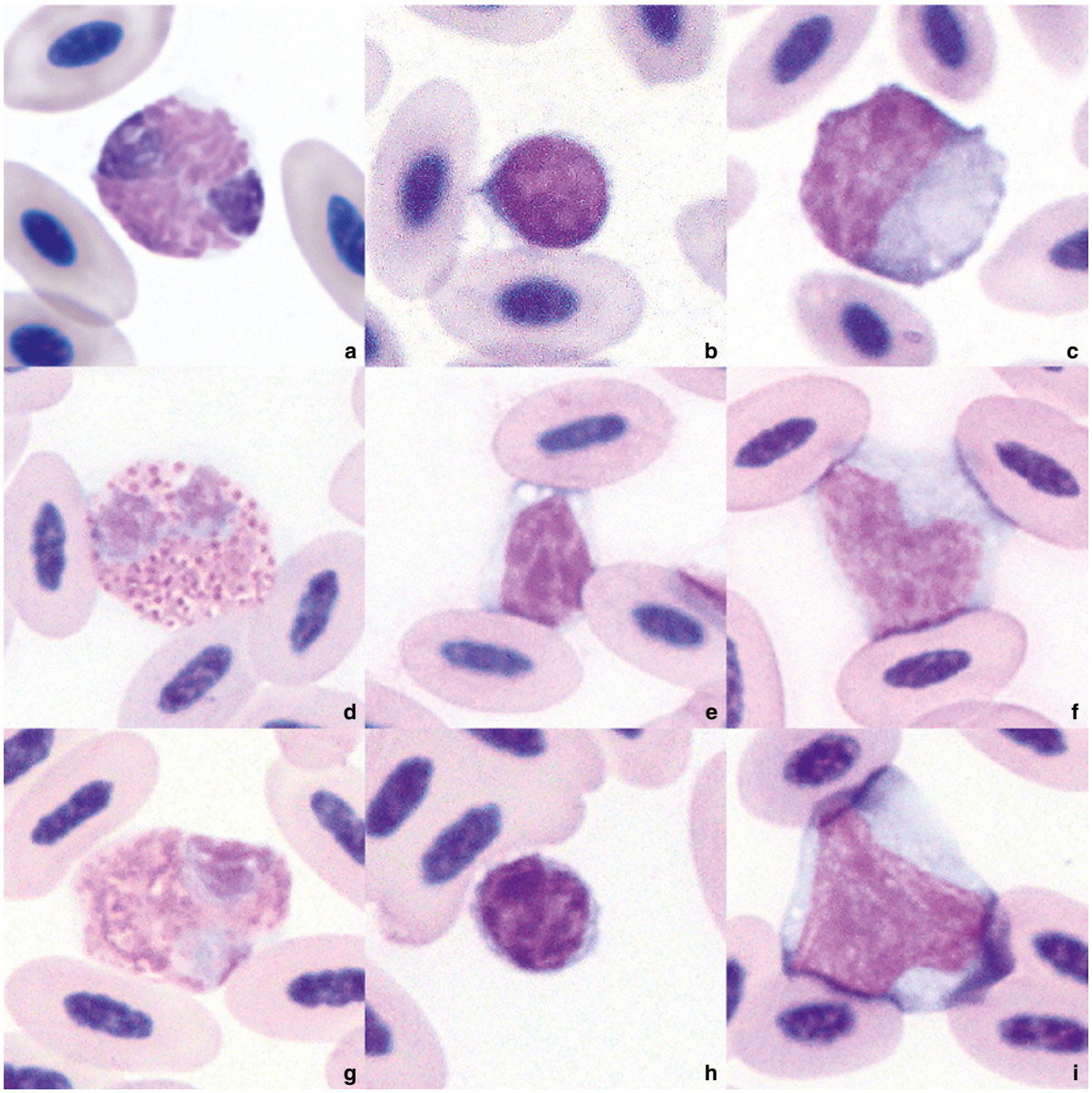


FIGURE 125.2 Selected normal leukocytes from three species of healthy, adult Anseriformes: Canada goose (*Branta canadensis*): (a) Heterophil; (b) Lymphocyte; (c) Monocyte. Black-bellied whistling duck (*Dendrocygna autumnalis*): (d) Heterophil; (e) Lymphocyte; (f) Monocyte. Wood duck (*Aix sponsa*): (g) Heterophil; (h) Lymphocyte; (i) Monocyte. Wright–Giemsa, 1000×. (**Sources:** Samples provided by the Ohio Wildlife Center and the Cameron Park Zoo.)

prior to ASVCP guidelines, insufficient number of target species, inability to prove health, limited statistical analysis, and/or inclusion of outliers.

Pre-analytical factors can affect reference intervals, and pre-analytical conditions (e.g., sampling method) must be reported and reviewed when establishing reference intervals. Capture method and time of day likely had stress-related and diurnal effects on hematologic values of mottled ducks. Ducks captured by hand net at night had significantly lower PCV and H:L ratio and significantly higher absolute WBC, eosinophil, and lymphocyte counts compared with ducks caught by rocket net during the day. The increased H:L ratio in the latter group suggests increased stress, but the effect of capture method and stress on hematologic values cannot be separated from a diurnal effect.[34]

Several biologic factors influence reference intervals in Anseriformes and these must be considered when designing or reviewing a reference interval study (Table 125.1). Reported effects of age on RBC, WBC, and thrombocyte concentrations vary. Increased PCV has been reported with aging in canvasback ducks (4–10-week-old ducklings versus adults >1 year) and trumpeter swans (*Cygnus buccinator*) (3–4 months versus 5–8 months).[21,32] Ferruginous ducks had a higher proportion of polychromatophils as juveniles than as adults.[2] This pattern is consistent with erythroid turnover and regeneration in neonates and juveniles. While

TABLE 125.1 Effect of sex on hematologic values in selected Anseriforme species. No significant differences in hematologic values between sexes in Anseriformes are reported, except those related to seasonal influences and breeding. Differences in WBC types between sexes were not reported.

	Canvasback ducks (*Aythya valisineria*)[23]	Lesser scaups (*Aythya affinis*)[23]	Tundra swans (*Cygnus columbianus*)[28]
Animals in study	Male: 26 Female: 28	Male: 21 Female: 9	Male: 44 Female: 66
	Mean ± SD	Mean ± SD	Mean ± SE
PCV (%)	M: 51.4 ± 2.5	M: 56.5 ± 3.1	M: 45.0 ± 0.5
	F: 53.1 ± 2.7	F: 58.0 ± 2.9	F: 44.6 ± 0.4
RBC (× 10^9/L)	M: 2.56 ± 0.18	M: 2.40 ± 0.12	
	F: 2.56 ± 0.16	F: 2.52 ± 0.13	
WBC (× 10^9/L)			M: 18.26 ± 1.77
			F: 19.7 ± 1.09

juvenile and adult wild tundra swans did not differ in their total WBC counts and differentials, juvenile mottled ducks and trumpeter swans demonstrated a significant decrease in total WBC count with aging toward adulthood.[32,28,34] Furthermore, a greater H:L ratio in adult versus juvenile mallards and ferruginous ducks has been reported.[2,11] It was suggested that a postjuvenile decrease in lymphoid organ activity might contribute to a decrease in peripheral lymphocytes.[2] Absolute eosinophil counts were significantly greater in adult ferruginous ducks than in first-year ducks, and it was presumed this was due to increased parasite exposure with increasing age.[2] Juvenile mottled ducks had significantly greater average absolute monocyte counts (1.1 × 10^9/L) compared with adults (0.7 × 10^9/L).[34] Only one study has explored aging changes in thrombocyte counts of Anseriformes. Five-day-old mallards had lower thrombocyte counts than adults, with thrombocyte counts reaching a plateau consistent with adult values (approximately 20 thrombocytes per 10 high-powered field) by 18 days of age.[11]

The literature overwhelmingly shows that there are no significant differences in hematologic values between sexes in Anseriformes, except those related to seasonal influences and breeding which will be discussed later. Wood ducks, black ducks, upland geese (*Chloephaga picta*), tundra swans, and trumpeter swan cygnets did not show significant differences in hematologic values between males and females.[28–30,15,32] Few studies have evaluated hematologic differences due to reproductive activity in Anseriformes. Both male and female mallards had significantly increased thrombocyte counts during pre-egg-laying and egg-laying stages compared with incubation, molting, and postreproductive stages.[11] Loss of a social partner had a stress-related effect on hematologic values in greylag geese (*Anser anser*), with decreased PCV during mate removal in both males and females. Isolated males also demonstrated elevated total WBC count and H:L ratio.[26]

Seasonal variation in hematologic values is common in Anseriformes and should not be confused with pathologic change. A few species have demonstrated higher RBC, PCV, Hgb, and MCHC and lower MCV during prenesting periods (winter and spring) compared with postnesting periods (summer and fall).[33,37] For male and female Canada Geese, RBC averaged 2.67–2.78 × 10^9/L, PCV averaged 51–52%, Hgb averaged 16.2–17.1 g/L, MCHC averaged 31.6–33.0 g/L, and MCV averaged 188.5–193.3 fL in the pre-nesting period (January to March), while in the post-nesting period (June to October) RBC averaged 2.18–2.30 × 10^9/L, PCV averaged 45–46%, Hgb averaged 13.6–14.0 g/L, MCHC averaged 29.9–30.7 g/L, and MCV averaged 198.8–210.1 fL.[37] For male and female mallards, RBC averaged 3.35–3.71 × 10^9/L, PCV averaged 49–50%, Hgb averaged 15.0–15.6 g/L, MCHC averaged 30.3–31.6 g/L, and MCV averaged 135.2–148.4 fL in the pre-nesting period (January–March), while in the post-nesting period (July–October) RBC averaged 2.01–2.51 × 10^9/L, PCV averaged 39–45%, Hgb averaged 11.4–13.5 g/L, MCHC averaged 29.2–29.9 g/L, and MCV averaged 186.6–199.6 fL.[37] Canvasback ducks in midmigration (November) were found to have lower average RBC (2.63–2.87 × 10^9/L) and higher average MCV (202–209 fL) than over-wintering ducks during December–January (RBC, 2.99–2.96 × 10^9/L; MCV, 186–187 fL) and February–March (RBC, 3.09–3.28 × 10^9/L; MCV, 165–168 fL).[21] It is suggested that these differences are due to hormonal changes that occur with altered photoperiod and nutritional changes during migration and nesting.

BONE MARROW COLLECTION AND EVALUATION

Bone marrow collection from the proximal tibiotarsus has been described in Canada geese (*Branta canadensis*), using a 14–16-gauge, 3-inch needle and a 10 mL syringe following sterile technique. Syringes and collection tubes should contain EDTA. Birds were restrained on their backs, and the needle was inserted 0.5 cm below and slightly to the right of the tibiotarsal anterior crest. This technique yielded adequate marrow cellularity for cytologic analysis and resulted in minimal, if any, lameness after the procedure.[8,39]

Very little work has been done to characterize normal bone marrow in Anseriformes. It is recommended to perform at least a 500-cell differential, as in other species. One study that evaluated 10 apparently healthy ducks (unstated species) native to Northern Iran found that precursor morphology and blood cell formation were similar to gallinaceous birds (see Chapter 123). Mean myeloid to erythroid ratio was 1.0. The erythroid series was composed predominantly of late polychromatophilic rubricytes (mean, 20.5%) with a low percentage of rubriblasts (mean, 0.8%). The myeloid series was composed predominantly of heterophilic myelocytes (mean, 14.8%). The specific myeloid series could be identified by secondary granules seen at the myelocyte stage. Lymphocytes were not observed in ducks, but plasma cells were present at a mean of 2.0%.[39] The authors surmised that peripheral blood lymphocytes likely arise from the spleen and gut-associated lymphoid tissue in many adult birds, as reported by Zinkl 1986.[41]

HEMATOLOGIC DISEASE

Heavy Metal Toxicity

Heavy metal toxicity is an unfortunate result of waterfowl and humans' coexistence and can occur via ingestion of or penetration by zinc or lead shot or ingestion of fishing weights. Ingestion of zinc-contaminated vegetation and sediment is another route of exposure. Experimentally induced zinc toxicity in mallards, which causes abnormal hematopoiesis and decreased RBC life span, caused a regenerative anemia characterized by decreased PCV and Hgb concentration (anemia) and increased reticulocyte counts (erythroid regeneration) compared with blood samples from control birds. Abnormal erythroid morphology included increased polychromasia, immature RBCs (basophilic and polychromatophilic prorubricytes), poikilocytosis, and binucleated RBCs.[14,25]

Lead toxicity typically results in microcytic, hypochromic anemia due to inhibition of enzymes involved in heme synthesis, including aminolevulinic acid synthase, aminolevulinic acid dehydratase, and ferrochelatase. Lead toxicity effects on hematologic values have been studied in a variety of free-ranging ducks in Argentina, trumpeter swans, and Canada geese. High blood lead concentrations were associated with significantly decreased PCV, RBC, Hgb, MCV, and MCHC and abnormal RBC morphologies including anisocytosis, poikilocytosis, polychromasia, hypochromasia, basophilic stippling, and increased numbers of immature rubricytes (Figure 125.3a,b).[3,12,18] Maturation arrest of erythroid precursors was also seen in the bone marrow of mallards.[3] Experimental lead intoxication in mallards caused increased mean total WBC counts, absolute heterophil counts, numbers of toxic heterophils and reactive lymphocytes, and decreased absolute lymphocyte counts compared with controls.[3,25] Similarly, increased blood lead concentrations in trumpeter swans were associated with a significantly increased total granulocyte count.[18]

Crude Oil Toxicity

Oil pollution is a serious hazard to waterfowl health. Oiling of plumage results in loss of thermal regulation and decreased feather integrity may hinder or prevent flight. Ingestion of oil-contaminated food, water, and preening of oiled feathers can affect metabolic function, suppress adrenocortical function, and induce hemolytic and/or hemorrhagic anemia in ducks and other aquatic birds.[16,17,24,35] Heinz bodies, which are microscopic evidence of oxidative damage to Hgb, and damaged organelles have been reported in RBCs of various species of aquatic and marine birds and likely also occur with crude-oil ingestion in Anseriformes.[17,24] In wild sea ducks

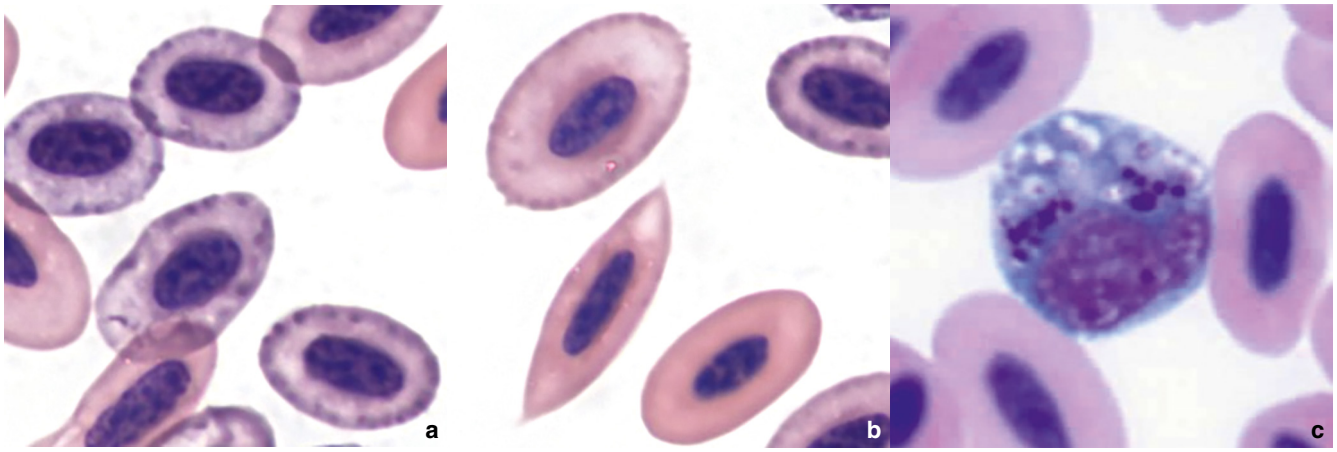


FIGURE 125.3 Abnormal erythrocyte and leukocyte morphology in Anseriforme blood. (a) Hypochromasia; (b) Anisocytosis and poikilocytosis in an anemic trumpeter swan (*Cygnus buccinator*) with lead toxicity. Wright's stain, 500×. Images courtesy of Dr. Dorothee Bienzle; (c) Metamyelocyte with primary granules in a duck (unknown species). Wright's stain, 1000×. (**Source:** Image courtesy of Dr. Kendal Harr.)

naturally affected by an oil spill, birds demonstrated regenerative anemias evidenced by reduced RBC count, PCV, and Hgb with increased polychromatophils. Marked multiorgan hemosiderin deposition on necropsy was presumed to be due to hemolysis.[40] It should be noted that other bird species had documented hematochezia due to coagulopathy, which may also induce regenerative, blood loss anemia.

Other anemias have been documented in research studies. Experimentally induced hypothyroidism in barnacle geese (*Branta leucopsis*) had significantly decreased PCV, by 18%, and Hgb concentration, by 22%, compared with control geese.[7]

Inflammatory Leukogram

Inflammatory changes in the leukogram of Anseriformes are similar to other species. Depending on severity and duration of the inflammatory process, either heterophilia or heteropenia may be seen, and both can be accompanied by a left shift and/or toxic change (Figure 125.3c). In some cases, lymphocytosis and monocytosis are seen with inflammatory response; or lymphopenia might occur concurrently as a stress response. Bienzle et al. reported cases of wild Anseriformes with hematologic abnormalities related to infectious and inflammatory processes. These included a septicemic teal (*Anas sp.*) with heteropenia, severe left shift, and toxic change; an Aleutian Canada goose (*Branta canadensis leucopareia*) with a mild left shift and moderate toxic change due to osteomyelitis and aspergillosis; and a black-bellied whistling duck with marked heterophilia, left shift, and toxic change due to septic osteoarthritis, valvular endocarditis, and hepatic necrosis. In the latter case, the total WBC count was 278 × 10^9/L and mature and immature heterophils each exceeded 100 × 10^9/L. Lymphocytosis, monocytosis, and eosinophilia were also present. This demonstrates the severe hematologic changes that can occur with inflammation in these species and the risk of misdiagnosis of leukemia if blood smears and complete case information are not evaluated appropriately.[5]

Leukemia

Few reports of leukemia in Anseriformes exist, especially in comparison to reports in domestic animals. Chronic T-cell lymphocytic leukemia was diagnosed in a black swan (*Cygnus atratus*) after severe lymphocytic leukocytosis was found incidentally on a health exam (reported as >1,000,000 total WBCs/μL [?] with 98% lymphocytes). Immunocytochemistry and immunohistochemistry confirmed CD3+ neoplastic lymphocytes in the peripheral blood, bone marrow, liver, spleen, heart, lungs, and kidneys. Neoplastic lymphocytes were described as small, round, and mature with a high nuclear to cytoplasmic ratio, mature condensed chromatin, and no overt nucleoli. The swan did not have a concurrent cytopenia of other cell lines and survived 433 days after initial evaluation.[38] This case contrasts with that of a 4-year-old Pekin drake diagnosed with lymphocytic leukemia and malignant lymphoma after presentation with depression, anorexia, and coughing; anemia, thrombocytopenia, and extreme leukocytosis (estimated at 2,000,000 total WBCs/μL) characterized by a marked lymphocytosis (99% lymphocytes) were found on clinicopathologic evaluation. Circulating lymphocytes were predominantly small. The duck survived 1 month after initial diagnosis, and on necropsy, well-differentiated, cleaved lymphocytes diffusely involved the liver, spleen, intestines, lungs, air sacs, kidneys, heart, ventriculus, proventriculus, and bone marrow. Erythroid hypoplasia was noted in the marrow at necropsy.[31]

Hemoparasites

Ducks, geese, and swans are found to be commonly infected with species of *Hemoproteus*, *Leukocytozoon*, and *Plasmodium* with one study reporting a >75% infection rate with at least one hemotropic parasite in surveyed waterfowl[1] (Figure 125.4; see Chapters 123 and 126). There are fewer reports of *Trypanosoma sp*. in waterfowl, and microfilaria are also occasionally seen in peripheral blood smears (Figure 125.4). *Plasmodium* and *Leucocytozoon sp*. are capable of causing severe anemia, weight loss, and death in susceptible birds, of which juveniles are often more susceptible than adults. Infections are typically

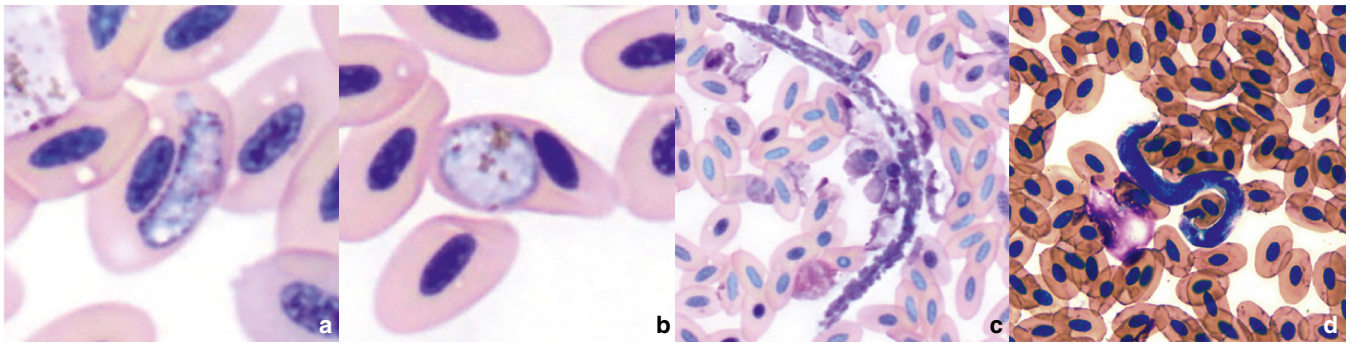


FIGURE 125.4 Hemoparasites of Anseriformes. (a) *Hemoproteus spp*. in a wood duck (*Aix sponsa*); (b) *Plasmodium spp*. in a northern pintail (*Anas acuta*); (c) *Microfilaria* in a wood duck. Wright's stain, 500–1000×. Images courtesy of Dr. Liza Bau-Gaudreault; (d) *Microfilaria* in a grebe (species unknown; note, grebes are in the order Podicipediformes, not Anseriformes). Wright's stain, 500×. (**Source:** Image courtesy of Dr. Kendal Harr.)

seasonal because transmission is vector-dependent: (*Hemoproteus sp*. via *Culicoides* and *Ornithomyia sp*. flies; *Plasmodium sp*. via *Culex* and *Aides sp*. mosquitoes; *Leukocytozoon sp*. via *Simulium sp*. black flies). Potential clinical signs include weight loss and anorexia or hyporexia, lethargy, weakness, lameness, and dyspnea.[1] Of particular importance, *Leucocytozoon simondi* appears to be a significant cause of anemia due to intravascular hemolysis in several duck species.[19,20,22,27]

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CHAPTER 126

Hematology of Raptors

JENNIFER JOHNS

Blood Collection
Erythron
Leukon
Reference Intervals
Hematologic Disease
Anemia
Inflammation
Common Disease Conditions
Coagulopathy
Hemoparasites

Acronyms and Abbreviations

CI, confidence interval; LD50, median lethal dose; LRL, lower reference limit; NA, not available; R, robust method; RI, reference interval; RT, robust method after Box–Cox transformation; TS/TP, total solids/total protein concentration; URL, upper reference limit.

The term "raptor" encompasses birds of prey, generally defined as carnivorous birds with a hooked beak and sharp talons that consume vertebrate species. Raptors include the families of generally diurnal birds Accipitridae (hawks, eagles, buzzards, harriers, kites, Old World vultures), Cathartidae (New World vultures, condors), Falconidae (falcons, forest falcons, kestrels, caracaras), Pandionidae (ospreys), and Sagittariidae (secretary bird, *Sagittarius serpentarius*); and the nocturnal owl families, Strigidae and Tytonidae.

BLOOD COLLECTION

Sample sites for blood collection in raptor species vary with patient size and potential use of sedation or anesthesia. The medial metatarsal vein, cutaneous ulnar vein, basilic vein, and jugular vein are common peripheral blood collection sites. The largest bore vein possible should be chosen to improve sample quality. Sample hemolysis can result from patient movement during collection, and hematomas may form. Lipemia is an uncommon sample condition for raptor species, but is reported as a postprandial effect. Capture, transport, confinement, and physical restraint may all contribute to a stress response that can alter hematologic values. In birds as a whole, blood volume is estimated at 6–12% of a bird's total body mass, with a recommended maximum blood collection volume of 1% of the patient's body weight to avoid deleterious effects.[8]

The anticoagulant of choice for hematologic evaluation of raptor blood is EDTA, as there is less leukergy and it does not result in staining artifact. In vitro hemolysis is reported infrequently and EDTA-anticoagulated blood is acceptable for smear preparation in raptors. However, as with other avian species, a common collection method is to place blood in a heparinized tube (preferably lithium heparin). A single sample can then be used for both hematologic and biochemical evaluation with maximal use of the sample volume. The minimum blood volume necessary for hematologic evaluation is removed, then the remaining sample is centrifuged to yield heparinized plasma for biochemical analysis. The potential downsides of heparin use are interference with smear staining and increased cell clumping which impairs accurate counting and morphologic evaluation. Small blood collection tubes for pediatric use (microtainers) are useful to ensure the correct ratio of blood to anticoagulant. A blood smear should be made prior to placing the blood sample in heparin anticoagulant, as staining artifacts can result in smears of heparinized blood and impair hematologic analysis. However, if very small blood volumes are collected,

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this may not be practical as the risk of the sample coagulating quickly is higher. Coverslip smears can be useful to reduce cell lysis due to blood smear preparation, but reference laboratories vary in their policies of accepting coverslip smears.

ERYTHRON

The average raptor mean red cell volume (MCV) ranges from 168–188 fL in different species, with RBC elliptical dimensions in blood smear evaluation reported as approximately 8 × 15 μm.[33] RBC life span is generally assumed to be similar to other avian species, ranging between 28 and 45 days.[39] As in other avian species, PCV measurement is used to measure oxygen-carrying capacity in raptors. Plasma from the spun hematocrit tube is then used to measure protein concentration via refractometry. Refractometers vary in accuracy of avian plasma protein measurement when compared to the biuret method standard; correlation to standard protein measurement is recommended prior to use of refractometers for samples from nonmammalian species.[7] Erythrocyte manual counting may be performed to calculate the mean cell volume using a hemocytometer-based Natt–Herrick staining technique or commercially available disposable pipette systems (see Chapter 123). Hemoglobin (Hgb) measurement is performed in some laboratories by lysing RBCs, centrifuging the sample to pellet nuclei and membranes, then measuring Hgb in the supernatant using the cyanmethemoglobin method. The Hgb concentration is then used to calculate the mean cell hemoglobin concentration (MCHC). Reticulocyte staining is performed using a 3:1 dilution of 0.5% new methylene blue stain to whole blood, incubating the blood for 10 minutes, and then preparing a smear. Ring-form reticulocytes are defined as RBCs containing aggregates of stained reticulum that form a ring, contiguous or discontiguous, around at least half of the nucleus. Ring-forms correlate more closely with polychromatophils in a Wright's-stained smear than other defined reticulocyte types[9] (See Chapter 123). Evaluation of RBC morphology includes semiquantitative assessment of polychromatophils (Figure 126.1a,b), anisokaryosis, and poikilocytosis including specific morphologic abnormalities, and hemic parasites (Figure 126.4). Polychromatophil and reticulocyte percentages in raptors generally range up to 5% in health; increased percentages indicate increased erythroid regeneration. Rubricytes are occasionally found and RBC more immature than rubricytes (Figure 126.1b,c) are generally not found in blood smears from healthy raptors.

LEUKON

As with other nonmammalian species, avian hematologic methods generally rely on manual counting of WBCs and thrombocytes. Three common methods of manual leukocyte counting are the two hemocytometer-based techniques using either Natt–Herrick or phloxine B stain, and a manual estimate from a stained blood smear. References vary as to the comparative accuracy of the three techniques for WBC counting in raptors and other birds.[8] Thrombocytes and RBCs can also be quantified via the Natt–Herrick technique; a 60-minute stain incubation improves differentiation between lymphocytes and thrombocytes.[3] Alternately, toluidine blue staining using a 1:100 ratio of blood to 0.01% stain can permit quantification of RBCs, WBCs, and thrombocytes. Smear estimation of thrombocyte concentration can be performed, but thrombocyte clumps (Figure 126.1d) and lysis can render the estimate useless. Leukocyte concentration estimate can be performed and substantial changes such as heterophilia and monocytosis can often be detected. Evaluation of WBC morphology may detect abnormalities such as immaturity and toxicity in heterophils, reactive changes in lymphocytes, and hemic parasites.

Heterophils have lobulated nuclei and contain orange-pink to brownish, rod-shaped to fusiform granules (Figure 126.2a–g). A mean diameter of 8.8 μm is reported for avian heterophils.[25]

Lymphocytes in healthy raptors vary from small to intermediate in size and generally have scant volumes of basophilic cytoplasm and rounded nuclei (Figure 126.2h–n). Thrombocytes can generally be distinguished from lymphocytes by their clear to pale blue cytoplasm, oval to slightly elongated nuclei, and smaller nuclear size. Monocytes are morphologically similar to those in other avian species and often have more rounded nuclei than the mammalian form (Figure 126.2o–u); vacuolated cytoplasm may be prominent if activated.

Eosinophils have bilobed nuclei and contain orange-pink granules that are generally round, but may be oblong and are more refractile (Figure 126.2v–ab). Eosinophils and heterophils are roughly the same size; a mean avian eosinophil diameter of 7.9 μm is reported.[25] Rare raptors (e.g., king vultures) have eosinophils with grayish granules, and raptors in the genus *Falco* have eosinophils with bluish cytoplasm that can appear vacuolated and poorly granulated and/or contain basophilic granules.[9,42] Owl eosinophils have been reported to have variable granulation, though cytochemistry and electron microscopy have not been used to confirm identification. For example, owls in the genus *Strix* have eosinophils with rounded bluish to dull pink granules and owls in the genera *Asio* and *Otus* have eosinophils with round bright pink/orange granules that often appear smaller than heterophils.[9] Basophils contain deep purple granules, but can appear hypogranulated or degranulated (Figure 126.2ac–ai), and are similar in size to heterophils.

REFERENCE INTERVALS

Many reference intervals (RI) used for raptors were not created in accordance with American Society for Veterinary Clinical Pathology guidelines and medical

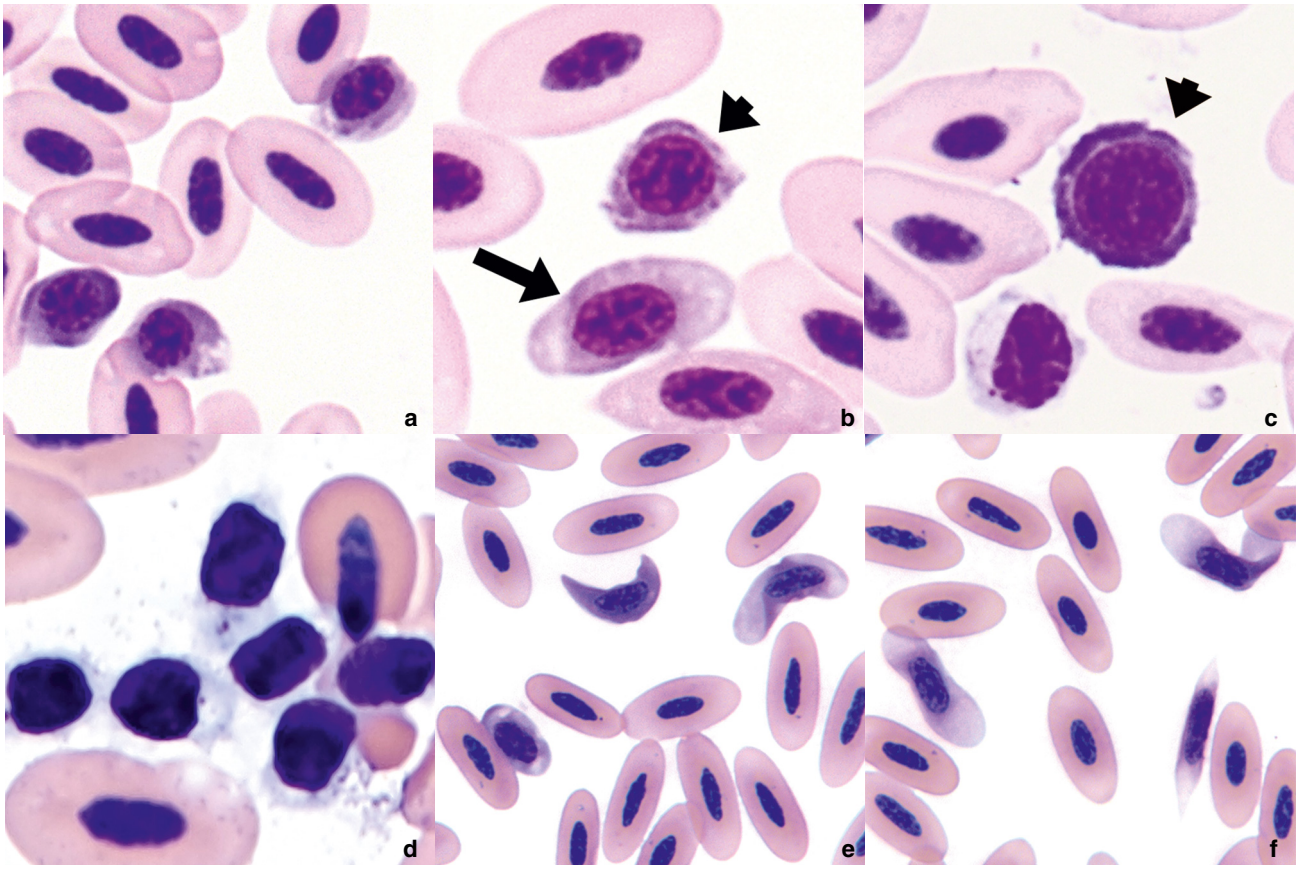


FIGURE 126.1 Normal and abnormal RBC morphology. All images taken using 100× (oil) objective with modified Wright–Giemsa stain. (a) Three polychromatophils (American kestrel, *Falco sparverius*); (b) Larger polychromatophil (arrow) and smaller polychromatophil (arrowhead) (red-tailed hawk, *Buteo jamaicensis*); (c) Immature erythroid cell (arrowhead) and thrombocyte (lower left) (red-tailed hawk, *B. jamaicensis*); (d) Thrombocyte clump (red-tailed hawk, *B. jamaicensis*); (e) and (f) Polychromasia and RBC morphologic abnormalities in confirmed lead poisoning (bald eagle, *Haliaeetus leucocephalus*). (**Source:** Images (e) and (f) courtesy of Dr. Nicole Stacy.)

care may be affected. Age is the most significant partitioning factor, affecting both erythroid and myeloid cells lines. Other partitioning factors may be masked by the stress response that is often present in raptor CBCs and additional significant factors may be identified as data that have been impacted when stress is identified, partitioned, and analyzed.

Reference intervals for PCV in raptor species vary with age. Age-related differences are consistently noted throughout the literature, with younger birds having lower PCV, Hgb concentration, and/or total protein concentrations than older birds.[6,10,17,23] This finding is commonly reported in other avian species and is hypothesized to reflect higher tissue oxygen demands in older, flighted birds. Younger birds have higher percentages of reticulocytes, rubricytes, and immature erythroid cells. Environment (e.g., altitude) can also affect erythroid values.[22]

Age-related differences are reported in total WBC count and other WBC parameters. Younger raptors often have higher WBC counts than older birds with increased heterophils accounting for a substantial part of the difference, but findings vary between species.[10,17,23] Owls generally have higher lymphocyte percentages than other raptors (Table 126.1), while heterophils generally predominate in other raptor species (Tables 126.2 and 126.3). The stress response should be considered when evaluating reference intervals for use diagnostically, as it likely impacts many of the published reference intervals. Boreal owl species tend to have lower WBC counts than temperate/tropical owls.[1]

Thrombocyte estimates based on smear evaluation are similar in raptors to those reported in other avian species, generally ranging from 20,000–50,000/µL. However, estimates are often impeded by clumping and cell lysis. Reference intervals for whole-blood clotting time in raptors range from roughly 2–10 minutes.[28] Species-specific reference intervals are suggested in one publication, as the reference interval for prothrombin time in barn owls (*Tyto alba*; 17–29 seconds) differed from that for Japanese quail (*Coturnix japonica*; 10–16.2 seconds), with the same method using avian kephalin.[49]

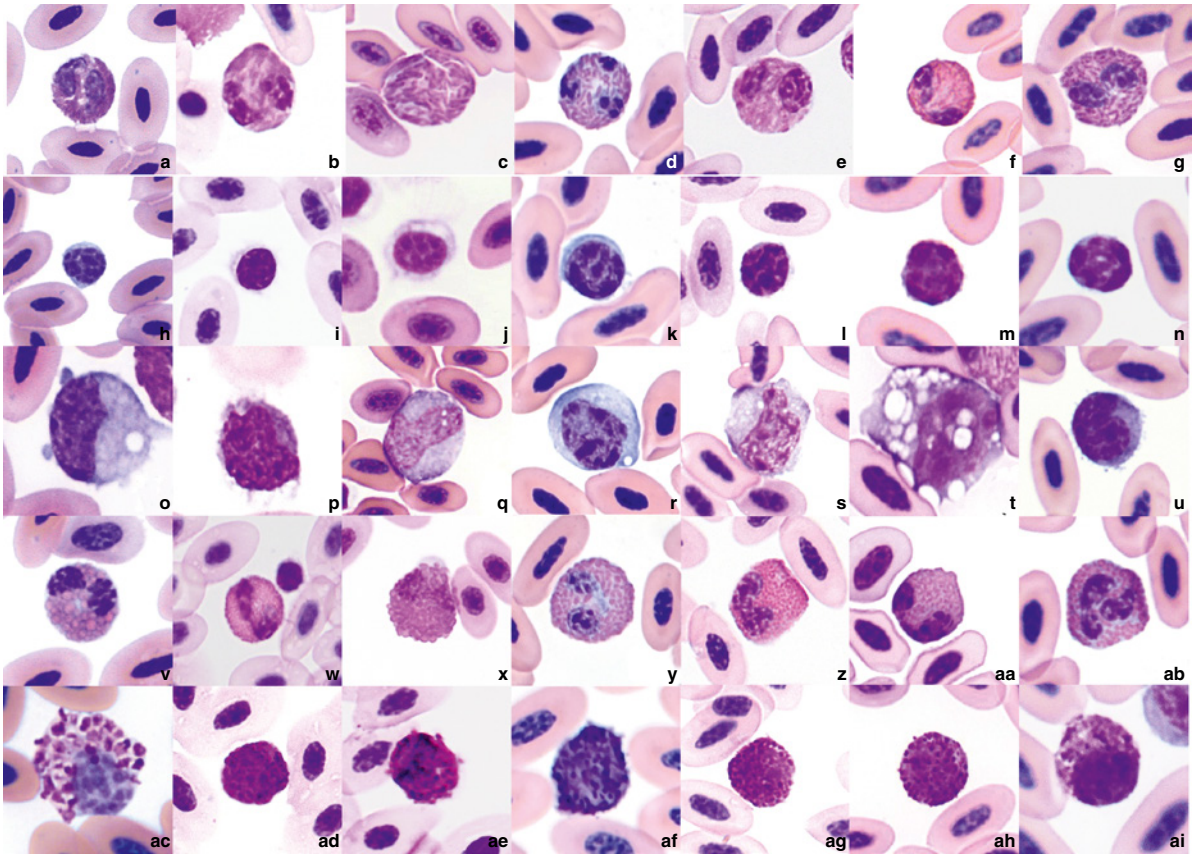


FIGURE 126.2 Normal WBC morphology. All images taken using 100× (oil) objective with modified Wright–Giemsa stain. (a)–(g) Heterophils: (a) Bald eagle (*Haliaeetus leucocephalus*); (b) Cooper's hawk (*Accipiter cooperii*); (c) Golden eagle (*Aquila chrysaetos*); (d) Great horned owl (*Bubo virginianus*); (e) Red-tailed hawk (*Buteo jamaicensis*); (f) Short-eared owl (*Asio flammeus*); (g) Turkey vulture (*Cathartes aura*). (h)–(n) Lymphocytes: (h) Bald eagle (*H. leucocephalus*); (i) Cooper's hawk (*A. cooperii*); (j) Golden eagle (*A. chrysaetos*); (k) Great horned owl (*B. virginianus*); (l) Red-tailed hawk (*B. jamaicensis*); (m) Short-eared owl (*A. flammeus*); (n) Turkey vulture (*C. aura*). (o)–(u) Monocytes: (o) Bald eagle (*H. leucocephalus*); (p) Cooper's hawk (*A. cooperii*); (q) Golden eagle (*A. chrysaetos*); (r) Great horned owl (*B. virginianus*); (s) Red-tailed hawk (*B. jamaicensis*); (t) Short-eared owl (*A. flammeus*); (u) Turkey vulture (*C. aura*). (v)–(ab) Eosinophils: (v) Bald eagle (*H. leucocephalus*); (w) Cooper's hawk (*A. cooperii*); (x) Golden eagle (*A. chrysaetos*); (y) Great horned owl (*B. virginianus*); (z) Red-tailed hawk (*B. jamaicensis*); (aa) Short-eared owl (*A. flammeus*); (ab) Turkey vulture (*C. aura*). (ac)–(ai) Basophils: (ac) Bald eagle (*H. leucocephalus*); (ad) Cooper's hawk (*A. cooperii*); (ae) Golden eagle (*A. chrysaetos*); (af) Great horned owl (*B. virginianus*); (ag) Red-tailed hawk (*B. jamaicensis*); (ah) Short-eared owl (*A. flammeus*); (ai) Turkey vulture (*C. aura*). (**Sources:** Images (a), (h), (o), and (v) courtesy of Dr. Laura Black. Images (d), (k), (r), (y), and (af) courtesy of Dr. Candice Pei-Hua Chu. Image (ac) courtesy of Dr. Nicole Stacy.

TABLE 126.1 Reference intervals for mixed-sex, variable aged barn owls (*Tyto alba*).[1]

Analytes	Units	n	Mean	SD	Median	Min.	Max.	RI[a]	LRL 90% CI	URL 90% CI	Method
PCV	L/L	22	0.48	0.04	0.49	0.41	0.57	0.41–0.57	0.38–0.44	0.54–0.59	R
RBC conc.	10^{12}/L	51	2.4	0.4	2.4	0.9	3.3	1.6–3.3	1.4–1.9	3.1–3.6	R
TS/TP	g/L	26	49	9	448	32	80	28–67	21–36	59–74	R
WBC conc.	10^9/L	51	13.1	5.9	11.2	5.4	28.5	5.4–28.5	4.5–5.8	23.8–33.7	RT
Heterophil	10^9/L	51	6.9	2.8	6.3	2.5	15.4	3.2–15	2.8–3.6	12–18.2	RT
Lymphocyte	10^9/L	51	3.1	2.1	2.7	0.2	9	0.3–8.6	0.2–0.5	7.1–10.0	RT
Monocyte	10^9/L	51	1.1	1.0	0.6	0	3.9	NR	NA	NA	RT
Eosinophil	10^9/L	51	1.6	1.5	1.0	0.2	7.1	NR	0.1–0.2	4.9–10.0	RT
Basophil	10^9/L	51	0.3	0.3	0.2	0	1.4	NR	0.0–0.0	0.8–1.5	RT

Conv. Units, conventional units; RI, reference interval; LRL, lower reference limit; URL, upper reference limit; CI, confidence interval; R, robust method; RT, robust method after Box–Cox transformation; NA, not available; NR, not reported by the editor; TS/TP, total solids/total protein concentration.
Editor's note: Reference intervals for monocytes, eosinophils, and basophils are withheld, as the standard deviation exceeds the median for these values. Readers are advised to look at minimum and maximum values with the caution that reference interval statistics have not been performed. Lymphocytes are retained so that readers note that animals with a significant stress response are included in these values. It is possible that anemic animals were included in this population. Communication from the Journal of Veterinary Clinical Pathology.

TABLE 126.2 Reference intervals for 41 adult, mixed-sex bald eagles (*Haliaeetus leucocephalus*).[20] Authors state that outliers were removed, but no detail as to how this was done is provided in the manuscript. Data should be used with caution in medical settings.

Analytes	Units	Mean	SD	Median	Min.	Max.	RI[a]	LRL 90% CI	URL 90% CI	Dist.
PCV	%	44	4	44	35	57	37–57	35–37	50–57	G
RBC conc.	10^6/mL	3.31	0.36	3.32	2.6	4.05	2.70–4.05	2.60–2.73	3.80–4.05	G
WBC conc.	10^3/mL	12.7	5	12.5	4.1	27.4	4.1–27.3	4.1–6	20.7–27.4	G
Heterophil	10^3/mL	10	1.4	10.28	6.35	11.81	6.35–11.81	6.35–8.13	11.68–11.81	G
Lymphocyte	10^3/mL	2.03	1.14	1.78	0.51	4.82	0.51–4.82	0.51–0.76	4.20–4.82	NG
Monocyte	10^3/mL	0.13	0.13	0.13	0	0.51	0–0.51	NA	0.51–0.51	NG
Eosinophil	10^3/mL	0.25	0.38	0.13	0	1.14	NR	NA	0.89–1.14	NG
Basophil	10^3/mL	NA	NA	0	0	0.13	0–0.13	NA	0–0.13	NG

Conv. Units, conventional units; Dist., distribution; RI, reference interval; LRL, lower reference limit; URL, upper reference limit; CI, confidence interval; G, Gaussian distribution; NG, non-Gaussian distribution. NA, not available.
Editor's note: Reference intervals for eosinophils are withheld, as the standard deviation exceeds the median for these values. Readers are advised to look at minimum and maximum values with the caution that reference interval statistics have not been performed. Readers should note lymphocytes lower values indicate that animals with a significant stress response are included in these values.

HEMATOLOGIC DISEASE

Stress leukograms are occasionally reported in raptors and are similar to corticosteroid-mediated stress leukograms in mammals, with heterophilia and lymphopenia noted. California condors (*Gymnogyps californianus*) are reported to have unusually pronounced stress leukograms with total WBC counts of 25–30 × 10^3/μL, potentially masking infection-related leukocytosis. A typical corticosteroid response is evident in Table 126.3. Without additional evidence (e.g., heterophil toxicity and immaturity) to support inflammatory disease, the diagnosis of concurrent inflammation is difficult in the face of a corticosteroid response.

Anemia

Anemia is common in debilitated raptors and has numerous known etiologies. Hypochromasia is often noted with severe or chronic anemia. Increased reticulocytes and appearance of more immature erythroid cells (Figure 126.1a–c) are consistent with regenerative anemia due to hemorrhage and/or hemolysis. Hemolytic anemia can be caused by acute intoxication (e.g., lead toxicity), hemoparasitism (e.g., *Plasmodium* or *Aegyptianella* infestation), and septicemia. Abnormalities including hypochromasia, microcytosis, basophilic stippling, nuclear abnormalities, and ballooning cytoplasm can raise suspicion for heavy-metal toxicosis (see Chapter 125 and Figure 125.3).

TABLE 126.3 Reference intervals for adult, mixed-sex black vultures (*Coragyps atratus*) in Brazil.[3]

Analytes	Units	n	Mean	SD	Median	Min.	Max.	RI[a]	LRL 90% CI	URL 90% CI	Dist.	Method
PCV	%	61	48.4	3.0	49.0	41	55	42–54	41.3–43.4	53.4–55.5	G	PUD
RBC conc.	10^6/mL	70	1.82	3.28	1.83	0.92	2.46	1.16–2.48	1.06–1.26	2.36–2.58	G	PUD
Hemoglobin	g/dL	20	10.5	0.9	10.7	8.36	12.1	8.5–12.5	7.9–9.1	11.9–13.1	G	PUD
MCV	Fl	61	262.6	50.2	264	195.93	453.7	203.2–402.6	195.9–211.6	361.7–458.2	G	PTD
MCHC	g/dL	20	21.5	2.0	21.9	16.72	25.98	17.4–26.2	16.1–19.3	24.5–27.6	NG	RUD
TS/TP	g/dL	61	3.8	0.5	3.8	2.3	5	2.4–4.8	2.3–3.1	4.6–5.0	NG	NP
WBC conc.	10^3/mL	69	13.25	5.35	12.76	6.16	27.72	5.93–27.14	5.44–6.53	23.39–30.77	G	RTD
Heterophil	10^3/mL	69	10.34	4.76	9.64	2.7	23.5	3.40–21.58	2.90–4.11	18.85–24.36	G	RTD
Lymphocyte	10^3/mL	68	1.74	1.33	1.50	0.16	6.87	0.19–5.16	0.14–0.27	4.23–6.15	G	RTD
Monocyte	10^3/mL	69	0.36	0.39	0.24	0.0	1.67	NR	0.0–0.0	1.22–1.67	NG	NP
Eosinophil	10^3/mL	68	0.71	0.70	0.52	0.0	3.31	NR	0.0–0.0	1.91–3.31	NG	NP
Basophil	10^3/mL	69	0.01	0.05	0.0	0.0	0.28	NR	0.0–0.0	0.07–0.28	NG	NP
H:L	none	66	9.3	8.5	6.2	0.93	32.33	NR	1.0–1.6	26.7–48.1	G	RTD
Thrombocytes	10^3/mL	61	14.14	4.79	NA	5.52	31.98	NA	NA	NA	NA	NA

Dist., distribution; RI, reference interval; LRL, lower reference limit; URL, upper reference limit; CI, confidence interval; G, Gaussian distribution; NG, non-Gaussian distribution; PUD, parametric untransformed data; RTD, robust transformed data; RUD, robust untransformed data; PTD, parametric transformed data; NP, nonparametric, NA, not available, NR, not reported; TS/TP, total solids/total protein concentration.
Editor's note: Reference intervals for monocytes, eosinophils, basophils, and H:L ratios are withheld, as the standard deviation exceeds the median for these values. Readers are advised to look at minimum and maximum values with the caution that reference interval statistics have not been performed. Lymphocytes are retained so that readers note that animals with a significant stress response are included in these values. It is possible that anemic animals were included in this population. Communication from the Journal of Veterinary Clinical Pathology.

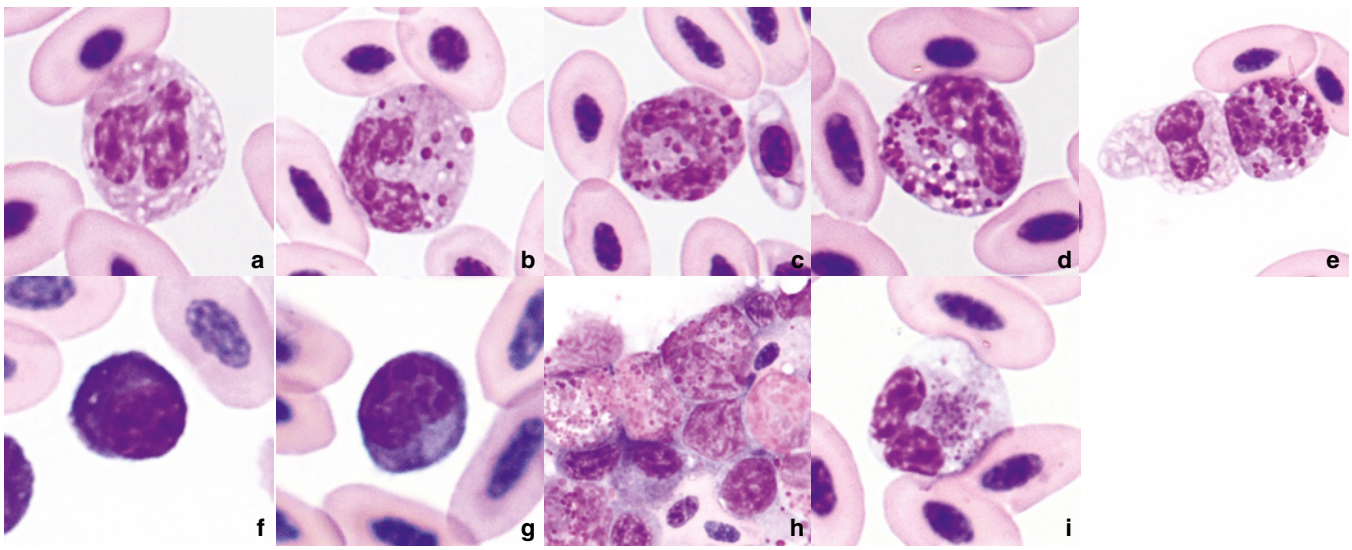


FIGURE 126.3 Abnormal WBC morphology. All images taken using 100× (oil) objective with modified Wright–Giemsa stain. (a)–(e) Toxic heterophils in red-tailed hawk (*Buteo jamaicensis*); (a) Mildly (1+) toxic heterophil; (b) Moderately toxic (2+) band heterophil; (c) Markedly toxic (3+) band heterophil; (d) Markedly toxic metamyelocyte; (e) Two variably toxic and immature heterophils; (f) Reactive lymphocyte in red-tailed hawk (*B. jamaicensis*); (g) Reactive lymphocyte in turkey vulture (*Cathartes aura*); (h) Bone marrow cytology with heterophilic hyperplasia in red-tailed hawk (*B. jamaicensis*); (i) *Chlamydia psittaci* in a toxic heterophil in red-tailed hawk (*B. jamaicensis*).

Nonregenerative anemia may be due to chronic/inflammatory disease, nutritional imbalance, viral infections, or chronic toxicosis and can be accompanied by hypochromasia. Anisocytosis and poikilocytosis are also seen in raptors with inflammatory disease.

Inflammation

Toxic changes in heterophils include sparse granulation, basophilic and/or foamy cytoplasm, and toxic granulation (small magenta-staining primary granules) (Figure 126.3a–e). Immature heterophils have

hyposegmented nuclei and are interpreted as a left shift when present (Figure 126.3). Heterophilia and increased heterophil:lymphocyte ratios are common in raptors with inflammatory disease and heavy-metal toxicosis. Monocytosis is common in subacute to chronic infectious and inflammatory disorders. Reactive lymphocytes have expanded pale to deep blue cytoplasm (Figure 126.3f,g) and may be plasmacytoid with perinuclear clearing. Bone marrow evaluation may be performed ante mortem or at fresh necropsy to assess the etiology of hematologic changes (Figure 126.3h).

Common Disease Conditions

Vultures (including condors) and bald eagles (*Haliaeetus leucocephalus*) are particularly prone to heavy-metal toxicoses due to carrion-scavenging behavior, but numerous nonscavenger raptor species are also affected. Experimental lead toxicosis induced in turkey vultures (*Cathartes aura*), Andean condors (*Vultur gryphus*), and other raptor species resulted in a substantial drop in PCV.[32] Marked reticulocytosis and basophilic stippling of RBCs are inconsistently reported possibly due to the stage of toxicosis, the blood smear staining and fixation method, and prolonged exposure to anticoagulants such as EDTA.[26] Hypochromasia, poikilocytosis, microcytosis, increased circulating immature erythroid cells, and bone marrow dyserythropoiesis are also reported in lead-poisoned birds (Figure 126.1e,f). Asymmetric cytoplasmic enlargement, aka ballooning or D cells, can be noted. Similar findings including hypochromasia, reticulocytosis, and dyserythropoiesis have been seen by the author and the editor with zinc toxicosis, presumably due to functional iron deficiency.

Methylmercury poisoning can occur in aquatic and piscivorous birds due to environmental contamination. As apex predators, raptors are vulnerable to secondary mercury poisoning with substantial accumulated concentrations due to the high bioavailability of mercury in consumed prey.[12] Experimental dosing of kestrels resulted in increased heterophil and total WBC concentrations, with heterophil counts exceeding reference intervals in the higher-dose group. Additionally, similar species may be exposed to petroleum toxicants after oil spills when bioaccumulation will occur. Petroleum intoxication causes anemia and WBC dyscrasia in all species of birds studied to date.[30] Heinz body hemolytic anemia has been identified in both natural and experimental petroleum exposure of birds; ultrastructural evaluation was required for accurate identification of avian Heinz bodies.[13,16]

Chlamydia psittaci is a gram-negative, obligate intracellular bacterium in the order Chlamydiales and the family Chlamydiaceae. Infection occurs in many avian species and is called chlamydiosis, previously termed ornithosis or psittacosis. *C. psittaci* was previously categorized by some in the controversial genus *Chlamydophila* along with other organisms, but reunification under the single genus *Chlamydia* is now generally accepted.[40] *Chlamydia avium* and *Chlamydia gallinacea*, two recently described infectious agents in poultry, pigeons, and psittacines, are not reported yet in raptors.[41] Clinical signs involve the respiratory and gastrointestinal tracts, e.g., anorexia, nasal discharge, coughing, dyspnea, and diarrhea. Hematologic abnormalities are not specific and indicate severe chronic inflammation, including heterophilia with evidence of left shift and minimal toxicity and monocytosis. Mild nonregenerative anemia consistent with anemia of chronic disease is typical. The infectious stage of *Chlamydia*, the elementary body, is approximately 0.2–0.3 μm in diameter. Host cells internalize the elementary bodies into membrane-bound vacuoles where they differentiate into coccoid to pleomorphic reticulate bodies approximately 0.6–1.0 mm in diameter. Chlamydial microcolonies then form via binary fission of reticulate bodies. Reticulate bodies subsequently transform into elementary bodies. Stains such as Giemsa, Gimenez, Macchiavello, methylene blue, and modified Ziehl–Neelsen can be used to detect chlamydial inclusions in smears or swabs. Elementary bodies are small, red/purple inclusions using Giemsa stain; larger, deep blue staining inclusion bodies may be seen in mononuclear cells or heterophils using Giemsa stain in blood smears or tissue samples (Figure 126.3i).[19] PCR is recommended as the most sensitive test for chlamydiosis, though quality assurance statistics will vary with primer sets.

Aegyptianella pullorum is a rickettsial infection that is most closely related to *Anaplasma sp*.[38] Argasid (soft) ticks serve as the biologic vector. Intraerythrocytic inclusions appear as smaller rounded, basophilic inclusions or as larger clusters or morulae composed of tiny cocci. Clinical disease due to *Aegyptianella*, including anemia, weakness, and hepatosplenomegaly, are described in several avian species, but reports of clinical disease in raptors are sparse.[45,46]

Aspergillus sp. infection is reported in numerous raptor species, with gyrfalcons (*Falco rusticolus*), immature red-tailed hawks (*Buteo jamaicensis*), goshawks (*Accipiter gentilis*), golden eagles (*Aquila chrysaetos*), bald eagles, and rough-legged hawks (*Buteo lagopus*) potentially more susceptible to clinical illness; prairie falcons (*Falco mexicanus*) and Harris's hawks (*Parabuteo unicinctus*) may be more resistant.[21] Concurrent disease and immunosuppression can predispose birds to clinical aspergillosis; lead poisoning of bald eagles and other raptor species is one example. Clinical illness may present either acutely or as a more chronic onset of clinical signs that can include dyspnea, exudative rhinitis, keratitis and periorbital swelling, and unilateral wing droop. Right ventricular dilation due to pulmonary hypertension may be detected during imaging with ascites and/or pulmonary congestion as potential sequelae. The typical hematologic profile reported with fungal infection of raptors is a mild to moderate nonregenerative anemia and leukocytosis characterized by moderate to severe heterophilia and monocytosis, with lymphocyte counts normal to decreased. Immature heterophils (left shift) can be seen, and increased plasma protein concentration, likely due to antibody production, is also reported. These hematologic abnormalities are nonspecific to

aspergillosis and other infectious/inflammatory disorders cannot be excluded. Additionally, immunosuppressed birds may have hypoproteinemia and lack leukocytosis.[5]

Coagulopathy

In avian species, coagulopathy is most commonly documented in raptors and usually due to intoxication (petroleum, heavy metals, rodenticides, other), though infectious causes and disseminated intravascular coagulation are possible (see Chapter 95). Anticoagulant rodenticides are a well-described threat to raptor species through both primary and secondary exposure routes. Biological action differs substantially between groups of anticoagulant rodenticides. The first-generation compounds include warfarin, chlorophacinone, and diphacinone. The need for multiple feeding exposures of these compounds for lethal toxicity led to the development of resistance in target rodent species. Second-generation anticoagulant rodenticides (e.g., brodifacoum, bromadiolone, difethialone, difenacoum, and flocoumafen) were subsequently developed and require only a single feeding exposure to kill target rodents. Potential for bioaccumulation in predatory wildlife differs by specific rodenticide type, with lower bioaccumulation risk for first-generation compounds and bromadiolone and flocoumafen, and higher risk for difethialone, difenacoum and brodifacoum.[36] Lower LD50 (median lethal dose) for a second-generation compound (brodifacoum) versus a first-generation compound (diphacinone) is reported in raptor species.[29] Of note, avian metabolism of anticoagulant rodenticides differs from that of mammals, and raptors (particularly owls) appear to have greater risk of bioaccumulation and toxicity due to limited detoxification ability.[35,48]

Anticoagulant rodenticide poisoning of raptors can result in hemorrhagic shock and blood-loss anemia with severe decreases in PCV and plasma protein.[28] Unlike mammalian species, a minority of poisoned birds present with excessive bleeding from minor wounds or pulmonary hemorrhage.[43] Confirmation of coagulopathy may be attempted clinically using either a whole-blood clotting time or activated clotting time. In rodenticide-poisoned birds, clotting times of 27 minutes and >60 minutes are reported (RI = 2–10 minutes).[28] Prothrombin time measurement can also be diagnostic for sub-lethal rodenticide poisoning, but avian-specific reagents and species-specific reference intervals are needed.[18] With appropriate medical support including vitamin K supplementation, PCV and plasma protein values steadily recover. PCV may normalize within a week's time.

Hemoparasites

Raptors are commonly infested with one or more hemic parasites depending on species and geographic location. The following parasites are commonly found in the peripheral blood of free-ranging raptors, though captive raptors may also have parasite burden.

Leukocytozoon is an apicomplexan parasite in the order Achromatorida, family Leucocytozoidae. *Simulium sp*. black flies serve as the definitive host of this avian parasite. Sporozoites are transmitted during insect feeding on birds and infect hepatocytes to become first trophozoites, then schizonts progressing to merozoites. Endothelial cells may also become infected at this stage. Merozoites then infect (a) macrophages and endothelial cells to become megaloschizonts, progressing through cytomere, schizont, and merozoite stages; and (b) circulating blood cells to become gametocytes. The gametocyte "sail" stage markedly distorts the host cell, impairing cell identification (Figure 126.4a–c). Staining differences between male and female gametocytes may be noted.[37] Organisms in both RBCs and WBCs are reported, and highly deformed, infected host cells typically appear to have nonlobulated nuclei. Interestingly, *Leukocytozoon sabrazesi* has been shown to infect thrombocytes.[50] A smaller, spherical form of *Leukocytozoon* organisms may also be seen in blood cells. While *Leukocytozoon* is often ubiquitous and nonpathogenic in adult raptors, it can cause intravascular hemolytic anemia and immature raptors tend to have heavier parasite burdens.[22] Hepatosplenomegaly and granulomatous inflammation have been reported in various avian species.[47] Combined hemoparasite infestations including *Leukocytozoon* have had severe consequences in raptors such as snowy owls (*Bubo scandiacus*).[11]

Hemoproteus is an apicomplexan parasite in the order Hemosporidia, family Hemoproteidae. Hemoproteus species identified in raptors include *Hemoproteus noctuae*, *Hemoproteus syrnii*, and *Hemoproteus tinnunculi*; infestation is most commonly reported in Strigiformes.[22] Avian *Hemoproteus* infection is frequently reported as an incidental finding and historically has been considered nonpathogenic in many avian species, but *Hemoproteus*-associated disease has been reported in several raptor species including snowy owls and goshawks.[24] As with other hemic parasites, *Hemoproteus* has the potential to cause anemia.[7] Deleterious clinical effects may be subtle, e.g., infestation is negatively correlated with weight in female American kestrels (*Falco sparverius*).[2] Treatment of a naturally infected population of female blue tits (*Cyanistes caeruleus*) resulted in increased survival to the next breeding season;[34] long-term effects on breeding and fitness may occur in raptors as well. The degree of parasitemia may reflect immune status in raptors, as reduction in parasite burden can occur following recovery from injury or illness.[8] Blood-sucking insects serve as both mechanical vectors and biologic intermediate hosts. Insect vectors inoculate sporozoites during feeding on birds; sporozoites develop into schizonts in numerous tissues including vascular endothelium, muscle, heart, spleen, and liver. Released merozoites can reinfect tissues becoming secondary schizonts or infect RBCs to become gametocytes. Pigmented gametocytes can be detected in peripheral blood smears where they appear as elongated structures, either microgametocytes or macrogametocytes. They occupy more than half of RBC cytoplasm and often wrap slightly around the erythrocyte nucleus, though typically the nucleus is still aligned along the axis of the cell (Figure 126.4d–f).[27] In aged or stored blood,

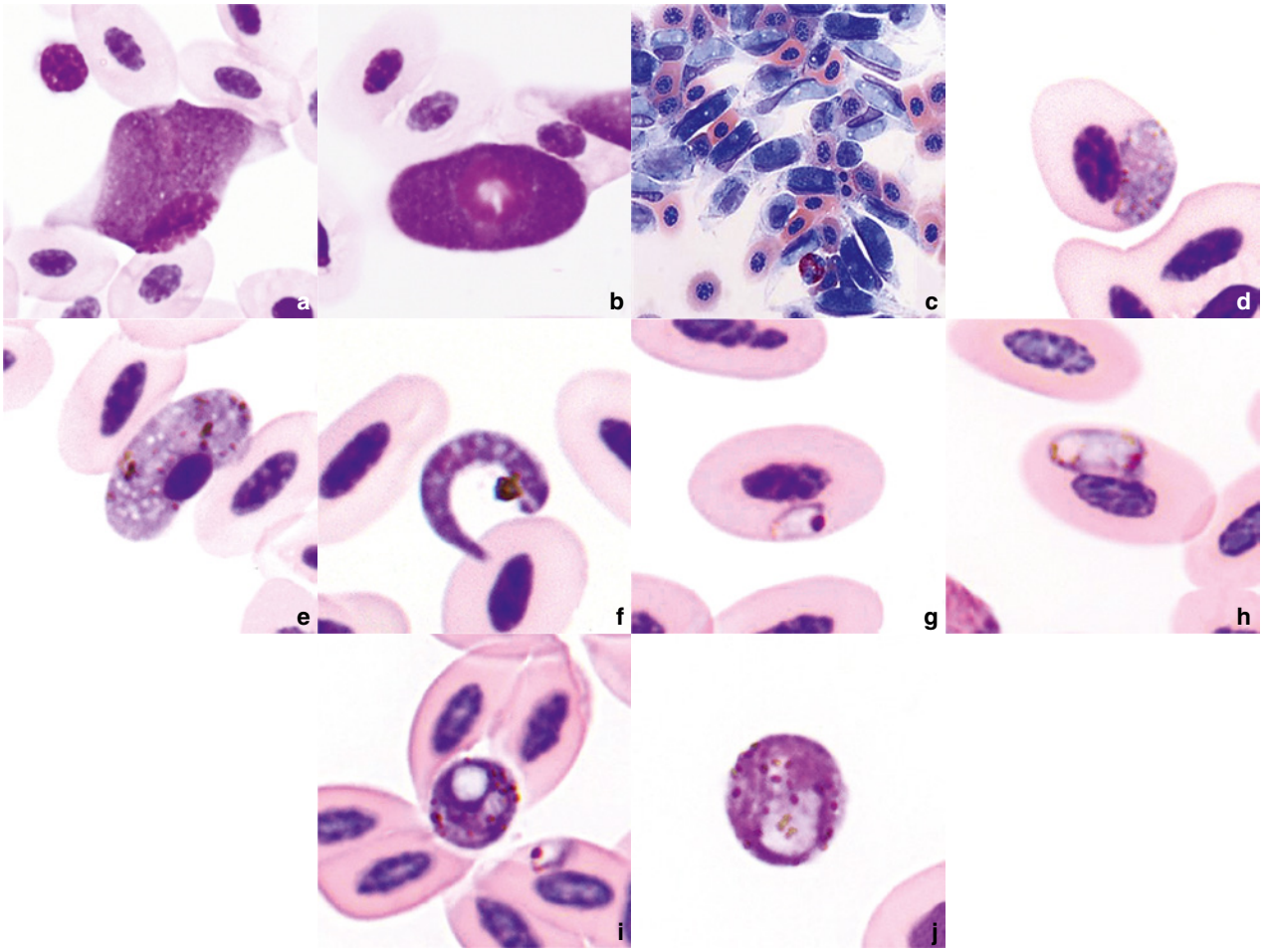


FIGURE 126.4 Hemic parasites in raptor species. All images taken using 100× (oil) objective with modified Wright–Giemsa stain. (a) and (b) *Leukocytozoon sp*. (Cooper's hawk, *Accipiter cooperii*); (c) *Leukocytozoon sp*. in owlet blood smear (barn owl, *Tyto alba*); (d)–(f) *Hemoproteus sp*. (American kestrel, *Falco sparverius*); (g)–(j) *Plasmodium sp*. (short-eared owl, *Asio flammeus*). (**Source:** Image (c) courtesy of Dr. Kendal Harr.)

gametocytes can appear in blood smears as either round and/or elongated extracellular structures (Figure 126.4f).[7]

Plasmodium is an apicomplexan parasite in the order Hemosporidia, family Plasmodiidae. Over 200 species of *Plasmodium* are characterized. *Plasmodium* infestation occurs in many avian species including various raptor species and is often reported as an incidental finding, but infection can be lethal especially to nonadapted species. Fatal hemolytic anemia caused by *Plasmodium relictum* infection is reported in raptors, with falcons (e.g., gyrfalcons) particularly affected.[22,37] Concurrent disease or immunosuppression (e.g., from captivity stress) may worsen parasitism levels. Conversely, parasitism may worsen immune compromise leading to co-infection. For example, in several captive snowy owls, *Plasmodium* was hypothesized to contribute to fatal outcome following infection with West Nile virus, due to impairment of T-cell function.[15] In a common buzzard (*Buteo buteo*) presenting for an open humeral fracture, severe *Plasmodium* parasitism was found with associated morbidity, including anemia and marked intraperitoneal hemorrhage due to splenic rupture.[14] *Culex sp*. mosquitoes are the primary insect vectors and serve as an intermediate host. In the initial stage of infection, an infected female mosquito feeding on a bird transmits *Plasmodium* sporozoites that parasitize host tissues and replicate into schizonts. The resulting merozoites infect RBCs, WBCs, and thrombocytes to become trophozoites that can mature into schizonts and gametocytes. Any or all of these stages may be seen in peripheral blood smears (Figure 126.4g–j). Gametocytes are irregularly rounded to elongated, often contain golden-brown granular iron pigment, and frequently displace the RBC nucleus. Trophozoites frequently contain a large vacuole that displaces the parasite nucleus eccentrically, resulting in the characteristic signet-ring morphology. Schizonts are rounded structures that contain fused to individual merozoites.

Babesia is an apicomplexan parasite in the order Achromatorida, family Babesiidae. Scattered reports of babesiosis in raptors describe infection with either of two species: *Babesia shortti*, potentially specific to

Falconidae, and *Babesia moshkovskii*, potentially specific to Accipitridae. Infestation may be subclinical, but fatalities are reported in several falcon and kestrel species; co-infection is often reported as a contributor to morbidity.[37] Ixodid ticks are presumed to serve as a biological vector. Sporozoites are transmitted from tick salivary glands during feeding and directly infect RBCs, becoming trophozoites. Trophozoites develop into merozoites, lysing-infected host cells in the process and releasing extracellular merozoites to infect additional RBCs. In peripheral blood smears, organisms appear as small, rounded to piriform inclusions that lack pigment.

Isospora (*Atoxoplasma*) is a coccidian parasite transmitted via the fecal–oral route. *Atoxoplasma* is now considered a junior objective synonym of *Isospora* and the genus name *Isospora* is recommended for use.[4] *Isospora* is a rare diagnostic finding in raptors and has been reported in spotted owls (*Strix occidentalis*).[31,37] Infestations are often fatal in passerine birds, but the pathogenicity is currently unknown in raptors. Following ingestion of sporulated oocysts by the avian host, organisms enter the bloodstream via the intestinal vasculature and infect mononuclear WBCs. Schizogony occurs in both tissue and circulating mononuclear cells, as well as in intestinal epithelial cells. They may be found in peripheral blood smears as rounded, pale-staining inclusions indenting the host cell nucleus.

Immature microfilariae may be noted extracellularly in peripheral blood smears from raptors, but are usually considered to be nonpathogenic.

Trypanosomes are unicellular, flagellated protozoa that may be found extracellularly in blood smears. A variety of blood-sucking arthropods serve as biological vectors for different *Trypanosoma* species. No clinical importance is currently ascribed to infection in raptors. Co-infection is more common with other microorganisms transmitted by *Simulium sp.*; thus, those that share the same insect vector.[44]

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CHAPTER 127

Hematology of Ratites

PHILLIP CLARK

Introduction
Blood Collection and Handling
Erythron
Leukon
Thrombon and Coagulation
Reference Intervals
Hematologic Disease
Hemoparasites

Acronyms and Abbreviations

IUCN, International Union for Conservation of Nature; ASVCP, American Society for Veterinary Clinical Pathology; MCV, mean corpuscular volume.

INTRODUCTION

The term ratite is a functional classification of birds that are flightless and have evolved with skeletal characteristics in which a keel is absent on the sternum, as there are no major flight muscles that require attachment. Taxonomically, these birds represent four orders within the Aves; namely, Apterygiformes, Casuariiformes, Rheiformes, and Struthioniformes. Tinamiformes are also considered by some authors to be ratites, but have not been considered in this chapter. Within these four orders, the International Union for Conservation of Nature (IUCN) currently recognizes five kiwi species, three cassowary species, three rhea species, two ostrich species, and a single species of emu[3] (Table 127.1). Each of these groups of ratites occurs naturally in distinct geographic regions with kiwi being restricted to New Zealand, cassowary to New Guinea and far north Australia, rhea to South America, ostrich to Africa, and emu to mainland Australia.

Several ratite species have been commercially produced. A boom in the farming, and associated value, of these birds occurred in the 1990s, which has subsequently subsided.[4] Many of these species are also held in zoological collections throughout the world. Consequently, hematologic assessment of ratites may be undertaken for health assessment of individuals or of a flock.

BLOOD COLLECTION AND HANDLING

Blood collection from ratites requires assessment of the species, situation, and in all instances the safety of the operator should be a paramount consideration. The large adult size and fractious nature of many ratites often necessitates chemical immobilization to allow safe blood collection.[21,32] Blood may be collected from standing, hooded, habituated ostriches with minimal physical restraint, from the brachial vein on the medial wing surface. Alternatively, blood can be collected from the medial metatarsal or jugular vein. The latter has been used to collect blood from emus physically restrained in sternal recumbency. In large adult ratites, blood is collected using a 21-gauge, 1-inch needle or 21-gauge butterfly catheter. In smaller ratites and juveniles of larger species, the jugular vein is the preferred site for venipuncture[4] using a 23–25-gauge needle. In most ratite species, blood volume is not a limiting factor with rhea weighing up to 25 kg, emu up to 55 kg, and ostrich up to 150 kg.[21]

EDTA causes hemolysis of ostrich blood and has been reported to cause hemolysis in other ratite species.[18] Consequently, EDTA is not recommended as an anticoagulant for ratites with the preferred anticoagulant being 3.2% buffered sodium citrate solution (blue top vacutainer tube), with the correct ratio of 1:9 anticoagulant to blood and correction for the dilution effect that liquid anticoagulant has on subsequent analyses. Alternatively,

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TABLE 127.1 Bird species considered to be ratites.

Common name	Scientific name	Order
Southern brown kiwi	*Apteryx australis*	Apterygiformes
North Island brown kiwi	*Apteryx mantelli*	Apterygiformes
Little spotted kiwi	*Apteryx owenii*	Apterygiformes
Great spotted kiwi	*Apteryx haastii*	Apterygiformes
Okarito kiwi	*Apteryx rowi*	Apterygiformes
Northern cassowary	*Casuarius unappendiculatus*	Casuariiformes
Southern cassowary	*Casuarius casuarius*	Casuariiformes
Dwarf cassowary	*Casuarius bennetti*	Casuariiformes
Emu	*Dromaius novaehollandiae*	Casuariiformes
Greater rhea	*Rhea americana*	Rheiformes
Lesser rhea	*Rhea pennata*	Rheiformes
Puna rhea	*Rhea tarapacensis*	Rheiformes
Common ostrich	*Struthio camelus*	Struthioniformes
Somali ostrich	*Struthio molybdophane*	Struthioniformes

heparin can be used as an anticoagulant; however, it typically interferes with staining and causes leukocyte clumping.[4] In all instances, it is recommended that blood smears be made immediately after blood collection to maintain the optimal morphology of ratite hematologic cells. A range of Romanowsky stains have proven suitable for staining blood films of ratites.

Analysis of blood should be undertaken according to standard techniques for handling avian blood.[6,7] Automated analysis is complicated, as with all avian samples, by nucleated erythrocytes (RBCs) and thrombocytes, as well as nucleated leukocytes (see Chapter 123).

ERYTHRON

Ratite RBCs, when examined using Romanowsky-stained blood films and light microscopy, are ovoid cells containing a centrally located ovoid nucleus composed of dense, darkly staining (dark blue-purple) chromatin and a moderate amount of evenly colored eosinophilic cytoplasm (Figure 127.1). Erythrocytes have an average length of 13.3–16.2 µm and width of 7.5–11.3 µm in the common ostrich,[26–28] the greater rhea,[14] and the emu.[11] Blood film examination of most ratites reveals a small proportion of polychromatophilic RBCs and occasional rubricytes.[4,15] Additional variant RBCs[9] have been reported in health including binucleated RBCs from a greater rhea,[14] anucleated RBCs (erythroplastids) from the southern cassowary and emu,[8] and multinucleated RBCs from a North Island brown kiwi.[19]

LEUKON

Ratite leukocytes (WBCs), when examined using Romanowsky-stained blood films and light microscopy, exhibit the general types of avian leukocytes; namely, heterophils, lymphocytes, monocytes, eosinophils, and basophils (Figures 127.2 and 127.3). The average length and width for heterophils are 13 × 12 µm (emu[11]) and 13 × 12 µm (greater rhea[14]); for lymphocytes 9 × 8 µm (emu) and 9 × 7 µm (greater rhea); and for monocytes 12 × 11 µm (emu) and 16 × 13 µm (greater rhea).

Heterophils are the most commonly encountered leukocyte in ratites. Typically, heterophils are irregularly round leukocytes with a lobed, basophilic nucleus and prominent acidophilic cytoplasmic granules. The nucleus is strongly basophilic (blue to purple) when adequately stained, but may exhibit an aqua-blue color with inadequate staining; and is commonly partially obscured by the presence of cytoplasmic granules. The cytoplasm contains robust, fusiform, red granules that are typically evenly distributed at moderate to high density. In some heterophils, these granules may exhibit a refractile central body in the granule's mid-section. There are no significant distinguishing differences in heterophil morphology between ratite species (Figures 127.2a and 127.3a,c,e).

Lymphocytes are usually the second most commonly encountered leukocyte in ratite blood. Morphologically, lymphocytes may vary in appearance. Typical small lymphocytes are the smallest WBC and have a round nucleus composed of dense, coarsely clumped chromatin, and a small, often incomplete cytoplasmic rim that is moderately to deeply basophilic. Larger and more variably shaped lymphocytes may also be encountered in the blood of healthy birds. These larger lymphocytes may be similar in size or larger than granulocytes and approach the size of monocytes. They have an irregularly round nucleus composed of moderately dense, irregularly clumped chromatin with a moderate amount of agranulated, moderately basophilic cytoplasm. Some lymphocytes may exhibit several punctate, azurophilic to basophilic cytoplasmic granules (Figures 127.2d,e and 127.3a). There are no significant distinguishing differences in lymphocyte morphology between ratite species.

Ratite monocytes are large, pleomorphic leukocytes with nuclei that may be ovoid, indented, horseshoe, or irregularly shaped and composed of fine to reticular chromatin. There is typically a moderate to large amount of gray to basophilic cytoplasm. Granules are typically not evident in the cytoplasm; however, small eosinophilic granules may be observed in some cells. One to several small vacuoles may be evident in the cytoplasm of some monocytes (Figure 127.2b). Although monocyte morphology in an individual's blood is often quite varied, there are no significant distinguishing differences in monocyte morphology between ratite species. As monocytes and large lymphocytes have a similar size and morphologic appearance when viewed in Romanowsky stained blood films, it may be difficult to distinguish between these two WBCs.

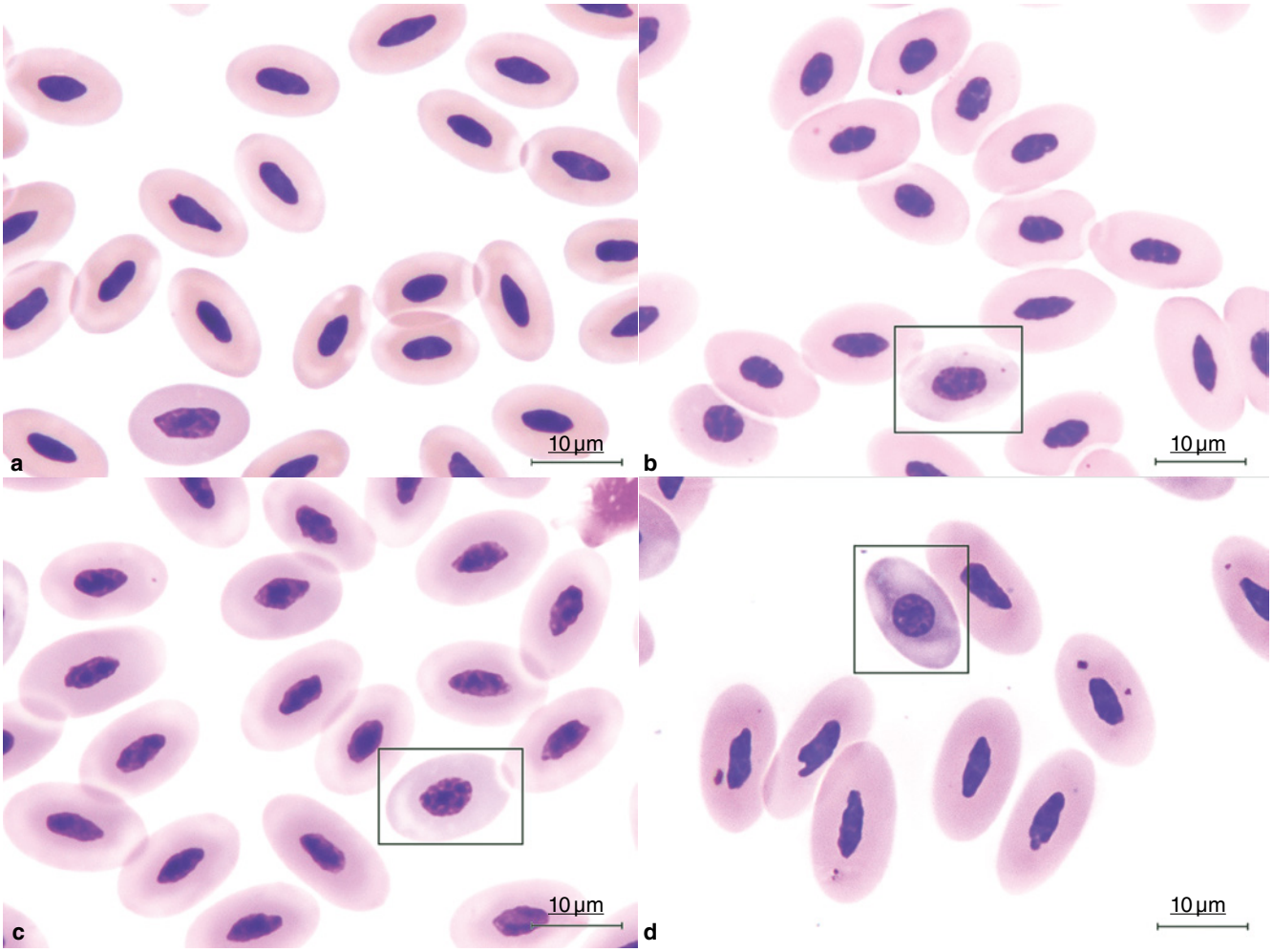


FIGURE 127.1 Erythrocytes. (a) Greater rhea (*Rhea americana*) erythrocytes with mild overlap; (b) Common ostrich (*Struthio camelus*) erythrocytes; (c) Emu (*Dromaius novaehollandiae*) erythrocytes; (d) Southern cassowary (*Casuarius casuarius*) erythrocytes. Bar = 10 µm. Boxes indicate polychromatophilic erythrocytes. Modified Wright's stain.

Eosinophils are far less commonly encountered in ratite blood than heterophils. Typically, eosinophils are irregularly round leukocytes with a lobed, basophilic nucleus and brightly acidophilic cytoplasmic granules. Eosinophils typically have two to three nuclear lobes that are partially obscured by cytoplasmic granules and commonly exhibit more darkly stained chromatin than the chromatin of adjacent heterophils. The cytoplasm is moderately basophilic and contains a moderate to high density of round to ovoid cytoplasmic granules that are more brightly eosinophilic than heterophil granules. Overall, eosinophils have a similar appearance in all ratite species with subtle variation in the shape and hue of the cytoplasmic granules (Figures 127.2c and 127.3d,f).

Typically, ratite basophils are irregularly round with prominent darkly staining, purple to black, round to ovoid basophilic granules that obscure the cytoplasm and much of the nucleus of the cell. When apparent, the basophilic chromatin of the nucleus often appears pale in comparison to the color of the cytoplasmic granules. However, in some birds, granules may fail to stain. In these cases, basophils exhibit regular, round vacuoles (or nonstaining granules) in a pale cytoplasm with occasional basophilic granules. In such cases, the round to ovoid or bilobed nucleus is apparent and composed of moderately dense chromatin. There are no significant distinguishing differences in basophil morphology between species of ratites (Figure 127.3b,c).

Leukocyte ultrastructure has been described for the ostrich.[5] Notably, heterophils had two granule types, type I were fusiform and type II had heterogenous shape and density and were smaller than type I. Ostrich eosinophils exhibited three granule types with the most common type being round in shape. The nucleus of both these acidophils was composed of peripheral heterochromatin and central euchromatin. Basophils were not observed in this study. Lymphocyte and monocyte ultrastructure revealed a similar distribution of peripheral heterochromatin in the nucleus of both cell types; however, monocytes had more invaginations of the membrane.

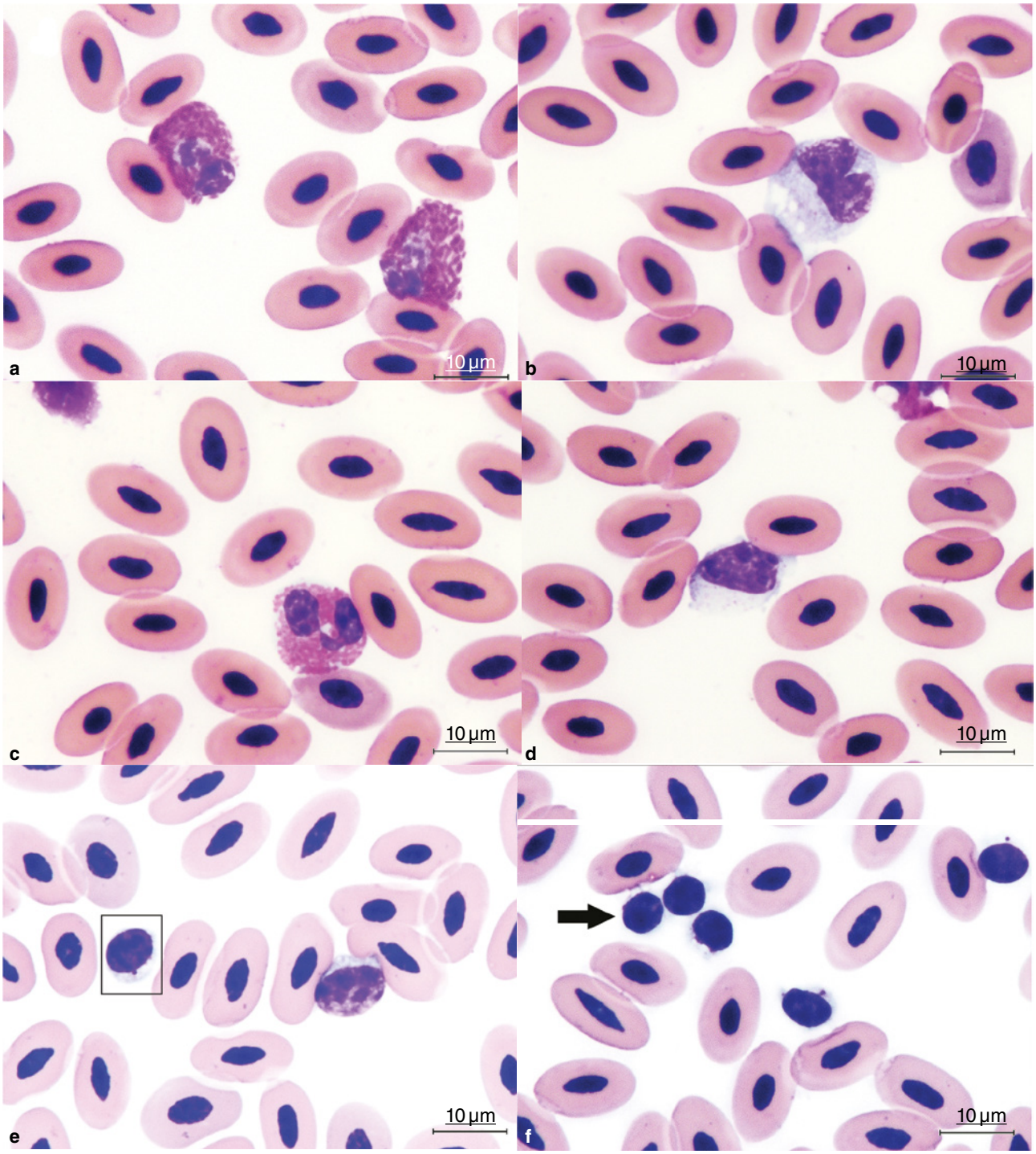


FIGURE 127.2 Hematologic cells from a common ostrich (*Struthio camelus*). (a) Two heterophils are shown amid RBCs; (b) A monocyte is portrayed amid RBCs; (c) An eosinophil is illustrated amid RBCs; (d) A lymphocyte is present amid RBCs; (e) A thrombocyte (box) and lymphocyte are demonstrated amid RBCs; (f) An aggregate of three thrombocytes (arrow) and two individual thrombocytes are evident amid RBCs. Bar = 10 µm. Modified Wright's stain.

THROMBON AND COAGULATION

Ratite thrombocytes, when examined in Romanowsky-stained blood films using light microscopy, are small, round or elongated, nucleated cells (Figure 127.2). The nucleus is either rounded or fusiform, reflecting the overall shape of the cell, composed of very dense chromatin giving a darkly basophilic, almost black appearance. A small amount of colorless to palely basophilic cytoplasm may be present in a thin rim around the rounded cells or tapers along the long axis of the fusiform cells. In some cases, fine cytoplasmic projections (pseudopodia) may be evident. Several small, punctate, eosinophilic, or azurophilic granules may be observed in

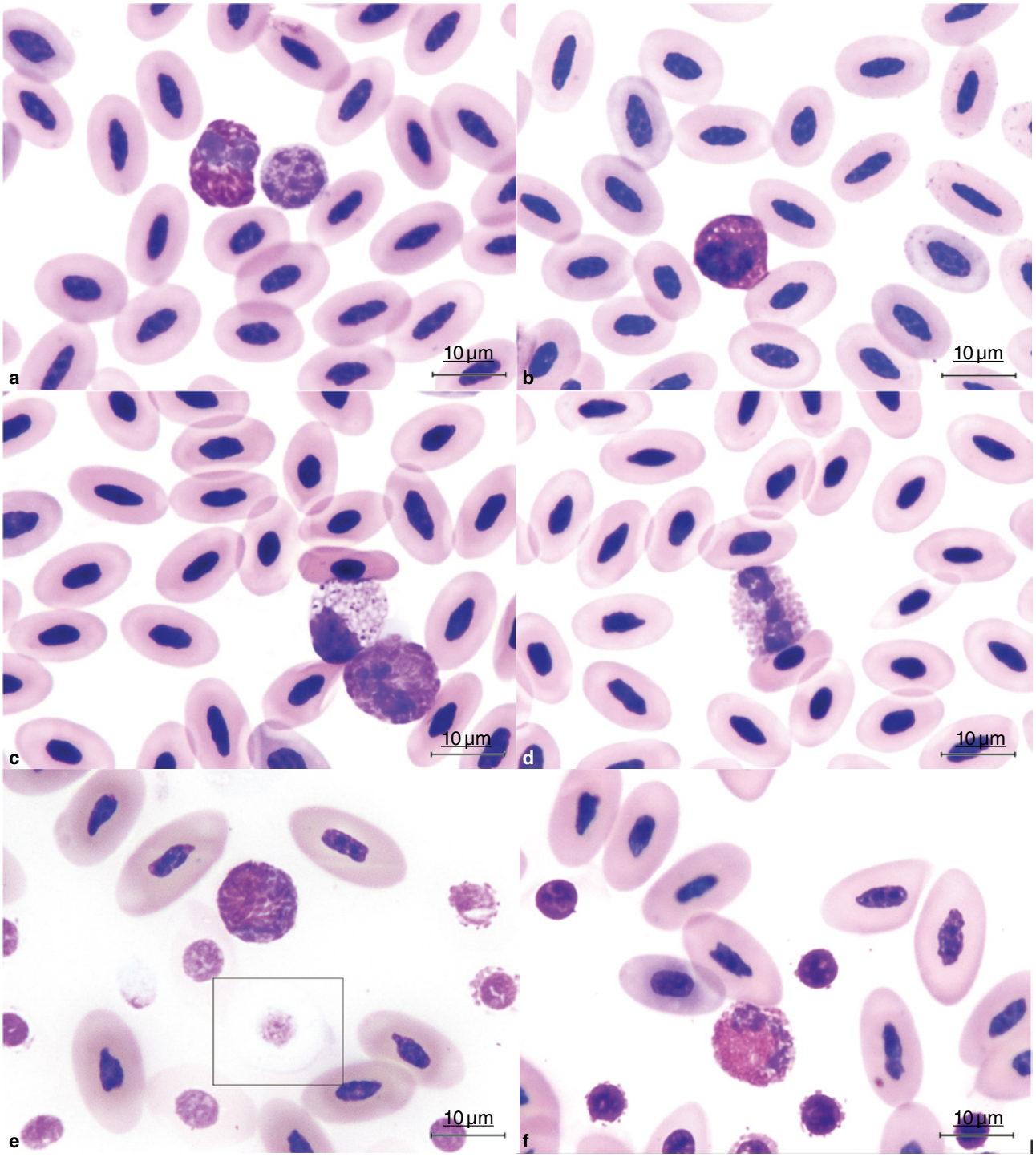


FIGURE 127.3 Comparative morphology of hematologic cells from ratites. (a) Blood from an emu (*Dromaius novaehollandiae*) showing a heterophil and lymphocyte amid erythrocytes; (b) Blood from an emu (*D. novaehollandiae*) showing a basophil amid erythrocytes. Note the dense basophilic cytoplasmic granules; (c) Blood from a greater rhea (*Rhea americana*) showing a basophil and a heterophil amid erythrocytes. Note the small number of basophilic granules and the large number of clear vacuoles or nonstaining granules evident in the basophil; (d) Blood from a greater rhea (*R. americana*) showing an eosinophil amid erythrocytes; (e) Blood from a southern cassowary (*Casuarius casuarius*) showing a heterophil amid erythrocytes including lysed cells devoid of hemoglobin (one illustrated by a box); (f) Blood from a southern cassowary (*C. casuarius*) showing an eosinophil amid erythrocytes and lysed cells. Bar = 10 µm. Modified Wright's stain.

the cytoplasm of some thrombocytes (Figure 127.2e,f). Importantly, individual thrombocytes must be distinguished from small lymphocytes. Typically, individual thrombocytes have a chromatin pattern that is more homogeneous and darker than that of lymphocytes; while thrombocyte cytoplasm is less basophilic than that of lymphocytes. Commonly, thrombocytes become aggregated during collection, transport, and storage.

Large aggregates at the leading edge of blood films or small aggregates distributed throughout the body of the blood film can be identified. Distinct, individual cell outlines may be difficult to discern in these aggregates.

REFERENCE INTERVALS

Most published studies on ratite hematology predate current American Society For Veterinary Clinical Pathology (ASVCP) guidelines for reference intervals and are not generated as medical reference intervals. Hematology data for some hematologic analytes have been published for a number of ratite species including the common ostrich,[4,26,28,31,34] emu,[24] southern cassowary,[34] lesser rhea,[30] greater rhea,[4,14,37] and three species of kiwi.[25]

Hematologic values are broadly similar across ratite species. Observed differences may be due to species as well as a range of preanalytical, analytical, and postanalytical factors.

The general characteristics of birds are typified by the mean erythrocyte values determined for 1240 individual birds encompassing 204 species which found an average PCV of 0.46 L/L, an average mean corpuscular volume (MCV)of 169 fL, and an average RBC concentration of 2.79×10^{12}/L.[17] In comparison to these general values, adult ratites are reported to have lower numbers of larger erythrocytes that result in an average PCV that is similar to the general avian average of 0.46 L/L. Ratite leukocyte values are characterized by a heterophil predominance with lesser numbers of lymphocytes and small numbers of monocytes, eosinophils, and basophils. It is rare for studies to report an accurate thrombocyte concentration due to the very common phenomenon of aggregation (clustering) affecting the accuracy of counts.

A number of physiologic partitioning factors have been reported to affect hematologic characteristics, including age, sex, and season.[7] However, it may be difficult to isolate the effect of a single physiologic factor; e.g., age, season, and nutrition may all be interrelated. The effect of age on hematologic values has been reported for the common ostrich by several authors.[26,31] Common ostrich chicks had a mean PCV of 0.37 L/L compared to adults that had a mean PCV of 0.48 L/L.[28] In contrast to some birds,[7] differences in hematologic values were not observed between sexes for emu[24] or lesser rhea.[31] Interestingly, two male emus, while incubating eggs, had a decrease in hematocrit from 0.57 to 0.47 L/L and 0.54 to 0.46 L/L, respectively.[4,10] In 2011, Samour et al.[31] reported increased total leukocyte concentration in ostrich chicks from $11.9 \pm 0.7 \times 10^9$/L at 1 month to $23.0 \pm 1.1 \times 10^9$/L at 12 months of age. By contrast, no statistical difference in total leukocyte concentration or heterophil concentration was detected for greater rhea; however, lymphocyte concentration was greater in juvenile birds.[31]

HEMATOLOGIC DISEASE

The hematologic response to disease is complex and depends on the inciting cause, magnitude, and duration of the disease process, as well as the bird's intrinsic hematologic characteristics and its ability to respond to a particular challenge. Notably, the effect of transport as a stressor of ratites has been investigated[2] with an increase in the heterophil:lymphocyte ratio (H:L) noted post-transport. While there have been a number of studies that documented hematologic values of ratites in health, there have been few published reports of the hematologic response to disease by ratites. Consequently, there are many ratite diseases that are yet to have the hematologic characteristics fully documented.

Anemia has been observed as the sole hematologic abnormality and also with other hematologic changes; notably with increased WBC concentration. Lesser rheas infested with the nematode, *Deletrocephalus dimidiatus*, had anemia (0.22 L/L) as the sole abnormality.[12] An emu with mucopolysaccharidosis IIIB had anemia (0.28 L/L), leukocytosis (33.6×10^9/L), heterophilia, and lymphocytosis.[15] A rare case of leukemia/lymphoma in an emu presented with an anemia (0.17 L/L) and an associated leukocytosis (112.6×10^9/L) including lymphocytosis (47.3×10^9/L) and many immature cells (54.0×10^9/L).[16]

Inflammation in ratites spans diverse etiologies, illustrating that ratites mount an effective leukocyte response often with significantly increased heterophil concentrations. An emu with a tarsometatarsal sequestrum and osteomyelitis following blunt trauma to its leg initially had a total WBC concentration of 41.7×10^9/L that subsequently increased to 68.9×10^9/L and then decreased after surgical removal of the bone fragments.[36] An emu infected with the bacteria, *Erysipelothrix rhusiopathiae*, had a total WBC concentration of 50.0×10^9/L with marked toxic changes in the heterophils and bacteria evident on blood smear evaluation.[20] Emus with encephalitis due to a filaroid nematode had total WBC concentrations of between 19.1 and 80.0×10^9/L.[22] A southern cassowary with serologic evidence of toxoplasmosis had a heterophil concentration (17.8×10^9/L) and a decreased lymphocyte concentration (0.6×10^9/L).[27]

In some cases, leukocytes may be consumed or sequestered at a severe inflammatory nidus, or not produced at all. Common ostriches with stomach impaction had a mean WBC concentration that was significantly less before (10.4×10^9/L) versus after (16.6×10^9/L) treatment.[40] Similarly, a common ostrich was reported with a leukopenia of 3.7×10^9/L and left shift with granule immaturity.[18] Exertional myopathy did not increase the total leukocyte concentration in emu[35] or greater rhea.[33]

HEMOPARASITES

A few hemoparasites have been recognized in ratite blood.[38] A number of hemosporidian species have been reported for Apterygiformes, Casuariiformes, Rheiformes, and Struthioniformes. *Leukocytozoon struthionis* has been described from Struthioniformes. Hemosporidians, yet to be identified to a species level, have been recognized in Casuariiformes and Rheiformes. Additionally, *Babesia kiwiensis* and *Hepatozoon kiwi* were found in the North Island brown kiwi.[29]

The clinical consequences of *Plasmodium sp*. are poorly characterized but may be significant. A 5-month-old emu, in Florida, with a *Plasmodium sp*. had a severe anemia (0.10 L/L) with 35% polychromatophilic RBCs and a 4% parasitemia.[13] *Plasmodium matutinum* has been recognized in a wide range of avian hosts including in North Island brown kiwi and great spotted kiwi in New Zealand.[39] *Plasmodium sp*. have been reported to cause the death of kiwi but no hematologic values were available.[1]

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CHAPTER 128

Hematology of Elasmobranchs

JILL E. ARNOLD and ALEXA DELAUNE

Sample Collection and Analysis
Erythrocytes
Leukocytes
Thrombocytes and Coagulation
Cytochemistry and Ultrastructure
Reference Intervals
Hematologic Response to Disease
  Physiologic Response
  Pathologic Response

Acronyms and Abbreviations

CEG (G1), coarse eosinophilic granulocyte; ER, endoplasmic reticulum; FEG (G3), fine eosinophilic granulocyte; G2, neutrophil; GT (G4), granulated thrombocyte.

Elasmobranchii is one of two subclasses of the class Chondrichthyes (cartilaginous fishes), the extant representatives of the jawed fishes. More than 800 species are divided into two superorders: Selachimorpha (sharks) and Batoidea (rays and skates).

SAMPLE COLLECTION AND ANALYSIS

Phlebotomy sites for hematology specimens include the caudal vein (sharks, skates, and rays) and the pectoral fin (wing) veins (skates and rays). Other phlebotomy sites may be used; however, hematologic values from different sites vary and may not be comparable.[18] Commercial blood collection tubes are appropriate for most species; however, EDTA may cause lysis in samples from some ray species.

Manual CBCs are performed as for teleost species except some cell counting diluent formulas may need to be adjusted for higher osmolality to prevent lysis. The stock formula of Natt and Herrick[19] (3.88 g NaCl, 2.50 g Na^2SO^4, 2.91 g $Na^2HPO^4{\cdot}12H^2O$, 0.25 g KH^2PO^4, 0.10 g methyl violet 2B, and 7.5 mL 37% formaldehyde) can be modified to simulate the physiologic conditions of marine elasmobranchs (E-NH diluent). The original formula supplemented with 31.5 g/L urea and 13.5 g/L sodium chloride (NaCl) will yield a final osmolality of 1190 mmol/kg.[2] An aliquot of the original formula (stable 2 years) is passed through low-ash, grade-42, filter paper before adding NaCl and urea (final solution stable approximately 24 hours). A 1:100 dilution is prepared by mixing 20 µL whole blood in 1980 µL E-NH diluent and loaded into the two chambers of an improved Neubauer hemocytometer. WBC counts (/µL or $\times10^9$/L) are determined by averaging the number of cells tallied in all nine large squares of both chambers and multiplying by 110. RBC counts ($\times10^6$/µL or $\times10^{12}$/L) can be determined from the same hemocytometer preparation by tallying the five small RBC squares, within the central large square, from both chambers, and multiply the average by 5000. Thrombocyte counts (/µL) can also be obtained from the same preparation by averaging the tallies of the central large square and multiplying by 1000. Elasmobranch blood is stable for up to 5 hours in EDTA, or a whole blood aliquot can be preserved at the time of collection in 10% neutral buffered formalin (1:5) for a month or longer for hemocytometer counts.[2,3] For formalin preserved samples, the 1:100 dilution for the hemocytometer is prepared by adding 20 µL of fixed cells (1:5) to 380 µL E-NH diluent (1:20), and cell counts are determined using the above mentioned formulas for whole blood. Total dissolved solids by refractometer and PCV using a microhematocrit centrifuge are performed using standard methods. Historically, fish hemoglobin (Hgb) was determined by spectrophotometer using Drabkin's cyanmethemoglobin

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method, followed by centrifuging to remove RBC nuclei; however, this method may not be readily available due to the hazardous chemical reagents. In 1990, HemoCue (HemoCue America, Brea, CA, USA) introduced the Hb201+ azide-methemoglobin whole blood portable analyzer which yielded higher hemoglobin concentrations in sandbar shark blood compared to the cyanmethemoglobin method;[2] therefore, method-specific reference intervals should be generated. Automation of differential cell counts is not currently possible for elasmobranchs.

ERYTHROCYTES

Elasmobranch nucleated RBC morphology is comparable to the avian counterpart except for much larger size (20–25 μm in length).[9] Mature cells have an ellipsoid shape, smooth cytoplasm uniformly filled with hemoglobin, and a central oval nucleus of condensed chromatin. Less mature, polychromatophilic cells are commonly observed on blood smears as well as occasional immature forms, including RBC in mitosis (Figure 128.1a). Compared to dogs, elasmobranch RBCs are much larger (approximately 7 μm in diameter for dogs vs ≥20 μm in sharks) and subsequently fewer in number ($5.9–8.4 \times 10^6$ cells/μL for adult dogs vs 0.5×10^6 cells/μL for sharks, respectively).[9,21,23] Erythropoiesis in adult sharks occurs in the splenic red pulp in the species studied.[9,27]

LEUKOCYTES

Elasmobranch lymphocytes, monocytes, neutrophils, and basophils resemble their mammalian counterparts in morphology, while there are some distinct differences in the eosinophilic granulocytes (Figures 128.1 and 128.2). WBC differentials typically reveal abundant lymphocytes, often the predominant cell type, and a small number of monocytes. Neutrophil numbers vary; commonly observed in shark species, but typically rare or not observed in rays and skates. Basophils are rarely observed on shark blood smears, but often found in low numbers in blood smears from ray species (Tables 128.1 and 128.2).

Elasmobranch granulocytes have been given multiple names in the literature. Neutrophils, similar to those in most mammals, have clear, colorless cytoplasm. The nucleus may be lobed or nonlobed, with both morphologies commonly observed on the same blood smear from apparently healthy animals (Figure 128.2). Mainwaring et al. describe this cell as granulocyte 2, or G2, in their G1–4 series.[17,21]

Several nomenclature systems for the eosinophilic cells have been proposed since the 1980s and this lack of a standardized naming system has led to confusion, especially for the two cells containing eosinophilic granules.[6] Cells with cytoplasm containing abundant elongate, reddish granules resemble avian heterophils. They have been termed fine eosinophilic granulocytes, or FEG, based on granule morphology, and as granulocyte 3 or G3 cells by Mainwaring et al., based on ultrastructure (Figure 128.3b).[13,17,21] Cells containing oval to round cytoplasmic granules, often with differing tinctorial properties than the heterophil, resemble avian eosinophils. They have been termed coarse eosinophilic granulocytes, or CEG, and as granulocyte 1 or G1 (Figure 128.3a).[13,17,21] Variations in size, shape, and tinctorial properties of the eosinophilic cells on Romanowsky-stained blood smears are observed in different elasmobranch species. The granule shape and color in the eosinophil vary by species more so than the heterophil and either have granules that are brighter red-orange than those in the heterophil (Figure 128.2j),granules that are lighter reddish-pink than the heterophils (Figure 128.2b), or show similar tinctorial properties to the heterophil but differ in shape (Figure 128.2f). The nuclei of elasmobranch eosinophilic granulocytes may be round, indented, or multilobed on the same blood smear (Figures 128.1c–e and 128.2c).

Lymphocytes are typically small with dark condensed chromatin and a small tag of blue cytoplasm. Low numbers of intermediate and large lymphocytes (>10 μm) may be found in blood smears from apparently healthy sharks. Antibody classes and structure are different than mammalian types and currently under investigation. Monocytes (phagocytes) are similar in morphology to those found in mammals and are differentiated from lymphocytes by more copious dark blue cytoplasm that is occasionally vacuolated.

THROMBOCYTES AND COAGULATION

Sharks, skates, and rays possess thrombocytes of similar size and morphology to those found in avian species. On Romanowsky-stained blood smears, thrombocytes appear as oval cells, with clear cytoplasm and a central oval nucleus of condensed chromatin. Rounded and spindle forms are sometimes observed. Like erythropoiesis, thrombocytopoiesis occurs in splenic red pulp. Pica studied thrombocyte form and function in torpedo rays (*Torpedo marmorata* bottom-dwelling rays) and a catshark (*Scyliorhinus stellaris*) and concluded that these cells appear functionally like mammalian platelets, although less abundant.[20] Thrombocytes form aggregates and adhere to glass. A study of hemostasis using five shark species, one skate, and one guitarfish found that elasmobranch blood clotted very slowly in glass tubes, often >48 hours; adding calcium chloride accelerated the clotting process but caused cell lysis.[15] Interestingly, thrombocytes from a Port Jackson shark (*Heterodontus portusjacksoni*) phagocytized latex particles.[22] The function of elasmobranch thrombocytes in response to infectious agents is unknown.

A second type of thrombocyte, first described by Mainwaring and Rowley as granulocyte type 4, or G4 cells, may be found in some elasmobranch species.[17] This cell resembles the typical thrombocyte in size and shape; however, the cytoplasm is filled with abundant azurophilic granules (Figures 128.2d and 128.3d). The

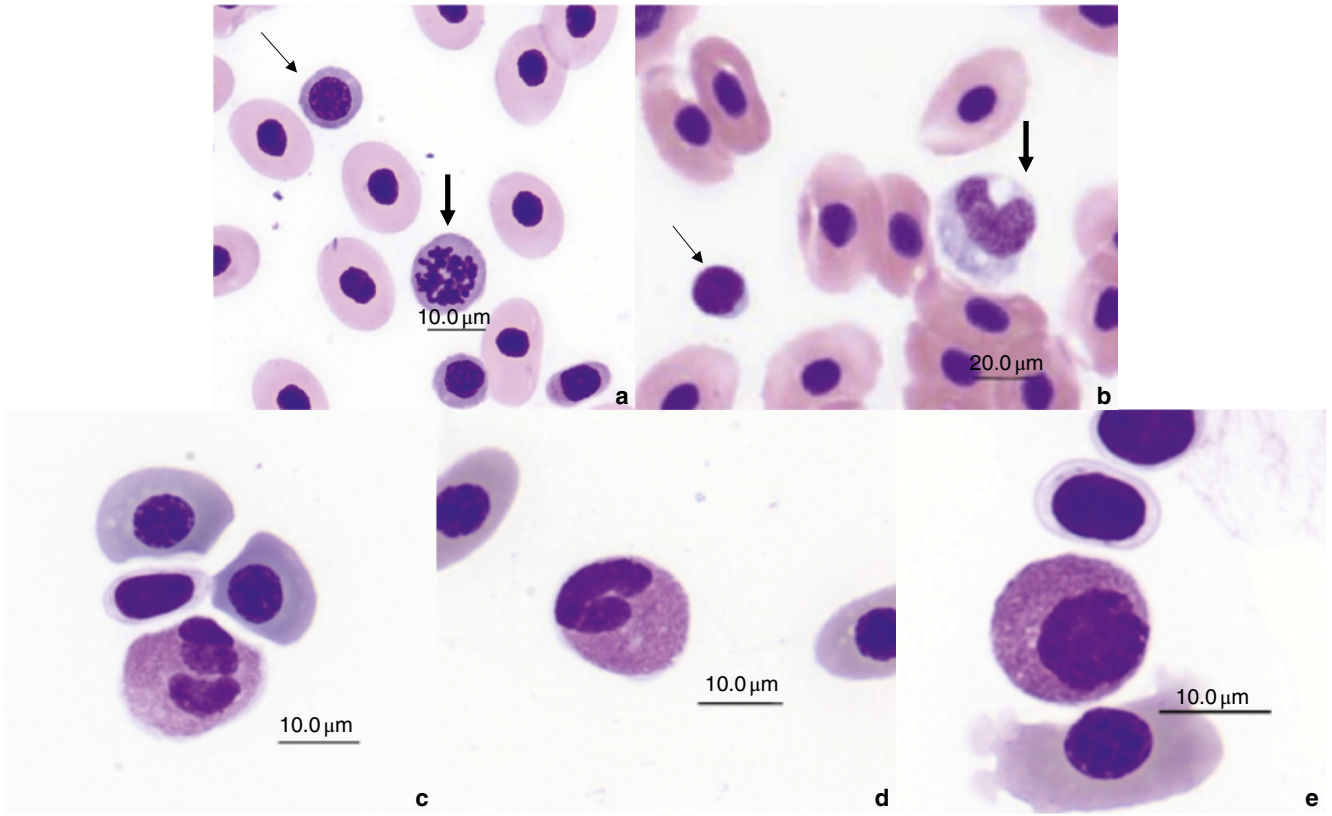


FIGURE 128.1 Elasmobranch blood cells. Blacknose shark (*Carcharhinus acronatus*): (a) Normal erythrocytes, polychromatophilic RBC or rubricyte (thin arrow), mitotic erythrocyte (thick arrow). Mitotic figures may be found in blood smears indicating erythroid regeneration and are not indicative of neoplasia. Cownose ray (*Rhinoptera bonasus*): (b) Lymphocyte (thin arrow), monocyte (thick arrow). Honeycomb stingray (*Himantura uarnak*): (c) Mature heterophil with segmented nucleus; (d) Heterophil band; (e) Immature heterophil. Modified Wright–Giemsa stain. 1000× magnification.

clinical significance of this cell is not known, and further investigation of this cell's identification and function is warranted.

CYTOCHEMISTRY AND ULTRASTRUCTURE

Multiple studies in elasmobranch blood cell cytochemistry were published in the mid-1980s, including the comprehensive observations by Hine, Wain, and Boustead for 24 elasmobranch species.[13] Hine summarized granulocyte cytochemistry findings as weak or negative for peroxidase, alkaline phosphatase, and beta-glucuronidase, but often strongly positive for acid phosphatase, and periodic acid–Schiff.[12] Reports vary for different species and future studies will aid in cell descriptions.

Ultrastructural studies (Figure 128.3) have described RBC cell maturation within the hematopoietic tissues, e.g., splenic red pulp, beginning with round cells containing a large nucleus with small amounts of condensed chromatin. The cytoplasm contains mitochondria and ribonucleoproteins, rough endoplasmic reticulum (ER), vacuoles, and Golgi complex. Successive developmental stages show maturation to an ellipsoid shape and the nuclear chromatin condenses.[27,28] Lymphocytes are described as having a central nucleus with dense patches of heterochromatin and occasional nucleolus. The limited cytoplasm contains few vesicles, vacuoles, mitochondria, rough ER, and occasional Golgi complex.[21] Monocytes have a prominent eccentric nucleus and the cytoplasm contains rough ER, Golgi complex, Golgi-derived vesicles, and heterophagosomes. Protoplasmic extensions are common.[21]

Rowley et al. summarized the granulocytes of dogfish (*Scyliorhinus canicular*) as G1 (eosinophil) with an irregular, sometimes bilobed nucleus, one population of unstructured electron-dense round-ovoid granules.[21] Hyder reported the presence of large, crystalline, membrane-limited granules containing

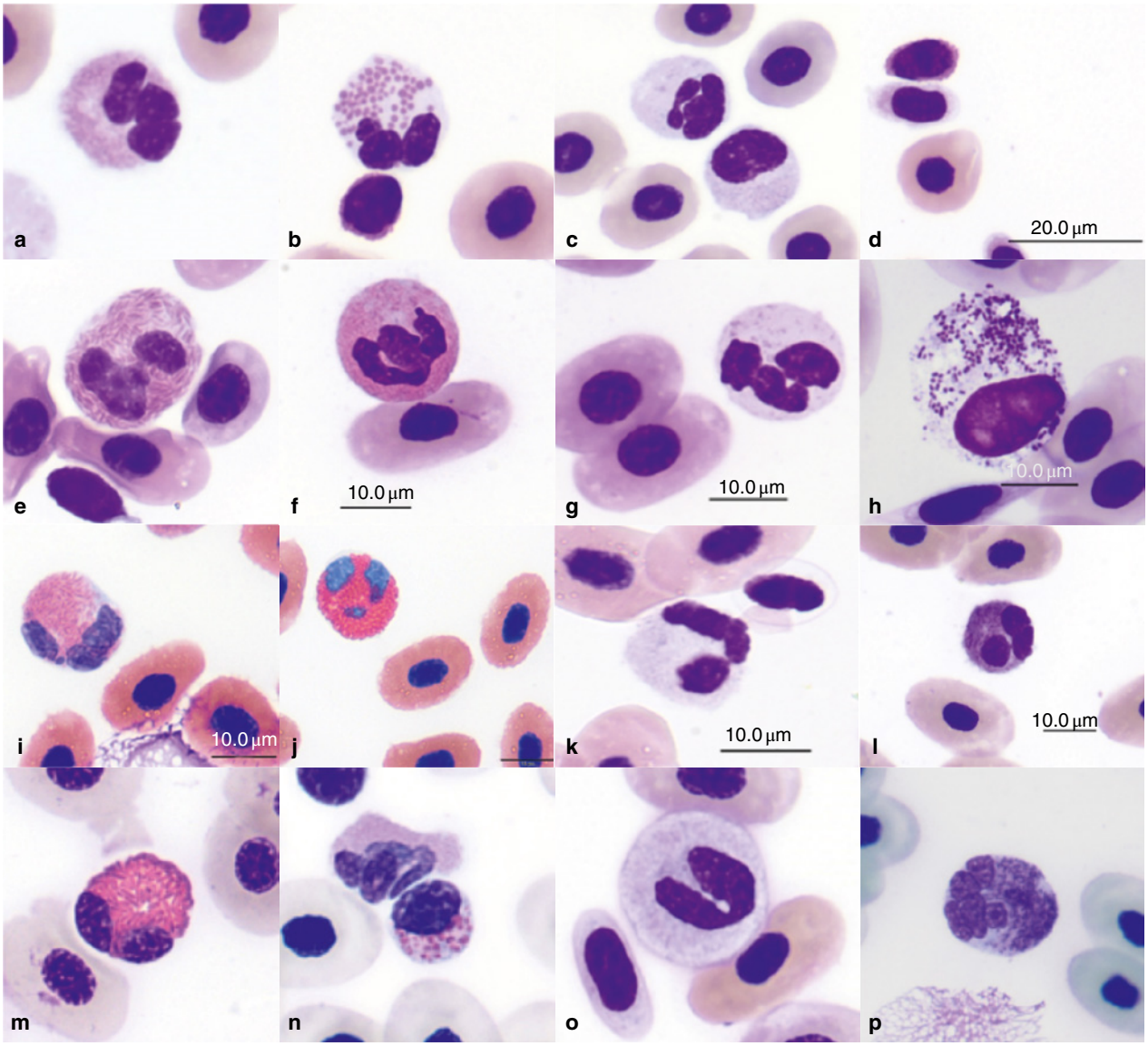


FIGURE 128.2 Normal elasmobranch granulocytes. Blacktip reef shark (*Carcharhinus melanopterus*): (a) Heterophil; (b) Eosinophil; (c) Mature and immature neutrophil; (d) Thrombocytes, granular and agranular. Whitespotted bamboo shark (*Chiloscyllium plagiosum*): (e) Heterophil; (f) Eosinophil; (g) Neutrophil; (h) Basophil. Southern stingray (*Dasyatis americana*): (i) Heterophil; (j) Eosinophil; (k) Neutrophil; (l) Basophil. Spotted eagle ray (*Aetobatus narinari*): (m) Heterophil; (n) Heterophil and immature granulocyte (probable eosinophil); (o) Neutrophil; (p) Basophil. Modified Wright–Giemsa stain. 1000× magnification.

elongated crystalloids in the nurse shark (*Ginglymostoma cirratum*) eosinophil, and compared these rhomboidal inclusions to the characteristic crystalline inclusions in mammalian eosinophil granules.[14] The G2 (neutrophil) nucleus typically appears clefted, or horseshoe-shaped, and the cytoplasm contains small heterogenous granules. The nucleus of the G3 (heterophil) is typically lobed and the granules are unstructured, electron-dense, and rod-shaped. Dogfish G4 granulocytes (granulated thrombocytes) have an elongate nucleus and cytoplasm filled with one population of square/cuboid electron-dense, unstructured granules.[21] Further investigation of the granulated G4 (granulated thrombocyte) is warranted to determine clinical significance. Editor's note: Further investigation of the G4 (granulated thrombocyte) as a granular lymphocyte is warranted.

Thrombocytes of nurse shark (*G. cirratum*) cytoplasm contain numerous agranular vesicles and small vesicles with electron-dense material.[14] Shark species that possess the second type of thrombocyte, G4 or granulated thrombocyte, such as the lesser-spotted dogfish (*S. canicula*) have cytoplasm filled with electron-dense granules (Figure 128.3d). Specific cytochemistry and ultrastructure studies may use different methods and have been misquoted in the literature. The reader is advised to access the original studies for accurate information and evaluation.

TABLE 128.1 Reference intervals for 33 adult, mixed-sex free-ranging sandbar sharks (*Carcharhinus plumbeus*) and 22 adult, mixed-sex aquarium-housed cownose rays (*Rhinoptera bonasus*).

Sandbar sharks

Measurand	Units	N	Mean	SD	Median	Min.	Max.	Dist.	RI	CI 90% of LRL	CI 90% of URL
PCV	%	32	27	3.6	27	20	35	G	20–33	19–22	31–34
RBC	$10^6/\mu L$	33	0.52	0.1	0.55	0.4	0.64	G	0.38–0.67	0.35–0.43	0.63–0.70
Hemoglobin	g/dL	33	6.3	0.6	6.2	5.3	7.6	G	5.2–7.5	5–5.5	7.1–7.7
MCV	fL	32	525	124	500	359	822	G	287–724	235–347	655–794
MCHC	g/dL	32	24	2.9	24	18	29	G	19–29	18–20	28–30
MCH	pg	33	124	21	122	92	167	G	87–159	78–95	148–169
TS/TP	g/dL	33	5.9	0.8	5.7	4.7	7.7	G	4.4–7.4	4–4.7	6.9–7.7
WBC	$10^3/\mu L$	33	22	4.8	21.5	14	32.2	G	13.6–30.3	11.3–15.3	27.4–32.4
Heterophil	$10^3/\mu L$	32	6.3	2.7	5.6	2.51	11.7	NG	1.2–10.9	0.2–2.4	9.2–12.2
Heterophil-segmented	$10^3/\mu L$	32	3.4	2.2	3	0.2	9.7	NG	0–6.9	0–0.3	5.5–8.1
Heterophil-nonsegmented	$10^3/\mu L$	32	2.8	1.7	2.8	0.5	6.9	NG	0–5.6	0–0.4	4.6–6.5
Lymphocyte	$10^3/\mu L$	32	9.3	3	6.5	3.3	15.2	G	4.1–14.5	2.8–5.5	13.1–15.9
Monocyte	$10^3/\mu L$	32	0.6	0.5	0.5	0	1.8	NG	0–1.8	NA	NA
Eosinophil	$10^3/\mu L$	32	4	1.8	3.6	0.9	9.1	G	0.5–7	0–1.5	5.9–8
Eosinophil-segmented	$10^3/\mu L$	32	2.7	2.1	2.5	0	6.7	G	0–6.3	NA	5.3–7.1
Eosinophil-nonsegmented	$10^3/\mu L$	32	1.3	1.6	0.6	0	7.5	NG	0–3.6	NA	2.4–4.9
Neutrophil	$10^3/\mu L$	32	2	0.9	1.7	0.7	4.4	NG	0–3.3	0–0.5	2.8–3.9
Basophil	$10^3/\mu L$	32	0	0	0	0	0	NA	NA	NA	NA

Cownose rays

Measurand	Units	N	Mean	SD	Median	Min.	Max.	Dist.	RI	CI 90% of LRL	CI 90% of URL
PCV	%	22	34	5	34	22	43	G	26–44		
TS/TP	g/dL	22	6.6	0.8	6.8	4.8	7.7	NG	5.3–8.3	4.7–5.9	7.8–8.7
WBC	$10^3/\mu L$	22	18.3	6.1	17.8	8	31.1	G	6.7–28.4	4.1–10.2	24.6–32.5
Heterophil	$10^3/\mu L$	22	1.9	1.2	1.8	0	4.2	G	0–4.1	0–0.3	3.3–4.7
Lymphocyte	$10^3/\mu L$	22	15.2	6.3	14.2	4.9	30.2	G	2.6–25	0–6.8	21–29.7
Monocyte	$10^3/\mu L$	22	0.2	0.2	0.2	0	0.7	G	0–0.6	NA	NA
Eosinophil	$10^3/\mu L$	22	0.7	0.6	0.6	0	2.3	NG	0–1.6	0–0.2	1.1–1.9
Neutrophil	$10^3/\mu L$	22	0.2	0.3	0.1	0	0.9	NG	0–0.6	NA	NA
Basophil	$10^3/\mu L$	22	0.09	0.1	0	0	0.6	NG	0–0.5	NA	NA

CI, confidence interval; G, Gaussian; NG, non-Gaussian; NA, not applicable; LRL: lower reference limit; URL: upper reference limit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; TS/TP, total solids/protein; SD, standard deviation; Min, minimum; Max, maximum; Dist, Distribution.
Source: Data courtesy of the National Aquarium. See text for further description.

REFERENCE INTERVALS

Interpretation of elasmobranch hematology results may be difficult for a variety of reasons including a lack of reference intervals for most species, preliminary identification and understanding of function of each leukocyte type, and individual species response to a variety of environmental factors. Additionally, results may be affected by different processing techniques or variation between technicians performing the CBCs. Biological factors such as age, sex, and season are essentially unexplored, though differences in species and in captive versus wild have been documented. For example, wild carpet sharks have higher PCV than captive carpet sharks (*Chiloscyllium punctatum*).[7] This finding demonstrates that it is important to generate reference intervals for wild and captive

TABLE 128.2 Hematologic cells found in elasmobranch species.

Scientific name	Common name	Monocyte	Lymphocyte	Neutrophil	Heterophil	Eosinophil	GT	Basophil
ORDER HETERODONTIFORMES: BULLHEAD SHARKS								
Heterodontus francisci	Horn shark[1] (>5)	●	●	●	●	a	●	ND
Heterodontus portusjacksoni	Port Jackson shark[4] (1)	ND	●	●	●	a	●	ND
ORDER ORECTOLOBIFORMES: CARPET SHARKS								
Eucrossorhinus dasypogon	Tasselled wobbegong[1] (1)	●	●	●	●	ND	ND	ND
Orectolobus maculatus	Spotted wobbegong[4] (1)	●	●	●	●	ND	ND	ND
Orectolobus ornatus	Ornate wobbegong[1] (2)	●	●	●	●	a	ND	ND
Chiloscyllium plagiosum	Whitespotted bamboo[1] (5)	●	●	●	●	a	ND	●
Chiloscyllium punctatum	Brownbanded bamboo[4] (1)	●	●	●	●	a	ND	ND
Ginglymostoma cirratum	Nurse shark[1] (>5)	●	●	●	●	c	ND	ND
Stegostoma fasciatum	Zebra shark[1] (>5)	●	●	●	●	a	ND	ND
Rhincodon typus	Whale shark[2] (5)	●	●	●	●	a	ND	●
ORDER LAMNIFORMES: MACKEREL SHARKS								
Carcharius taurus	Sand tiger shark[1] (>5)	●	●	●	●	a	●	ND
ORDER CARCHARHINIFORMES: GROUND SHARKS								
Cephaloscyllium ventriosum	Swell shark[1] (5)	●	●	●	●	a	●	ND
Mustelus canis	Dusky smooth-hound[1] (>5)	●	●	●	●	c	●	ND
Triakis semifasciata	Leopard shark[1] (>5)	●	●	●	●	b	●	ND
Carcharhinus acronatus	Blacknose shark[1] (5)	●	●	●	●	b	●	ND
Carcharhinus brevipinna	Spinner shark[1] (1)	●	●	●	●	b	●	ND
Carcharhinus leucas	Bull shark[1] (1)	●	●	●	●	b	●	ND
Carcharhinus limbatus	Blacktip shark[1] (1)	●	●	●	●	b	●	ND
Carharhinus melanopterus	Blacktip reef shark[1] (5)	●	●	●	●	b	●	ND
Carcharhinus obscurus	Dusky shark[1] (1)	●	●	●	●	b	●	ND
Carcharhinus plumbeus	Sandbar shark[1] (>5)	●	●	●	●	b	●	ND
Galeocerdo cuvier	Tiger shark[1] (1)	●	●	●	●	b	●	●
Negaprion brevirostris	Shortnosed lemon shark[1] (>5)	●	●	●	●	c	●	ND
Rhizoprionodon terraenovae	Atlantic sharpnose shark[1] (1)	●	●	●	●	a	●	ND
Sphyrna tiburo	Bonnethead shark[1] (5)	●	●	●	●	c	●	ND
Triaenodon obesus	Whitetip reef shark[1] (>5)	●	●	●	●	b	●	ND
ORDER CHIMAERIFORMES: RATFISH								
Hydrolagus colliei	Spotted ratfish[3] (1)	●	●	●	●	a	D	ND

(*Continued*)

TABLE 128.2 (Continued) Elasmobranch Cell List: Skates and Rays

Scientific name	Common name	Monocyte	Lymphocyte	Neutrophil	Heterophil	Eosinophil	GT	Basophil
ORDER PRISTIFORMES: SAWFISHES								
Pristis microdon	Greattooth or freshwater sawfish[1] (>5)	●	●	Rare	●	a	ND	ND
Pristis pectinata	Smalltooth sawfish[1] (3)	●	●	Rare	●	a	ND	●
Rhynchobatus laevis	Smoothnose wedgefish[1] (>5)	●	●	Rare	●	a	ND	●
ORDER RHINFORMES: WEDGEFISHES								
Rhina ancylostoma	Bowmouth guitarfish[3] (4)	●	●	ND	●	a	ND	●
ORDER RHINOBATIFORMES: GUITARFISHES								
Trygonorrhina fasciata	Southern fiddler ray[3] (5)	●	●	●	●	a	ND	●
Platyrhinoidis triseriata	Thornback guitarfish[1] (4)	●	●	●	●	a	ND	ND
ORDER RAJIFORMES: SKATES								
Raja eglanteria	Clearnose skate[1] (3)	●	●	ND	●	a	ND	●
ORDER MYLIOBATIFORMES: STINGRAYS								
Urobatis jamaicensis	Yellow stingray[3] (5)	●	●	ND	●	a	ND	ND
Paratrygon aiereba	Discus/Ceja freshwater stingray[3] (4)	●	●	ND	●	a	ND	●
Potamotrygon castexi	Vermiculate river stingray[3] (3)	●	●	Rare	●	a	ND	●
Potamotrygon henlei	Bigtooth river stingray[3] (5)	●	●	ND	●	a	ND	●
Potamotrygon leopoldi	White-blotched river stingray[3] (5)	●	●	ND	●	a	ND	●
Potamotrygon tigrina	Tiger stingray[3] (3)	●	●	ND	●	a	ND	ND
Dasyatis americana	Southern ray[1] (>5)	●	●	Rare	●	a	ND	●
Dasyatis centroura	Roughtail ray[1] (>5)	●	●	Rare	●	a	ND	●
Dasyatis say	Bluntnose stingray[1] (1)	●	●	ND	●	a	ND	●
Dasyatis violacea	Pelagic stingray[1] (5)	●	●	Rare	●	a	ND	●
Himantura chaophraya	Freshwater whipray[1] (2)	●	●	ND	●	a	ND	ND
Himantura dalyensis	Freshwater whipray[1] (3)	●	●	ND	●	a	ND	ND
Himantura granulata	Mangrove whipray[3] (2)	●	●	ND	●	a	ND	●
Himantura uarnak	Honeycomb stingray[1] (5)	●	●	ND	●	a	ND	●
Taeniura lymma	Blue-spotted ribbontail stingray[3] (5)	●	●	ND	●	a	ND	ND
Taeniura meyeni	Round ribbontail stingray[1] (2)	●	●	ND	●	a	ND	●
Gymnura altavela	Spiny butterfly ray[1] (>5)	●	●	Rare	●	a	ND	●
Aetobatus narinari	Spotted eagle ray[1] (>5)	●	●	Rare	●	a	ND	●
Myliobatis freminvillei	Bullnose eagle ray[1] (>5)	●	●	ND	●	a	ND	●
Rhinoptera bonasus	Cownose ray[1] (>5)	●	●	Rare	●	a	ND	●

● indicates presence, numbers in parentheses are the number of smears reviewed for that species.
a. Eosinophil granules brighter than heterophil.
b. Eosinophil granules paler than heterophil.
c. Granules of eosinophils and heterophil similar in pigment; differ in shape.
ND = None detected on observed slides.
GT = granulated thrombocyte.
Copied with permission from Haines and Arnold.[11] Slides provided by: [1]National Aquarium; [2]Georgia Aquarium; [3]John G Shedd Aquarium; [4]Underwater Adventures.

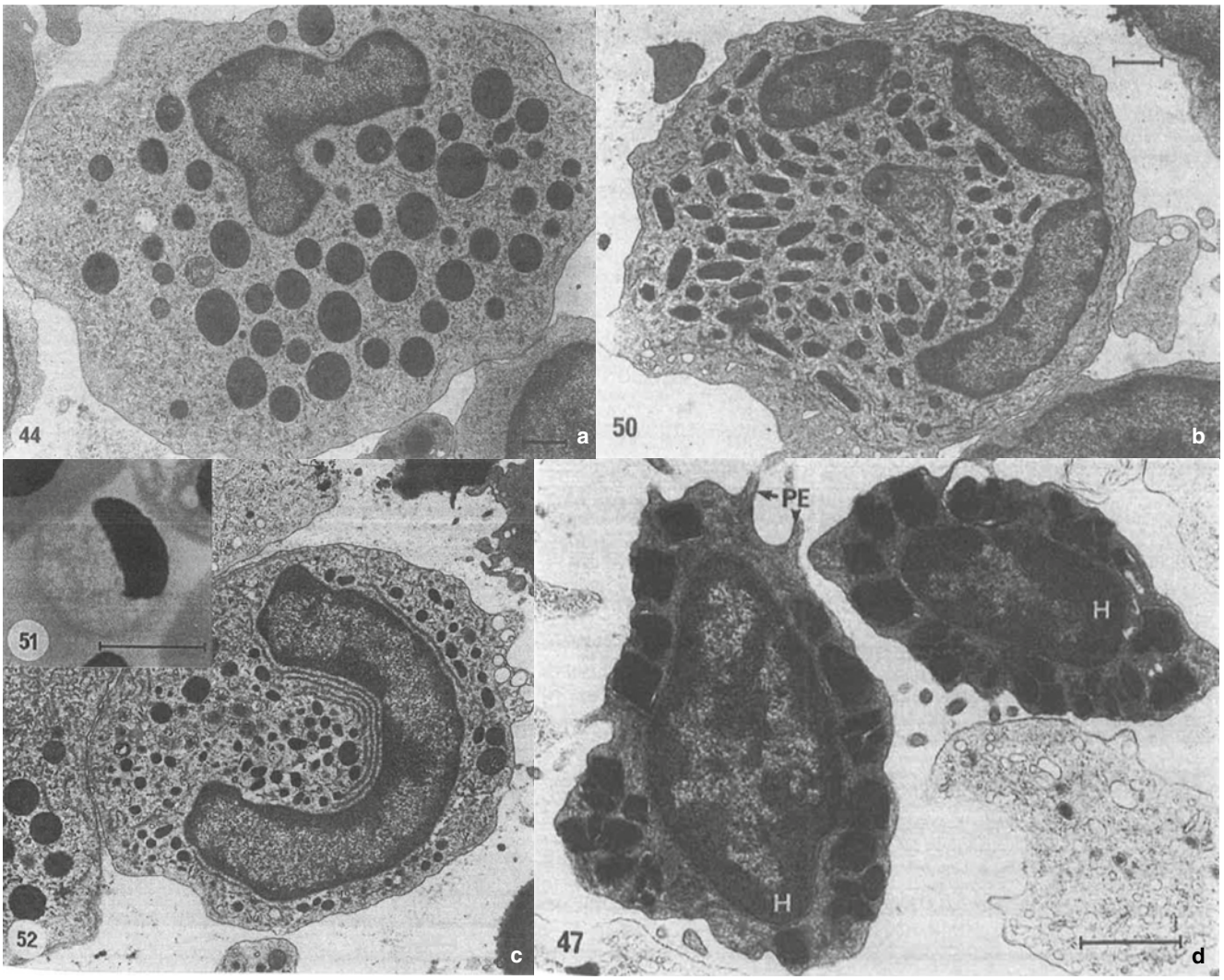


FIGURE 128.3 Elasmobranch granulocyte ultrastructure descriptions by Mainwaring and Rowley.[17,22] Lesser-spotted dogfish (*Scyliorhinus canicula*): (a) Eosinophil, G1 eosinophilic granulocyte showing characteristic round electron-dense granules; (b) Heterophil, G3 eosinophilic granulocyte with elongate electron-dense granules; (c) Neutrophil, G2 granulocyte showing numerous small granules; (d) Described as a granulated thrombocyte, G4 eosinophilic granulocyte showing angular granules and protoplasmic extensions (PE), heterochromatin (H). (Source: Adapted with permission from Rowley.[21])

populations, as they may not be interchangeable. Although established reference intervals may not be available for an individual, the clinician may find serial CBCs to be a useful diagnostic tool. When interpreting hematology results for an individual or group of elasmobranchs, it is helpful to analyze trends.

Reference intervals were calculated for 33 free-ranging sandbar sharks (*Carcharhinus plumbeus*) and 22 aquarium-housed cownose rays (*Rhinoptera bonasus*), following American Society for Veterinary Clinical Pathology guidelines (Table 128.1). Animals were briefly manually restrained following the National Aquarium Animal Care and Welfare protocols. The sandbar sharks were collected via bottom-set longlines or gill nets in the Delaware Bay near Cape May, NJ and Lewes, DE, during summer months of 1985–1999. The baited lines or nets were set for no more than 30 minutes. Immediately upon landing, the sharks were manually restrained briefly for blood collection, body measurements, and tagging. All sharks sampled were active and appeared healthy. Samples for the 22 cownose rays were obtained during regularly scheduled physical examinations at the National Aquarium between 2007 and 2009. Each ray was netted individually, transferred to a holding tank, and manually restrained for blood collection and examination.

Blood samples were collected from the caudal vein and immediately transferred to EDTA (sharks) or lithium heparin (rays) tubes. Hemocytometer counts were determined using the modified Natt and Herrick diluent described in the earlier text. PCV using a microhematocrit centrifuge (>11,000 g) was determined for all but one sandbar shark, due to a broken capillary tube. Total plasma solids were read by refractometer. Hemoglobin

for the sandbar sharks was measured using a hand-held Spencer-AO hemoglobinometer (American Optical). This device was discontinued mid-1990s, as was the Unopette 5859 HGB (Becton Dickinson Corporation). RBC evaluation for the cownose ray samples was restricted to PCV, per National Aquarium laboratory protocols. Blood smears were stained either with Wright–Giemsa, or a combination of May–Grünwald and Wright–Giemsa. WBC differentials were determined for all but one sandbar shark, due to a broken slide.

Reference intervals were generated de novo using the robust method with MedCalc® statistical software version 19.5 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2020) after outliers were removed for leukocytosis or anemia, detected by the Tukey method. Data were tested for normal distribution using the Shapiro–Wilk test. Double-sided 90% confidence intervals for the lower and upper reference limits were determined.

HEMATOLOGIC RESPONSE TO DISEASE

Physiologic Response

Dogfish (*S. canicular*) exposed to confinement stress have decreased PCV, Hgb concentrations, and WBC counts.[24] Mako sharks (*Isurus oxyrinchus*) and sandbar sharks (*C. plumbeus*) have increased hematocrit due to capture stress.[4,25] In contrast, no change in PCV is noted after capture in gummy sharks (*Mustelus antarcticus*).[10] The differences in response to capture stress illustrate the importance of evaluating elasmobranchs on an individual basis and caution should be used when making assumptions between species. Leukocytosis attributed to stress is common in clinical settings.

Pathologic Response

Decreased PCV (anemia) has been reported in several cases. In clinical practice, anemia has been noted due to parasitism, trauma, gastrointestinal ulcerations, cardiac disease, and other chronic disease (Delaune, personal communication). Lesser-spotted dogfish (*S. canicula*) treated with enrofloxacin had decreased PCV, possibly due to oxidative stress.[26] Spiny dogfish (*Squalus acanthias*) and cownose rays (*R. bonasus*) had decreased PCV after a 30–40-day quarantine period.[16,8] Mandelman and Farrington hypothesize that the reason for the apparent decreased PCV in spiny dogfish may be attributed to an artificially increased PCV postcapture and transport, due to RBC swelling.[16]

Multiple causes for elevated PCV (polycythemia) have also been posed. Lemon sharks (*Negaprion brevirostris*) have increased PCV during exercise.[5] Wild and captive epaulette sharks (*Hemiscyllium ocellatum*) and grey carpet sharks (*Chiloscyllium punctatum*) have increased PCV after exposure to anoxic conditions.[7] Both captive and wild carpet sharks have increased Hgb concentrations immediately post exposure to anoxic conditions, though wild and captive epaulettes demonstrate no change in Hgb concentration after exposure to anoxic conditions.[7] The authors hypothesize that polycythemia (increased PCV and Hgb concentrations) post anoxia exposure may be attributed to RBC release from hematopoietic organs, hemoconcentration, accelerated RBC maturation in circulation, or an increase in oxygenated RBC, as blood is diverted from muscles and/or gastrointestinal tract into the peripheral blood.[7]

Interpreting WBC counts and differentials is difficult, as the function of leukocyte types is not entirely understood. In clinical practice, leukocytosis may be observed due to stress, parasitism, bacterial or fungal infections, and trauma. Male whitespotted bamboo sharks (*Chiloscyllium plagiosum*) with amputated claspers had a heterophilic leukocytosis with toxic changes, while with sharks with normal claspers did not.[1] Heterophils (FEGs) and monocytes from nurse sharks (*G. cirratum)* demonstrated in vitro phagocytosis).[14] With suspected inflammatory or infectious conditions, a shift in the WBC differential results to a heterophil predominant differential versus the expected lymphocyte predominant differential, i.e., an inverted heterophil:lymphocyte ratio, may occur. Some elasmobranchs that are apparently healthy will have an acute leukocytosis of unknown origin. It is important to note that not all clinically ill elasmobranchs will respond with a leukocytosis or shifted differential. As stated in the earlier text, trends in individual hematology results are often helpful when managing clinical cases.

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CHAPTER 129

Hematology of Salmonids

JERE STERN

Blood Collection
Laboratory Analysis
Hematopoiesis
Erythrocytes
Leukocytes
Thrombocytes
Reference Intervals and Partitioning Factors
Hematologic Disease
  Anemia
  Stress
  Inflammation
  Hemoparasites

Acronyms and Abbreviations

ASVCP, American Society for Veterinary Clinical Pathology; MS-222, tricaine methanesulfonate; ICM, intermediate cell mass; PAS, periodic acid–Schiff; PRV, piscine orthoreovirus.

Many of the most commonly farmed fishes are salmonids, particularly Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*).[17] While the use of automated hematology in these species is limited by nucleated erythrocytes and thrombocytes, hematology can be a useful diagnostic technique in fisheries management. Unfortunately, the paucity of valid baseline clinical information including for salmonid hematology has limited its diagnostic utility.

BLOOD COLLECTION

As most fish respond poorly to handling, sedation is frequently required. Salmonids, like many other teleosts, have somewhat soft oval-shaped cycloid scales and, as such, should be handled gently in order to mitigate associated trauma. Cutaneous mucus is important as both a physical barrier to invading organisms and as a part of humoral immunity, with IgM found in the cutaneous mucus of Atlantic salmon (*S. salar*);[42] handling trauma may damage or remove cutaneous mucus, predisposing to potentially fatal secondary infection. Tricaine methanesulfonate (MS-222) is the only anesthetic approved for use in food fish and requires a 21-day withdrawal period. MS-222 is typically administered by immersing fish in a water bath, prepared with water similar to living conditions, preferably from the same tank. Recommended added concentrations are broad as are buffering recommendations, and other sources should be referenced for dosing guidelines. It is important to note that MS-222 is acidifying and the preparation should be buffered with sodium bicarbonate.[10]

Salmonids are most commonly bled from the caudal tail vein.[8] Cardiac puncture should be limited to individuals that will not be recovered after blood collection. Blood should be collected into a heparinized or EDTA-treated container to prevent blood clotting and maintain cellular morphology. Heparin best maintains WBC morphology in these species, though it can induce both thrombocyte and WBC clumping, affecting differential counts. Erythrocytes in EDTA-stored samples tend to swell, affecting the PCV.[13] Serum will quickly gel in a significant number of samples within half an hour and so plasma is required to ensure that the sample taken can be analyzed for biochemistry.

LABORATORY ANALYSIS

As all salmonid blood cells are nucleated with significant overlap in cell size between cellular lineages, automated methodologies utilized in mammalian

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samples have limited use in fish. Instead, manual counting methods with a hemocytometer are needed (see Chapters 123 and 128). Sources of error in manual counting methods include dilution with anticoagulant and other matrices, sample settling, variable hemocytometer filling, cell identification, and counting of low cell numbers. Differentiation between activated thrombocytes and WBCs, particularly lymphocytes, may be difficult. While thrombocytes are typically oval, they become small and round when activated, resembling a lymphocyte. These manual methods utilize a diluent that differentially stains RBCs and WBCs. Natt–Herrick (Bioanalytic GmbH, Umkirch, Germany) and Rees–Ecker (RICCA Chemical Company, Arlington, TX) diluents are both effective in salmonids. A 1:100 or 1:200 dilution of blood to filtered diluent is prepared, and cells are allowed to stand for at least 5 minutes prior to filling the hemocytometer. As in avian species, allowing the cells to stain longer may result in better staining and more accurate results (see Chapter 128).

Information regarding RBC density may be obtained from manual RBC counts or from a spun PCV, and hemoglobin (Hgb) concentration can be measured utilizing the cyanmethemoglobin method.[7] In manual hemocytometer methods, RBCs are counted in five secondary squares of the center primary square. This raw count is multiplied by 5000 for a 1:100 dilution or by 10,000 for a 1:200 dilution, providing the number of RBCs per microliter of blood. Leukocytes can be counted in the four corner primary squares. This raw count is multiplied by 250 for a 1:100 dilution or by 500 for a 1:200 dilution, providing a number of WBCs per microliter of blood. Repeating the described methods on both sides of the hemocytometer is recommended and only results that agree within <10% are accepted. Flow cytometry has been investigated for salmonid blood cell quantification.[34] Based on forward and side scatter, flow cytometry can sort blood into the following three groups: erythrocytes, lymphocytes and thrombocytes, and granulocytes.

A WBC estimation can be performed when examining a well-made blood smear with even cell distribution.[22] Blood film evaluation should only be done using a well-made smear in the monolayer with even WBC distribution. Care should be taken when preparing these smears, as non-mammalian WBCs tend to lyse, altering differential counts. A variety of gentle blood smear techniques have been described, including gentle push smear techniques and coverslip preparation. Salmonid blood film evaluation, as in other species, should initially be scanned on low power to ensure the slide is well-prepared with even WBC distribution, to assess thrombocyte clumping at the feather edge, and investigate for hemoparasites. The hematologist should become familiar with the cell types present as magnification is increased. A 200-WBC differential should be performed in the monolayer, and these percentages are then applied to the WBC count to provide absolute counts for each leukocyte type.

HEMATOPOIESIS

Primitive hematopoiesis begins in the yolk sac blood islands at 2 days post fertilization in rainbow trout (*O. mykiss*) and then in the intermediate cell mass (ICM), from days 7–12 to day 15 post fertilization in rainbow trout.[20] The ICM is ventral to the notochord and forms when the posterior mesoderm migrates medially to fuse at midline.[20] Hematopoietic production is higher in the ICM than in the yolk sac, and proerythroblasts form alongside primitive endothelial cells of the trunk vasculature.[20] These proerythroblasts proliferate before entering circulation. A second wave of myelopoiesis occurs in the posterior ICM.[30] Hematopoietic cells then colonize the pronephros, surrounding vessels between renal tubules and glomeruli. The pronephros is the primary site of adult erythropoiesis and myelopoiesis, with the spleen serving as a secondary site.[20] Erythroid precursors are released from the kidney into circulation at an immature stage and rubriblasts, prorubricytes, and rubricytes can all be seen on blood film evaluation, especially in fry.

The pronephros is the site of B-cell lymphopoiesis in salmonids.[20] As in all gnathostomes (jawed vertebrates), T lymphocytes develop in the thymus.[20] While thrombocytes are present in circulation early in development, the cellular equivalent to the megakaryocyte, the maturation stages, and the primary site of thrombopoiesis have not been identified.

ERYTHROCYTES

Salmonid RBCs resemble those of birds and reptiles, as they are oval with abundant pale eosinophilic cytoplasm and a central oval nucleus with clumped chromatin. They can be distinguished from other animal families by nuclear morphology (Figure 129.1). The cytoplasm is typically homogeneously pale pink (or salmon colored), though rare to occasional small pale staining or clear areas, associated with degenerating organelles as in other species, are occasionally observed. Small amounts of anisocytosis and polychromasia are present in normal salmonid blood smears. Polychromatophils have a slight blue tinge to the cytoplasm, appear slightly rounder, and have a more open nuclear chromatin pattern. Polychromatophils comprise <5% of total RBCs, while immature RBCs (rubriblasts, prorubricytes, and rubricytes) are typically <1% of total RBCs in blood samples from apparently healthy fish. These round erythroid precursors have variably basophilic cytoplasm with a central round nucleus that is larger in size than that in mature RBCs (Figure 129.1m–p). As in other nonmammalian species, erythroid precursors undergoing mitosis can be seen in peripheral blood.

Erythrocyte size can be evaluated with impedance technology or microscopically, by direct measurement of RBCs on a blood film, and is highly variable, even within the same family and species.[6,14] Erythrocyte size

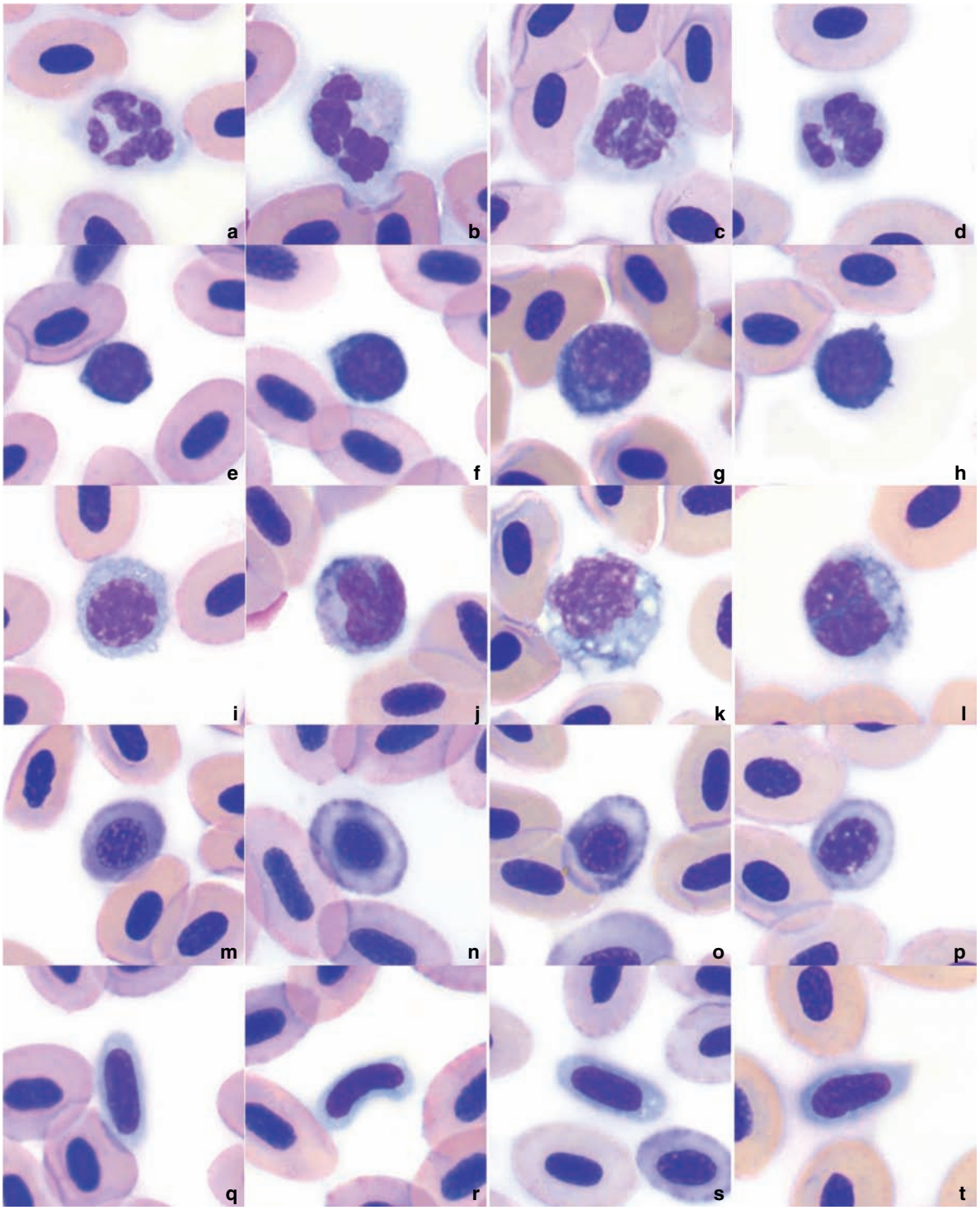


FIGURE 129.1 Leukocytes, immature erythrocytes, and thrombocytes from four salmonid species. King or Chinook salmon (*Oncorhynchus tshawytscha*): (a) Neutrophil; (e) Lymphocyte; (i) Monocyte; (m) Rubricyte; (q) Thrombocyte. Pink salmon (*Oncorhynchus gorbuscha*): (b) Neutrophil; (f) Lymphocyte; (j) Monocyte; (n) Basophilic polychromatophil; (r) Thrombocyte. Coho salmon (*Oncorhynchus kisutch*): (c) Neutrophil; (g) Lymphocyte; (k) Monocyte; (o) Basophilic polychromatophil; (s) Thrombocyte. Sockeye or red salmon (*Oncorhynchus nerka*): (d) Neutrophil; (h) Lymphocyte; (l) Monocyte; (p) Rubricyte; (t)

in teleosts has been previously correlated to Hgb concentration, oxygen delivery, and, ultimately, aerobic swimming ability.[27] Fish with higher Hgb concentration have smaller RBCs. Additionally, triploid salmonids, or fish with three sets of chromosomes, have both longer and wider RBCs than those found in their

diploid counterparts (fish with two sets of chromosomes).[6,14] This difference is significant as induction of triploidy in farmed fish has been pursued as a method of sterilization, minimizing the impact of escaped farmed fish on wild populations.[5] Triploidy can be induced by duplicating the maternal genome using both heat and pressure treatments, among other techniques.[5]

LEUKOCYTES

The three types of WBCs documented in salmonids are neutrophils, lymphocytes, and monocytes[48] (Figure 129.1). Lymphocytes predominate with fewer but roughly equal numbers of neutrophils and monocytes.[48] Lymphocytes are round with a small amount of basophilic cytoplasm. Monocytes are larger with pale basophilic to gray, sometimes vacuolated, cytoplasm and a round to indented nucleus. Neutrophils are moderate in size with pale gray cytoplasm and a nucleus that varies in shape, but are typically segmented in apparently healthy salmon.

Neutrophils exhibit diffuse, weak to strong positive staining with periodic acid–Schiff (PAS), peroxidase, and Sudan black B stain with increased granular staining associated with cellular maturity.[7,48] Lymphocytic cells do not stain with Sudan black B or peroxidase, though some lymphocytes contain a few PAS-positive cytoplasmic granules, likely consistent with antibody.[7,48] Neither thrombocytes nor RBCs stain with Sudan black B, peroxidase, or PAS.[7,48]

Lymphocytes, the most common leukocyte in salmonid peripheral blood, are important for both innate and humoral immunity. B lymphocytes secrete antigen-specific antibodies with three heavy-chain immunoglobulin classes, IgM, IgT, and IgD, represented.[21] T lymphocytes may express either αβ- and γδ-T-cell receptors, and subpopulations include cytotoxic (CD8), helper (CD4), and regulatory (Treg, Th17).[40]

THROMBOCYTES

Thrombocytes are small, usually oval to spindloid cells with a small amount of colorless to pale basophilic cytoplasm and a small condensed nucleus (Figure 129.1o–t). Thrombocytes may be confused with lymphocytes, though thrombocytes have more condensed chromatin and lighter cytoplasm, and usually have a more oval shape. Thrombocytes can be enumerated on the hemocytometer and are easily differentiated from RBCs, as they are smaller and have a higher nucleus to cytoplasm ratio. Unfortunately, thrombocytes tend to clump, precluding an accurate estimate. If the sample is not clumped, the number of thrombocytes in the center primary square is multiplied by 1000 for a 1:100 dilution or by 2000 for a 1:200 dilution, providing a number of thrombocytes per microliter of blood.[2]

REFERENCE INTERVALS AND PARTITIONING FACTORS

Currently, no salmonid reference intervals generated in concordance with the American Society for Veterinary Clinical Pathology (ASVCP) reference interval guidelines exist for clinical use. Significant seasonal variations in RBC size are reported that are likely associated with the spawning season, though temporal associations with RBC size are not consistent across salmonid species.[15,39] For example, when the length of RBCs of rainbow trout (*O. mykiss*) were measured with an ocular micrometer, RBCs were largest in late summer and smallest in early spring.[15] On the other hand, RBCs of Atlantic salmon (*S. salar*) are largest during early summer and smallest during early autumn, based on a calculated mean cell volume (MCV).[39] Hematocrits also exhibit seasonal and inter and intraspecies variability. In general, less active fish have lower hematocrits, while more active fish have higher hematocrits.

HEMATOLOGIC DISEASE

Anemia

The regenerative response to peripheral anemia results in increased proportions and total numbers of polychromatophils and immature RBCs. A pre- or nonregenerative anemia is characterized by a lack of immature RBCs in the face of decreased erythroid mass. Decreased RBC mass has been associated with a variety of bacterial infections, including *Aeromonas* and *Streptococcus* species,[3] *Vibrio anguillarum*,[46] and *Renibacterium salmoninarum*.[9] *Aeromonas salmonicida* is the causative agent of furunculosis, which may result in septicemia, hemorrhage, and death. *V. anguillarum*, along with other *Vibrio sp.*, causes vibriosis. The virulence of *V. anguillarum* is, at least in part, associated with the production of hemolysins and other membrane-damaging toxins.[35] *R. salmoninarum*, the causative agent of bacterial kidney disease, causes diffuse granulomatous reactions within the reticuloendothelial tissues, resulting in a progressive infection that may ultimately result in death.[9]

Viral causes of anemia include infectious salmon anemia virus,[16] viral hemorrhagic septicemia,[37] and erythrocytic inclusion body syndrome.[44] Infectious salmon anemia virus, caused by an orthomyxovirus, is of economic importance in the farming of Atlantic salmon (*S. salar*) and causes severe anemia, leukopenia, and ascites.[16] In viral hemorrhagic septicemia, caused by a novirhabdovirus, endothelial cells are targeted by the virus, resulting in hemorrhage.[25] Erythrocytic inclusion body syndrome, caused by piscine orthoreovirus-2 (PRV-2), results in severe anemia in affected salmonids.[44] Both PRV-1 and PRV-2 have been shown to infect and replicate within salmonid RBCs, with resultant cytoplasmic viral inclusions, though only PRV-2 causes

anemia, while PRV-1 infection results in heart and skeletal muscle inflammation.[44]

Proliferative kidney disease, caused by the myxozoan *Tetracapsula bryosalmonae*, has been reported to significantly decrease RBC mass.[1,24] Vitamin-E deficiency may also result in anemia.[33] A variety of toxicants have been noted to result in anemia, with phenylhydrazine commonly utilized to induce hemolytic anemia in the research setting.[23] Other toxins associated with decreased erythrocyte mass include cadmium,[28] nitrites,[29] and bis(tri-n-butyltin) oxide[11] (see Chapter 130).

Stress

Sampling conditions must be considered, as stress may result in RBC and WBC redistribution. Splenic contraction in response to catecholamine release secondary to exercise or handling can increase PCV by up to 20%.[26] Increased catecholamine concentrations also induce an RBC sodium–hydrogen counter-transporter.[4] As sodium enters the RBC, water follows osmotically and RBCs swell.[4] Acutely stressed salmon with increased circulating cortisol concentrations have decreased circulating WBCs and/or lymphocytes and increased circulating neutrophils.[31,32,36] Antibody production by lymphocytes and reactive oxygen species production by neutrophils are reduced with increased concentration, as well.[38,45] A dorsal aorta cannulation technique for blood technique has been described to limit handling and stress during blood collection, though this method is impractical for use outside the research setting.[41]

Inflammation

Inflammatory responses in salmonids are characterized by a neutrophilia in the first 24 hours followed by an increase in monocyte numbers after several days.[18] Toxic neutrophils have increased cytoplasmic basophilia and vacuolization. Primary granules can be observed with severe toxicity.[43] Toxic neutrophils can be associated with both infectious (e.g., bacterial septicemia, mycotic infections) and noninfectious (e.g., tissue necrosis) causes of inflammation, though bacterial disease typically results in more severe toxic changes.

Hemoparasites

Several hemoparasites may be found in salmonid blood cells or hematopoietic precursors. Developmental stages of *T. bryosalmonae*, the causative agent of proliferative kidney disease, may be seen on peripheral blood evaluation.[24] These trypanosomes are leaf-like in shape with a single nucleus, kinetoplast, and flagellum. A diffuse granulomatous response affects the kidney and may affect other organs, including the spleen. Anemia results due to reduced erythropoietin production by the nephron.[1]

The hemoflagellate, *Cryptobia (Trypanoplasma) salmositica*, may be identified on blood film evaluation. Trypanoplasms are elongate and thin with two flagella and a kinetoplast.[47] Transmission occurs via the leech *Piscicola salmositica* or directly between fish. Clinical signs of cryptobiosis include anemia, anorexia, splenomegaly, ascitic distension (dropsy) and generalized edema.[47] The degree of anemia is directly related to the degree of parasite burden.[47] The cause of anemia is likely multifactorial, including secretion of hemolysins by *C. salmositica* and hematopoietic tissue necrosis.[47]

Developmental stages of the microsporidian, *Nucleospora* (*Enterocytozoon*) *salmonis*, may be seen in the nucleus of hematopoietic cells in the spleen, kidney, and peripheral blood.[12] Spores are oval and colorless to pale, with one nucleus and a coiled polar tube.[12] Affected fish are often anemic with a severe lymphocytosis, with death associated with heavy parasite burdens.[19] Circulating lymphocytes often contain intranuclear stages of the microsporidium.[19]

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CHAPTER 130

Hematology of Ictaluridae

PATRICIA GAUNT

Blood Collection and Sample Preparation
Erythrocytes
Leukocytes
Thrombocytes and Coagulation
Hematopoiesis
Reference Intervals
Hematologic Disease
  Anemia
    Methemoglobinemia
  Leukocyte Changes in Disease

Acronyms and Abbreviations

ASVCP, American Society for Veterinary Clinical Pathology; CCA, channel catfish anemia; MS-222, tricaine methanesulfonate.

The Ictaluridae family is primarily composed of 10 catfish species within the genus *Ictalurus*. The ictalurid catfish originated in the freshwaters of North America and include the commonly known blue, channel, flathead, and white catfish, as well as various species commonly known as bullheads (e.g., brown bullhead [*Ameiurus nebulosus*], black bullhead [*Ameiurus melas*], and yellow bullhead [*Ameiurus natalis*]). Three ictalurid catfish, *Ictalurus punctatus* (channel), *Ictalurus furcatus* (blue), and *I. punctatus*♀ × *I. furcatus*♂ hybrids, are the most commonly cultured catfish for food in the USA. Currently, over 75% of commercially raised catfish in the USA are channel–blue catfish hybrids, representing a sustainable food fish with faster growth rate and increased disease resistance. Catfish is the leading aquaculture industry in the USA. Commercial catfish production generates over 27% of the value of aquaculture production in the USA. From the first commercial production in ponds in the 1960s, catfish production has grown to reach annual sales of roughly 319 million pounds in 2016. Mississippi leads the USA in production with 34,700 acres in 2017.

To manage the health of ictalurid catfish in aquaria, natural, and commercial settings, veterinarians can utilize hematology as a nonlethal diagnostic test. Although a considerable amount of information on ictalurid hematology is available because of their commercial importance, additional information is still needed to define their hematologic responses.

BLOOD COLLECTION AND SAMPLE PREPARATION

To minimize handling, stress, and excitement, catfish are usually anesthetized prior to venipuncture. Anesthesia allows positioning of the fish and an unobstructed approach to a stabilized blood collection site. Tricaine methanesulfonate (MS-222) is the only anesthetic approved for use in fish in the USA. For a quick procedure, MS-222 is administered by placing catfish in a water bath with final concentration of 100–300 mg MS-222 per L water, preferably buffered. While alkaline water may not require buffering, American Veterinary Medical Association guidelines recommend that water with low alkalinity (<50 ppm) be buffered with sodium bicarbonate to a pH between 7.0 and 7.5. Once the catfish begins to enter stage two of anesthesia, with total loss of reaction to external stimuli and a slight decrease in opercular rate, it is removed from the water bath and immediately sampled. This anesthetic level is sufficient

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in duration for an experienced phlebotomist to complete the venipuncture, with rapid return to the water environment and quick recovery from anesthesia. Anesthetic agents used in other countries for venipuncture include eugenol, benzocaine, and metomidate. All anesthetics should be used according to label directions in accordance with the country's regulations.

The most common venipuncture site for catfish is the caudal vein, which is approached laterally just ventral to the lateral line, or ventrally via the ventral tail region (Figure 130.1). An alternate site for venipuncture is the dorsal aorta, accessible via the buccal cavity. However, this blood collection site requires heavier sedation and a highly skilled phlebotomist. Cardiocentesis is another option for obtaining a blood sample, especially larger volumes, but there is a greater risk of fatal injury. The maximal, non-lethal sample volume is approximately 1% of body weight. Small blood volumes are collected using a small gauge needle (e.g., 21–23 g) attached to a 1–3 cc syringe. Small fingerling catfish may require a 25g needle. Collection using vacuum collection tube systems (e.g., Becton Dickinson Microtainers, Franklin Lakes, NJ, USA) can be used for larger volumes. Blood should be placed in an appropriately sized tube, to avoid an excessive K-EDTA to blood ratio, as soon as possible, thereby decreasing thrombocyte and coagulation factor activation. EDTA has not been associated with hemolysis in catfish, so heparin and other anticoagulants are typically avoided for hematologic analysis.

Blood smears should be prepared immediately with blood without anticoagulant, or as soon as possible with blood collected in EDTA. Blood prepared for hemocytometer blood cell counting methods, e.g., Natt–Herricks stain, should be counted as soon as possible. Blood can be preserved for later counting by transferring 100 µL whole blood to a tube containing 400 µL 10% neutral buffered formalin (1:5 dilution) and mixed well[3] (see Chapter 128). Blood cells are reportedly stable for counting with Natt–Herricks solution for up to 1 month following formalin fixation.[3] As a general reminder, formalin solutions and vapors should be kept away from unstained blood smears (during their preparation, storage, and shipping), as any fixation adversely affects the Romanowsky staining of blood cells.



FIGURE 130.1 Lateral approach to venipuncture site for caudal vein in a stocker-sized hybrid catfish (*Ictalurus punctatus* ♀ × *Ictalurus furcatus* ♂).

ERYTHROCYTES

Mature RBCs (Figure 130.2k) are the most numerous blood cell and are oval to elliptical with eosinophilic cytoplasm using Romanowsky stains.[12,19] The nucleus is generally centrally located, round to oval in shape, and has condensed chromatin. Erythrocytes vary from 10–12 µm on their long axis. Immature RBCs, polychromatophils, appear rounder than mature cells, the cytoplasm stains more basophilic, and the nuclear chromatin is less condensed (Figure 130.2i,j). There are currently no standardized methods for evaluating the erythropoietic response in blood (e.g., reticulocyte staining, grading degree of polychromasia, rubricyte counts) of ictalurid catfish.

LEUKOCYTES

Neutrophils and lymphocytes are the predominant leukocytes in healthy catfish blood, followed by monocytes with rare basophils.[12,18] Eosinophils have not been definitively identified in healthy catfish. Functionally, catfish WBCs are analogous to mammalian WBCs. Channel catfish neutrophils are capable of bacterial phagocytosis and oxidative metabolite release. Monocytes exhibit in vitro phagocytosis of beads, but do not adhere well to plastic or glass surfaces. T and B lymphocytes have been identified.[7]

On Romanowsky-stained blood smears, neutrophils are round to oval and are approximately 11 µm in diameter.[18] The nucleus is eccentrically located and typically has minimal segmentation, so nuclear shapes range from oval to banded or irregular (Figure 130.2a–c). While catfish neutrophils may resemble mammalian bands and myelocytes because of their hyposegmentation, there is no current evidence that these represent abnormally immature cells. Low numbers of band neutrophils and rare myelocytes (identified using mammalian categorization) may be identified in blood smears of apparently healthy fish and do not necessarily indicate a left shift (Figure 130.2b,c). The moderate amount of cytoplasm on Romanowsky stains appears colorless to lightly basophilic, with low numbers of fine to coarse clear to lightly eosinophilic granules. Ultrastructurally, neutrophil cytoplasm contains many dense granules and small amounts of rough endoplasmic reticulum (ER).[6] On cytochemical staining, catfish neutrophils are strongly positive for peroxidase, periodic acid–Schiff (PAS), and Sudan black B.[7,18,21] Though pale staining granules may be found, heterophils have not been definitively identified in ictalurid catfish.

Lymphocytes (Figure 130.2d) are variable in size (small to large), with diameters that range from 5–9 µm.[7,18] The nucleus is more centrally located with variably condensed chromatin. The sparse to moderate cytoplasm typically stains lightly basophilic, with cytoplasmic blebs sometimes observed. Ultrastructurally, catfish lymphocytes have few cytoplasmic organelles as distinguishing features.[6] Lymphocytes from catfish do not stain with routinely used cytochemical stains.[18]

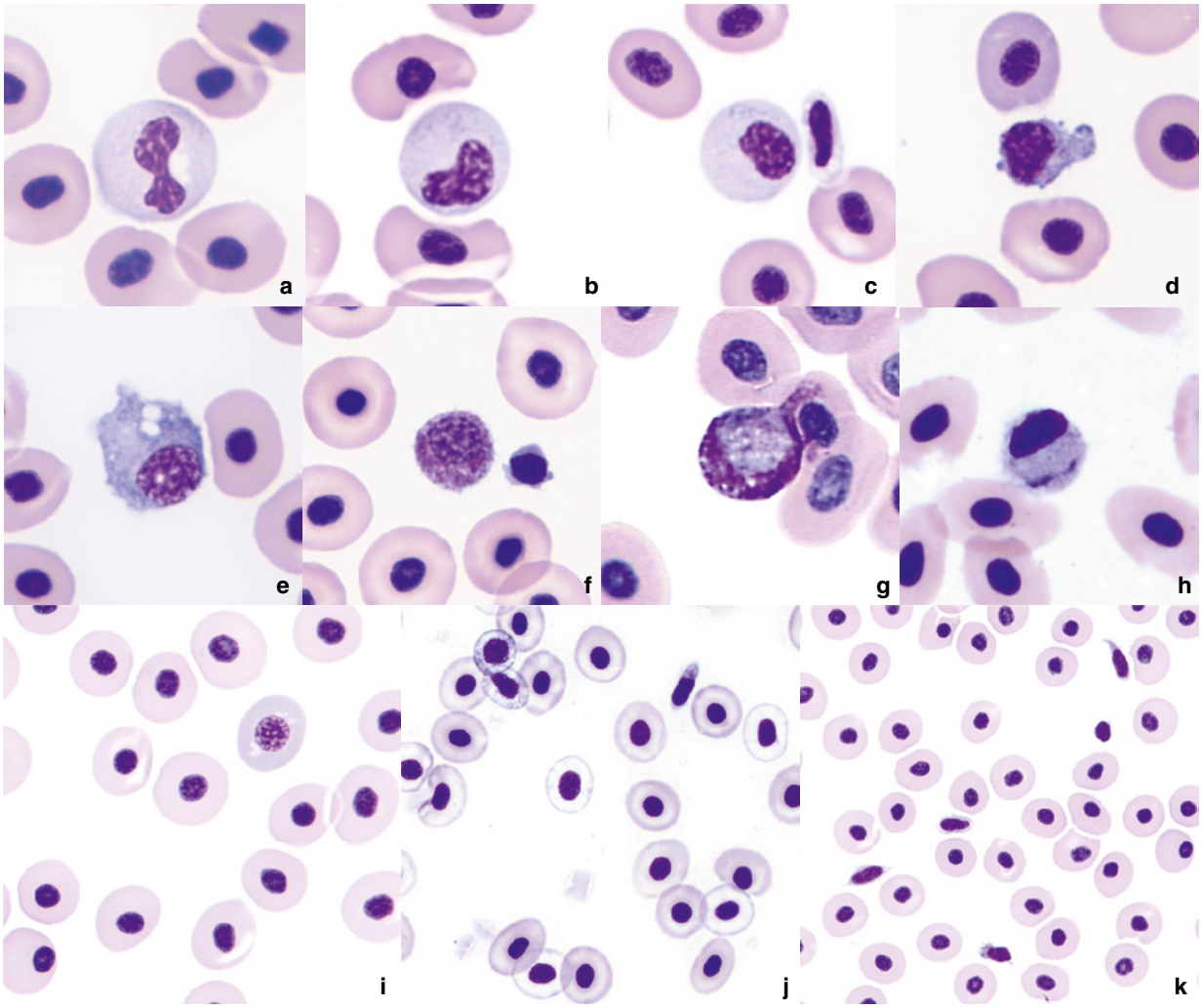


FIGURE 130.2 Blood cells of channel catfish (*Ictalurus punctatus*). Fish were assessed as healthy unless otherwise noted. (a) Neutrophil with irregularly shaped, bilobed nucleus; (b) Neutrophil with band-shaped nucleus; (c) Neutrophil with oval nucleus (possible metamyelocyte); (d) Small lymphocyte with cytoplasmic blebs; (e) Monocyte with cytoplasmic basophilia and clear vacuoles; (f) Basophil with round nucleus and cytoplasmic purple granules; (g) Basophil, Modified Wright-Giemsa stain (**Source:** Millipore Sigma, St. Louis, MO, USA); (h) Neutrophil with prominent linear basophilic inclusions in cytoplasm (possible Döhle bodies) and increased cytoplasmic basophilia from a catfish with acute *Edwardsiella ictaluri* infection; (i) Mature RBCs and a polychromatophil from a healthy fish; (j) Erythrocytes from an anemic catfish affected with channel catfish anemia (CCA) exhibiting hypochromasia; (k) Thrombocytes with oval to fusiform nuclei. Blood smears stained with Wright-Giemsa stain. All images are originally 1000× magnification except g which is 500× magnification. (**Source:** Images courtesy of Dr. Shannon Dehghanpir, Louisiana State University; (g) courtesy of Dr. Kendal Harr.)

Monocytes (Figure 130.2e) are oval with a diameter of approximately 12 µm, similar in size or larger than an RBC, and variably shaped nuclei that range from oval to indented to lobulated.[18] The nuclear chromatin has incomplete condensation. The cytoplasm is moderate in amount and stains basophilic, with few to absent clear vacuoles. Ultrastructurally, the cytoplasm contains ER, vacuoles, and granules.[6] Ictalurid monocytes are strongly positive for nonspecific esterase and reported as negative to weakly positive for peroxidase.[18,21]

Basophils (Figure 130.2f,g) occur in very low numbers in the peripheral blood and are relatively small leukocytes, with a diameter of 7 µm.[18] The nucleus is round in shape and centrally located, although the nuclear features are typically obscured by the granulated cytoplasm on Romanowsky stains. The cytoplasm is moderate in amount and contains many small purple-staining granules that vary in size and shape. In addition, the color of basophil granules may vary with aqueous versus alcohol-based Romanowsky stains (Figure 130.2g) (see Chapter 117). The cytoplasm of ictalurid basophils exhibits metachromatic staining reaction with toluidine blue, with conflicting reports of positive staining with PAS.[18,21]

THROMBOCYTES AND COAGULATION

Thrombocytes (Figure 130.2k) range in length from 9–11 μm and vary in shape from oval to elongate to fusiform.[18] The nucleus is oval to fusiform in shape with condensed chromatin, while the cytoplasm stains colorless with occasional clear vacuoles/granules. As in other vertebrates with thrombocytes, differentiating oval thrombocytes from small lymphocytes on Romanowsky smears can be challenging. One report indicated that catfish thrombocytes are less fusiform and more rounded when blood smears were prepared 2 hours or more after blood collection.[7] Ultrastructurally, the cytoplasm contains a canalicular system, glycogen granules, and vacuoles (some with phagocytosed debris).[18] Catfish thrombocytes are weakly positive for peroxidase, PAS, and nonspecific esterase.

There are no published studies that detail the physiology and diagnostic testing of the coagulation system in ictalurid catfishes. One preliminary study with citrated plasma from bullhead catfish indicated the presence of fibrinogen and rapid coagulation of plasma following addition of thrombin from other animals.[14] The prothrombin time (PT) and partial thromboplastin time (PTT) were dependent on the species origin (e.g., rabbit, dog, human, catfish) of thromboplastin used as reagent. The shortest times for PT and PTT in citrated catfish plasma were obtained using thromboplastin prepared from catfish brain. Catfish thromboplastin is not a standardized reagent, which limits studies of routine coagulation tests in catfish. Thromboelastographic studies of blood coagulation have not been reported in ictalurid catfish at the time of publication.

HEMATOPOIESIS

The primary sites for erythropoiesis, granulopoiesis, and thrombopoiesis in ictalurids are the head and trunk kidneys. The head kidney consists of bilaterally fused lobes that are anterior to the swim bladder.[12] It is composed primarily of hematopoietic, lymphoid, and inter-renal tissues. The inter-renal cells have an endocrine function similar to the adrenal glands of mammals.[9] Renal tubules and glomeruli initially form in the head kidney of ictalurids, but undergo degeneration during the fingerling stage and are absent in adult ictalurids. The fused portion of trunk kidney is posterior to the swim bladder and the bilateral lobes of trunk kidney extend cranially along the swim bladder. The corpuscles of Stannius are located on the lateral margins of the trunk kidney in ictalurids and are endocrine tissue. The trunk kidney is composed mostly of nephrons with glomeruli and tubules, and fewer hematopoietic and lymphoid aggregates. There is uncertainty as to whether the larger trunk kidney is equivalent in hematopoietic mass to the head kidney, and whether these two sites are similar in their ability to support basal and increased erythropoiesis, granulopoiesis, and thrombopoiesis. The spleen is primarily a lymphoid organ in ictalurids, but may contain low numbers of hematopoietic cells.[10]

The erythroid and granulocytic precursors in catfish are easily identified on Romanowsky-stained impression smears of the head and trunk kidney.[10] Some studies have applied a nomenclature system to the individual stages of these precursors, but this has not been formalized. The thrombopoietic precursors in catfish are not well described or identified on routinely stained smears, especially given their similarity to lymphoid cells. Hemosiderin granules are also present on smears of the head and trunk kidneys of ictalurids.

REFERENCE INTERVALS

While many reports of hematology reference values for ictalurid catfish are published, all are plagued by small sample sizes, some by misclassification of leukocyte types, inclusion of thrombocytes as a leukocyte subtype, and other issues. Seasonal variance of the circulating erythrocyte mass is a complicating factor in establishing reference intervals for pond-reared catfish. A study of channel catfish revealed a lower mean PCV (15%) in the winter compared to that (26%) in the summer months, reflecting the increased erythropoiesis and oxygen demand associated with a higher metabolic rate at warmer temperatures.[15] The two most recent reports on hematology reference values sampled 40 channel catfish, but utilized percentiles and statistics that are not in accordance with the published American Society for Veterinary Clinical Pathology (ASVCP) guidelines.[11,18,19] Therefore, there currently are no acceptable reference intervals for hematology values of ictalurid catfish.

HEMATOLOGIC DISEASE

Anemia

Channel catfish anemia (CCA) is a syndrome of severe anemia, including PCVs as low as 1%, in pond-raised channel catfish. Typically, the catfish with the most severe anemia are identified in ponds with lethargic and anorexic fish that exhibit marked pallor of lips, gills, and viscera. Channel catfish anemia is characterized by hypochromic erythrocytes (Figure 130.2j), hypoferremia, decreased hepatic iron concentrations, decreased transferrin saturation, absence of siderophages in the head kidney, and decreased hepatic expression of hepcidin.[5,13] Administration of parenteral iron to catfish with severe anemia attributed to CCA resulted in dramatic recovery from the anemia and abnormal iron status. All of these findings are consistent with iron deficiency, but the actual cause of altered iron metabolism in CCA remains unknown. Previously hypothesized causes of CCA included use of feeds with nutritional deficits, oxidized ingredients, toxins (e.g., mycotoxins, gossypol

from cottonseed meal), or iron chelation in pond water, but none of these have been definitively substantiated. Rare cases of severe anemia in channel catfish were attributed to commercial catfish feed.[16] Experimentally, severe anemia could be induced in healthy catfish by feeding the incriminated batch of feed, and the anemia resolved after feeding unaffected feed. No toxins, including heavy metals, mycotoxins, peroxides, pesticides, were identified in this feed, and there are no recent reports of feed-related anemia in catfish.

Anemia of inflammatory disease is likely common in fish, and has been documented in channel catfish with *Edwardsiella ictaluri* infection. This bacterial infection, known as enteric septicemia of catfish, initially causes intense neutrophilic and mononuclear cell infiltrates in the intestines.[9] In experimental *E. ictaluri* infections, PCV and RBC concentration are decreased by day 8, although the anemia was not characterized as nonregenerative.[2,16,17] Another study of *E. ictaluri* infection demonstrated a marked upregulation of hepcidin in hepatocytes within hours, and later in other tissues (e.g., intestine, olfactory sac).[13] Catfish hepcidin is considered to have the same role in iron regulation as in mammals, with iron sequestration likely contributing to the anemia. A similar pattern of hepcidin upregulation and hepcidin-induced iron sequestration likely occurs in the many other infectious and noninfectious inflammatory diseases reported in ictalurid catfish.

Hemorrhage is not commonly reported in ictalurid catfish and is not well-documented clinically with hematologic data. In a model of acute hemorrhage using bled catfish, increased numbers of polychromatophils and earlier erythroid precursors were present after 1 week in both the blood and head kidney, but less so on smears of trunk kidney and spleen.[10] Blood loss could occur in catfish from trauma (e.g., biting injury from other catfish, spearing injury from predatory birds) or even nosocomially from excessive venipuncture at research facilities. Moderate anemia occurs in channel catfish with proliferative gill disease caused by *Henneguya ictaluri*. This anemia was attributed to marked hemorrhage from damage to the vascular gill filaments, but was not characterized as regenerative.[4] However, the roles of other factors, such as iron sequestration due to inflammatory disease, were not ruled out as primary or contributing causes of this anemia.

Methemoglobinemia

Nitrite toxicosis is a major water quality concern of catfish farmers, since increased nitrite concentrations can occur in ponds, especially during the fall and spring. Nitrites from the fishes' nitrogenous wastes and protein breakdown products from uneaten feed, dead fish, and decaying algae accumulate in the ponds. Nitrites oxidize hemoglobin to methemoglobin with conversion of up to 90% of total hemoglobin in experimental fish, resulting in severe cyanosis and increased mortality rates.[5,19] In healthy catfish, methemoglobin concentrations are typically 5% or less of total hemoglobin. A moderate to severe anemia may also occur and is attributed to shortened RBC life span from oxidant-induced membrane damage and increased metabolic stress when pathways such as cytochrome b_5 reductase (NADPH methemoglobin reductase) are used to counter oxidants in the RBCs.[20] Anemia may exacerbate the hypoxic effects of methemoglobinemia. Diagnosis of nitrite toxicosis in catfish is based on clinical detection of methemoglobinemia by presence of chocolate-brown discoloration of the lips, gills, and viscera. On water quality testing, increased nitrite and decreased chloride concentrations with resultant increase in the nitrite:chloride ratio are confirmatory. The nitrite:chloride ratio in pond water should be 1:10 or less. Chloride plays a key role in preventing nitrite toxicosis, since chloride competes with nitrite for absorption from water by the gills, and thereby inhibits nitrite uptake. Chloride supplementation to lower the nitrite:chloride ratio of pond water is used as both a treatment and preventative of nitrite toxicosis, typically by adding sodium chloride (common salt) to the pond water.

Leukocyte Changes in Disease

Leukocyte changes attributed to stress have been documented in ictalurid catfish following presumed stressful circumstances (e.g., long-distance transportation from ponds to lab) and administration of cortisol.[7,8] These studies relied on relative neutrophilia and lymphopenia, based on changes in percentage differential counts from blood smears. Using flow cytometry, lymphopenia in cortisol-dosed catfish was attributed to decreased B-lymphocyte numbers.[1]

The primary inflammatory leukocyte changes identified in ictalurid catfish include leukocytosis, neutrophilia, monocytosis, and lymphopenia. These changes were documented in catfish with bacterial infections such as *Flavobacterium columnare*, *E. ictaluri*, and parasitic infections such as *Ichthyophthirius multifiliis*. Absolute neutrophilia, monocytosis, and lymphopenia have been documented.[7,17] It is difficult to assess the presence of immature neutrophils in blood due to hyposegmentation in blood smears from normal catfish. No standard exists for the morphologic determination of a "left shift" in catfish. However, the author has observed increased amounts of basophilic cytoplasmic inclusions in neutrophils from catfish with acute *E. ictaluri* infection (Figure 130.2h). These basophilic inclusions are more numerous and often linear, compared to the fine to coarse cytoplasmic granules observed in neutrophils of healthy catfish, and could represent Döhle bodies. The cytoplasm is also more diffusely basophilic, representative of another toxic change. There are no ultrastructural studies to confirm that these inclusions represent retained rough ER and increased free ribosomes. Other evidence of toxic changes such as vacuolization have not been reported in ictalurid catfish.

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CHAPTER 131

Hematology of Cyprinidae

ILZE K. BERZINS and ALEXANDER E. PRIMUS

Restraint, Blood Collection, and Storage
Laboratory Analysis
Erythron
Leukon
Thrombocytes and Hemostasis
Cytochemical and Ultrastructural Features
Hematopoiesis
Reference Intervals
Partitioning Factors
Hematologic Disease
Anemia
Physiologic Changes
Inflammatory Disease

Acronyms and Abbreviations

ER, endoplasmic reticulum; FDA, Food and Drug Administration; HSC, hematopoietic stem cells; N:C, nuclear to cytoplasmic ratio; PAS, periodic acid–Schiff; SVC, spring viremia of carp; TMS and MS-222, tricaine methanesulfonate; USP, unit of potency.

Cyprinidae is the largest family of predominantly freshwater teleost fishes with over 350 genera and 2500 species (http://www.catalogueoflife.org), including such groups as carp, goldfish, zebrafish and related danios, true minnows, chubs, barbs, shiners, rasborines, and others. It is a morphologically diverse family with members ranging in size from a few centimeters to over 3 meters. They lack jaw teeth, but they have pharyngeal teeth, one to three rows with up to eight teeth per row, which are used for species identification. Their lips are thin and lack folds, ridges, or papillae. The mouth can be sucker-like which may be surrounded by sensory barbels, and often have a protrusible upper jaw. Fin rays are soft except in a few genera where they are fused and spine-like in the dorsal fin. They lack a true stomach and are physostomes with the gas bladder connected to the alimentary canal by a pneumatic duct. This enables the animal to gulp air to fill the gas bladder. Gills are the main respiratory organ in cyprinids, but also play a role in excretion of nitrogenous wastes, ion regulation, and osmotic balance. Cutaneous gas exchange is limited in this group. Hemoglobin is the oxygen-carrying protein.

Cyprinidae are native to North America, Africa, and Eurasia. They primarily eat invertebrates and vegetation, and are egg-layers, but most species do not guard the nest. They are important food fish, recreational angling sport fish, bait fish, and are popular in the aquarium hobby as well as used in research settings. Some, such as the Asian carp (several species collectively called Asian carp), have recently become invasive species in North America, whereas the zebrafish (*Danio rerio*) is an important model species for scientific research.[5,13]

RESTRAINT, BLOOD COLLECTION, AND STORAGE

Fish can be physically restrained without using anesthetics, but this can result in excessive stress which may affect blood values. Handling for as little as 20 seconds can increase PCV up to 25%.[4] If not using a general anesthetic, try to collect blood in less than 30 seconds. When possible, try to collect without completely removing the

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fish from water, keeping head and gills submerged by using a sling. In the field, active collection methods involve moving sampling gear to collect fish including electrofishing, stunning, trawls, cast nets, and angling. Passive techniques are stationary and allow fish to move into them including gill nets, traps, and bait lines. All of these may induce a stress response.

Anesthetic agents can also result in physiologic changes, but, when used properly, can help lessen stress. Tricaine methanesulfonate (TMS, MS-222) is the most common and is currently the only United States Food and Drug Administration (FDA) fully approved anesthetic for finfish. Tricaine will acidify the water, which can damage gills, but adding sodium bicarbonate (baking soda) to buffer will reduce or eliminate adverse effects.[15] Specifically, a weight ratio of 1:1 or 2:1 bicarbonate to tricaine is often used. MS-222 concentration of 100–150 mg/L at an intermediate temperature range of 15–23 °C (41–73.4 °F) is recommended for most procedures in cyprinids[11] and typically achieves rapid induction. Ideally, stage 2 anesthesia (loss of equilibrium) is achieved within 20 seconds.[14] Lower concentrations are not recommended, as it takes longer to sedate the animal, which could lead to increased stress. It is important to note that induction time is inversely related to temperature. Other immobilization methods using chemical (quinaldine, benzocaine, metomidate, clove oil and its derivatives) and nonchemical (electrical currents, lower temperatures) options are reviewed in Ross and Ross[17] and may not be recommended. For prolonged procedures, keeping the gills perfused (water running over the gills) with well-aerated water using a pump or moving the fish forward (ram ventilation) is recommended. Fish can then be recovered in a container of fresh water, again well oxygenated with good branchial perfusion.

In larger fish (>8 cm), blood is usually collected using a ventral or lateral approach to the caudal vertebral vessels located ventral to the peduncular vertebrae. The artery and vein are in close proximity and either or both may be sampled. On a ventral approach, a 22–23 g needle attached to a 1–6 cc syringe is inserted (under the scale if necessary) along the ventral midline of the caudal peduncle posterior to the anal fin either at a slightly oblique anterodorsal angle or perpendicular angle, and directed toward the vertebral bodies. Once contacting the vertebrae, the needle is withdrawn slightly, and the plunger is gently drawn back. Vigorous aspiration should be avoided, as this may result in vessel collapse or hemolysis of fragile blood cells. On a lateral approach, the needle is inserted a few millimeters below the lateral line near the base of the caudal peduncle and directed toward the midline and under the vertebral bodies (see Chapter 130). The lymphatic system in fish is extensive and lymphatic fluid dilution will affect results, complicating diagnostic use. Blood can also be collected from the two-chambered heart or bulbus arteriosus of teleosts, but there is a greater risk of damage to the fish. Other collection sites include the Cuvierian duct (a vessel within the posterior oral cavity), dorsal aorta, and branchial vessels.

In smaller fish (<8 cm), collection of small blood volumes from the bulbus arteriosus of anesthetized fish with a small, 23 gauge needle attached to a 1 cc syringe or capillary tube after exposure of the body cavity is possible. Another suggested blood collection method in anesthetized fish is to sever the tail with a scalpel blade and apply a heparinized capillary tube to the caudal vessels.[12,15] However, this method not only results in death, it likely results in tissue fluid contamination and cell lysis. Diagnostic value of potential results should be assessed prior to use of this technique. For these methods, the fish should be immediately euthanized once blood has been collected.

Total estimated blood volume in teleosts is 5% of body weight (kg), which is less than the 10% estimate in many mammals. However, up to 30% of total blood volume from healthy fish could potentially be collected for a nonlethal sample due to differences in fish hemodynamics.[12] Lithium heparin and EDTA are both used as anticoagulants in fish. Fish blood tends to clot rapidly, so it may be necessary to coat the needle and syringe with an anticoagulant prior to collection. Heparin is more commonly used and appears to be effective at 50–100 USP units/mL or 0.3–0.75 mg/mL in most cases.[12,15] Cyprinid blood cells retain morphology with heparin, though some heparin artifact may be present.[14] Mixing rapidly is important and blood clots may form, as heparin does not prevent clotting initiated by the alternate coagulation pathway, which can result from vessel/tissue trauma during blood collection.[12] EDTA at 4–5 mg/mL final concentration is sometimes used as an alternative, as it more reliably prevents clotting, but it is not recommended when using MS222 because the combination of these compounds often causes hemolysis.[5]

Samples should be processed rapidly, as clinically significant changes can occur in whole blood after 1 hour at room temperature or 1–3 hours when refrigerated. Storing whole blood can result in artifactual results. Hematologic counts should be completed within hours of collection. A recently described method which has been evaluated for elasmobranch and several teleost species uses formalin fixation with blood evaluation days to weeks later[1] (see Chapter 128). This method remains to be validated for other species.

LABORATORY ANALYSIS

Nucleated RBCs complicate automated hematologic analysis and while newer techniques are starting to address this issue, they are not readily available.[8] Total RBC and WBC counts are performed similarly to bird and sharks (see Chapters 123 and 128). Packed cell volume is determined using a microhematocrit tube and manual centrifugation. In a study specifically designed to establish reference intervals for koi (*Cyprinus carpio*), samples were spun at 19,000 g for 5 minutes, though >11,000 g is likely satisfactory (Table 131.1).[19]

Hemoglobin (Hgb) concentration is measured using the cyanmethemoglobin method. RBC indices, including

TABLE 131.1 Minimum and maximum hematologic values for 19, 4–5 year, mixed-sex, healthy koi (*Cyprinus carpio*) held at 20–22°C at the University of Minnesota with outliers removed according to ASVCP guidelines.

Measurand	Units	Mean	SD	Median	Min.	Max.	Dist.
PCV	%	44	6.6	46	32	56	G
Hemoglobin	g/dL	11.4	1.9	11	8.7	14.5	G
TS/TP[a]	g/dL	1.032	0.003	1.032	1.026	1.039	G
WBC	$10^3/\mu L$	6.32	1.4	6.3	4.1	9.1	G
Heterophil-segmented	$10^3/\mu L$	0.8	0.43	0.71	0.26	1.9	NG
Heterophil-nonsegmented	$10^3/\mu L$	0.11	0.11	0.09	0	0.41	NG
Neutrophil	$10^3/\mu L$	0.01	0.02	0	0	0.08	NG
Lymphocyte	$10^3/\mu L$	5	1.2	5	3.5	7.9	G
Monocyte	$10^3/\mu L$	0.41	0.27	0.39	0.14	1.2	NG
Eosinophil	$10^3/\mu L$	0	0	0	0	0	NA
Basophil	$10^3/\mu L$	0	0	0	0	0	NA

[a] $n = 18$ due to removal of one outlier due to sample quality/data entry.G, Gaussian; NG, non-Gaussian; NA, not applicable; TS/TP, total solids/protein; WBC estimate performed, Hgb determined using a HemoCue Hb 201+ analyzer without centrifugation. MedCalc statistical software version 19.4.1 (MedCalc Software Ltd., Ostend, Belgium; https://www.medcalc.org; 2020).

mean cell volume (MCV) and mean cell hemoglobin concentration (MCHC), are calculated from the total RBC count, PCV, and Hgb concentrations using standard formulas. Blood smears can be stained with either Wright's or Diff-Quik™ stains for differential counts and blood cell morphology evaluation. WBC morphologic and cytoplasmic characteristics, particularly the granulocytes, are variable between fish species. Cytochemical staining using commercial kits for human WBCs and transmission electron microscopy to evaluate cellular ultrastructure can be used to delineate appropriate identification,[20] but standardization is still far from complete.

ERTYHRON

Cyprinid RBCs are oval, with a centrally located, irregularly oval basophilic nucleus with densely clumped chromatin and pale eosinophilic cytoplasm using Romanowsky stains. Koi RBCs measure 10–12 µm by 3–4 µm[19] and those in zebrafish are smaller, approximately 7 by 10 µm[13] Reticulocytes are often smaller in size, with larger, rounded nuclei, a higher nuclear to cytoplasmic (N:C) ratio, and more basophilic cytoplasm (Figure 131.1a). Erythropoiesis can occur in the peripheral blood, so immature forms can be present and can contribute to a mild to moderate degree of anisocytosis and polychromasia (Figure 131.1b,t). Maturation of RBCs is evidenced by increased cell size and Hgb content, and mature cells have higher MCV and MCHC values. The erythron may be affected by stress and environmental conditions.

LEUKON

The most abundant granulocytes have been called both heterophils and neutrophils in the literature.[19] Heterophils are defined as granulocytes with variably staining granules, while neutrophils have granules that appear white, clear, or are inapparent on Romanowsky stains. Cytoplasm is abundant and is often colorless to light blue or gray, and filled with abundant numbers of fine cytoplasmic granules. While granule color may vary with aqueous versus alcohol-based stains, apparently healthy cyprinids may have both cell types on the same slide (Figure 131.1k–o). These cell types are counted separately, though the clinical significance, especially of the neutrophil, is unknown. Cytochemical stains and ultrastructural analysis are warranted to determine underlying functional differences and appropriate classification. Polymorphonuclear cells typically measure 10–12 µm in diameter in koi[19] and 9–10 µm in zebrafish,[13] and are the predominant granulocyte involved in inflammation. They are generally round and contain eccentric nonsegmented to bilobed nuclei with moderately condensed chromatin. The nuclei have less lobation than in mammals and these should not be mistaken for band or immature cells, as this has not been confirmed and may vary by species. Heterophils with round nuclei are presumed to be more immature, consistent with mammals; but this has not been confirmed.

Lymphocytes, typically the predominant leukocyte, function in both humoral and cell-mediated immunity in cyprinids[13] (see Chapter 129). They are usually small, round cells with a rim of light to medium blue cytoplasm, but may be elongated (Figure 131.1e,f). They frequently have pseudopods or blebs along the cell margin (Figure 131.1g). The nucleus is typically rounded with deeply basophilic condensed chromatin, but may occasionally be oval, elongate, or pleomorphic. Lymphocytes have the highest N:C ratio of all leukocytes. As they become reactive, they increase in size and the nucleus may contain large amounts of clumped chromatin (Figure 131.1h). Koi lymphocytes can be classified as small (<8 µm), medium (8–10 µm), or large (>10 µm) based on their diameter.[19] In zebrafish, they average 5–8 µm in diameter.[13]

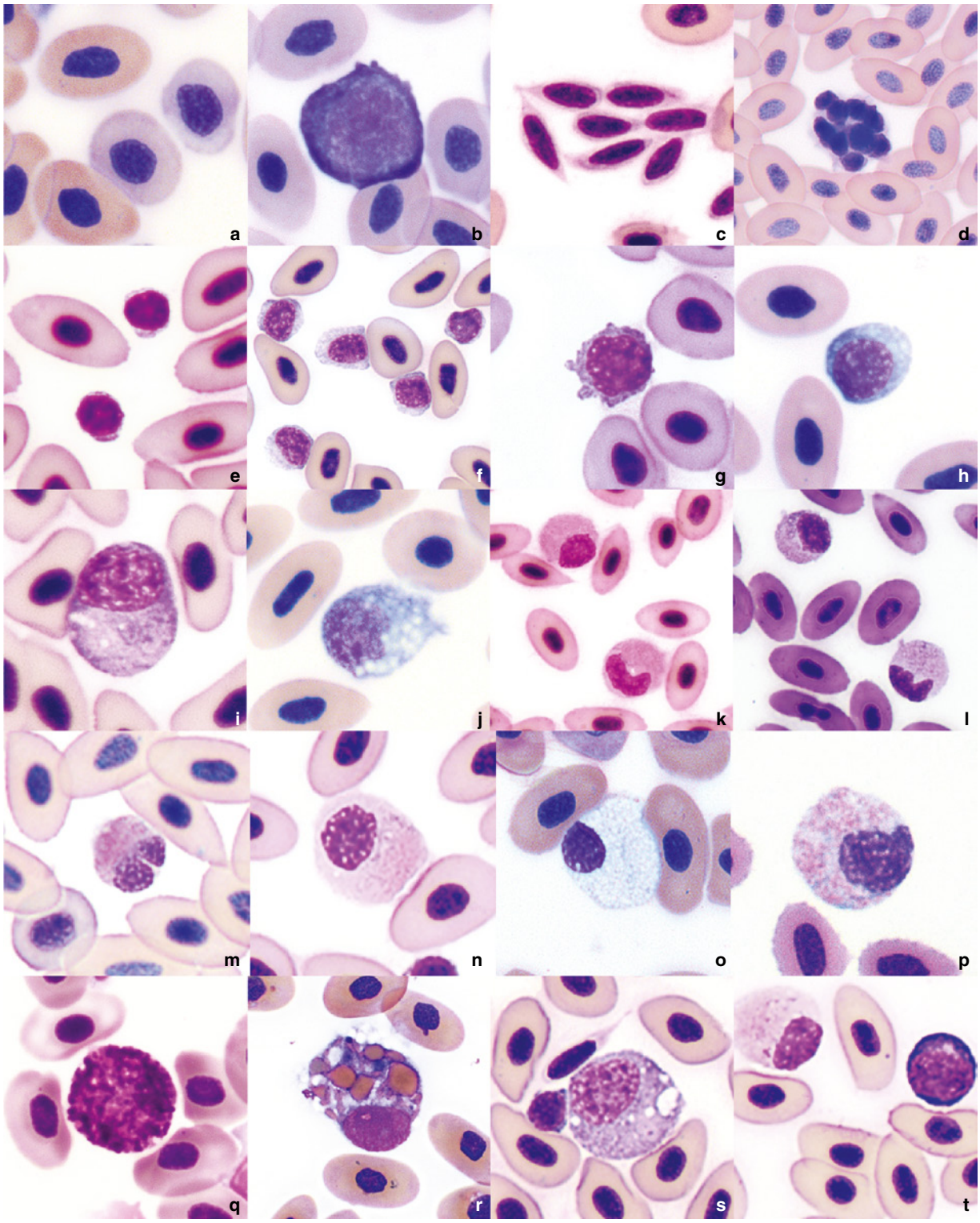


FIGURE 131.1 Magnification under oil, 1000×. Wright–Giemsa stain unless indicated otherwise. All photos, except (p), (q), and (r), are from koi (*Cyprinus carpio*); (q) and (r) are from goldfish (*Carassius auratus*); and (p) is from a mummichog (*Fundulus heteroclitus*). (a) Mature RBCs and reticulocytes showing polychromasia; (b) Erythroid precursor (presumptive prorubricyte); (c) Thrombocytes, spindloid; (d) Reactive thrombocytes forming a clot; (e) Small lymphocytes; (f) Mildly reactive lymphocytes; (g) Pseudopodia on a lymphocyte; (h) Plasmacytoid lymphocyte; (i) Monocyte; (j) Monocyte; (k) Heterophil (likely mature and immature); (l) Heterophils with Diff-Quik™ stain; (m) Mature heterophil; (n) Immature heterophil; (o) Nonstaining granulocyte/neutrophil; (p) Eosinophil; (q) Basophil; (r) Erythrophagia in a fish with buoyancy problems and previous surgery; (s) Monocyte, lymphocyte, thrombocyte; (t) Immature heterophil and erythroid precursor (rubricyte). (**Source:** Photo credits: Dr. Nicole Stacy, Dr. Kendal Harr, and Ms. Jill Arnold. Basophil picture is courtesy of NC State Veterinary Teaching Hospital, Clinical Pathology Laboratory.)

Monocytes are usually the largest leukocyte, measuring 14–16 µm in koi[19] and 12–18 µm in zebrafish.[13] They are round to irregular in shape with a large, often eccentric heterochromatic indented round to horseshoe-shaped nucleus. Monocytes are phagocytic cells, termed macrophages when they migrate into tissue, with round, oval, or lobed nuclei, moderately condensed chromatin, and abundant gray to moderately basophilic cytoplasm (Figure 131.1i). There may be clear or variably colored, round vacuoles in the cytoplasm. Reactive cells may have numerous vacuoles which distort the cell (Figure 131.1j). Cellular margins may appear ragged, due to presence of pseudopodia. Larger amounts of cytoplasm, lower N:C ratio, nuclear morphology, and presence of cytoplasmic vacuoles help differentiate monocytes from large lymphocytes.

Eosinophils are less common than heterophils, though similar to slightly smaller in size. Nuclei are round, multilobed, or irregular and contain fine, mildly condensed chromatin. Cytoplasm is slightly to moderately basophilic with pink, distinct round to rarely elongate granules. The larger granule size and color differentiate them from heterophils (Figure 131.1p). They may not be found in blood smears from healthy fish and there may be species variation in normal eosinophil counts.

Basophils are rare. In koi, they are round and are typically smaller than heterophils, measuring 8–10 µm.[19] They contain large round, eccentrically located nuclei with condensed chromatin. Cytoplasm is abundant and on Wright's stain it is light blue-gray and contains moderately large, round to ovoid, dark basophilic granules that may obscure the nucleus (Figure 131.1q). But again, there can be stain variation with basophil granules staining more poorly with Diff-Quik™. Degranulated basophil granules may appear as empty vacuoles.

THROMBOCYTES AND HEMOSTASIS

Thrombocytes are involved in coagulation and clot formation. They are smaller than RBCs, oval, and have a central, elongate nucleus with clear or light blue-gray-staining cytoplasm. The N:C ratio is higher than in RBCs. Cell shape does vary with species, maturity, and degree of reactivity, from fusiform to oval to irregularly round. They are 3–4 µm in length in zebrafish.[13]

CYTOCHEMICAL AND ULTRASTRUCTUAL FEATURES

Cytochemistry, marker gene expression, immunohistochemistry, flow cytometry, fluorescent stains, and transmission electron microscopy are some methods that can be used to elucidate cell lines; however, use is limited on a routine basis. Upon cytochemical staining of koi whole blood, heterophils (as designated in this chapter) had strong peroxidase activity and reacted with acid phosphatase, periodic acid–Schiff (PAS), and Sudan Black B. Lymphocytes were positive for acid phosphatase and PAS. Monocytes were also positive for acid phosphatase and PAS, but were also positive for α-naphthyl butyrate esterase and β-glucuronidase activity. Eosinophils were positive for naphthol AS-D chloroacetate esterase activity. Basophils were reported to stain with PAS.[19]

Ultrastructurally,[19] koi RBCs contained a nucleus rich in heterochromatin and the cytoplasm was filled with electron-dense hemoglobin, a few mitochondria, and endoplasmic reticulum (ER). Heterophil nuclei contained peripheral heterochromatin and central euchromatin and there were numerous granules, mitochondria, and ER in the cytoplasm. The cytoplasmic granules were elongate, 0.2–0.4 µm in diameter, and consisted of a homogenous matrix with rare thin, needle-shaped crystalloids. In lymphocytes, the nucleus contained both heterochromatin and euchromatin. There were numerous free ribosomes, some mitochondria, and ER in the cytoplasm, and microvilli were often observed on the cell membrane surface. In monocytes, the peripheral rim and central area of the nucleus contained heterochromatin aggregates with strands of euchromatin. The cytoplasm was vacuolated and contained fewer free ribosomes than lymphocytes, numerous mitochondria, and some ER. Pseudopodia were often noted on the cell membrane surface. Eosinophil nuclei contained heterochromatin with small aggregates of euchromatin, and mitochondria and ER were present in the cytoplasm. Cytoplasmic granules were circular to oval, 0.5–1 µm, and had a fine granular matrix with one or more dense, bar-shaped crystalloids. Basophil nuclei contained abundant heterochromatin with small strands of euchromatin, and some mitochondria, ER, ribosomes, and granules were present in the cytoplasm. The granules were round to oval, 0.2–0.4 µm, with a core of dense crystalloid material. Small folds were noted in the cell membrane. Thrombocyte cytoplasm was filled with microtubules and large canaliculi and possible vacuoles with rare mitochondria, polyribosomes, and membrane-bound granules (Figure 131.2).

HEMATOPOIESIS

Fish hematopoiesis is similar to other vertebrates and occurs in several phases. However, cyprinids do not have bone marrow. The zebrafish model is highly conserved and can serve as a general overview for the cyprinids.[5,13] There are multiple phases or waves that take place in different locations. In the first wave, about 12–24 hours post fertilization, primitive myelopoiesis occurs in the anterior lateral mesoderm and primitive erythropoiesis occurs in the posterior lateral mesoderm, which subsequently forms the intermediate cell mass. Cells from these areas develop into either hematopoietic stem cells (HSC) or vascular endothelial precursors. Cell circulation begins after 24 hours post fertilization. The next waves are known as definitive and create erythromyeloid progenitors and pluripotent HSCs, which migrate to the embryonic anterior and caudal

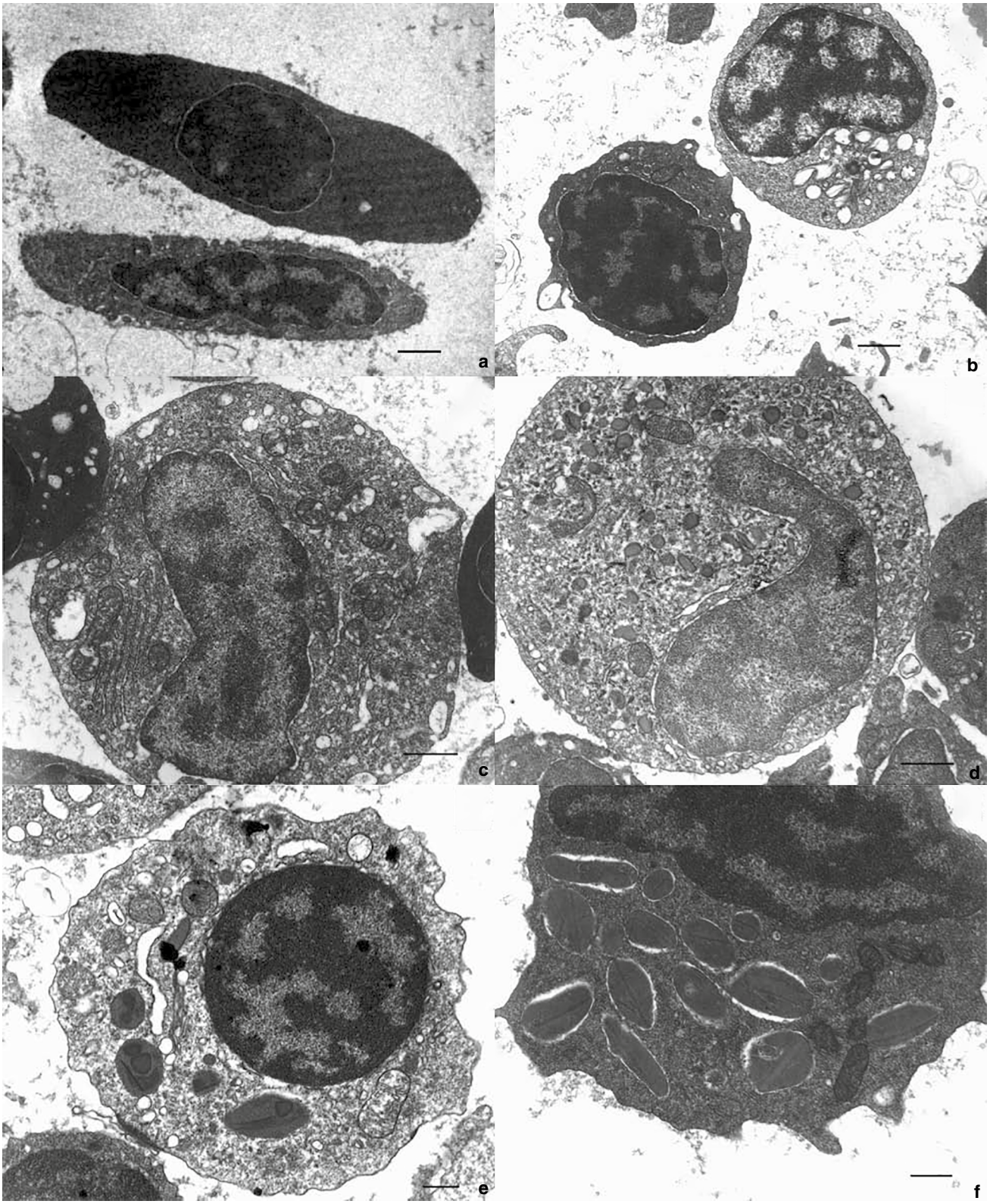


FIGURE 131.2 Ultrastructure of koi blood cells. (a) Erythrocyte with dense cytoplasm and a thrombocyte with an elongated nucleus and cytoplasmic microtubules (bar = 1lm). (b) Canaliculi are visualized as clear, membrane-bound vacuoles in the cross-section of a thrombocyte (left). The lymphocyte (right) has a thin rim of cytoplasm containing a few mitochondria (bar = 1lm). (c) Monocyte with large amounts of vacuolated cytoplasm containing scattered mitochondria and endoplasmic reticulum (bar 1 =lm). (d) Neutrophil with lobulated nucleus and numerous, small cytoplasmic granules (bar =1lm). (e) Basophil with numerous monoribosomes and fewer cytoplasmic granules with crystalloid cores (bar = 500 nm). (f) Eosinophil with densecytoplasm, scattered mitochondria, and large rice-shaped granules that have a characteristic needle-shaped crystalloid core (bar = 400). (**Source:** From [19], figure 3 (p.81) / with permission of John Wiley & Sons.)

kidney, becoming hematopoietic tissue. An HSC subset migrates to the developing thymus to become lymphoblasts. These become the primary hematopoietic sites in adult fish. The anterior or head kidney is predominantly hematopoietic tissue with few renal tubules, the trunk or posterior kidney contains more renal tubules with less interstitial hematopoietic tissue. The spleen, also a site of erythropoiesis, and anterior kidney filter foreign particles and degenerate blood cells.

REFERENCE INTERVALS

Further work on reference intervals in cyprinids is needed. There are multiple reference tables available, but many are compilations or do not meet recent American Society for Veterinary Clinical Pathology (ASVCP) reference interval guidelines.[9] Most do not state outlier removal, do not thoroughly describe the animal population or method which likely contributes to clinically significant variation between publications, or provide confidence intervals[19] instead of medical reference intervals. The issue is further complicated given the enormous diversity and numerous species in this family which inhabit a variety of warm water and temperate conditions. These poikilotherms can tolerate a wide variety of temperatures, which in turn impact hematologic variables. A recent paper[20] summarizes multiple studies on hematologic values of juvenile common carp, *C. carpio*, but authors express concerns on the limited number of individuals and variable genetic and environmental factors. To minimize variability, the authors generated a reference interval from a distinct population of juvenile carp, 5–8 months old, from rearing ponds sampled during a specific time frame, the end of September through October over 9 years. Ranges were wide indicating potential high biological variability. There is no mention of outlier removal, so diseased animals may have been included in the population. The authors note that some hematologic parameters of juvenile carp were quite stable (Hct, lymphocyte counts), some moderately variable (RBC, Hgb, MCV, MCH, and MCHC) and some highly variable (neutrophil, monocyte, and platelet counts). They suggest that the goal should be to develop ranges that are relevant for fish of similar age, size, reared under similar conditions, and sampled in similar time frames or seasons at the same temperature.

In light of the paucity of medical reference intervals for cyprinids in the literature, a de novo reference interval for a low number of koi was generated by the authors (Table 131.1). Blood was collected once in November from adult, healthy, mixed sex koi (4–5 years) held at the University of Minnesota as part of an aquaponics program. They were housed in three 1000 L rectangular tanks (50 animals divided among the three systems), each part of an A-frame nutrient film technique aquaponics system in an enclosed greenhouse. Filtration included a foam pad for mechanical filtration and a wet–dry biofilter with a plastic bio-ball substrate. The fish had been in these systems for at least 3 years and maintained on a standard commercial diet (Skretting, USA). They were originally obtained from a commercial breeder at approximately 2 years of age. Water temperature was 20–22 °C, pH was 7.2, and total ammonia nitrogen was 0.5 ppm. Fish were individually anesthetized in a separate holding container with buffered MS-222. Blood was collected from the caudal vessels by use of a lateral or ventral approach using a heparin-coated syringe and a 23 g needle into Li-heparin blood tubes. Blood smears, PCV, total protein values, and manual hemocytometer counts were done within 24 hours. Blood smears were stained with both Wright–Giemsa and Diff-Quik™ stains for comparison (Figure 131.1k,l). Differentials were conducted using Wright–Giemsa-stained slides, though both stains could be used diagnostically. Hemoglobin was analyzed using a HemoCue Hb 201+ Analyzer.

Partitioning Factors

Although much less is known about the factors that influence cyprinid blood values compared to mammals, some intrinsic and extrinsic factors that influence hematology include age, sex, breed, temperature, water quality, handling stress, hypoxia, reproductive status, season, nutrition, population densities, and disease.[2,3,6,7,8,10,11,12,16,18]

Both age and sex appear to affect hematologic values in common carp (*C. carpio*).[2] Hemoglobin, RBC, WBC, and MCHC increased in older fish (2–3 years) compared to younger fish (9 months to 1 year). Differences were correlated with the animal's length and size. White blood cell count, MCV, and PCV were noted to be higher in males than females. Hematologic profiles of common carp (*C. carpio*) spawners from eight breeds reared under identical conditions and collected during the breeding season revealed significant differences for Hgb, PCV, MCV, and MCH. However, values for RBC concentration, total WBC counts, and leukocyte differentials did not differ between breeds.[18] Hence, breed-specific reference intervals may be needed for some analytes.

Cyprinids compose a large, diverse group of fish capable of inhabiting highly variable environmental conditions. Age, sex, age, time of sampling, and other factors significantly impact hematologic reference values. Lack of method standardization and statistical evaluation further hinders comparison. While literature review is always a recommended starting point, the materials and methods of cyprinid reference interval studies should be thoroughly detailed, as most do not present true medical reference intervals, regardless of title. Establishing study-specific reference intervals is encouraged and standardization of testing and analytical specifications according to guidelines is advised.

HEMATOLOGIC DISEASE

Anemia

Anemia is a common sequela to disease and follows similar patterns of regeneration and lack thereof as found in mammals (see Chapters 129 and 130). However, as poikilotherms, cyprinids will have a decreased regenerative

response at colder temperatures and recovery may, therefore, be extended when the temperature is low. Immune-mediated hemolytic anemia has not been reported in cyprinids, though there is evidence that macrophages will phagocytize older RBCs. For example, a goldfish presented with recurrent buoyancy problems that were determined on radiographs to originate in the swim bladder, not the gastrointestinal tract as is more common. Surgery was performed to correct the swim bladder defect. The follow-up CBC revealed monocytes/macrophages exhibiting erythrophagia, presumed to be part of the postsurgical healing process (Figure 131.1r).

Physiologic Changes

In zebrafish, leukocyte differential cell counts changed depending on the type of stress.[11] Acute stress, simulated by alternating short intervals of air exposure by suspending the fish in a net or swimming at low water levels with the dorsum exposed to air, resulted in lymphopenia, monocytosis, and neutrophilia. Chronic stress, simulated with a combination of tank changes, overcrowding, chasing, and dorsal body exposure, for variable times intervals over 5 days, induced lymphopenia and monocytosis, but no significant change in neutrophil populations. Changes were postulated to be due to increased endogenous cortisol concentrations. Increased erythropoiesis was noted in the common carp (*C. carpio*) when exposed to air for up to 4 hours.[6] Approximately one-third of mature RBCs were replaced by immature RBCs and the authors suggest that this can increase the fish's tolerance to stress.

Inflammatory Disease

Cyprinids are susceptible to a number of viral, bacterial, parasitic, and fungal/oomycete infections which can impact hematologic values (Table 131.2). Several viruses

TABLE 131.2 Physiologic and pathologic factors that affect hematologic values in common carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*).[12] NCR: Not clinically relevant.

Measurand	Increased value	Decreased value
PCV, Hct, Hgb	Stress hemoconcentration	Stress
	Age—mature fish	Age—juvenile fish
	Males	Females
	Increased temperature (especially in small fish)	Decreased temperature
	Severe hypoxia	Hemolysis, Hemodilution, Lymph dilution
		Nutritional deficiencies, infectious disease, toxicity
Methemoglobin	Low temperature, toxicity	NCR
Mean RBC volume	Stress	Sample artifacts
	Immature cells	
	Improper/prolonged storage	
RBC count	Increased constant temperature	Anemia (nutritional deficiencies, infectious disease, toxicity)
Immature, dividing, and degenerative RBCs	Stress	Nonregenerative anemia
	Cyclic temperatures hypoxia	
	Regenerative anemia	
WBC count	Stress	Stress
	Increased temperature (maximum value at 25 °C)	*A. salmonicida* and other severe infections
	Infectious disease	
Lymphocytes	Increased temperature (maximum value at 25 °C)	Stress
	Viral disease	Decreased at temperatures >25 °C
		Infectious disease
Monocytes	Stress	NCR
	Infectious disease	
Thrombocytes	Stress	Stress
	Increased temperature (>25 °C)	
Granulocytes	Stress	*A. salmonicida* and other severe infection
	Increased temperature (>25 °C)	
	Infectious disease	

are specific to cyprinids and likely impact hematologic values including: koi herpesvirus (Cyprinid herpesvirus-3), which causes disease in koi, common carp, and hybrids; carp pox (Cyprinid herpesvirus-1), which infects several cyprinid species; herpes viral hematopoietic necrosis disease (Cyprinid herpesvirus-2), which infects goldfish; carp edema virus aka koi sleepy disease (poxvirus), which infects carp, carp hybrids, and koi; and spring viremia of carp virus (rhabdovirus), which infects a number of cyprinid species.[16] There is a scarcity of published information on the hematologic effects of these diseases and further research is warranted. Spring viremia of carp may cause anemia and neutrophilia, monocytosis, eosinophilia, and basophilia, potentially for months after infection.[16] *Aeromonas salmonicida* or other *Aeromonas* species may cause severe ulcerative disease and mortality which can result in either leukocytosis or leukopenia, heterophilia or heteropenia, and monocytosis. Eosinophilia was noted in zebrafish with helminth infestation.[3]

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CHAPTER 132

Hematology of Lizards, Crocodilians, and Tuatara

CHARLOTTE HOLLINGER and JEAN A. PARÉ

Blood Collection and Processing
Erythron
Leukon
Thrombon
Hematopoiesis and Bone Marrow Assessment
Reference Intervals and Interpretation
Hematologic Disease
Anemia
Inflammation
Neoplasia
Hemoparasites and Inclusions

Abbreviations and Acronyms

PAS, periodic acid Schiff

The suborder Sauria (Lacertilia), within the order Squamata, is an assemblage of roughly 5600 lizard species, spanning more than 40 families.[64] All share a similar elongated quadrupedal body plan, although limbs may be reduced, vestigial, or even absent in some species. Lizards vary in size from the Jaragua dwarf gecko (*Sphaerodactylus ariasae*), weighing a mere fraction of a gram, to the Komodo dragon (*Varanus komodoensis*) that can weigh up to 200 kg.[64] Lizards occupy a wide range of habitats across all continents, except Antarctica, and have evolved a variety of biological and ecological adaptations to survive as ectotherms in different environments. The order Crocodilia includes the morphologically similar crocodiles, alligators, gharial, false gharial, and caimans. Crocodilians are ancient reptiles more closely related to birds than to lizards, but sharing some general anatomic and physiologic similarities to lizards including ectothermia. All 23 crocodilian species are aquatic and carnivorous, with the saltwater crocodile (*Crocodylus porosus*) being one of the world's largest predators. The tuatara (*Sphenodon punctatus*), native to New Zealand, is the only extant species of the Rhynchocephalia, a reptilian order distinct but closely related to lizards. Tuataras are long-lived, cold-adapted, lizard-like primitive reptiles that possess unique anatomical features among which is a parietal, or third, eye. For the purposes of this book, crocodilians and the tuatara will be treated alongside Sauria. Because sick reptiles display a limited array of nonspecific clinical signs, blood collection for hematology and biochemistry is particularly useful and provides valuable clinical information for health assessment of these rather stoic animals.

BLOOD COLLECTION AND PROCESSING

Venipuncture requires an understanding of anatomy, technical skill, and aseptic technique. Logistical considerations include body size, venipuncture sites, and both patient and handler safety. The small size of some species, such as geckos and anoles, may preclude blood sampling. Only limited research has been performed to assess total blood volume and safe volume parameters for blood withdrawal in lizards. The total blood volume likely approximates 5–8% body weight, based on data for snakes and turtles, yet values as low as 2–3% were reported from early experimental studies in sand lizards (*Lacerta agilis*).[17] Quick withdrawal of up to 10% total blood volume is empirically considered tolerable across vertebrates, and has been assessed directly in Siamese crocodiles (*Crocodylus siamensis*) without observed adverse effect.[5] In addition to hemodynamic compromise, potential complications of venipuncture include infection, vascular trauma, and collateral damage to surrounding soft or bony tissues. Some lizards and tuataras are capable of caudal autotomy (voluntary self-amputation of the tail) in response to

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real or perceived attack. This potentially life-saving defensive mechanism, which may occur prior to the animal being restrained or the tail being grasped, comes with a variety of physiologic and social costs, even if the tail regenerates.[2,20]

Appropriate physical, and sometimes chemical, restraint is essential for successful venipuncture, and care must be taken to minimize risk to both the patient and the handlers. Reptiles are surprisingly strong, with powerful jaws and sharp claws. Helodermatid lizards, e.g., Gila monster (*Heloderma suspectum*) and beaded lizard (*Heloderma horridum*), are highly venomous, and other lizard species such as varanids likely have toxin-secreting glands.[16,65] Wounds (e.g., bites and scratches) incurred during handling of any lizard pose human health risks including blood loss, anatomic compromise, and potential infection that warrant immediate medical attention.[12] Handling of the larger crocodilians is best left to experienced staff.

Accessible venipuncture sites are limited in lizards and site selection is based on several factors, including animal size and behavior. In larger lizards, tuataras, and smaller crocodilians, the ventral caudal/coccygeal/tail vein is commonly used. This vein runs just ventral to caudal vertebrae and is accessed through a ventral midline or a lateral approach. In male lizards, the needle is inserted distal enough from the vent to avoid the hemipenes, which are absent in crocodilians and tuatara. Tail autotomy in many skinks, geckos, and other lizards is problematic, so the tail should be avoided in these species. In chameleons, tail discoloration may occur distal to the sampling site, but resolves within weeks.[37] A ventral abdominal vein, which runs along the ventral coelomic midline just beneath the coelomic membrane, is accessible for venipuncture in most lizards. However, this vessel cannot be visualized (blind stick). Careful and slow needle insertion in a sedated lizard is warranted to avoid lacerating the vein and causing a hemocoelom. Jugular venipuncture techniques have been described as an alternative for small lizards and chameleons.[9,14,47] Approaches for cephalic vein and brachial plexus sampling are uncommonly used. Blood sampling using digit lacerations or amputations, the postorbital sinus, and cardiocentesis are not recommended due to potential risks, including pain, infection, or damage to neighboring tissues.[60] In large crocodilians, blood can be collected from the postoccipital spinal venous sinus (supravertebral sinus, internal jugular vein, occipital sinus) caudal to the occiput and dorsal to the spinal cord, but risk exists for sample contamination with cerebrospinal fluid as well as for potential injury to the spinal cord.[48,50]

Lizards possess a closed circulatory system with a chambered heart and a circulatory tree distributed throughout the body. The blood of lizards is red, except for five species of aptly named green-blooded skinks in the genus *Prasinohaema*, native to New Guinea. In these lizards, the highest serum biliverdin level among any amniote species imparts a nonpathological bright lime-green color to the blood.[1] The lymphatic system differs from that of mammals in the absence of lymph nodes and existence of one or more lymph hearts, often located in the pelvic region.[32] Due to the close association of lymphatic and vascular networks, lymph-contaminated blood samples are common. This lymph component decreases erythroid density (decreased PCV, Hgb, etc.) and increases small lymphocyte numbers. A sample with visible lymph contamination, seen as clear or white fluid entering the needle hub and syringe upon venipuncture, is best avoided for hematologic assessment.

The anticoagulant of choice is dependent on test and species. For CBCs, either lithium heparin or K-EDTA is commonly used. EDTA has been used repeatedly in lizards, crocodilians, and tuatara blood samples with no hemolysis reported and is recommended at this time for CBC evaluation. Studies suggest that EDTA may be preferable to lithium heparin in green iguanas (*Iguana iguana*) and Chinese water dragons (*Physignathus concincinus*) due to better cell morphology, less WBC and thrombocyte clumping, and cell counts more similar to those from assessment of non-anticoagulated blood.[27,36] Anticoagulation with heparin is often linked to blue or pink discoloration of blood films and increased clumping of WBCs and thrombocytes versus EDTA-anticoagulated blood,[31] potentially affecting cell identification and quantification. Both EDTA and heparin anticoagulation are known to cause some changes to hematologic values in comparison to non-anticoagulated blood;[27] smear preparation from non-anticoagulated blood immediately after collection can negate potential artifacts and also yield information from patients of extremely small body size, in which collection of only a drop of blood is feasible. The coverslip method for blood film preparation is recommended by some authors due to perceived benefits in regard to reptilian blood cell fragility and cell distribution.[60] When a small blood volume is anticipated, heparin anticoagulation has the benefit of better facilitating plasma chemistry in addition to hematology. There is increased plasma yield in comparison to serum, and sodium- and potassium-EDTA salts confound electrolyte results.

A lizard CBC includes quantitative (PCV, RBC, Hgb, and WBC concentration) and qualitative assessment of RBC, WBC, and thrombocyte lineages, as in other vertebrates (see Chapter 123). Qualitative morphologic assessment is a key component of reptile health evaluation and requires well-made and representative blood films, with appropriate cell distribution and staining quality. Blood smears should be made as soon as possible after collection. Due to the risk of spurious findings, clotted samples should be avoided. A variety of Romanowsky-type stains (e.g., modified Wright–Giemsa) can be used for microscopic assessment, including rapid stains (e.g., Diff-Quik), though the latter may stain cell granules less reliably. Diagnostic accuracy and interpretation rely on the hematologist's familiarity with the specie's cell morphology under the stain conditions used.

ERYTHRON

Lizard RBCs are nucleated oval cells as in all vertebrates except mammals. When compared to birds and mammals, lizards have fewer circulating RBCs and lower

PCVs, but comparatively larger RBCs with lower corpuscular hemoglobin concentrations.[30] Lizard RBC sizes vary across species with dimensions reported in the range of 13–19 µm length and 8–12 µm width. These RBC sizes are generally smaller than that of snakes and turtles, and RBC numbers are reciprocally higher.[13,29] Crocodilian RBCs are on par with lizards, while the length and width of tuatara RBCs (mean 23 × 14 µm) exceeds that of squamates.[11,21] Mature RBC nuclei are centrally located and rounded to oval with variably irregular margins, dense chromatin, and surrounding eosinophilic cytoplasm (Figures 132.1 and 132.2). The nucleus of aged RBCs is smaller and denser (pyknotic).[60] Mild anisocytosis, polychromasia, and poikilocytosis are common in health (Figure 132.3). Erythroplastids (RBCs lacking nuclei) occur in low numbers and have no known clinical significance.[31] Low numbers (<2%) of circulating immature RBCs (e.g., rubricytes) are also common in health. Reptile RBCs are reported to be long-lived, though studies in lizards are lacking.[54]

Erythrocyte lineage maturation is similar to that in mammals with notable exceptions of nuclear retention and mild cell-size enlargement (versus progressive diminution) in terminal maturation. Polychromatophils are oval, but rounder than mature RBCs, with mild cytoplasmic basophilia and less-dense chromatin. Rubricytes are smaller round cells with a round nucleus, clumped chromatin, and a concentric ring of darker basophilic cytoplasm, which must be differentiated visually from similarly sized lymphocytes. Earlier erythroid precursors in circulation (prorubricytes and rubriblasts) are uncommon in health, but increase in pathologic states (e.g., erythroid regeneration) (Figures 132.3 and 132.4). These larger round cells with dark basophilic cytoplasm, round nuclei, looser chromatin, and, in blasts, a visible nucleolus, are similar to those of mammals. Mitotic rubricytes are occasionally present in circulation in health and increase in regenerative states.

LEUKON

Heterophils, lymphocytes, monocytes, basophils, and occasionally eosinophils are typically found in health (Figures 132.1 and 132.2). Other circulating WBCs, including plasma cells and macrophages (siderocytes, melanophages), are inconsistently seen and can indicate disease (Figures 132.3 and 132.4). The WBC differential of many lizards is primarily lymphocytic, although heterophils are reported as predominant in some species, such as Gila monsters.[8,60] Both heterophil- and lymphocyte-predominant leukograms are reported in crocodilians,[38,43,51,59] and lymphocytes are reported as predominant in tuataras.[11]

Mature heterophils are large, round granulocytic cells with colorless cytoplasm containing many eosinophilic pleomorphic granules (rod-, spindle-, or angular-shaped). Cell sizes in the range of 9–23 µm have been reported for various lizards as well as tuatara and the Nile crocodile (*Crocodylus niloticus*).[21] Heterophil granules range from neutral or muted to brightly eosinophilic depending on staining method. Degranulated-appearing heterophils are a relatively common finding and likely relate to sample handling or slide preparation factors, such as prolonged storage, underfixation, or staining.[28,60] Nuclear morphology varies across species: round to oval (e.g., most crocodilians, monitor lizards) or lobulated/segmented (e.g., green iguana, bearded dragon, tuatara). Chromatin in mature heterophils is densely clumped. Cytochemical staining patterns of lizard heterophils vary between species including variable reports of positive or negative staining for benzidine peroxidase (peroxidase).[8,11,28,43,46] For expected staining patterns, a thoughtful literature review is recommended. Heterophils are considered phagocytic, but may be capable of oxidative response in at least some species of lizards (e.g., green iguana, red-headed rock agama (*Agama agama*)).[4,28]

Lymphocytes are comparable to those in other vertebrates, consisting of predominantly small cells with high nuclear to cytoplasmic ratio and mildly basophilic cytoplasm (Figures 132.1 and 132.2). Size variation is common (5–15 µm).[21] Nuclear morphology is round with densely clumped chromatin. In many species, lymphocytes are the predominant circulating WBC in health. Differentiation of lymphocytes from thrombocytes is a frequent visual challenge (Figure 132.3). Distinguishing features of lymphocytes include mild cytoplasmic basophilia, slightly larger nuclei, and more clumped chromatin. Cytochemical staining with periodic acid–Schiff (PAS) typically stains reptile thrombocytes positively, likely staining glycogen, and most lymphocytes negatively. An exception to this pattern was seen in lymphocytes of American alligators, which stained PAS-positive, reflecting probable antibody production.[43] Reactive lymphocytes are characterized by larger size and more abundant deeply basophilic cytoplasm (Figure 132.3). Granular lymphocytes contain low numbers of distinct small eosinophilic cytoplasmic granules. Plasma cells are also occasionally identified (<1% of WBCs in health), and are classically characterized by deeply basophilic cytoplasm, paranuclear clearing, eccentric nuclear position, and coarsely clumped chromatin. Rarely, plasma cells containing clear or pale basophilic immunoglobulin Russell bodies (Mott cells) may be found (Figure 132.4g). The presence of reactive lymphocytes and/or plasma cells suggests immunologic stimulation. Circulating lymphocyte concentration in reptiles is also influenced by factors other than disease, such as seasonality and environmental conditions.[13,63]

Monocytes are often the largest leukocytes, with cell size ranging up to 25 µm diameter.[60] These cells are round with moderate to large quantities of pale-gray and subtly grainy cytoplasm. Nuclear shape is variably round, oval, indented, or mildly lobulated, with fine chromatin. Cytoplasmic vacuolation of occasional monocytes is common and may reflect cellular reactivity or in vitro artifact (Figure 132.3). Circulating monocytes containing phagocytized material can herald underlying pathology (e.g., hemolysis, inflammation), but must be interpreted in light of sample handling (Figure 132.4). Macrophages containing melanin pigment (melanomacrophages) are normally found in many reptilian tissues

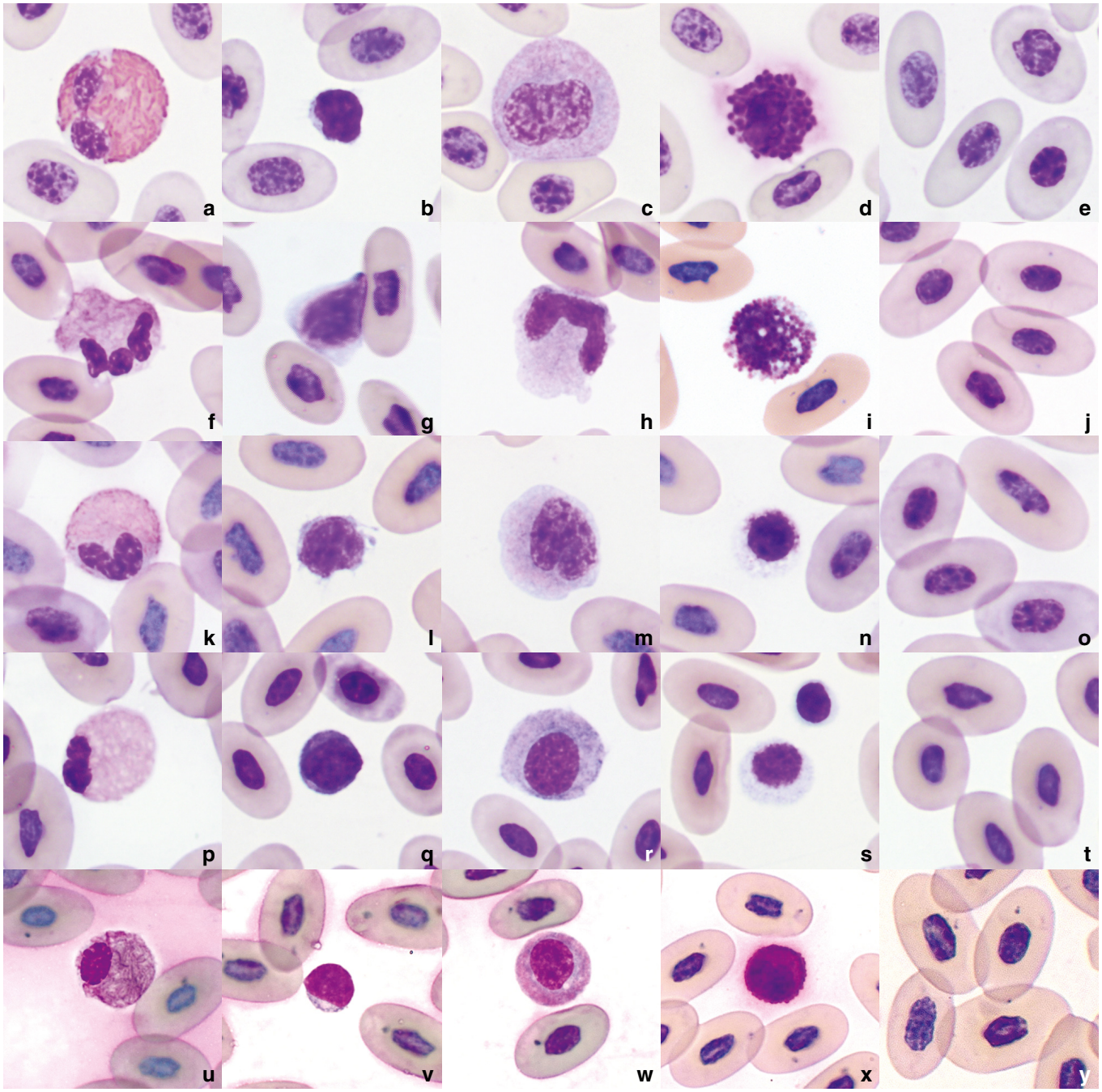


FIGURE 132.1 Common blood cells of iguanid and gekkonid lizards in health. (a)–(e) Green crested basilisk (*Basiliscus plumifrons*); (f)–(j) Bearded dragon (*Pogona sp.*); (k)–(o) Common chuckwalla (*Sauromalus ater*); (p)–(t) Spiny-tailed lizard (*Uromastyx sp.*); (u)–(y) Leopard gecko (*Eublepharis macularius*). Columns left to right: Mature heterophil; Small lymphocyte; Monocyte; Basophil; Erythrocytes. Eosinophils are more rare and not pictured (Figure 132.3). (u) Illustrates heparin artifact. 1000× modified Wright–Giemsa stain. (**Source:** Photo (i) is courtesy of Dr. Nicole Stacy. Photos (u)–(y) are courtesy of Dr. Kendal Harr.)

and can rarely be found on blood films. When increased, melanomacrophages can indicate underlying nonspecific disease. Azurophilic monocytes are also identified occasionally in lizards and crocodilians, and are visually distinguished from standard monocytes by azurophilic cytoplasmic granules ranging from fine dust-like stippling to distinct purple granules (Figure 132.3). The nucleus is round to oval with clumped chromatin. The cellular origin of azurophils has been controversially classified as granulocytic or monocytic and may vary between reptile species. In most lizards and crocodilians, cytochemical stains support a monocytic lineage (azurophilic monocytes), being variably positive for acid phosphatase, alpha-naphthyl butyrate esterase, and PAS, and negative or only weakly positive for Sudan Black B and peroxidase.[28] Strong staining of azurophils for peroxidase and Sudan Black B, similar to that found in snakes and mammalian neutrophils, was a

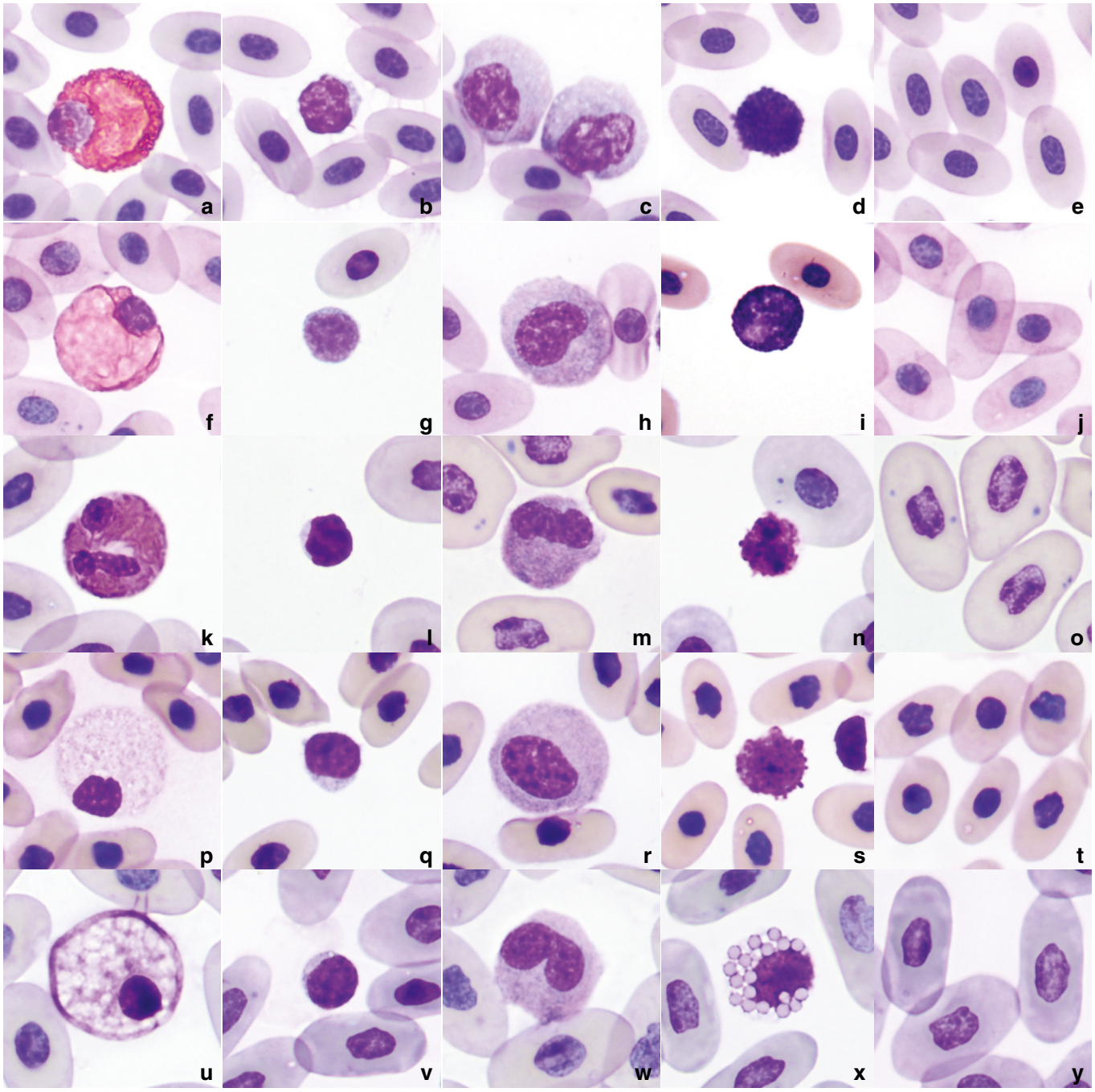


FIGURE 132.2 Common blood cells of monitor lizards, crocodilians, and Chinese crocodile lizards in health. (a)–(e) Komodo dragon (*Varanus komodoensis*); (f)–(j) Grey and lace monitors (*Varanus sp.*); (k)–(o) Caiman lizard (*Dracaena guianensis*); (p)–(t) Cuban crocodile (*Crocodylus rhombifer*); (u)–(y) Chinese crocodile lizard (*Shinisaurus crocodilurus*). Columns left to right: Mature heterophil; Small lymphocyte; Monocyte; Basophil; Erythrocytes. (x) Possible basophil, eosinophil not excluded. 1000× modified Wright–Giemsa stain. (**Source:** Photo (i) is courtesy of Dr. Kendal Harr.)

unique finding in Gila monsters.[8] Due to unclear clinical significance and limited diagnostic value, separate classification and enumeration of azurophils in lizards is typically not necessary.

Eosinophils are present in low numbers or absent in health and are round granulocytic cells with clear to light blue cytoplasm containing many distinct round granules that stain variably eosinophilic, blue, or green across species. Eosinophil granule-staining properties are highly variable across species (Figure 132.3a–e). Cell sizes similar to heterophils (9–20 µm) are reported.[60] The nucleus is variably round to oval, lobed, central, or eccentric, depending on species. Though the distinction between heterophils and eosinophils ("eosinophilic granulocytes") is unclear in early references, eosinophils of lizards measured the smallest across reptile taxa.[21] Peroxidase staining has been suggested to help differentiate eosinophils from heterophils; however, variable

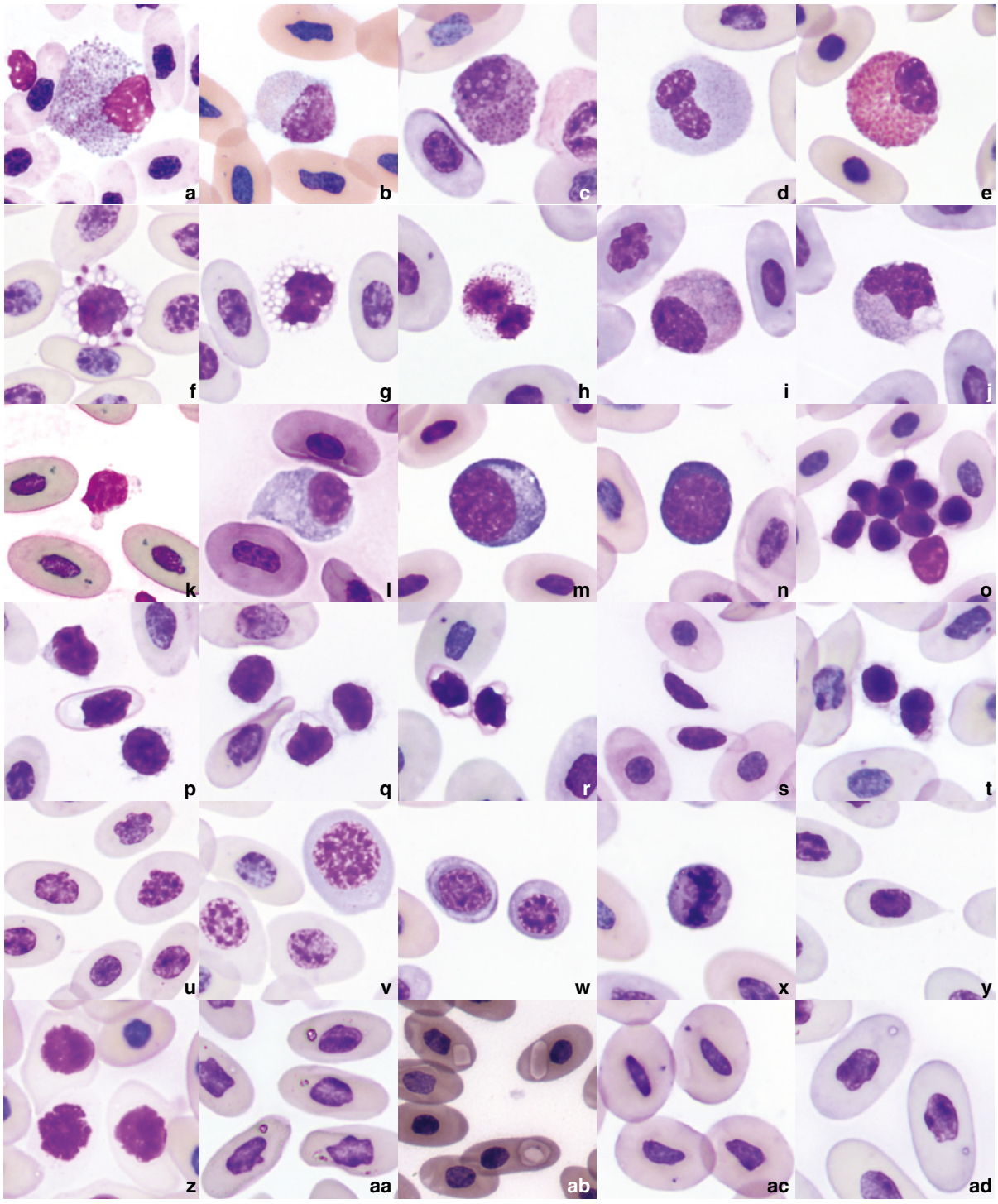


FIGURE 132.3 Other common hematologic findings in health. (a)–(e) Eosinophils of green Iguana (*Iguana iguana*), bearded dragon (*Pogona sp.*), common chuckwalla (*Sauromalus ater*), caiman lizard (*Dracaena guianensis*), and Cuban crocodile (*Crocodylus rhombifer*); (f),(g) Basophils of green crested basilisk (*Basiliscus plumifrons*), with variable partial or nonstaining granules (compare to Figure 132.1d); (h) Basophil of caiman lizard with finely stippled staining (compare to Figure 132.2n); (i),(j) Monocytes of Chinese crocodile (*Shinisaurus crocodilurus*) lizard with (i) azurophilic staining or (j) vacuolation; (k) Small lymphocyte of leopard gecko (*Eublepharis macularius*) with cytoplasmic blebs; (l) Plasmacytoid lymphocyte of bearded dragon; (m) Reactive lymphocyte of spiny-tailed lizard (*Uromastyx sp.*); (n) Early erythroid precursor, presumed rubriblast, of common chuckwalla for comparison to reactive lymphocyte; (o) Thrombocyte cluster in Komodo dragon (*Varanus komodoensis*); (p),(q) Thrombocytes of green crested basilisk showing (p) oval and (q) round morphologies with comparison to two small lymphocytes (above and below oval thrombocyte in (p); (r)–(t) Thrombocytes of caiman lizard, Cuban crocodile, and Chinese crocodile lizard with short pseudopodia in (t) suggesting activation; (u) Normal variability of nuclear contour in green crested basilisk; (v) Normal variable morphology of circulating RBCs from shrunken dense nucleus (upper left) to polychromatophilic (upper right) in green crested basilisk; (w) Basophilic rubricyte precursors in common chuckwalla; (x) Mitotic figure in common chuckwalla; (y) Pyriform RBC in green crested basilisk; (z) Artifactually lysed RBCs in Cuban crocodile; (aa) Refractile artifact in bearded dragon; (ab) Hemoglobin crystals in green iguana; (ac) Punctate basophilic inclusions in spiny-tailed lizard; (ad) Punctate vacuoles in Chinese crocodile lizard. 1000× modified Wright–Giemsa stain. (**Source:** Photo (b) is courtesy of Dr. Nicole Stacy. Photos (a), (k) and (ab) are courtesy of Dr. Kendal Harr.)

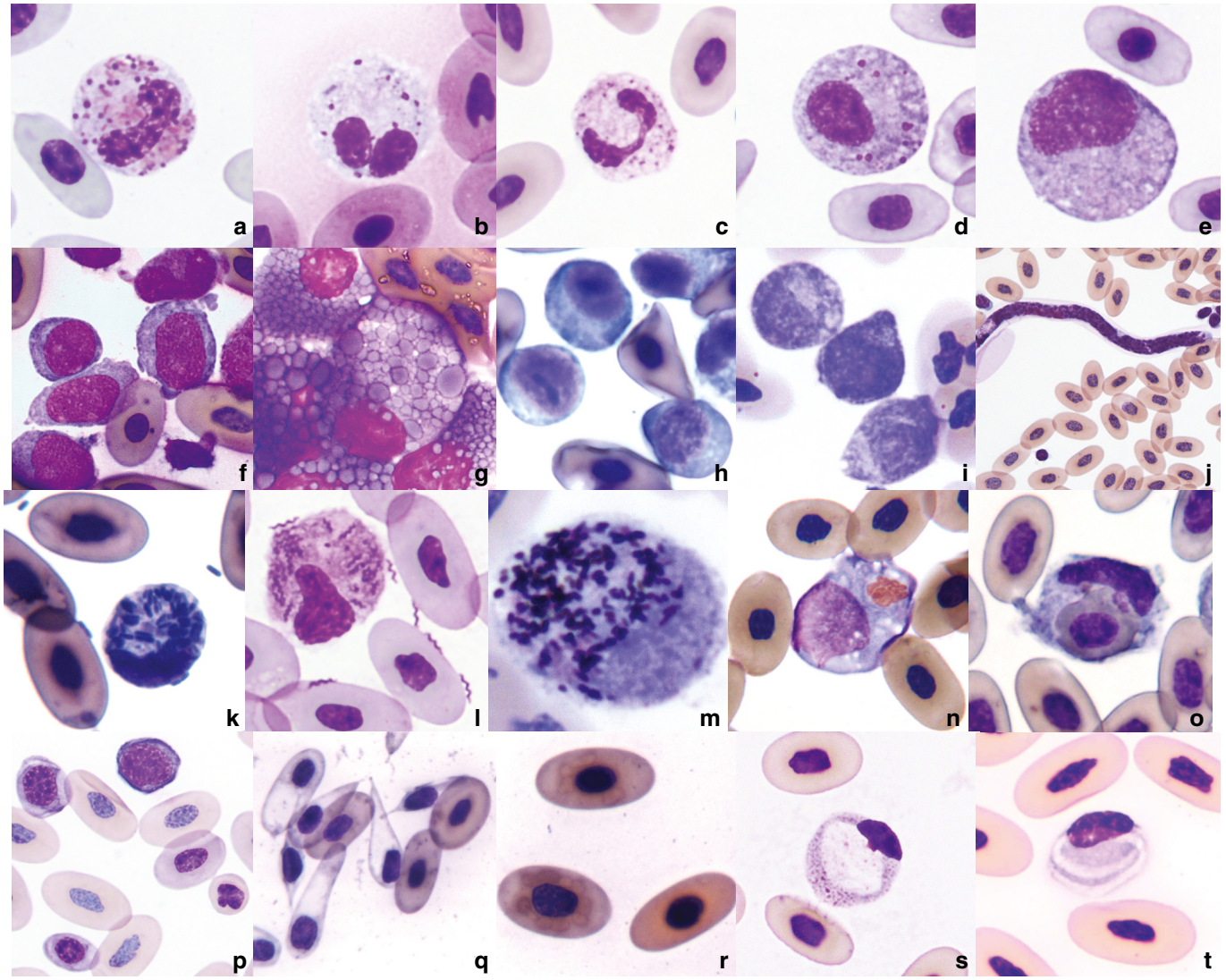


FIGURE 132.4 Hematopathology in lizards. (a)–(c) Toxic change affecting green crested basilisk (*Basiliscus plumifrons*), bearded dragon (*Pogona sp.*), and spiny-tailed lizard (*Uromastyx sp.*) (compare to heterophils in Figure 132.1); (d),(e) Left-shifted and toxic myelocyte and early promyelocyte of prehensile-tailed skink (*Corucia zebrata*); (f) Leukemoid reaction in bearded dragon positive for atadenovirus, for which leukocytosis resolved after 3 months; (g) Increased circulating Mott cells in a bearded dragon; (h) Myeloid leukemia in panther chameleon (*Furcifer pardalis*); (i) Lymphoid leukemia in bearded dragon; (j) Microfilaria in a chameleon; (k) Bacteremia in bearded dragon with intracellular and extracellular bacilli; (l) Spiriliform bacteremia confirmed molecularly as helicobacteremia in Grand Cayman blue iguana (*Cyclura lewisi*); (m) Melanomacrophage in bearded dragon; (n) Monocyte containing phagocytized heterophil granules in monitor lizard (*Varanus sp.*); (o) Erythrophagocytosis in panther chameleon; (p) Erythroid regenerative response in common chuckwalla (*Sauromalus ater*) including small atypical round erythrocyte (lower right) consistent with mild dysplasia; (q) Hypochromasia and poikilocytosis in atadenovirus-positive, bearded dragon; (r) Possible Heinz bodies in a green iguana (*Iguana iguana*) with renal failure; (s),(t) Oval to linear cytoplasmic clearing in apparently healthy red tegu (*Salvator rufescens*) and Savannah monitor (*Varanus exanthematicus*), which are of unknown composition and clinical significance, pending further analysis. 1000× modified Wright–Giemsa stain. (**Source:** Photos (i) and (m) are courtesy of Dr. Nicole Stacy. Photos (f)–(h), (j), (k), (n), (o), (q), (r), and (t) are courtesy of Dr. Kendal Harr.)

patterns are reported and review of species-specific literature is again recommended to assess expected cytochemical staining.[28]

Basophils are often present in low number, but vary substantially. They are smaller round cells (7–16 µm) with clear cytoplasm containing variably staining dark basophilic to purple or nonstaining granules.[21] Basophilic-staining granules often obscure the nucleus, which is round and central. Poorly staining granules appear as clear vacuoles and are commonly attributed to artifact from water-based stains or other components of rapid stains (Figure 132.3).

THROMBON

Thrombocytes are important in primary hemostasis of lizards and additionally have mild phagocytic activity. The cells are small and variably elliptical, with colorless to pale blue cytoplasm, central round-oval nuclei, and dense chromatin (Figure 132.3). Low numbers of small eosinophilic granules are occasionally present. Differentiation from lymphocytes can be difficult; thrombocytes generally have less basophilic to colorless cytoplasm, wispier or indistinct cytoplasmic margins, and denser nuclear chromatin. As mentioned in the earlier text, with few exceptions, PAS staining has been used to differentiate these cells; thrombocyte cytoplasm stains positive (pink) and that of lymphocytes typically does not stain.[60] Activated thrombocytes can show irregular margins with cytoplasmic extensions (pseudopodia) and vacuolation. Thrombocyte clumps in vitro are common and should be noted, as well as communicated in reporting of thrombon mass adequacy.

Thrombocytopenia is recognized but uncommon; assessment must include blood smear evaluation for clumps causing pseudothrombocytopenia. As in other taxa, excess consumption, destruction, or decreased production are the primary underlying processes. Associations between nuclear polymorphism in thrombocytes and severe inflammatory disease as well as prolonged anorexia have been proposed.[31]

HEMATOPOIESIS AND BONE MARROW ASSESSMENT

There are few studies of lizard hematopoiesis. As in birds, the yolk sac is a primary site of embryonic erythropoiesis. The bone marrow is the primary site of postnatal hematopoiesis and consists of a reticular network and blood sinuses, in which erythropoiesis occurs intravascularly and granulopoiesis extravascularly.[67] Other organs such as the spleen, liver, and mucosal-associated lymphoid tissues can contribute substantially in some disease states.[40] Erythrocytes are physiologically removed by phagocytes, primarily in the spleen.

Though uncommonly performed, and complicated by lack of procedure optimization, poor cellular exfoliation, and impact of extramedullary hematopoiesis, bone marrow in lizards, crocodilians, and tuataras is most reliably collected from the femur or tibia. In smaller lizards, a spinal needle is inserted through the trochanteric fossa or through the tibial crest and bone marrow is aspirated with a similar method as in other species (see Chapter 141). Erythropoiesis, granulopoiesis, and lymphopoiesis follow cellular morphology and progression similar to that in other vertebrates. Low numbers of macrophages, lymphocytes, and plasma cells are also observed. Like mammals, T lymphocytes are derived from the thymus. B lymphocyte origin is unclear in lizards. Thrombopoiesis is comparable to that in birds, consisting of a developmental progression from thromboblasts, to immature thrombocytes, to mature thrombocytes. Thromboblasts resemble rubriblasts; developing thrombocytes become progressively smaller, less basophilic, and elliptical, with dense nuclear chromatin. Interpretation of hematopoietic abnormalities in these species follows mammalian classifications.

REFERENCE INTERVALS AND INTERPRETATION

Reference intervals for individual species are often not available or are incompletely described. Adherence to best practices for reference interval generation is only recently more common and published "reference intervals" must be critically evaluated.[15] When no appropriate reference interval is available, results from the same animal over time or similarly housed comparator animals of the same species may provide pertinent data for comparison.

In addition to familiarity with species variations and preanalytic factors common in homeothermic vertebrates, lizards are ectotherms that rely heavily on environmental parameters, having evolved behaviors and strategies to best utilize the specific biotopes they inhabit. Hematologic values may fluctuate, and the sparse sets of "reference interval" values published for lizard species seldom account for factors that would likely impact interpretation of cell counts. Factors include intrinsic (age, sex, reproductive status) and extrinsic (season, temperature, other environmental conditions, diet, and social dynamics) variables. Furthermore, preanalytic variables such as venipuncture site, lymph dilution, and sample handling play a large role. The requirement for manual methods yields inherent imprecision and error. Techniques used to determine cell counts vary and interobserver differences in cell description and identification are a concern. Chronic and acute stress related to captivity and capture may also impact hematologic variables,[10] though this is challenging to document, especially in wild populations in which health status and other variables are unclear. Examples of recognized differences in hematologic ranges generated from healthy populations based upon extrinsic and intrinsic variables other than disease include: differences in total and differential WBC counts in free-ranging versus managed Texas horned lizards (*Phrynosoma cornutum*), or captive green iguanas housed under different conditions;[28,44] effects on RBC density or lymphocyte concentration by manipulation of environmental temperature in Cunningham's skinks (*Egernia cunninghami*) and saltwater crocodiles;[39,63] differences in seasonal variations in RBC, lymphocyte, and eosinophil concentrations in various species; and many other differences in hematologic findings between life stages, sexes, and reproductive status in a wide variety of species.[13,59]

HEMATOLOGIC DISEASE

Quantitative and qualitative hematologic assessment contributes to health screening of lizards, crocodilians, and tuataras in the same manner as in other vertebrate

species. Clinically significant findings can include anemia, inflammation, atypical cell populations, and infectious agents. A working knowledge of species-specific or related genera-specific findings, natural history, and literature review is necessary for correct interpretation.

Anemia

The response of the erythrocytic lineage to disease manifests in lizards similarly to other vertebrates, though specific disease conditions are generally less well described in comparison to mammals (see Section III). The major pathologic mechanisms of anemia (common) and erythrocytosis (rare) are conserved across taxa. Increased erythroid precursors most commonly reflect a regenerative response (Figure 132.4). In lizards, this response decreases mean cell hemoglobin concentration and, unlike in mammals, may also decrease mean cell volume due to the smaller size of precursor versus mature RBCs. Mildly increased polychromasia due to precursor circulation (rubricytosis) is reported with ecdysis. Dyserythropoiesis with dysplastic maturation evident in circulating blood is uncommon as a primary pathology. However, dysplastic changes, including anisocytosis, binucleation, and mild poikilocytosis, may be seen with intense regeneration, or may be secondary to failure of erythropoiesis in pathologies such as inflammation, neoplasia, and sepsis. Fusiform to pyriform RBCs occur commonly in low numbers as preparation artifacts. Reticulocyte enumeration can be used to assess regenerative response. Reticulocytes are identified by a ring of aggregated reticulum encircling the nucleus with new methylene blue vital staining. Polychromatophils are expected to stain as reticulocytes, but, due to a maturation spectrum, not all reticulocytes are polychromatophilic with routine stains. The regenerative response of lizards is sluggish compared to that in mammals. Anemia persisting more than 1 month without regeneration is considered nonregenerative.[60]

Inflammation

Leukocyte responses in disease are comparable to those seen in other vertebrates, though cellular responses are more affected by extrinsic factors and may therefore seem less predictable, e.g., low temperatures are associated with lower WBC counts. The most common WBC shift is heterophilia due to inflammation. Overwhelming inflammation can cause heteropenia. Established inflammation can result in monocytosis. Other shifts include responses to physiologic states and stressors (e.g., catecholamines, glucocorticoids, reproductive cycles), infectious agents (e.g., parasitism causing eosinophilia), metabolic derangements (e.g., lymphopenia with starvation), and a variety of pathologic and extrinsic variables (neoplasia, toxins, environment, etc.).

With significant bone marrow stimulation, left shift of the heterophil lineage is characterized by progressively more immature precursors (bands, myelocytes, metamyelocytes, promyelocytes, and myeloblasts) displaying increasingly immature features: subtly larger cell and nuclear size, abnormal nuclear lobulation, increased cytoplasmic basophilia, immature (primary) or pleomorphic cytoplasmic granules, and a higher nuclear to cytoplasmic ratio (Figure 132.4). In species with lobulated mature nuclei, immature cells have progressively less segmentation (from band-shaped to indented to rounded). In species with round mature nuclei, changes with immaturity are more subtle and may be difficult to assess (i.e., larger nuclear size, looser chromatin).

Toxic changes signifying accelerated heteropoiesis are common in cases of severe inflammatory stimulation. These morphologic changes represent alterations in normal cell development in the bone marrow and are characterized by a spectrum of increased cytoplasmic basophilia, cytoplasmic vacuolation (foaminess), reduced and/or more variable cytoplasmic granules (degranulation), and increased abnormal, often purple or blue, cytoplasmic granules (Figure 132.4). Both the degree of the toxic morphology and proportion of cells affected provide insight regarding severity. Artifactual heterophil degranulation is distinguished from toxic change by the absence of other morphologic alterations characteristic of toxicity (e.g., cytoplasmic basophilia, abnormal granulation).

The presence of circulating bacteria is a critical hematologic finding (Figure 132.4). Care must be taken to exclude contamination from sampling or staining. With this exception of in vitro artifact, phagocytized bacteria signify bacteremia and typically a poor prognosis. Infection with extracellular spiral bacteria was recognized in a rhinoceros iguana (*Cyclura cornuta*),[57] and a molecularly confirmed *Helicobacter sp*. causes infection and disease in Grand Cayman blue iguanas (*Cyclura lewisi*) and introduced green iguanas.[7] A chlamydial infection with classic chlamydial bodies in monocytes is reported in a flap-necked chameleon (*Chamaeleo dilepis*) with concurrent poxviral infection.[35]

Neoplasia

Although infrequent by mammalian standards, hematopoietic neoplasms have been identified as one of the most common malignancies in lizards.[18,33] Of these, lymphoid neoplasms are most common. Leukemias are recognized by leukocytosis and either atypical cell morphology (acute leukemia) or well-differentiated morphology (chronic leukemia). Acute leukemias have been more commonly reported (Figure 132.4). Species diagnosed with lymphoid leukemia include the Egyptian spiny-tailed lizard (*Uromastyx aegypticus*), Savannah monitor (*Varanus exanthematicus*), green tree monitor (*Varanus prasinus*), inland bearded dragon (*Pogona vitticeps*), Gila monster, Northwestern alligator lizard (*Elgaria* [*Gerrhonotus*] *coerulea principis*), and a chameleon.[18,19,26,56] Myeloid leukemias affecting bearded dragons, unspecified geckos,[18,24] and unclassified leukemias are also reported.[23] Diagnostic classification of hematopoietic proliferations follows the same scheme as in mammals, though ancillary testing (immunochemistry, flow cytometry) is more limited and infrequently employed.[19]

Hemoparasites and Inclusions

Many commonly identified RBC inclusions in lizards are of no clinical significance, except to recognize and differentiate from pathology (Figure 132.3). These include: pleomorphic refractile cytoplasmic foci attributed to staining artefact; small, discrete, round, basophilic cytoplasmic inclusions corresponding ultrastructurally to degenerate organelles;[6] and rounded to angular pale crystalline inclusions linked ultrastructurally to polymerized Hgb.[60] The latter inclusions have been documented in both healthy and ill iguanas as well as other reptiles, and may increase with disease, but are not of clear clinical significance.[28,58] Larger intraerythrocytic distinct clear rounded vacuoles have occasionally been observed in ill reptiles. Ultrastructural assessment in a septic alligator showed the vacuoles to be empty or contain amorphous particles. An association with septicemia is proposed.[53]

Hemoparasites are a relatively common finding in lizards, particularly in wild or wild-caught animals, and also occur in crocodilians and tuataras. The clinical importance of hemoparasites must be interpreted considering both intrinsic factors (e.g., immunosuppression, concurrent disease) and extrinsic factors (e.g., husbandry, temperature, season, age).

Lizards are host to a variety of protozoal parasites. Hemogregarine gamonts are common incidental findings in the cytoplasm of low numbers of RBCs in health. The gamonts are oval elongated or plump (sausage-shaped) with pale basophilic to colorless cytoplasm and a small basophilic to purple nucleus. These variably displace the nucleus and contain no heme pigment. Genera include *Hepatozoon*, *Hemogregarina*, *Karyolysus*, and others,[42,60] but definitive identification by molecular or other methods is pursued in relatively few cases and they are often reported simply as "hemogregarines." These protozoa are host-adapted and usually considered clinically incidental; statistical associations of hemoparasitism with hematologic changes, such as increased circulating erythroid precursors or monocytosis, have been occasionally reported,[3,8] but are not consistent findings and may reflect other host factors or comorbidities. *Hepatozoon tuatarae* infestation in tuataras varied with body size and tick burden.[22]

Hemococcidia are eimerial parasites that are considered potentially pathogenic. The life stages of hemococcidia (e.g., *Schellackia*, *Lainsonia*, *Lankesterella sp*.) include sporozoites in the peripheral blood and schizogony in the intestine.[45] Zoites infect WBCs or RBCs, and are rounded to oval structures with colorless cytoplasm and a small nucleus (resembling systemic *Isospora* (*Atoxoplasma*) of birds).

Hemosporida (e.g., *Plasmodium*, *Hemoproteus*, *Saurocytozoon sp*.) are considered potential pathogens, though light infections may be clinically tolerated. The life cycles and related morphologies recognized on blood films in this group vary and resemble corresponding organisms in birds. *Plasmodium sp*. undergo both sexual and asexual replication in circulation, recognizable as zoites (merozoites, trophozoites), gamonts, and schizonts (meronts). While there are no *Plasmodium* species described from crocodilians or tuatara, malaria in lizards is common and often apparently subclinical, but may impact performance and therefore population health.[55] The intraerythrocytic stages of *Hemoproteus sp*. are limited to transient zoites and predominant gamonts. Hemoglobin breakdown pigment (hemozoin) is described in both *Plasmodium* and *Hemoproteus sp*. infections. By contrast, the circulating intracellular gamonts of *Saurocytozoon sp*. closely resemble *Leukocytozoon sp*. of birds, causing cellular distortion and lacking heme pigment.

Infection by hemoflagellates such as *Trypanosoma* or *Leishmania sp*. appears to be subclinical. Trypomastigotes are morphologically comparable to those seen in other taxa, with a kinetoplast and undulating membrane. *Leishmania sp*. can be recognized by extracellular flagellated promastigotes and/or amastigotes in circulating cells, including RBCs and thrombocytes. A kinetoplast is described, but appears variably apparent on light microscopy.[60]

Sauroplasma (*Aegyptianella*, *Sauroplasma sp*.) are piroplasmid parasites characterized by small round or ring-shaped inclusions in RBCs (resembling those of *Babesia* or *Theileria sp*. in mammals).[62] They must be differentiated from common nonparasitic inclusions and pathogenicity is uncertain.

Microfilaria are commonly identified organisms in lizard blood and a good reminder for low-magnification screening (Figure 132.4). A variety of filariid genera are reported, such as *Foleyella* in chameleons and agamas, but in many cases the parasites are not definitively identified.[49] The infestations are often subclinical, but a high parasite burden, inopportune anatomic location of adult worms, and other factors can lead to clinical disease.[34,41]

A few viral infections in lizards induce recognized hematologic findings. Intraerythrocytic iridoviruses have been documented in chameleons, agamas, skinks, geckos, bearded dragons, and other species.[25,52,61] Thought to be possible piroplasms (pirhemocytonosis) in early reports, infected RBCs contain variably sized and pleomorphic eosinophilic or basophilic cytoplasmic viral inclusions that enlarge as infection progresses, and may be accompanied by poorly staining globoid structures (vacuole, albuminoid body).[25,52,61] Iridoviral pathogenicity is incompletely understood and may be dependent on temperature, among other factors; polychromasia, hypochromasia, and anemia have been reported in some cases.[25] Poxviral infection, seen as eosinophilic to blue-green staining pleomorphic intracytoplasmic inclusions, has been reported in circulating monocytes, lymphocytes, and thrombocytes of free-ranging flap-necked chameleons.[35] Pale basophilic intracytoplasmic inclusions ultrastructurally linked to rhabdovirus infection have also been reported in a caiman lizard (*Dracaena guianensis*) with unknown clinical significance.[66]

Although not associated with inclusions, Agamid adenovirus-1, which is overrepresented in bearded dragons and associated with variable clinical signs, is also frequently associated with leukocytosis and anemia in clinically affected lizards (K. Harr, personal communication).

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CHAPTER 133

Hematology of Serpentes

LAURA J. BLACK and MARJORIE BERCIER

Blood Collection and Preanalytical Error
Erythrocytes
Leukocytes
Thrombocytes and Coagulation
Reference Intervals
Bone Marrow and Collection
Hematologic Disease
Anemia
Physiologic Changes
Inflammatory Changes
Viral Disease
Coagulopathy
Hemoparasites
Neoplasia

Acronyms and Abbreviations

ASVCP QALS, American Society for Veterinary Clinical Pathology Quality Assurance and Laboratory Standards; CNS, central nervous system; IBD, inclusion body disease; IUCN, International Union for Conservation of Nature; MCHC, mean corpuscular hemoglobin concentration; RT-PCR, real-time polymerase chain reaction.

Snakes are part of the class Reptilia, order Squamata, and infraorder Serpentes. The idea that snakes are a group distinct of lizards is a common misconception. Snakes are part of the fourth divergent clade of squamate evolution (after geckos, skinks, plated lizards, night lizards and teiids, lacertids, amphisbaenids), called Toxicofera for their shared anatomical trait of venom glands.[36] Along with snakes, iguanids, agamids, chameleons, monitors, helodermatids, and anguids are also included in Toxicofera. Therefore, a ball python (*Python regius*) is more closely related to, and a better model for, a bearded dragon (*Pogona vitticeps*) than a prehensile-tailed skink (*Corucia zebrata*). More than 2900 snake species are currently recognized, inhabiting all continents except Antarctica. As of 2020, the International Union for Conservation of Nature's (IUCN) Red List of Threatened Species listed 126 snake species as endangered, and 63 as critically endangered.[16]

By understanding their taxonomy, one can better evaluate individual species' hematologic values and morphologic changes on a peripheral blood film. Additionally, understanding the biology of these ectotherms is important not only for the individual patient's well-being, but for insight into environmental changes associated with global climate warming and emergence of disease. Snakes, just like any reptiles, are often the victim of the human–animal interface crisis and are harmed by illegal trade, poaching, introduced and invasive species, habitat destruction, emerging infectious diseases, and global warming.

BLOOD COLLECTION AND PREANALYTICAL ERROR

Venipuncture sites in snakes are few and include the ventral coccygeal vein, the jugular veins, and the heart. Because of the close anatomic association of lymph and blood vasculature, lymphatic fluid contamination may occur during blood sampling. This can result in dilution of the sample, resulting in decreased PCV, WBC counts, and possibly low thrombocyte counts. Lymphocytes may appear significantly increased and caution should be used to not overinterpret as a lymphoid leukemia. Unlike other species, puncture of the three-chambered heart, with the intent of sampling the single ventricle, is often done in the awake animal by experienced reptile

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practitioners. With cardiocentesis, pericardial fluid may be concurrently sampled and can also have a dilutional effect. As with any sample, if the findings and hematologic values do not correlate clinically, a repeat sample should be obtained and analyzed prior to diagnostic interpretation.

Hemolysis definitively due to K-EDTA has not been documented in snakes at this time and several species including the king cobra (*Ophiophagus hannah*), eastern diamondback rattlesnake (*Crotalus adamanteus*), Burmese python (*Python bivittatus*), and rat snake (family Colubridae, subfamily Colubrinae) have not shown significant hemolysis when preserved in EDTA.[1,3,13,26] Preservation with a minute volume of formalin in a Natt–Herrick stain preparation may decrease clumping of all cells and provide improved preservation, though this has not been tested with snake species. The procedure was originally done to preserve fish hematologic samples[2] (see Chapter 128). Any refrigerated whole blood samples should be warmed to room temperature (20 °C) prior to making blood films or mixing with reagents for cell counts or other analysis.[30]

ERYTHROCYTES

Mature, oval erythrocytes have a similar morphology to other reptile species with a central, irregularly oval, condensed nucleus and are larger than their mammalian counterparts. For example, yellow rat snake (*Pantherophis alleghaniensis,* formerly *Elaphe obsolete quadrivitatta*) RBC dimensions are 18 × 10 µm and side-striped palm viper (*Bothriechis lateralis*) RBC dimensions are 22 × 12 µm, while a dog (*Canis familiaris*) RBC averages 7 µm in diameter.[30] In one study of wild spectacled cobras (*Naja naja*), measured mean cell volume (MCV) was 300–400 fL.[9] Hemoglobin concentration (Hgb) and PCV tend to be lower than mammalian species and the larger MCV is inversely proportional to RBC count.[5] Significant variation in PCV in healthy snakes may be due to species, wild-caught versus captive-bred populations, seasonality, hibernation, reproductive status, ambient temperature, ecdysis, and sex.[10,19,26] Additionally, ophidian RBC life span is longer than mammalian erythrocytes, with averages up to 600–800 days.[29] Unlike other reptiles, erythroplastids (anucleate RBCs) are rarely found in snakes and have no known clinical significance.[28] As in turtles and other reptile species, RBCs may contain small, round or irregular, deeply basophilic cytoplasmic inclusions that have been determined to be degenerate organelles in most cases. These degenerate organelles also have no known clinical significance and must be differentiated from viral inclusions and breakdown products found in septic animals[5,28] (Figure 133.3d).

Low numbers of polychromatophils, rubricytes, and mitotic figures are normal in young snakes or in snakes undergoing ecdysis[5,29] (Figure 133.1m–o). Polychromatophils are smaller and have smoother chromatin than mature RBCs. These cells enlarge as their chromatin condenses during maturation, which is opposite of their mammalian counterparts.[28] Reticulocytes (polychromatophils) can be quantified in peripheral blood films with new methylene blue staining, which results in basophilic precipitated RNA that rims the nucleus. Samples from healthy snakes typically have <5% reticulocytes.[5] Rubricytes may be quantified on Romanowsky-stained smears to assess erythroid regeneration and are rare (<3 rubricytes/100 WBC) in healthy snakes.

LEUKOCYTES

The snake heterophil has a similar function to the neutrophil in innate immunity and phagocytic activity, though function may be decreased in comparison to mammalian neutrophils in that peroxidase staining is weaker to absent in snakes.[1] Heterophils are found in lower numbers than lymphocytes and often azurophils in some healthy snakes.[19,21,25,26] Heterophils (10–23 µm) are smaller in size than azurophils, but larger than lymphocytes.[28] Heterophils are round with colorless cytoplasm which contains few to many clear to pale pink-orange (heterophilic) rod-shaped granules (Figure 133.1a–c). These granules often occupy the majority of the cytoplasm, but the round nucleus is usually still visible. The nucleus has the same condensed chromatin pattern seen in mature mammalian neutrophils, but in mature cells, the nucleus is round to oval lacking elongation or segmentation. Immature heterophils, which correlate with left shifting indicating accelerated bone marrow release, cannot be readily identified using standard diagnostic light microscopy.

Upon ultrastructural evaluation of eastern diamondback rattlesnake (*C. adamanteus*) heterophils, partially or completely degranulated heterophils were identified in addition to fully granulated heterophils.[1] On Romanowsky stains, these partially granulated heterophils have colorless to slightly gray or blue, apparently vacuolated cytoplasm. This is thought to be a preanalytical artifact of the collection/storage process as these samples are commonly collected into lithium heparin anticoagulant, though toxic change is also possible.

Lymphocytes are the predominant leukocyte in circulation in most snake species.[6,19,21,25,26] Their nuclei may be round, oval, triangular, or pleomorphic containing condensed to lacy chromatin and usually no distinct nucleoli. Lymphocytes have a scant to moderate amount of typically pale basophilic cytoplasm and sometimes exhibit cytoplasmic blebs (Figure 133.1d–f). Small lymphocytes (8–10 µm) are often smaller than an RBC, with intermediate and large lymphocytes (12–18 µm) slightly larger. Small and intermediate sized lymphocytes predominate, but a few large lymphocytes may be found, particularly with antigenic stimulation. Some may contain a few small magenta granules, consistent with granular lymphocytes. Numbers of granular lymphocytes will increase with disease. When antigenically stimulated, lymphocytes may exhibit deeply basophilic cytoplasm and sometimes a perinuclear clearing (Golgi

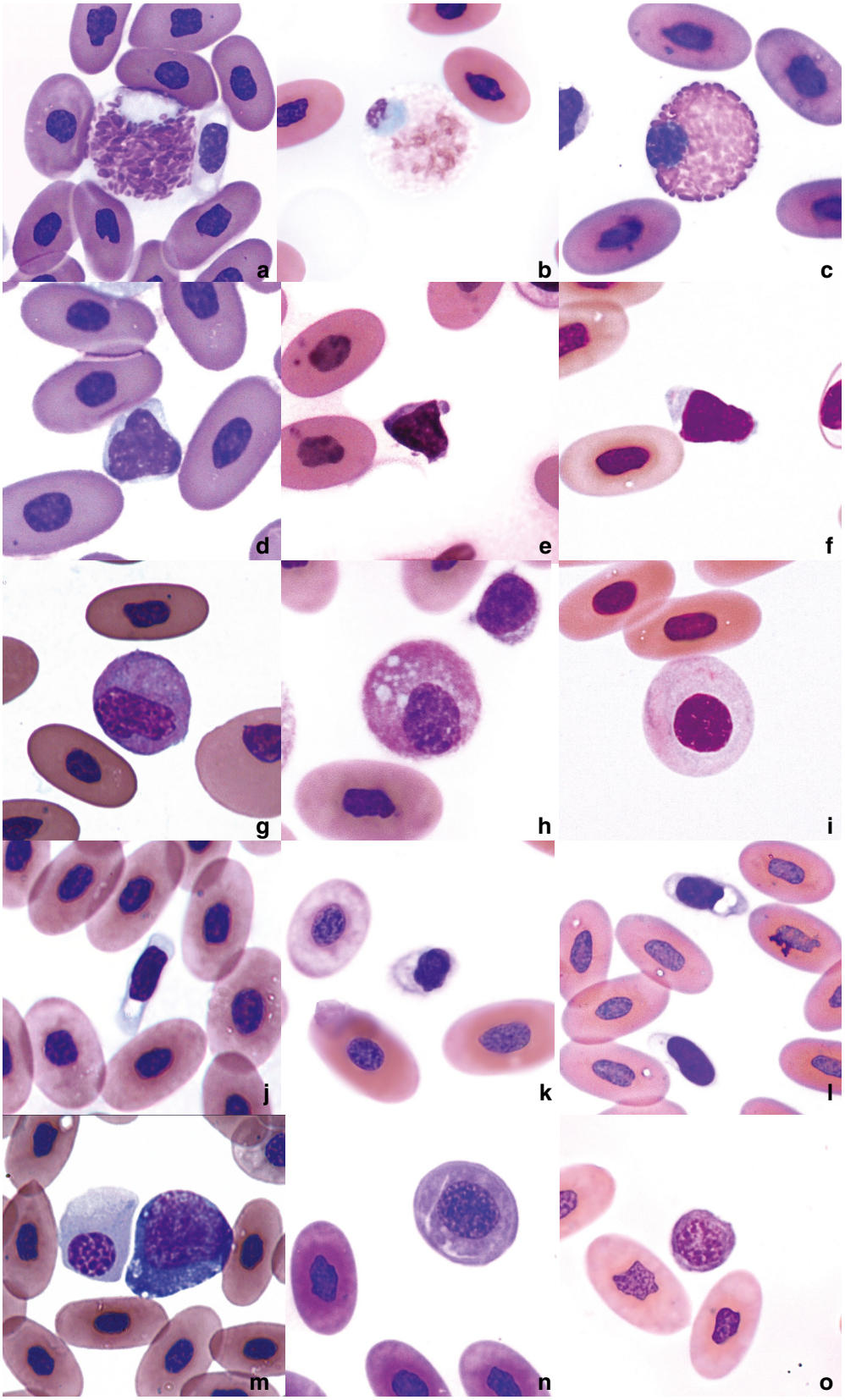


FIGURE 133.1 Normal blood cells from selected snake species. Wright–Giemsa stain. King cobra (*Ophiophagus hannah*): (a) Heterophil; (d) Small lymphocyte; (g) Azurophil; (j) Thrombocyte; (m) Rubricyte (left) and probable prorubricyte (right). Rattlesnake (*Crotalus sp.*): (b) Heterophil; (e) Small lymphocyte; (h) Mildly reactive azurophil; (k) Thrombocyte; (n) Rubricyte. Ball python (*Python regius*): (c) Heterophil; (f) Small lymphocyte; (i) Azurophil; (l) Two thrombocytes; (o) Rubricyte. (**Source:** King Cobra and rattlesnake images are courtesy of Drs. Nicole Stacy and Ball python images courtesy of Kendal Harr.)

zone). Plasma cells or, rarely, Mott cells can be seen in peripheral circulation with antigenic stimulation and inflammation[32] (Figure 133.2d).

Azurophils are large, monocytoid circulating immune cells which may predominate in individuals with an inflammatory leukogram.[19,21,25,26] While they are monocytoid in appearance, they stain strongly with peroxidase and often lack positive staining with naphthyl butyrate esterase, making their cytochemical characteristics more similar to the mammalian neutrophil.[1] Cytochemically, snake azurophils commonly stain positive for benzidine peroxidase, Sudan Black B, and periodic acid–Schiff (PAS), though there is some variation in the literature.[1] The azurophil lineage is not yet clearly established. These leukocytes are most prominently found in snakes, compared with other reptilian species. Azurophils are round or oval with a moderate to large amount of basophilic cytoplasm that contains a significant diffuse dusting of azurophilic (fine, magenta) granules. The nucleus is round or oval and often eccentric with clumped chromatin, but not as condensed as that seen in granulocytes (Figure 133.1g–i). During inflammation, azurophils may become vacuolated, phagocytic, or even binucleated (Figure 133.2f,g).

Given that azurophils are cytochemically different from monocytes and therefore functionally different, they should be enumerated separately from monocytes on a differential cell count, if present. Monocytes are rarely found in circulation in snakes, and most commonly not counted in differential cell counts from healthy snakes.[1,6,19,21,25,26] They are similar in appearance to azurophils except lacking the fine magenta granulation and have similar morphologic characteristics to those found in mammals. When monocyte-like cells were identified in the blood of yellow rat snakes, they were assumed to be degranulated azurophils[3] and were not definitively identified in eastern diamondback rattlesnakes[1].

Eosinophils are absent in most snake species studied.[1,6,29] Presence in individual species should be confirmed with cytochemical stains and ultrastructural evaluation. These granulocytes are larger than heterophils with a rounded or sometimes elongated nucleus and condensed chromatin. They have pale blue-gray cytoplasm that contains many rounded or plump bright pink to salmon color granules which sometimes appear refractile. Eosinophils may not always have eosinophilic granules. Since eosinophils are quite rare in snakes (if present at all in certain species), their function and kinetics have not been well defined.[26]

Basophils may not be seen in some animals and are typically found in low numbers.[1,26] Increased basophil numbers may be found in diseased animals. They have the same cytologic appearance as mast cells, but as mast cells reside within solid tissues, the circulating cells are identified as basophils. Basophils are small and round with a moderate amount of colorless to pale blue-gray cytoplasm which contains many dark purple round granules that typically obscure the nucleus. Some cells may be degranulated or nonstaining (especially if stained with an aqueous Romanowsky stain), and the cytoplasm appears vacuolated. In these cells, the nucleus is readily visible and round or oval with condensed chromatin (Figure 133.2j–l). Cytochemically, basophils are similar to mammalian basophils and have been reported to stain PAS-positive.[1,26]

THROMBOCYTES AND COAGULATION

Thrombocytes are the equivalent of mammalian platelets, with some fundamental differences. Thrombocytes are whole cells which contain a condensed nucleus and mature from a distinct cell line with a thromboblast progenitor, not a megakaryocyte.[28] Thrombocytes are often easy to differentiate from lymphocytes in most snake species, as they are elongated or oval with colorless to pale basophilic cytoplasm and sometimes contain single or multiple cytoplasmic vacuoles, especially when activated (Figure 133.1j–l). They may exhibit cytoplasmic blebbing. Thrombocytes readily clump in heparinized samples, making them difficult to quantify, and K-EDTA is preferred for thrombocyte evaluation. On peripheral blood films or in hemocytometer preparations, thrombocytes may aggregate and comparison with the morphology of clumped thrombocytes may aid in differentiation of lymphocytes from individual thrombocytes (Figure 133.2n). Thrombocytes can become phagocytic when activated.[28]

Coagulation in snakes is considered similar to other reptiles; however, there is minimal literature regarding snake species specifically.

REFERENCE INTERVALS

Published ophidian hematologic reference values performed according to American Society for Veterinary Clinical Pathology Quality Assurance and Laboratory Standard (ASVCP QALS) guidelines are currently limited. Hematologic reference intervals can be significantly influenced by factors such as species, age, gender, reproductive status, season, ambient temperature, geographic range,[35] restraint method,[3] and ecdysis. In one study that compared hibernation and seasonal factors of captive and wild viperid snakes, no differences were observed in PCV or total and differential WBC counts in pre- versus posthibernation samples.[10] However, given that snakes do not drink or eat during hibernation, changes due to dehydration are possible. Hematologic values can vary between captive versus wild-caught animals of the same species. Underlying etiologies may include parasitism in wild animals, and stress and husbandry issues in captive animals. In a study comparing hematologic values of wild-caught versus captive-bred king cobras (*O. hannah*), captive-bred individuals had higher PCV (mean 33% vs. 20%), Hgb concentration (10 g/dL vs. 6.5 g/dL), and lymphocyte counts, while heterophil and azurophil counts were lower.[26] The captive cobras in this study also had lower prevalence of *Hepatozoon* infestation as detected by light microscopy.

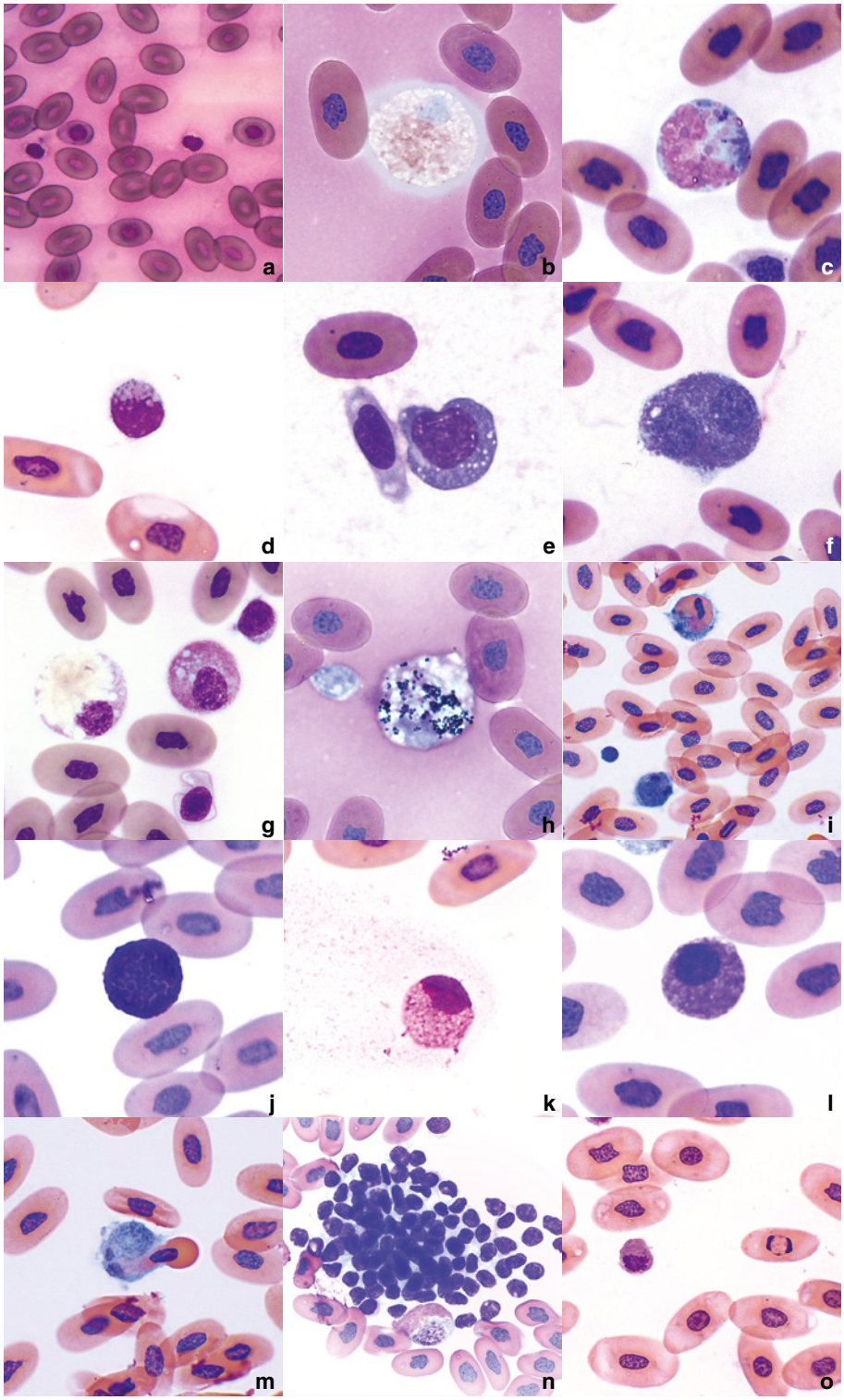


FIGURE 133.2 Artifact and activated cells in selected snake species. Wright–Giemsa stain. (a) Heparin binds to background protein resulting in a dark pink to purple haze that distorts cell morphology; (b) Ethiopian viper (*Bitis parviocula*): Heparin artifact and a heterophil; (c) Emerald tree boa (*Corallus caninus*): Moderately toxic heterophil; (d) Ball python (*Python regius*): Mott cell; (e) King cobra (*Ophiophagus hannah*): Thrombocyte (left) and atypical lymphocyte (right); (f) Emerald tree boa: Binucleated azurophil typical of marked reactivity; (g) Rattlesnake (*Crotalus sp.*): Probable toxic heterophil (left) and a reactive azurophil (right); (h) Ethiopian viper (*Bitis parviocula*): Melanomacrophage; (i) Ball python (*Python regius*): Siderophage (lower left) and erythrophagia consistent with acute and chronic hemorrhage; (j) Ball python: Basophil; (k) Ball python: Basophil with nonstaining granules, Quik Stain; (l) Emerald tree boa: Basophil; (m) Ball python: Active erythrophagia; (n) Emerald tree boa: Microclot containing clumped activated thrombocytes and a heterophil; (o) Ball python: Mitotic figure in a probable RBC (left) and a probable nuclear inclusion (virus unidentified). (**Source:** Some images are courtesy of Drs. Nicole Stacy and Kendal Harr.)

It is not possible to differentiate disease state from housing and husbandry issues in many of these comparative studies.

Reference intervals are available through many sources including online databases, but caution in their medical use is recommended, as many neither have outliers removed, use incorrectly applied statistics, nor account for partitioning factors. Ideally, reference intervals should be developed utilizing the ASVCP QALS guidelines (https://www.asvcp.org/page/QALS_Guidelines). Concurrent sampling from a healthy individual with similar signalment (i.e., age, sex, housing/ambient temperature, restraint, reproductive status, etc.) may be compared to the suspected ill animal; however, this is not a replacement for medical reference intervals. Without reliable reference intervals, CBC interpretation can be challenging and potentially result in under- or overinterpretation when used without caution. Hematologic values for the same patient over time may also be used to monitor progression of disease or response to therapy.

Editor's note: In order to capture a perceived lymphocyte predominance in snakes, reference interval generation for ball pythons seen at a veterinary clinic was attempted prior to this chapter's publication. Upon review of history and follow-up clinical data, it was found that over half of the 28 pythons, originally included as they had been listed at the time of sampling as apparently healthy, were dead within 3 years. Additional pythons were lost to follow-up. Eight of the dead animals had confirmed arenavirus, nidovirus, or other disease. Reference interval generation was therefore suspended.

BONE MARROW AND COLLECTION

Hematopoietic cells are synthesized in the marrow spaces of adult snakes similarly to mammalian species. Snake bone marrow is typically quite fibrous and not readily amenable to fine-needle or core biopsy collection. There is minimal marrow in the ribs, leaving the vertebra as the only sampling site. If a sample is collected, an acellular or hypocellular sample should not be overly interpreted. Confirmed successful techniques for antemortem sampling have not been reported.

HEMATOLOGIC DISEASE

Anemia

Causes of anemia are the same for snakes as other vertebrate species and include hemorrhage, hemolysis, or decreased production.[5] Because of the long RBC life span, regenerative processes also take longer to develop and become apparent clinically. Signs of regeneration include increased polychromasia and erythroid precursors including rubricytes and may be as immature as rubriblasts[30] (Figure 133.1m). Mitotic figures within polychromatophils can be found in cases of active regeneration[28] (Figure 133.2o). In one case report, a corn snake (*Elaphe guttata*) was found to develop signs of regeneration approximately 20 days after mild intraoperative hemorrhage.[12] Erythrophagia after surgical and other trauma is seen with some frequency in practice (Figure 133.2i,m). Generally, a regenerative response may take up to several weeks[30] and can be dependent on other factors such as ambient temperature, concurrent inflammation, immunosuppression, or other stressors. Repeat manual evaluation of peripheral blood films and PCV is recommended to monitor the erythron, and if sufficient time has passed, a nonregenerative anemia may be diagnosed. Anemia of chronic disease is likely the most common cause of nonregenerative anemia in snakes and can develop from a number of factors including poor environment especially including suboptimal temperature, inappropriate diet, chronic infection, neoplasia, etc.

Hypochromasia, associated with decreased mean corpuscular hemoglobin concentration (MCHC), may be seen with iron deficiency or chronic inflammation. Iron deficiency can develop secondary to the same etiologies as in other species including malabsorption from gastrointestinal disease, inappropriate diets (iron-deficient), blood loss, and chronic inflammation. Basophilic stippling is rare and may point to a regenerative response such as seen in posthibernation reptiles. It is also reported in patients with lead toxicosis and iron deficiency.[3,5] Some forms of poikilocytosis will be seen secondary to similar disease processes as established in mammalian species. For example, schistocytes occur with conditions that result in erythrocyte fragmentation. Increased numbers of RBCs with fusiform or teardrop shapes have been associated with septicemia or chronic infectious disease.[28]

Physiologic Changes

Noninflammatory, physiologic causes of a heterophilia include gravidity, excess endogenous or administration of exogenous glucocorticoids, summer seasonality, and free-living status. Lymphopenia is expected in response to glucocorticoids, though this may be difficult to detect.

Inflammatory Changes

A standard inflammatory leukogram is usually represented by a heterophilia with or without toxic change, and azurophilia. Etiologies are many and commonly include bacterial or parasitic infections, tissue injury, or necrosis.[4,28] Heterophils will exhibit toxic morphologic change, related to accelerated release from bone marrow and activation in the peripheral blood.[5,28] These toxic changes consist of (in order of increasing severity): degranulation or partial degranulation with cytoplasmic basophilia, foamy vacuolation, retention of cytoplasmic round magenta primary granules, color change in ragged, activated lysosomes and vacuoles, and sometimes giantism (Figure 133.2c). The degree of toxicity may reflect disease severity; however, snakes with

bacterial infection may present with no evidence of toxic change.[28] Heteropenia may also be seen with acute, overwhelming inflammation. Often toxic changes are seen, but may be absent very early on.

Azurophilia, and less commonly melanophages and hemosiderophages, may be seen with inflammation or hemorrhage[11,32] (Figure 133.2h). Azurophilia in snakes is more commonly associated with acute as opposed to chronic inflammation. Azurophils can demonstrate phagocytic activity and were found in higher numbers in *Hepatozoon*-infected king cobras than in their *Hepatozoon*-negative counterparts. The azurophils contained phagocytosed-free *Hepatozoon* gamonts, documented by electron microscopy.[26] *Chlamydia* inclusions have also been identified in circulating phagocytes of emerald tree boas with pneumonia and confirmed by PCR.[28] Significant, though nonspecific, changes in WBC counts were found in corn snakes (*Pantherophis guttatus*) experimentally infected with three different genogroups of ferlaviruses, which are associated with highly pathogenic outbreaks of respiratory disease.[23] Total WBC, lymphocyte, and azurophil counts were significantly greater than at baseline starting at day 16 postinfection, whereas heterophilia was identified on days 28 and 49. Basophilia has also been reported in cases of viral infection and parasitism.[5]

Viral Disease

Inclusion body disease (IBD) is one of the most important diseases of captive boid snakes (families Boidae and Pythonidae) worldwide.[7] Antemortem IBD diagnosis can be challenging. In addition to the animal's signalment, history, and clinical signs, inclusion detection on blood smears may be diagnostic. Intracytoplasmic inclusions are characteristically found in lymphocytes of infected snakes and are diagnostic for IBD. In lymphocytes and other peripheral blood cells (heterophils, azurophils, thrombocytes, RBCs),[7,17] oval or round inclusions are homogenously pale blue to slightly aqua in color (Figure 133.3c–e). They vary in size and may take up the majority of the cytoplasm, pushing the nucleus to the periphery in a half moon shape. They can sometimes be difficult to differentiate from the background cytoplasm and care must be taken when evaluating peripheral blood films from snake species, even in patients with absent clinical signs (Figure 133.3a,b). A buffy coat preparation may aid in diagnosis. Not all IBD-positive snakes will display inclusions in peripheral blood cells and additional diagnostics such as RT-PCR or histopathology may be confirmatory. The DeRisi Lab at University of California San Francisco has ongoing research on IBD and the novel snake arenavirus[30] (http://derisilab.ucsf.edu/index.php?news=125). Affected snakes can have leukocytosis and lymphocytosis.[7] Additional information on antemortem and postmortem diagnosis of IBD is available elsewhere including details on histologic appearance and distribution of lesions.[20,22,24]

Boid snakes afflicted with IBD show high morbidity and mortality and over 10 boid species have been reported to be infected. IBD has also been suspected in a few viperid and colubrid species,[17] but has not been confirmed.[7,17] Clinical presentation spans from asymptomatic to severe neurological disease and death. The duration of latent infections varies between pythons and boas and remains unknown in many species.[7] After the onset of clinical signs, pythons will generally succumb from fatal neurological disease within a few weeks, whereas boas will mostly die within months.[14,20,34] Clinical signs include incoordination, disorientation, torticollis, disequilibrium, opisthotonus, loss of righting reflex, anisocoria, and flaccid paralysis.[7,24] Intermittent regurgitation followed by anorexia has been predominantly described in *Boa sp*.[7,24] Although little is known about the clinical course of the disease, IBD appears to be invariably fatal.[34] As a probable consequence of immunosuppression, affected animals usually die from secondary infections and septicemia with encephalitis, pneumonia, hepatitis, enteritis, or osteomyelitis, and from neoplastic processes, such as lymphoma and leukemia.[14,27]

Iridoviral infections (aka snake erythrocyte virus and formerly pirhemocytonosis) are frequently reported in chelonians and amphibians and less commonly in Serpentes. The family Iridoviridae is composed of two genera, Ranavirus and Iridovirus. A third group of iridoviruses known to infect reptiles are erythrocytic iridoviruses.[38] Long thought to be caused by protozoal hemoparasites (*Toddia* and *Pirhemocyton*), eosinophilic intraerythrocytic inclusions were first documented to be of iridoviral origin by electron microscopy in a fer-de-lance viper (*Bothrops asper*) and later confirmed by sequencing from a diseased peninsula ribbon snake (*Thamnophis sauritus sackenii*) in Florida.[18,20] Severe anemia is the hallmark of erythrocytic iridoviral infections. The fer-de-lance viper presented with macrocytic and hypochromic anemia, polychromasia, and immature circulating RBCs left shifted to the rubriblast stage.[18] Two types of intracytoplasmic inclusions can be seen in the erythroid series. The first consists of punctate, granular pink to dark purple (acidophilic) aggregates ultrastructurally consistent with iridoviral particles. The second inclusion type is a translucent hexagonal crystal, suspected to be lipid and protein representing cellular and viral by-products. In the fer-de-lance viper, 14% of erythroid cells contained both inclusion types, 0.5% had iridoviral particles only, 0.5% had crystalline material only, and 85% were unaffected.[18] In the ribbon snake, 60% of RBCs were hypochromatic, with 95% of these displaying either inclusion type.[20]

Coagulopathy

Causes of thrombocytopenia are more likely due to peripheral consumption than decreased production.[5] If a thrombocytopenia is suspected, correlation with clinical findings, thorough evaluation of a peripheral blood film, and gross examination of the collection tube for clots are recommended. Repeat sampling may also be indicated. Immature thrombocytes are larger with less-condensed chromatin. An increased number of

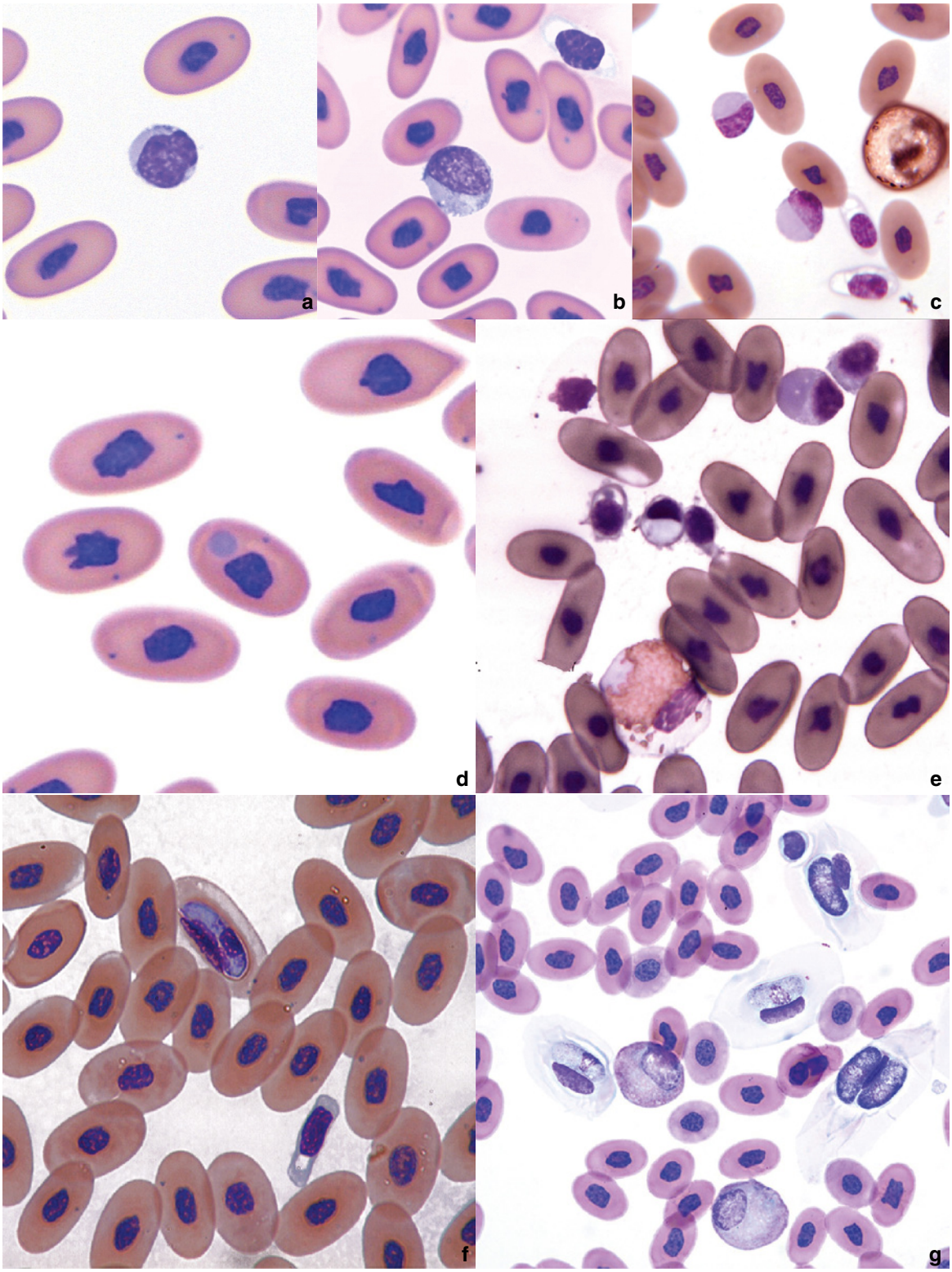


FIGURE 133.3 Infectious agents found selected snakes. Wright–Giemsa stain. Inclusion body disease caused by arenavirus may be diagnosed using hematology. The inclusions may be small and indistinct, but the large inclusions that push the nucleus into a half moon shape are pathognomonic. Boa constrictor (*Boa constrictor*) (note: all inclusions were not found in the same animal): (a) Small cytoplasmic inclusion in a lymphocyte; (b) Arenavirus inclusion is moderately sized and flattening the nucleus. Note the Golgi body to the right; (c) Large blue, ground-glass inclusions in two lymphocytes with the classic half-moon nuclear shape, two thrombocytes, and a heterophil also present; (d) Arenavirus-induced inclusions must be differentiated from the more common small punctate dark blue inclusion that is commonly seen in healthy snakes; (e) In severe infections, inclusions may be found in all cells including lymphocytes, thrombocytes, RBCs, and heterophils; (f) Wild python, species unknown (*Python sp.*): *Hepatozoon sp.* in a RBC; (g) Indigo snake (*Drymarchon sp.*): Five *Hepatozoon* organisms causing RBC lysis and two azurophils and one lymphocyte. (**Source:** Images a, c, and e are courtesy of Dr. Kendal Harr; f is courtesy of Dr. Nicole Stacy.)

immature thrombocytes correlate with a regenerative response to thrombocytopenia or increased thrombocyte demand (i.e., secondary to consumption or destruction).[29]

Hemoparasites

Most common reptilian hemoparasites have an indirect life cycle; therefore, they are more frequently seen in wild-caught specimens than captive-bred individuals, especially when housed indoors. Most are considered nonpathogenic and infestations usually have a good prognosis.

Hemogregarines are commonly found and often do not cause clinical disease. The term "hemogregarines" refers to four different genera, *Hemogregarina*, *Hepatozoon*, *Karyolysus*, and *Hemolivia*, which are morphologically similar and PCR is needed for differentiation.[29] However, *Hepatozoon* are more commonly found in snake species.[29] The intraerythrocytic, oblong gamonts have pale basophilic cytoplasm and a central round to oval nucleus with dark purple chromatin. They may displace or wrap around the RBC nucleus (Figure 133.3f,g). Otherwise, healthy individuals generally do not exhibit anemia and infection is subclinical.[26] Although anemia and inanition can be seen, reptilian hemogregarines only rarely cause clinical disease.

Hemoproteus sp. is a malaria-inducing hemoparasite seen in snakes. Gametocytes, meronts, and trophozoites can be found in RBCs or extracellularly. The gametocytes are round, oval, or elongated with pale basophilic cytoplasm and a round purple-blue nucleus. They characteristically contain brown or golden pigment consistent with hemoglobin breakdown products and this finding can help differentiate from *Hepatozoon* (hemogregarines).[29] Like hemogregarine infections, *Hemoproteus* infection generally does not produce clinical signs, but can contribute to hemolytic anemia in immunocompromised individuals.

Neoplasia

Hematopoietic neoplasia has not been commonly reported in snakes. Lymphoid neoplasia may be the most common hematopoietic neoplasia of snakes, but caution in interpreting increased lymphocyte counts is advised, as infectious conditions and general antigenic stimulation may result in marked lymphocytosis. Repeat sampling from different sites so as to avoid lymphatic dilution may be necessary. Other diagnostics such as cytochemical staining and flow cytometry are not currently readily available for reptile species, but may be offered by some laboratories. Cytomorphology, including visible nucleoli, mitotic figures in lymphocytes, and a monomorphic population, is important to determine neoplastic versus reactive lymphoid cells.

Myeloid neoplasms are rarely seen in snakes, though prevalence of neoplasia appears to be highest in snakes in comparison to other reptile groups.[11] A presumptive granulocytic leukemia in a hognose snake was further investigated with cytochemistry and found to be strongly positive for toluidine blue and periodic acid–Schiff and weakly positive for Alcian blue, supporting a basophilic leukemia.[33]

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CHAPTER 134

Hematology of Testudines

JENNIFER D. STEINBERG and STEPHEN J. DIVERS

Sample Collection
Erythrocytes
Leukocytes
Thrombocytes and Hemostasis
Bone Marrow
Reference Intervals
Hematologic Disease
  Anemia
  Inflammation
  Neoplasia
  Hemoparasites and Inclusions

Acronyms and Abbreviations

N:C, nuclear:cytoplasmic; NBE, naphthyl butyrate esterase; ANAE, α-naphthyl acetate esterase; NaF, sodium fluoride; PAS, periodic acid–Schiff; ACP, acid phosphatase; PER, benzidine peroxidase; CAE, chloroacetate esterase; SBB, Sudan Black B; TB, toluidine blue; FP, fibropapillomatosis.

Testudines, also referred to as Chelonia, are poikilothermic nonmammalian vertebrates that belong to the class Reptilia. Over 300 species occupy terrestrial, fresh-water, and marine habitats worldwide, and include turtles, tortoises, and terrapins. Considered one of the most ancient reptiles, they are highly adapted to diverse environmental conditions and, therefore, there is marked inter- and intraspecies hematologic variability.

SAMPLE COLLECTION

The optimal site varies with size and species, level of sedation, medical condition, and skill of the phlebotomist. Possible locations include the jugular (preferred), femoral, brachial, and coccygeal veins, as well as the subcarapacial and postoccipital sinus.[11]

ERYTHROCYTES

As with other nonmammalian vertebrates, all circulating blood cells of testudines retain their nuclei. This precludes enumeration via an automated hematology analyzer and necessitates a manual method (see Chapters 123 and 128 for details on these techniques). On Wright-stained blood films, mature RBCs have an ovoid appearance with orange-pink cytoplasm and round to ovoid deeply basophilic nuclei (Figure 134.1). Testudine RBCs are relatively large compared to those of other species, achieving lengths approximating those of heterophils. Mean cell volume ranges from 310 fL, median in Hermann's tortoises (*Testudo hermanni boettgeri*), to 975 fL, mean in male green sea turtles (*Chelonia mydas*).[4,29] Unlike mammalian RBCs, mature testudine RBCs are larger than immature forms. Polychromatophilic RBCs contain faintly to deeply basophilic cytoplasm and more dispersed nuclear chromatin (Figure 134.1b,e). Rubricytes vary in size depending on the maturation stage, but typically have a round, less-elongate profile, with basophilic cytoplasm, indicating a lower Hgb concentration and relatively high nuclear to cytoplasm (N:C) ratio (Figure 134.1c,e,g). Nuclei are round to irregularly shaped and chromatin is coarsely granular to loosely clumped. Increased numbers of polychromatophilic cells, rubricytes, mitotic figures (Figure 134.1i), and binucleate cells (Figure 134.1h) may indicate an attempt at regeneration in anemic patients. The long RBC life span (up to 11 months) in Testudines may mute this response,

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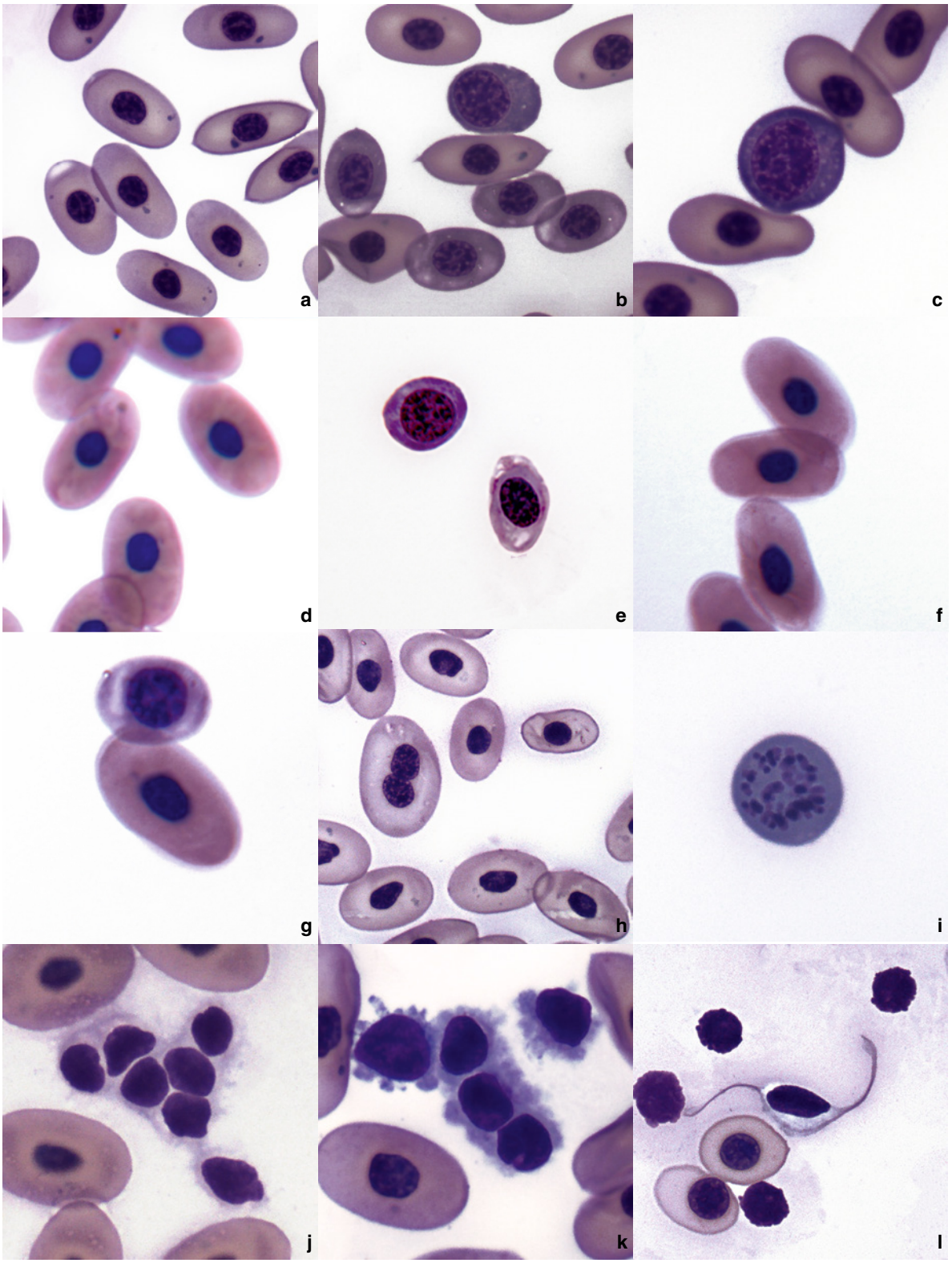


FIGURE 134.1 Erythrocytes and thrombocytes from selected Testudine species. Wright's stain, 1000× unless otherwise specified. Red-eared slider (*Trachemys scripta elegans*): (a) Normal RBCs, note the fully hemoglobinized cytoplasm, centrally located nuclei, and small basophilic inclusions; (b) Polychromatophilic RBCs are smaller and rounder, the chromatin is less-condensed, and the cytoplasm is more basophilic than mature RBCs; (c) Stage 1 rubricyte with a round profile, dispersed chromatin, and dark blue cytoplasm. Loggerhead sea turtle (*Caretta caretta*): (d) Normal erythrocyte; (e) Polychromatophilic RBC (lower right) and a stage 2 rubricyte (upper left). Yellow-footed tortoise (*Chelonoidis denticulatus*): (f) Normal RBC; (g) Stage 3 rubricyte. Box turtle (*Terrapene carolina*): (h) Binucleate RBC is slightly larger than normal RBCs. Annam leaf turtle (*Mauremys annamensis*): (i) Circulating mitotic figure which may be found during erythroid regeneration. Kemps Ridley sea turtle (*Lepidochelys kempii*): (j) Clumped thrombocytes with a high N:C ratio, scant nonstaining cytoplasm, and a round nucleus with densely clumped chromatin. *T. scripta elegans*: (k) Activated thrombocytes that appear to have pseudopodia and a more dispersed chromatin pattern. Desert tortoise (*Gopherus agassizii*): (l) Spindle-shaped thrombocyte amidst free nuclei with heparin artifact in the background. (**Source:** Photos (d)–(g): courtesy of Dr. Kendal Harr.)

however.[5] Erythrocytes from poikilotherms are believed to have significantly longer life spans than mammals, though there is little recent research on the subject. Erythrocytes from apparently healthy chelonians may contain small, punctate, basophilic cytoplasmic inclusions (Figure 134.1), thought to be either degenerating organelles or hemoglobin precipitates; neither having pathologic significance.[14,15]

LEUKOCYTES

Testudine leukocytes consist of heterophils, lymphocytes, monocytes, eosinophils, and basophils. Heterophils average 15.9 ± 2.8 µm and contain numerous linear, pink-orange, refractile granules that are acicular in shape and often appear to "melt" together[29] (Figure 134.2). Nuclei are round to ovoid and eccentrically located with densely clumped chromatin. In some cells, the granules obscure visualization of the nuclei. Cytochemical staining of leukocytes can vary among species. As an example, heterophils of the yellow-bellied slider (*Trachemys scripta scripta*) and loggerhead turtle (*Caretta caretta*) stain positively with acid phosphatase (ACP) with and without tartaric acid, peroxidase (PER), chloroacetate esterase (CAE), Sudan Black B (SBB), and periodic acid–Schiff (PAS); however, those of *T. scripta* are positive for naphthyl butyrate esterase (NBE), with and without sodium fluoride (NaF), whereas those of *C. caretta* are negative.[7,14] Heterophils in yellow-headed temple turtles (*Hieremys annandalii*) stain positively with PER, SBB, ACP, PAS, as well as α-naphthyl acetate esterase (ANAE).[8] These staining characteristics are similar to those of mammalian granulocytes implying similar functionality.

Lymphocyte appearance has been conserved among vertebrate species. Mature lymphocytes are generally approximately 7.5 ± 1.8 µm in diameter and contain a thin rim of basophilic cytoplasm and a round nucleus[29] (Figure 134.2). Distinguishing small lymphocytes from thrombocytes can present a challenge for the unseasoned and, occasionally, for the veteran hematologist. In general, lymphocytes have a more dispersed chromatin pattern and more deeply basophilic cytoplasm when compared to thrombocytes. Reactive change results in increased cytoplasmic basophilia, possibly with a perinuclear clear area (Figure 134.3h). Granular lymphocytes with eosinophilic to azurophilic cytoplasmic granules can be found on blood smears from normal chelonians (Figure 134.3i). Occasionally, larger, immature-appearing cells that contain more abundant cytoplasm, granular chromatin, and variably prominent nucleoli can be identified in the circulation from apparently healthy animals. While these reactive changes may be found in low numbers in blood smears from clinically healthy animals, the prevalence may increase significantly in sick animals and, thus, a search for a source of inflammation or antigenic stimulation is warranted.

Monocytes contain moderate amounts of blue-gray cytoplasm and a round to ovoid to reniform nucleus with loosely clumped to coarsely granular chromatin (Figure 134.2). They measure 13.4 ± 2.3 µm in diameter in green sea turtles.[29] Some cells may have an ameboid appearance, often attributable to pseudopodia. Monocytes in loggerhead turtles and yellow-bellied sliders stain positively with NBE with NaF.[7,14] Monocytes in the latter also stain positively with ACP with and without tartaric acid. When activated, monocytes often contain cytoplasmic vacuoles, possibly with phagocytized pigment (Figure 134.3k), and occasionally cellular components (Figure 134.3j-l). Azurophils, PER-positive phagocytic cells that contain magenta granules, are most often seen in snakes and are rarely encountered on chelonian blood smears; though they have been reported in desert tortoises (*Gopherus agassizii*)[6–9] (see Chapter 133).

Eosinophils are as large if not larger than heterophils, with a mean diameter of 18 ± 3.1 µm.[29] They contain a round to ovoid, eccentrically located nucleus and moderate to large numbers of round to ovoid, pink-orange granules. Eosinophil appearance seems to be the most variable among WBCs between turtle and tortoise species (Figure 134.2). The granules tend to be round, discrete, and less-refractile/duller in appearance when compared to heterophil granules. Eosinophil granules in *T. scripta*, *C. caretta*, and *H. annandalii* stain positively to varying degrees with ACP, NBE (with NaF), CAE, SBB, and PAS.[7,8,14] Eosinophil granules of *H. annandalii* also stain with PER.

Basophils are typically smaller (14.4 ± 1.3 µm) than heterophils and eosinophils and contain moderate to large numbers of fine, purple to deeply basophilic granules resembling mammalian mast cells[29] (Figure 134.2). Nuclei are round to ovoid, often centrally located with a dense chromatin pattern. Basophils can undergo degranulation between sample collection and slide preparation and may stain variably in aqueous-based and alcohol-based Romanowsky stain.

THROMBOCYTES AND HEMOSTASIS

Assessing primary hemostasis poses several challenges due to the tendency of thrombocytes to form clumps (Figure 134.1j,k) and their similar appearance to small lymphocytes. The latter becomes particularly important when preparing blood smears from chilled blood, as cell shrinkage makes differentiation between these two cells more difficult. Thrombocytes contain small amounts of nonstaining to pale basophilic cytoplasm and a round to oval nucleus. The chromatin is densely clumped, and nucleoli are not visible. Thrombocytes of loggerhead sea turtles and yellow-bellied sliders stain positively with PAS, presumably binding to glycoproteins.[7,14] Additionally, these cells also stain positively with NBE with and without fluoride in yellow-bellied sliders. When activated, the cytoplasm may become vacuolated or exhibit irregular cytoplasmic margins (Figure 134.1k). A study in healthy Hermann's tortoises (*Testudo hermanni*) demonstrated an average of 3–6 thrombocytes per 100× field; however, whether this value is appropriate for all Testudines remains to be determined.[2]

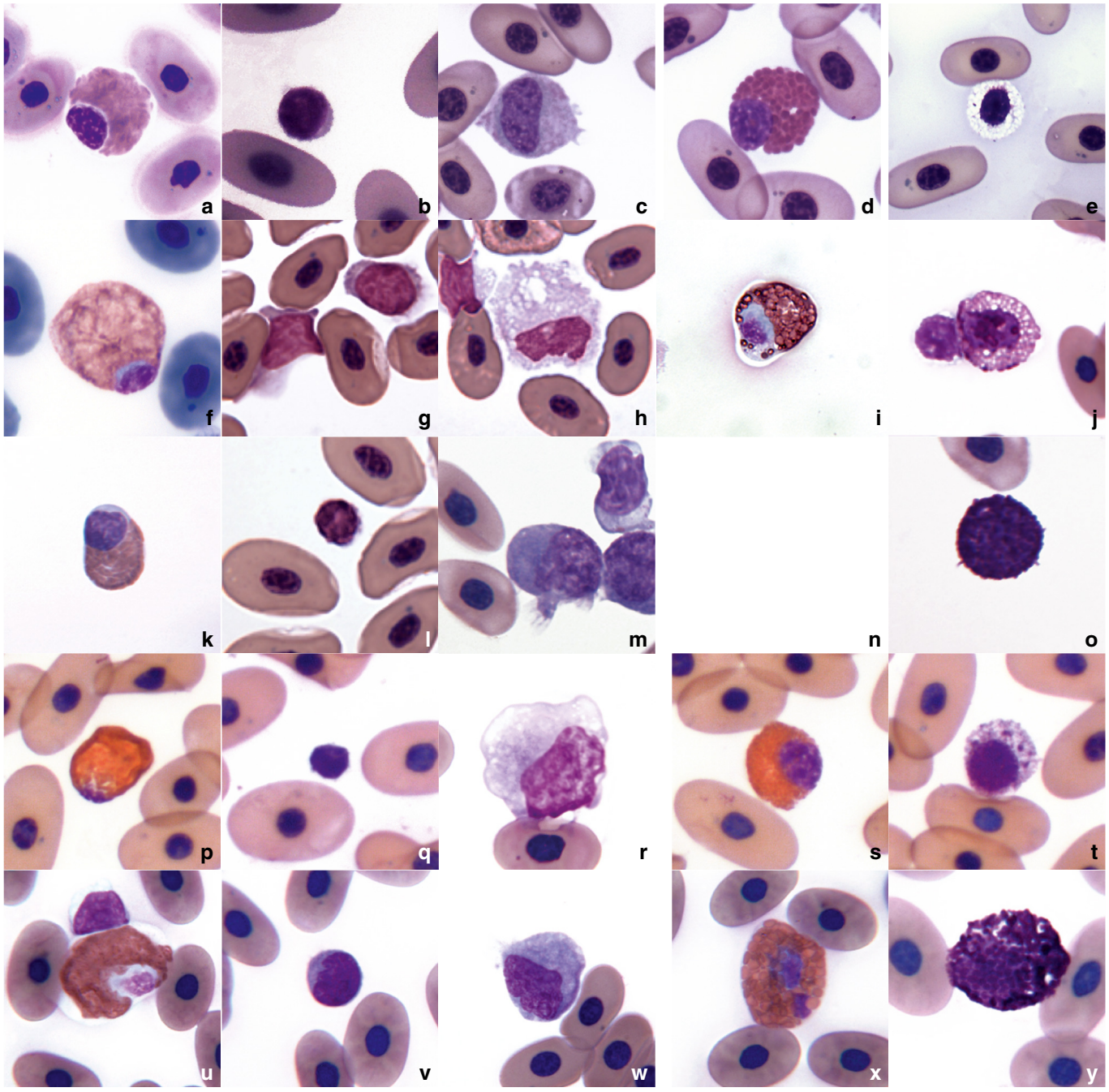


FIGURE 134.2 Normal leukocytes from selected Testudine species. Modified Wright's stain, 1000× unless otherwise specified. Red eared slider (*Trachemys scripta*): (a) Heterophil; (b) Lymphocyte; (c) Monocyte with blue-gray granular cytoplasm and slightly indented nucleus; (d) Eosinophil with easily discernible individual round granules versus those in the heterophil; (e) Degranulated or nonstaining basophil which appears "vacuolated" due to the lack of stained granules. Loggerhead sea turtle (*Caretta caretta*): (f) Heterophil; (g) Two intermediate-sized lymphocytes which can be found in samples from normal animals; (h) Monocyte with vacuolated blue-gray granular cytoplasm; (i) Eosinophil; (j) Basophil with clear granules and purple demarcation. Yellow-footed tortoise (*Chelonoidis denticulatus*): (k) Mature heterophil with a distinct round nucleus and muted pink/orange oblong to spindle granules, not classified as left shifted; (l) Small lymphocyte; (m) Monocytes with blue-gray cytoplasmic pseudopodia; (n) Eosinophil, not pictured; (o) Well-stained basophil such that the nuclear morphology is obscured. Snapping turtle (*Chelydra serpentina*) using Wright-Giemsa stain; (p) Heterophil with indistinct granulation; (q) Small lymphocyte; (r) Monocyte; (s) Eosinophil with round granules; (t) Partially staining basophil with a round nucleus, similar to a mast cell. Leopard tortoise (*Stigmochelys pardalis*): (u) Heterophil with indistinct granulation and a clear rim of cytoplasm with adjacent lymphocyte; (v) Lymphocyte with irregular, cleaved nucleus; (w) Monocyte; (x) Eosinophil with distinct round granules; (y) Basophil. (Photos a, f-q, s-y courtesy of Drs. Kendal Harr and Nicole Stacy.)

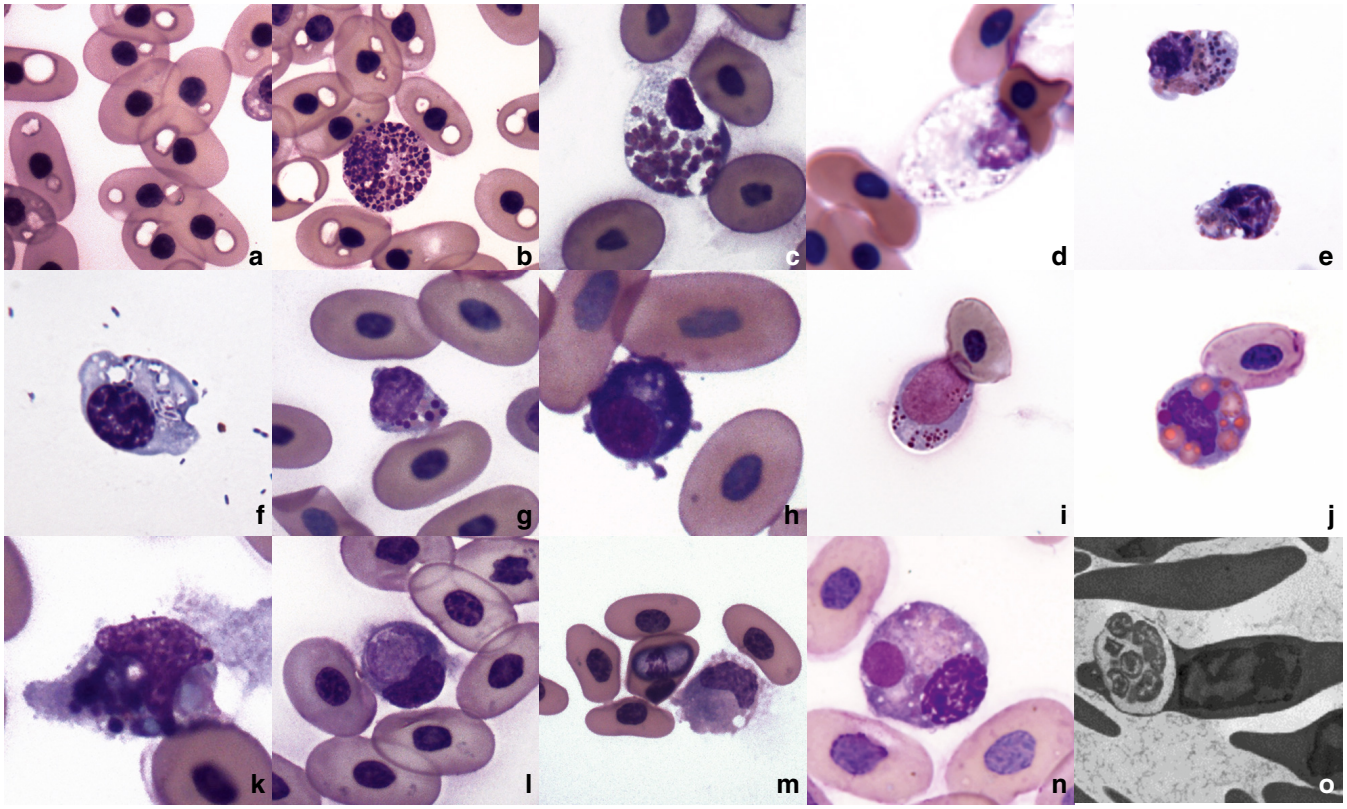


FIGURE 134.3 Abnormal cell morphology and infectious agents found in Testudines. Wright's stain, 1000× unless otherwise specified. Red-eared slider (Trachemys scripta elegans): (a) These non-staining refractile cytoplasmic structures in the RBC have been associated with sepsis in other species. These are not hemic parasites. See Chapter 133; (b) Toxic heterophil, marked. This cell contains almost exclusively primary granules and is found with aberrant RBC vacuolation described in (a). Loggerhead turtle (Caretta caretta): (c) Eosinophil for comparison. These should not be confused with toxic degranulation seen in heterophils; (d) Moderate toxic change with refractile degranulation and purple granule remnants. Leopard tortoise (Stigmochelys pardalis): (e) Markedly toxic heterophils in a tortoise with evidence of sepsis; (f) Monocyte with phagocytized bacteria from the same animal as in (e). Green sea turtle (Chelonia mydas): (g) Myelocyte with primary granules. (h) Plasmacytoid-reactive lymphocyte with deeply basophilic cytoplasm and perinuclear clear area in the same animal as (g). Loggerhead sea turtle (C. caretta): (i) Granular lymphocyte, found in higher numbers in virally infected turtles; (j) Circulating phagocyte which has vacuoles containing heterophilic debris. A dysplastic myeloid precursor could not be ruled out and NBE staining may have been useful in determining the origin of this cell. The sea turtle was afflicted with fibropapilloma virus; (k) Pigment-laden monocyte/macrophage (possible siderophage) containing abundant, globular, blue-black pigment. Box turtle (Terrapene carolina): (l) Cytophagic monocyte which has engulfed another leukocyte. Annam leaf turtle (Mauremys annamensis): (m) Intraerythrocytic basophilic, ovoid hemogregarine gametocyte fills and distorts the RBC cytoplasm. Adjacent activated monocyte. Eastern box turtle (Terrapene carolina carolina): (n) Monocyte containing a round magenta intracytoplasmic Iridovirus inclusion. Reprinted with permission from Dr. Michael Fry. Desert tortoise (Gopherus agassizii): (o) Presumptive intraerythrocytic apicomplexan in a desert tortoise, transmission electron micrograph. Courtesy of Dr. Sabrina Clark. Images d-f, i, and j courtesy of Dr. Kendal Harr.

BONE MARROW

On occasion, it may be necessary to submit bone marrow to permit a more detailed hematologic evaluation; however, there are very few descriptions of bone marrow cytology from healthy or diseased turtles. The bone marrow collection technique depends upon the size and nature of the patient. Diagnostic samples can be collected from the femoral or tibial medullary cavities of large chelonians; although many have limited medullary tissue.[11] A suitably sized Jamshidi or spinal needle with a stylette (or possibly a hypodermic needle for very small patients) can be placed into the medullary cavity and moderate aspiration used to obtain a sample. An alternative site is the plastrocarapacial bridge that laterally connects the plastron and carapace, but again the medullary tissue is limited. It is generally necessary to drill through the outer layer of keratin and bone to gain access to the medullary cavity and marrow beneath.

Hematopoiesis occurs primarily in bone marrow in adult turtles and tortoises with erythropoiesis localized to

blood sinuses and myelopoiesis to the extravascular spaces.[13] Maturation in both series in chelonian species follows a similar progression to those of mammalian and avian counterparts with the distinction that mature RBCs are larger than precursors. Though thrombopoiesis has been studied in avian, amphibian and fish species, the origin of thrombocytes in Testudine has yet to be elucidated.

The earliest identifiable erythroid precursor is the rubriblast, a round cell with deeply basophilic cytoplasm, a round nucleus, and relatively high N:C ratio. The chromatin pattern is dispersed, and nucleoli are round to ovoid and occasionally multiple. Reptilian rubriblasts have distinctive peripheral pinocytotic vesicles involved in ferritin acquisition for Hgb synthesis. Prorubricytes, the subsequent maturation stage, are similarly round and basophilic; however, the chromatin is more condensed, and nucleoli are absent. As maturation progresses to the rubricyte stage, cells maintain a round profile with paler cytoplasm due to Hgb synthesis. Nuclear size decreases, chromatin continues to condense, and rubricytes may be released into circulation. The rubricyte can be further subclassified as basophilic or stage 1 (Figure 134.1b,c,e,g) early polychromatic or stage 2 and late chromatic or stage 3 depending on N:C ratio and degree of hemoglobinization. At this point, cells increase in size to form the polychromatic erythrocyte. These cells are ovoid with nonuniform lightly basophilic cytoplasm and a rounded nucleus. The mature RBC (metarubricyte) is similar in size to the polychromatic RBC, but contains fully hemoglobinized, pink-orange cytoplasm and a round to ovoid, pleomorphic nucleus with condensed chromatin. Erythroid precursors and mature erythrocytes, when stained with SBB, had uniformly dark gray to black cytoplasm with scattered black cytoplasmic granules.[13]

Developing granulocytes originate from myeloblasts, cells characterized by a moderate amount of basophilic cytoplasm devoid of granules. Nuclei are large, often centrally located with granular chromatin and large, prominent nucleoli (Figure 134.3e). The progression to progranulocyte involves a decrease in cell size and cytoplasmic basophilia with increasingly condensed chromatin. Secondary granules specific to eosinophils and heterophils become apparent in myelocytes and metamyelocytes and increase in number as maturation progresses. Later stage precursors may be more difficult to differentiate than in mammals due to round nuclei in normal, mature cells. It can also be difficult to differentiate myeloblasts and monoblasts based on cytologic appearance on Wright-Giemsa stained preparations.[13]

REFERENCE INTERVALS

PCV varies with species, sex, age, nutritional status, disease status, and season[2,4,6,8,10,16,17,29,32,33] (Table 134.1). Chinese male yellow-marginated box turtles (*Cuora flavomarginata*) have higher PCVs than females of the same species.[33] There is a seasonal variation in polychromatophil and rubricyte numbers in certain species, with higher numbers in the winter, spring, and summer relative to autumn in geometric tortoises (*Psammobates geometricus*).[30] Juveniles of this species tend to have larger numbers of circulating rubricytes and polychromatophils relative to adult females.

Prior to reference generation, samples must be critically inspected to ensure quality. Blood vessel proximity to lymph and cerebrospinal fluid compartments often results in dilution, impacting the hemogram by falsely decreasing the PCV, Hgb, and RBC concentration, which can also vary with venipuncture site.[18] Inclusion of lymph-diluted samples in reference intervals results in increased variability and decreased diagnostic acuity. Additional and important factors must be considered, including health status information obtained after reference interval publication. While seasonal variation is

TABLE 134.1 Hematology data for adult, male and female, free-ranging desert tortoises (*Gopherus agassizii*) in the Mojave Desert over a 5-year period, divided by season and sex (PCV are presented), which were found to be statistically different.[9] Median PCV values generated in the field as sample quality issues during transport were noted by the author. Underlying endemic disease, including Mycoplasma, was further identified in this well-studied population after the reference intervals were published in 1999.[1,31]

	Winter	Spring	Summer	Fall
PCV (%), Males	30	30	29	28
PCV (%), Females	22	25	25	23
Total WBC/μL	970–10,520	1420–10,860	1500–10,920	390–10,120
Heterophil/μL	600–7260	490–6830	720–7160	170–6920
Lymphocyte/μL	20–1880	0–3190	60–2750	10–3820
Monocyte/μL	0–570	0–340	0–320	0–320
Eosinophil/μL	0–560	0–860	0–950	0–710
Basophil/μL	0–2700	0–4360	60–3570	30–3760
Azurophil/μL	0–900	0–660	0–560	0–620

well illustrated in Table 134.1, ELISA testing for Mycoplasma endemic in the desert tortoise population has improved since reference interval generation.[31] It is likely, considering the outlier statistics used, that diseased animals were included in this population.[9]

Much like PCV, total and differential WBC concentrations vary with species, age, sex, reproductive status, and season (Table 134.1).[6,8,9,26,33,34] For example, juvenile loggerhead sea turtles have higher median WBC and heterophil counts than nesting adults[6] (Table 134.2). Absolute WBC counts decrease with age in red-eared slider turtles (*Trachemys scripta elegans*).[34] Heterophil numbers exhibit seasonal variations in certain species, with numbers increasing during the warmer, active season (spring through summer).[9]

Lymphocyte numbers tend to attain minimum values during brumation in hibernating species.[9] Peak lymphocyte counts occur in the summer in desert species and in years with ample rainfall.[6,9,33] Monocyte counts decrease during the summer months and reach peak values during the winter/periods of hibernation.[9,33] Conversely, in red-eared slider turtles, monocyte numbers increase from spring into late summer.[35] Monocyte counts also decrease with age in certain species.[2] Female yellow-marginated box turtles have higher monocyte counts than males.[33] Eosinophil and basophil numbers vary with species, season, and geographic location.[6,9] For example, eosinophils and basophils occur with higher frequency in freshwater than marine chelonian species. Male *C. flavomarginata* have higher eosinophil counts than females.[33] Like lymphocytes, basophil numbers decrease during hibernation and are often absent from blood samples from hibernating desert tortoises.[9] Given the aforementioned variability in cell counts, it is frequently more useful to review serial samples from an individual, rather than rely entirely on limited published values, especially those that are not in accordance with ASVCP guidelines (Table 134.3).

HEMATOLOGIC DISEASE

Anemia

Artifact must be differentiated from true anemia. Given the proximity of lymphatic or cerebrospinal fluid compartments to certain venous vessels, lymph dilution can occur with sample collection, but appears to be less common with jugular samples. Marked decreases in PCV (<20%) with minimal polychromasia in conjunction with increased small lymphocyte numbers are suspect for lymphatic contamination.[11] Phlebotomists can often, but not always, appreciate the clear to white lymphatic fluid and dilution during blood collection; such observations should be included with sample submission. Clinicians and biologists should consider discarding lymph diluted samples, as these may generate erroneous values that may lead to misdiagnosis.

Nonregenerative anemia occurs with a variety of disease processes in Testudines including parasitic infestation, bacterial and viral infection, poor husbandry, and chronic renal, hepatic, and gastrointestinal disease.[25] Stranded green sea turtles and chronically debilitated loggerhead sea turtles often present with anemia, either regenerative or nonregenerative, and an underlying cause must be appropriately identified and treated.[12,19,28] Fibropapillomatosis exacerbates this anemia, which worsens with tumor burden.[32] Fibropapillomatosis is a herpes-viral-induced neoplasm that affects all species of marine turtles. It results in the growth of single to multiple external and visceral fibroepithelial, plaque-like to verrucous growths. Lesions can impair vision, mobility, and feeding, depending on the anatomic location. As these animals have presumptive, concurrent vascular trematode infections, the anemia may be the result of peripheral consumption associated with vasculitis and chronic disease from spirorchid granuloma formation as well as the chronic disease that is FP. Several species of tortoises with intranuclear coccidian infection can present with anemia.[25] Anemia can also occur in tortoises infected with siadenovirus and other herpes strains.[24,28] Regenerative anemias can occur with nonfatal traumatic injuries (e.g., boat strikes, hit by car, predation). Severe ectoparasite burdens with ixodid and argasid ticks can cause blood loss anemia secondary to skin wounds, typically in terrestrial species.[25]

Inflammation

Leukocyte responses depend on the nature and severity of the disease process. Leukocytoses are most often associated with inflammation and antigenic stimulation, infection, and stress. For example, in addition to anemia, patients with intranuclear coccidian infection may present with leukocytosis.[25] Hematologic responses to siadenovirus infections vary as patients may present with leukocytosis or leukopenia.[24] Monitoring of serial CBCs can provide additional useful information regarding the patient's health status, as leukocyte trends may be as valuable as absolute values in evaluating responses to treatment. For example, there is an inverse relationship between total WBC count and health status in green sea turtles, as cell counts decrease with convalescence.[19]

Heterophilia occurs with inflammation, infection, and trauma as specifically documented in unhealthy stranded green sea turtles, presumably due to bacteremia and parasitism.[12,19,28] Disruption of bone marrow maturation due to severe inflammation and infection results in toxic change. Mild toxic change is characterized by increased cytoplasmic basophilia, and moderate to marked signs include retention of primary granules (Figures 134.3b,d,e). As heterophil nuclei are not lobulated, it is difficult to detect a left shift akin to those seen in mammals. With severe inflammation and infection, early myeloid precursors can enter the circulation. Heteropenia has also been documented in

TABLE 134.2 Loggerhead sea turtle (*Caretta caretta*) reference intervals. The population is wild-caught, male and female, juvenile turtles in the summer and fall months. These reference intervals were generated using previously published data shared by the authors,[17] with all outliers removed according to American Society for Veterinary Clinical Pathology (ASVCP) guidelines. Unfortunately, in the previous publication, outlier statistics were applied once, but not applied repetitively until no outliers remained.

Measurand	Units	n	Mean	SD	Median	Min.	Max.	Distribution	Reference Interval	CI 90% of LRL	CI 90% of URL
PCV	%	167	30	4.7	31	18	40	G	21–40	20–22	39–41
WBC conc.	$10^3/\mu L$	167	9.5	2.5	9	4	16	NG	3.5–15.1	3.0–4.1	14.3–15.7
Heterophil	$10^3/\mu L$	167	5.1	2.7	4.7	0	13	NG	0.5–11.5	0–1.3	10.6–13
Lymphocyte	$10^3/\mu L$	167	3.7	1.9	3.3	0.9	9.2	NG	1.1–8.2	0.9–1.2	7.2–9.2
Monocyte	$10^3/\mu L$	167	0.2	0.2	0.1	0	0.8	NG	0–0.8	0–0	0.8–0.8
Azurophil	$10^3/\mu L$	167	0.1	0.2	0	0	1.2	NG	0–0.5	0–0	0.4–1.2
Eosinophil	$10^3/\mu L$	167	0.4	0.4	0.2	0	1.6	NG	0–1.3	0–0	1.3–1.6
Basophil	$10^3/\mu L$	167	0	0.1	0	0	0.4	NG	0–0.3	0–0	0.2–0.4

G, Gaussian; NG, Non-Gaussian; n, Number of turtles; CI, Confidence interval; LRL, Lower reference limit; URL Upper reference limit

Testudines and is likely to occur in severe infection as in other species. Tortoises with siadenovirus infection can present with either heteropenia or heterophilia.[24] Heteropenia with toxic change, likely as a result of secondary bacterial infection, can occur with herpes viral infections in spur-thighed tortoises (*Testudo graeca)*, possibly due to secondary bacterial infection.[20] An asymptomatic, prolonged heteropenia associated with fenbendazole toxicosis has been reported in Hermann's tortoises.[21]

Lymphocytosis occurs with wound healing, spirorchid infection, and rarely siadenoviral infections. In addition to heteropenia, significant lymphocytosis has been reported in spur-thighed tortoises with herpes viral rhinitis infections.[20] It is likely that other viral diseases and chronic inflammation will also induce lymphocytosis. Lymphopenia is a feature of stress/glucocorticoid responses, and can also occur with malnutrition and immunosuppression.

Monocytosis occurs with acute and chronic infections, including fibropapillomatosis, fungal infections, and siadenovirus.[16,24,32] Eosinophilia has been reported in patients with helminthiasis and less commonly siadenovirus infections.[24] Allergic response has been minimally studied in Chelonia and further research is warranted.

Neoplasia

Lymphoid neoplasia occurs rarely in Testudines.[3,22,27] Both lymphomas and leukemias of B- and T-cell origin have been reported in turtles and tortoises. Patients with leukemia can present with marked leukocytosis and >140,000 lymphocytes/μL. Cells can be intermediate to large in size (8–18 μm in diameter) depending on immunophenotype. Immunocytochemistry using CD3 and BLA36 have been performed on air-dried blood smears to distinguish between T-and B-cell variants, respectively.[3,27] Quality assurance should include species specific controls.

TABLE 134.3 Hematology Reference Intervals, for yellow marginated box turtles (*Cuora flavomarginata*). Values are from the spring, though authors did identify sex and season as partitioning factors.[33]

Analyte	Median (RI)
N	52; 17M, 35F
PCV%	27(21–35)F
	30(27–35)M
Total WBC $10^3/\mu L$	4.05(0.6–8.6)F
	3.15(1.2–7.4)M
Heterophils $10^3/\mu L$	2.39(0.3–5.59)F
	1.63(0.58–3.54)M
Lymphocytes $10^3/\mu L$	0.95(0.22–2.38)F
	1.12(0.4–2.23)M
Monocytes $10^3/\mu L$	0.26(0–0.76)F
	0.07(0–0.21)M
Eosinophils $10^3/\mu L$	0.01(0–0.06)F
	0.01(0–0.03)M
Basophils $10^3/\mu L$	0.44(0–1.82)F
	0.47(0.07–0.70)M

N, individuals in study.

Hemoparasites and Inclusions

Care must be taken to distinguish between staining/preparation artifacts and true viral inclusions/parasites. Small basophilic inclusions thought to be degenerate organelles are commonly found in blood smears from apparently healthy Testudines and should not be interpreted as abnormal (Figure 134.1). Semiaquatic freshwater turtle RBCs and rarely WBCs may contain hemogregarine parasites, usually of the genus *Hemogregarina* (Figure 134.3m).[11] Though these parasites

are not host-specific, they rarely cause primary disease in infected animals. Iridoviral inclusions have been demonstrated in heterophils and monocytes in eastern box turtles (*Terrapene carolina carolina*) (Figure 134.3n). Intraerythrocytic *Anaplasma* morulae have been identified in the peripheral blood of anemic gopher tortoises[23] (Figure 134.3o).

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CHAPTER 135

Hematology of Amphibians

PERRY BAIN and KENDAL E. HARR

Hematopoiesis
Erythron
Leukon
Thrombocytes
Hematologic Disease

Acronyms & Abbreviations

ASVCP, American Society for Veterinary Clinical Pathology.

Amphibians (class Amphibia) are ectothermic, tetrapodic vertebrates and include 8177 known species of frogs and toads (order Anura), salamanders and newts (subclass Caudata, order Urodela), and caecilians (order Gymnophiona).[10] Their life cycle typically includes an aquatic stage followed by metamorphosis into an adult form. Many species are capable of regenerating lost limbs and degenerating body parts, which makes them interesting research subjects. Study of amphibian pathobiology is important for the preservation of wildlife populations, the health of captive animals kept as pets, and to aid in research studies.

Sample collection has been thoroughly described in a recent review article.[9] Contamination of the blood sample with lymphatic fluid during collection may decrease the erythroid mass and artifactually increase lymphocyte number.

In general, amphibian blood cell morphology is similar to that of reptiles; however, the cells are significantly larger. Comparative hematology studies have been performed only in a few species and the vast majority of reference intervals have low numbers or do not have outliers removed in accordance with American Society for Veterinary Clinical Pathology (ASVCP) guidelines.[5,9,12] These reference intervals cannot be used for medical use due to the risk of misdiagnosis.

HEMATOPOIESIS

Amphibian species vary in their sites of hematopoiesis, but common anatomic sites in adult amphibians include the spleen, liver, bone marrow, and kidney.[8] In most studied species of adult frogs, erythroid cells are produced in the spleen, while lymphoid, myeloid, and thrombocytic cell lines develop in the bone marrow.[8] In the South African clawed frog (*Xenopus laevis*), erythropoiesis and thrombopoiesis take place in the liver, while the granulocyte and monocyte/macrophage cell lines originate in the bone marrow.[16] In the axolotl (*Ambystoma mexicanum*), hematopoiesis is only found in the spleen and liver in adults.[11] In newts, hematopoiesis does not occur in bone marrow, but in the spleen, liver, kidney, and thymus.[8]

ERYTHRON

Mature amphibian RBCs are ovoid or elliptical in shape, biconvex with red to red-orange hemoglobin-containing cytoplasm and contain nuclei (Figure 135.1). Immature forms are rounded and have bluer cytoplasm. Occasional anucleate RBCs (erythroplastids) may be encountered on blood films, and lungless salamanders of the genus

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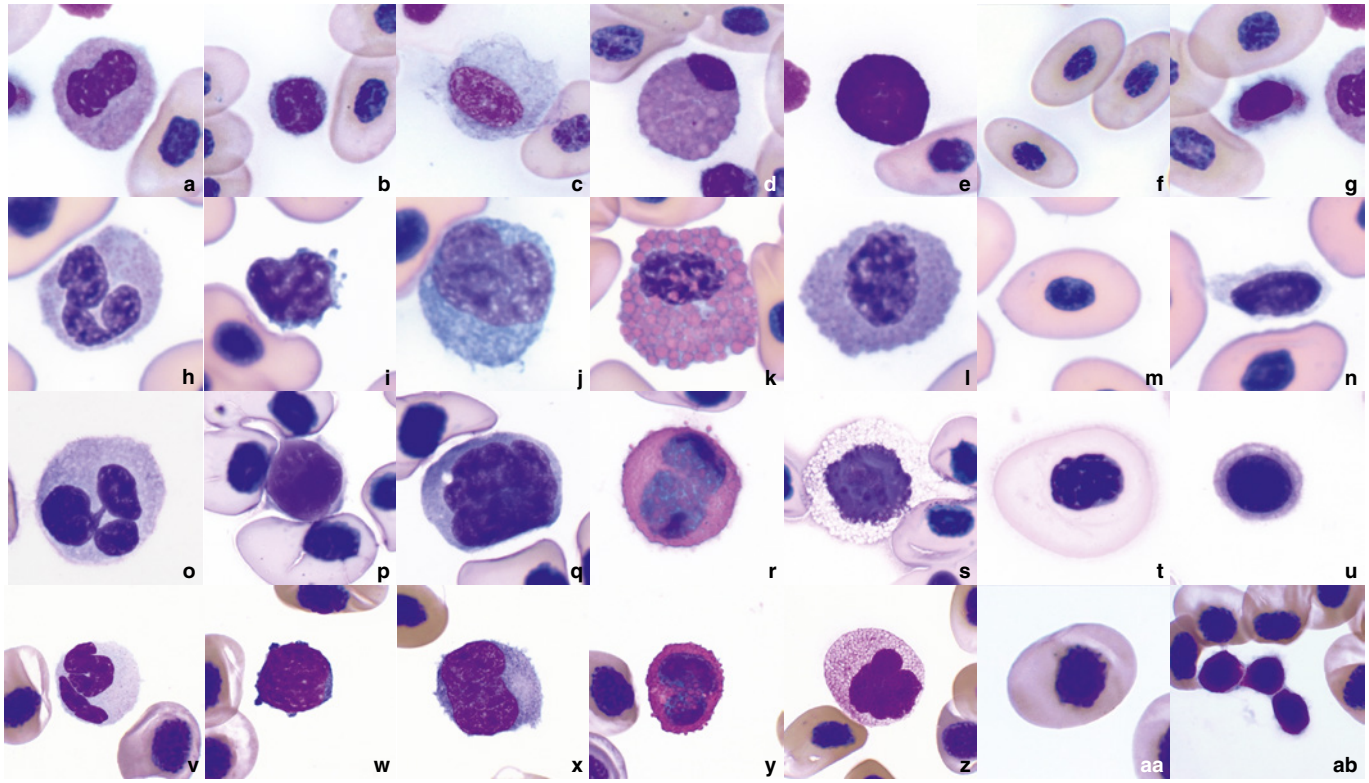


FIGURE 135.1 Normal amphibian blood cells from selected species. Argentine horned frog (*Ceratophrys ornata*), aka Pac-Man frog in the pet trade: (a) Heterophil; (b) Lymphocyte; (c) Monocyte; (d) Eosinophil; (e) Basophil; (f) Erythrocyte; (g) Thrombocyte. African clawed frog (*Xenopus laevis*): (h) Neutrophil/heterophil; (i) Lymphocyte; (j) Monocyte; (k) Eosinophil; (l) Basophil; (m) Erythrocyte; (n) Thrombocyte. Eastern tiger salamander (*Ambystoma tigrinum tigrinum*): (o) Neutrophil; (p) Lymphocyte; (q) Monocyte; (r) Eosinophil; (s) Basophil; (t) Erythrocyte; (u) Thrombocyte. Axolotl (*Ambystoma mexicanum*): (v) Neutrophil; (w) Lymphocyte; (x) Monocyte; (y) Eosinophil; (z) Basophil; (aa) Erythrocyte; (ab) Three thrombocytes.

Batrachoseps have been found to have a majority of anucleate circulating RBCs (>90%)[6] (Figure 135.2e). The clinical significance of this finding is unknown.

Among amphibian species, RBCs exhibit considerable size variation, but their RBCs are generally larger than those of the birds and reptiles. The largest reported amphibian erythrocyte size is found in the amphiuma (*Amphiuma tridactylum*), with a measured RBC size of 70 × 50 µm.[14] Average RBC sizes reported vary between species in the same genus, e.g., 13 × 10 µm in Western clawed frogs (*Xenopus tropicalis*), 18 × 11 µm in African clawed frogs (*X. laevis*); though some of the variation may be due to measurement technique.[12] Other frog species have similar sizes reported, e.g., 18 × 11 µm in the white-lipped tree frog (*Litoria infrenata*). Typically, the urodele amphibians have larger RBCs than the anuran amphibians, but definitive, species-specific RBC sizes remain poorly defined. Some species have individuals with marked ploidy and either diploid, triploid, or tetraploid erythrocytes, which increases the RBC size variation as found in axolotl (*A. mexicanum*).[9,11] Erythroid mass and number may be measured by PCV and direct hemocytometer cell count (see Chapters 123 and 128). Amphibians typically have lower reported PCV values compared to birds and reptiles.[3,5,9,17]

Immature RBCs are recognized by increased cytoplasmic basophilia and a rounder cell shape (Figure 135.2a). These cells are typically smaller in size than mature RBCs.[1,4,9,15] The nuclei of immature RBCs (rubricytes) typically have more open (lighter-staining) chromatin patterns in comparison to mature RBCs (metarubricytes), and their chromatin becomes more condensed as the cells age[4] (Figure 135.2c). While occasional rubricytes may be found on blood smears from normal amphibians, increased numbers of immature RBCs are evidence of a regenerative response in anemic amphibians, those with hemoparasites (such as *Hepatozoon sp*. and hemogregarines), and those with secondary polycythemia due to decreased oxygen perfusion.[9,17]

LEUKON

Granulocytes observed in amphibians include neutrophils, eosinophils, and basophils. Amphibian neutrophils, or heterophils in some species, are granulocytes with myeloperoxidase-containing granules that do not stain or stain only weakly with typical hematology stains (e.g., Wright–Giemsa)[9] (Figure 135.1). In species in which these cells have visible red-orange granules, these cells may be referred to as heterophils (Figure 135.1a). Mature neutrophils typically have segmented or bilobed nuclei, but immature neutrophils may be seen in the peripheral blood in response to inflammation, and may have band-shaped, reniform, or oval nuclei (Figure 135.2f).

Amphibian lymphocytes have round to irregularly round or indented nuclei, small amounts of basophilic cytoplasm, with a high nucleus to cytoplasm ratio (Figure 135.1). Azurophilic granules may be found in lymphocyte cytoplasm in Ranidae frogs as well as axolotl, and the authors presume in many species of amphibians[4] (Figure 135.2d). Lymphocytes have been reported as the predominant leukocyte type in some species or individuals, including the American bullfrog (*Rana catesbeiana*), Australian tree frogs (*Litoria sp*.), the Western clawed frog (*Xenopus tropicalis*), and the California tiger salamander (*Ambystoma californiense*).[3,5,12,17]

Amphibian monocytes are similar in appearance to those seen in other species. They are large leukocytes with variably shaped nuclei (ranging from round to oval, bilobed, trilobed, or amoeboid). Monocytes have basophilic cytoplasm, which sometimes contains discrete vacuoles[1,4,9,15] (Figure 135.1).

Amphibian eosinophils display prominent, bright red-orange, round cytoplasmic granules with Wright–Giemsa stain (Figure 135.1). Eosinophils may show less segmentation than neutrophils/heterophils and contain bilobed or round nuclei, though they are not immature. Amphibian basophils are plentiful in aquatic species and may have a segmented or round to oval nucleus. Basophil granules are classically seen as round, dark purple/basophilic granules on blood films, but their granules may be nonstaining or clear, sometimes referred to as "degranulated" basophils, especially in aqueous stains (Figure 135.1). Clear or nonstaining basophil granules may be the result of partial dissolution of the granules when water-based hematology stains are used (Diff-Quik and similar stains).[4]

WBC counts may be estimated from cell density on a blood film, but this technique has limited accuracy due to the uneven WBC distribution on the blood film. These values should be considered an estimate and not a quantitative value as produced by automated hematology analyzers. Amphibian WBC counts are performed manually, utilizing Natt and Herrick stain to identify WBCs in a hemocytometer. A potential error source is the misidentification of thrombocytes for lymphocytes, thus artifactually inflating the WBC count.[15] Natt-and-Herrick-stained lymphocytes have more basophilic cytoplasm compared to thrombocytes, however.[4] Inactivated amphibian thrombocytes are oval to elongated and distinctly different than round lymphocytes which exhibit a higher nucleus to cytoplasmic ratio and more basophilic cytoplasm in comparison to thrombocytes.[1,4] Lymphocytes may be difficult to distinguish from thrombocytes in the blood film and hemocytometer in samples with activated cells. Phloxine staining may be used to visualize and count neutrophils/heterophils and eosinophils in a hemocytometer, which can then be used to calculate the total WBC count after a WBC differential is performed.[1] Using the phloxine method avoids the difficulty of differentiating thrombocytes and lymphocytes in the hemocytometer, as this is done while performing the differential cell count on a stained blood film. The phloxine method is less useful for species which have significant numbers of basophils and neutrophils (rather than heterophils), as these cell types stain minimally with phloxine dye.[1] Thus, the indirect calculation of total WBC count is more likely to be inaccurate and imprecise.

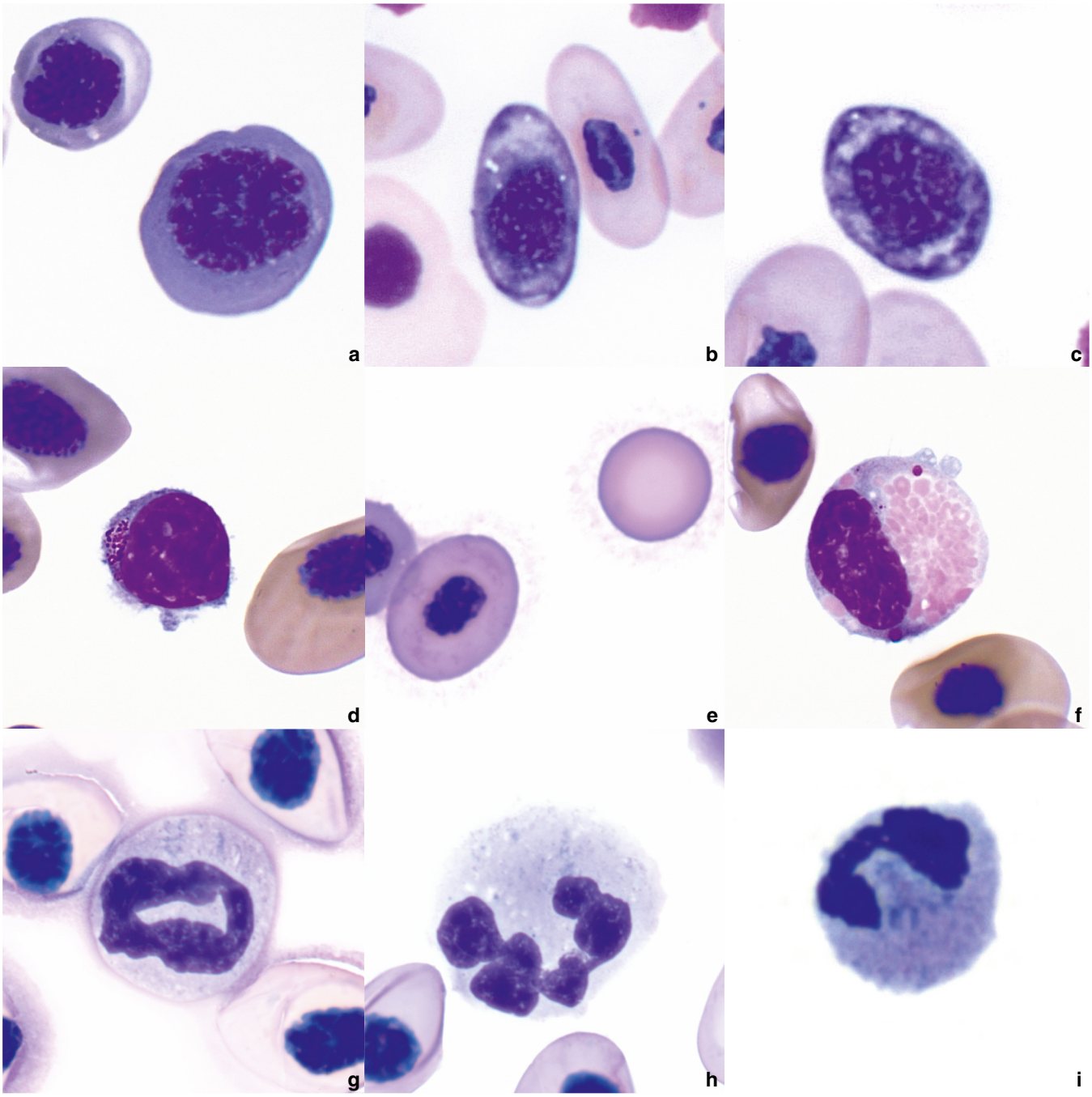


FIGURE 135.2 Abnormal amphibian blood cells. Axolotl (*Ambystoma mexicanum*): (a) Immature erythrocytes including probable rubricytes. Argentine horned frog (*Ceratophrys ornata*): (b) Immature, basophilic erythrocyte; (c) Rubricyte. American toad (*Anaxyrus [Bufo] americanus*): (d) Granular lymphocyte; (e) Anucleate erythrocyte or erythroplastid. Axolotl (*Ambystoma mexicanum*): (f) Toxic, immature heterophil/neutrophil with cytoplasmic basophilia, purple primary granules, and mild vacuolation with an oval nucleus. Eastern tiger salamander (*Ambystoma tigrinum*) with confirmed sepsis: (g) Toxic neutrophil with a circular nucleus exhibiting Döhle bodies and cytoplasmic basophilia; (h) Toxic neutrophil with a hypersegmented nucleus from the same salamander as (g). Mud puppy (*Necturus sp.*) with confirmed bacterial infection: (i) Toxic neutrophil.

THROMBOCYTES

Amphibians have nucleated thrombocytes, the functional equivalent of mammalian platelets. These are typically the smallest blood cells found on the blood film. They are round to oval or spindle-shaped and have small amounts of clear to pale blue cytoplasm[4,9] (Figure 135.1n,u). Activated thrombocytes may have increased numbers of eosinophilic cytoplasmic granules. Immature thrombocytes may be encountered, and these have rounder nuclei and more basophilic cytoplasm

than mature thrombocytes.[1,4,9] Thrombocytes often cluster together on the blood film, impairing estimation of their numbers (Figure 135.1ab). Enumeration of thrombocytes is uncommonly done, but is possible using a Natt-and-Herrick-stained sample on a hemocytometer, or they can be estimated on a stained blood film. With the Natt and Herrick stain, thrombocytes are smaller than RBCs and have pale basophilic cytoplasm, compared to the more deeply basophilic leukocytes.[9] If clotting occurs in the sample tube, thrombocytes will be trapped within the clot and are therefore less numerous on blood film preparations or in the hemocytometer.

HEMATOLOGIC DISEASE

Abnormalities of WBC number and morphology are not as well-studied as those of mammals or birds.[1] In general, leukocyte responses may be interpreted in a similar way to those of other species. Neutrophilia is generally expected in response to inflammation, though decreased numbers may result in severe, acute inflammatory disease. Toxic changes consistent with those found in mammals, including increased Döhle bodies, cytoplasmic basophilia, and vacuolation, are commonly seen in amphibian's immune response to infectious agents; especially typical gram-negative pathogens found in aquatic environments, such as *Vibrio* and *Aeromonas* (Figure 135.2f–i). The presence of circular neutrophil nuclei and hypersegmented nuclei has been found in diseased animals. The clinical significance of these findings, if any, is unknown.

Eosinophils may increase in parasitic disease, and lymphocytes may increase due to an immune response, fear/excitement, viral disease, or lymphoid neoplasia.[4] Hemoparasites identified in Amphibia include extracellular *Trypanosoma sp.*,[13] *Hepatozoon sp.*,[2] and intraerythrocytic rickettsial organisms.[7]

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CHAPTER 136

Hematology of Invertebrates

JILL E. ARNOLD

Hematologic Methods
- Phlebotomy Sites by Body Type
- Anticoagulants and Sample Handling
- Laboratory Analysis

Hemocytes
- Arthropoda/Crustacea—Lobsters, Crabs
- Arthropoda/Chelicerata—Horseshoe Crabs and Spiders
- Mollusca/Bivalvia—Oysters, Clams
- Echinodermata/Echinoidea—Urchins and Starfish
- Hematopoiesis

Hematologic Response to Disease
- Physiologic
- Inflammation and Intoxication

Acronyms and Abbreviations

N:C, nucleus to cytoplasm ratio.

Current estimates of named living animal species are approximately 1,382,400, of which 58,000 (4%) are vertebrates and 1,324,400 (96%) are invertebrates represented in 30 phyla.[4] Corals, worms, snails, clams, lobsters, spiders, and sea stars illustrate the diversity of only a few representatives. Respecting the magnitude of this group of animals, only four selected phyla will be represented including Arthropoda/Crustacea (lobsters, crabs), Arthropoda/Chelicerata (horseshoe crabs, spiders), Mollusca/Bivalvia (oysters, clams), and Echinodermata/Echinoidea (sea urchins, sand dollars).

The United Nations Food and Agriculture Organization 2015 world aquaculture report described the global annual aquaculture production by relative percent, tons, and US dollar value by food category. Crustaceans (crabs, lobsters, and shrimp) represented 10% (7.4 million tons) and were valued at US$38.5 billion. Bivalves (clams, cockles, mussels, oysters, and scallops) represented approximately 21% (16.4 million tons), valued at US$17.9 billion. Nine species of invertebrates categorized as "other animals," which include the edible urchins, made up 1 million tons and were valued at US$3.9 billion.[25]

The commercial value of the horseshoe crab (*Limulus polyphemus*) is due to its hematologic characteristics and the unique ability of the hemocytes (often called amebocytes) to detect lipopolysaccharides and 1,3-β-D-glucan in the cell walls of bacteria and fungi, respectively. This high sensitivity is the basis of the Limulus Amebocyte Lysate test, used to assure safety of injectable drugs and implantable medical devices. Horseshoe crabs are also used in the eel and whelk fisheries industry as bait.[17] The combined fishing pressures have resulted in decreasing Atlantic populations which may also be impacted by climate change, resulting in an International Union for Conservation of Nature designation of vulnerable.

Hematology for health assessment is not widely practiced for invertebrates. Further development of hematologic techniques is needed to address the challenges of working with viscous hemolymph, the diversity of hemocyte characterization for so many species, and to consider the rapid changes in hemocyte distribution due to internal and external factors, as well as life stage for species that molt. Similar to early attempts to develop hematology methods for avian and reptilian species,

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invertebrate hematology is becoming an important diagnostic tool as techniques are developed.

HEMATOLOGIC METHODS

Phlebotomy Sites by Body Type

Hemolymph, derived from Latin for blood/clear water, is comprised of cells, water, dissolved organic salts, and proteins. The cells, termed hemocytes, move freely in and around the tissues and organs. Circulatory systems vary in complexity with the general principal that a pumping organ, or developed heart, sends hemolymph to the gills and oxygenated hemocyanin is returned to the heart. The animals discussed in this chapter have an open circulatory system and lack vasculature.

Typical phlebotomy sites for crustaceans are the ventral abdominal sinus, at the first or second abdominal segment in lobsters[3] (*Homarus americanus*) (Table 136.1a), and the arthrodial membrane at the base of the chelae (large claw) or swimming leg of crabs (Figure 136.1). Hemolymph is collected from the pericardial sinus of the horseshoe crab (*L. polyphemus*) through the arthrodial membrane between the prosoma and opisthosoma (Table 136.1c). Hemolymph is drawn from the adductor muscle of hard-shelled oysters and clams (class Bivalvia) after drilling a hole in the shell or directly from the heart after shucking the shell open (Table 136.1b). The adductor muscles of soft-shell clams (e.g., *Mya arenaria*) may be accessed from partially opening the shell. Coelomic fluid is collected from sea urchins and sand dollars (class Echinoidea) from the peristomal membrane adjacent to the mouth, with care to avoid damage to the internal organs by angling the needle 45° away from the oral/anal axis, directing the needle toward the body wall[22] (Table 136.1d).

Anticoagulants and Sample Handling

Given the variety of terrestrial and aquatic invertebrate species, it is understandable that there are no standardized anticoagulants. Multiple formulations are found in the literature (Table 136.2). Techniques reported to prevent or slow cell aggregation include collecting hemolymph into syringes prefilled with ice-chilled solutions of anticoagulant or seawater, keeping syringes and sample tubes on ice (for marine species), and using formalin or formaldehyde to fix the cells.[2,3]

Laboratory Analysis

Hemolymph's viscous nature presents challenges for preparing smears and techniques for vertebrate blood must be modified. Various slide preparation techniques have been reported including staining a cytocentrifuged, hemolymph pellet with H&E, and toluidine blue. Lobster (*H. americanus*) hemolymph, drawn directly into a syringe containing cold formalin, was prepared using coverslips, methanol fixed, and stained with Giemsa for 30 minutes, followed by

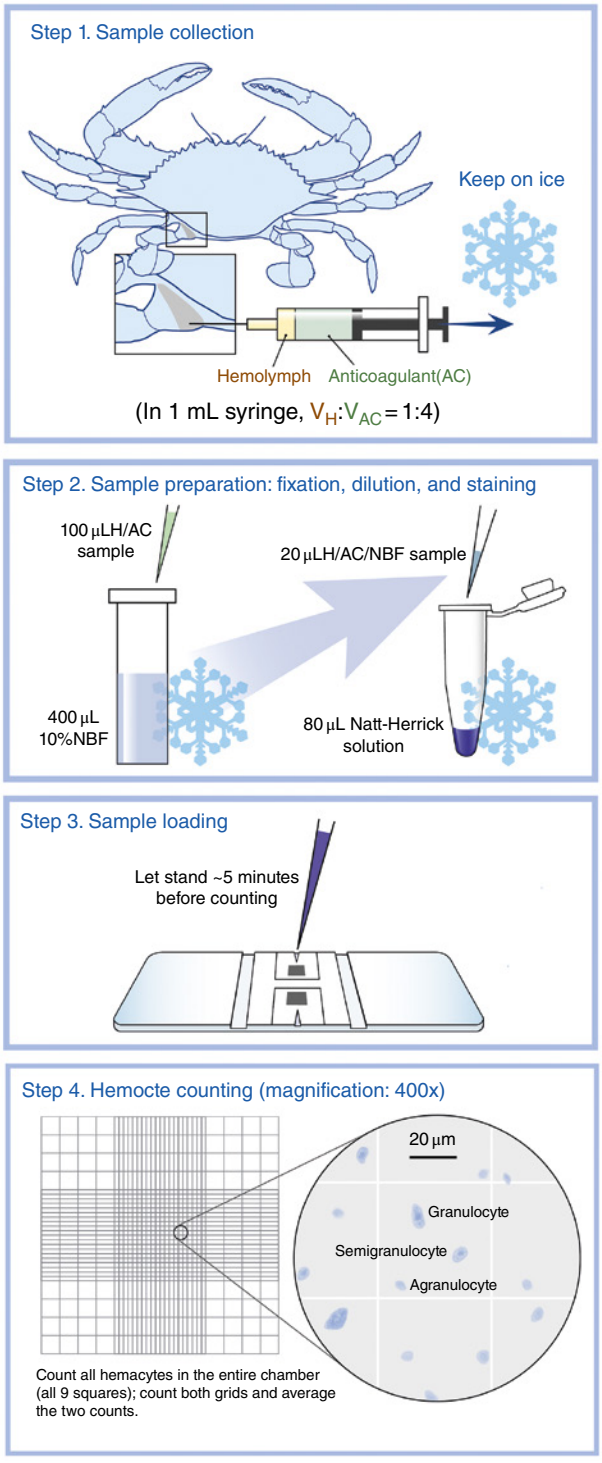
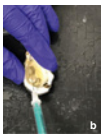
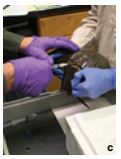


FIGURE 136.1 Blue crab (*Callinectes sapidus*) phlebotomy and hemocyte count. (Step 1) Hemolymph is drawn from the arthrodial membrane at the base of the swimming leg into a syringe containing chilled anticoagulant; (Step 2) Contents of the syringe are quickly transferred to vial (not shown) and a 100 µL aliquot is mixed with 400 µL 10% neutral buffered formalin to fix the cells. The final 1:100 dilution for cell counting is prepared by adding 20 µL fixed cells to 80 µL Natt and Herrick's solution; (Step 3) Neubauer hemocytometer is loaded; (Step 4) All hemocytes are tallied in the nine large squares from both grids and averaged. Cells × 110 = total hemocyte count/µL. (**Source:** Illustration by Wenjing Wu, CMI 2020©Wenjing Wu.)

TABLE 136.1 **Hemolymph collection for four invertebrate species, illustrating different phlebotomy sites based on body type. (a) American lobster (*Homarus americanus*); (b) Pacific oyster (*Crassostrea gigas*); (c) American, or Atlantic, horseshoe crab (*Limulus polyphemus*); (d) Sand dollar (*Dendraster excentricus*). Sand dollar image courtesy of Monterey Bay Aquarium.**

Species	Phlebotomy site	Sample handling	Photo
Lobster	Ventral abdominal sinus	Hemolymph drawn into syringe prefilled with chilled anticoagulant (1:5)[3]	
Oyster	(A) Heart (shown) from a shucked oyster (B) Adductor muscle from drilled hole in shell (not shown)	(A) Hemolymph drawn into sterile syringe, then fixed in chilled 10% neutral buffered formalin (1:5) (B) Hemolymph into syringe prefilled with chilled seawater	
Horseshoe crab	Heart/pericardial sac via the arthrodial membrane	Hemolymph drawn into syringe prefilled with chilled anticoagulant[27] (1:4), then fixed in chilled 10% neutral buffered formalin (1:5)	
Sand dollar	Coelomic fluid via the peristomial membrane, taking care not to damage internal organs	(A) See horseshoe crab (B) Fluid collected into ice cold anticoagulant[22]	

rinsing in distilled water (pH 4) until the stain color transitions to pink.[9] Slides for lobster hemocytes can also be prepared using a cytocentrifuge, by loading 100–200 µL of hemolymph-anticoagulant solution or formalin-fixed cells into the sample cup, resulting in a diagnostic sample.[3] The slides can be stained with Wright–Giemsa or other Romanowsky stains. Formalin-fixed hemocytes stained in any Romanowsky stains may be very dark blue, and better viewing of cellular features can be obtained by diluting the methylene blue or similar purple stain in distilled water at a ratio of 1:50 (Figure 136.2).

Automated hemocyte counts using flow cytometry have been reported for Pacific oysters (*Crassostrea gigas*)[11] and purple sea urchins (*Strongylocentrotus purpuratus*).[22] Manual total hemocyte counts can be performed by standard methods using hemocytometers. If the hemolymph or coelomic fluid is collected directly into anticoagulant solution, this initial dilution must be included in determining the final multiplication factor for the number of cells tallied (Figure 136.1). Some reports employ no special cell counting diluent beyond anticoagulant for lobsters, blue crabs, and tarantulas.[3,27,23] Others have used formalin solutions for lobsters and Pacific oysters.[2,13] Cell counting diluents for birds, reptiles, and fish may be used.[18]

This chapter's author uses the Natt and Herrick formula, supplementing the stock solution with NaCl (0.14 g/10 mL) and urea (0.32 g/10 mL) for marine species whose hemolymph osmolality values approximate the surrounding seawater. Attempt to collect 0.5 mL hemolymph directly drawn into 1.5 mL chilled anticoagulant (0.3 M NaCl, 0.1 M glucose, 30 mM Na citrate, 26 mM citric acid, 10 mM EDTA; pH = 5.4)[27], for approximately 1:4 dilution. The sampling dilution factor may be variable based on hemolymph volume acquired. The syringe contents are quickly transferred to a chilled sterile vial. Then 100 µL anticoagulated hemolymph is immediately transferred to a vial containing 400 µL chilled 10% neutral buffered formalin (1:5 dilution). The second dilution factor, 5, is standard using this technique. The vials are brought to ambient temperature for transport to the lab. Prepare the 1:100 final dilution by adding 20 µL of the formalin-fixed cells to 80 µL Natt and Herrick stain. Load the Neubauer hemocytometer and tally all the hemocytes in the nine large squares of both grids, average, and multiply by 110 for hemocytes/µL (Figure 136.1).

Hemolymph total dissolved solids can be measured by refractive index as for vertebrate plasma. Hemocyanin is the respiratory pigment of cephalopods (octopus, squid, cuttlefish), gastropod mollusks (snails, slugs, limpets), and arthropods (horseshoe crabs and spiders) and can be measured by spectrophotometric methods.[7] Hemocyanin is an extracellular, copper-based oxygen-carrying protein that rapidly releases oxygen. In contrast to the gastropod mollusks, the bivalve mollusks (clams and oysters) and echinoderms

TABLE 136.2 Anticoagulant formulas for invertebrates, converted to per liter from the (published formula concentrations).

Chemical (per liter)	Lobster[3]	Blue crab[27]	Urchin[22]	Spider[23]	Horseshoe crab[7]
Calcium Chloride dihydrate ($CaCl_2$-$2H_2O$)	1.5 g				
Sodium chloride (NaCl)	28.4 g	17.5 g (0.3 M)	29.2 g (500 mM)	8.2 g (0.14 M)	3 g (3%)
Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	8.7 g				
Magnesium chloride hexhydrate ($MgCl_2.6H_2O$)	5.5 g				
Potassium chloride (KCl)	0.7 g		1.9 g (26 mM)		
Tween 80	0.5 mL				
37–40% formaldehyde	1.25 mL				25 mL
Glucose ($C_6H_{12}O_6$)		18.0 g (0.1 M)		18.0 g (0.1 M)	
Dextrose ($C_6H_{12}O_6$)					18.16 g (100 mM)
Sodium citrate dihydrate ($C_6H_5Na_3O_7.2H_2O$)		8.8 g (30 mM)		8.8 g (0.03 M)	
Citric acid monohydrate ($C_6H_8O_7.H_2O$)		5.5 g (26 mM)		5.5 g (26 mM)	
Citric acid anhydrous ($C_6H_8O_7$)					9 g (47 mM)
EDTA ($C_{10}H_{16}N_2O_8$)		2.9 g (10 mM)		2.9 g (10 mM)	2.9 g (10 mM)
EGTA ($C_{14}H_{11}N_2O_{10}$)			19 g (50 mM)		
TRIS ($C_4H_{11}NO_3$)			1.2 g (10 mM)		
Solution pH	7.6	5.4	7.4	4.7	4.6

Prepare solutions by adding the chemical amounts to a 1-liter volumetric flask and q.s. to mark with distilled or deionized water. Lobster (*Homarus americanus*), blue crab (*Callinectes sapidus*), urchin (*Strongylocentrotus purpuratus*), spider (*Lasiodora sp.*), and horseshoe crab (*Limulus polyphemus*).

(urchins) lack hemocyanin and utilize cell-bound hemoglobin within nucleated cells that are structurally like that of vertebrates.[8]

HEMOCYTES

Arthropoda/Crustacea—Lobsters, Crabs

While terminology varies somewhat in the literature, the consensus is that three cell morphology types, large granule, small granule, and hyalinocytes, can be consistently identified in crustacean hemolymph (Figure 136.2). Large granule hemocytes have a low N:C ratio and the cytoplasm is filled with granules (>1 μm), which store prophenol-peroxidase. The nucleus is usually eccentric. These cells have a primary role in phagocytosis of foreign material and fungal encapsulation. Small granule hemocytes also have a central nucleus, low N:C ratio and the cytoplasm contains fewer, smaller granules (<1 μm) that contain lysosomal enzymes. One main function of this cell is phagocytosis of bacteria. Hyaline hemocytes, in contrast, have a high N:C, and the cytoplasm contains abundant small electron-dense deposits in the cytoplasm. They serve a primary role in coagulation and are rich in transglutaminase activity, essential for clotting reactions.[3,15]

Hemocyte differential counts vary among species and are subject to rapid change depending on environmental factors, especially for species that molt. For example, the blue crab (*C. sapidus*) is reported to have changes in both total and differential hemocyte counts during the molting process. Intermolt crabs averaged $6 \times 10^3/\mu L$ hemocytes. Average total hemocyte counts increased to $14 \times 10^3/\mu L$ in late premolt and then returned to intermolt levels during ecdysis (molt). The differential counts showed marked

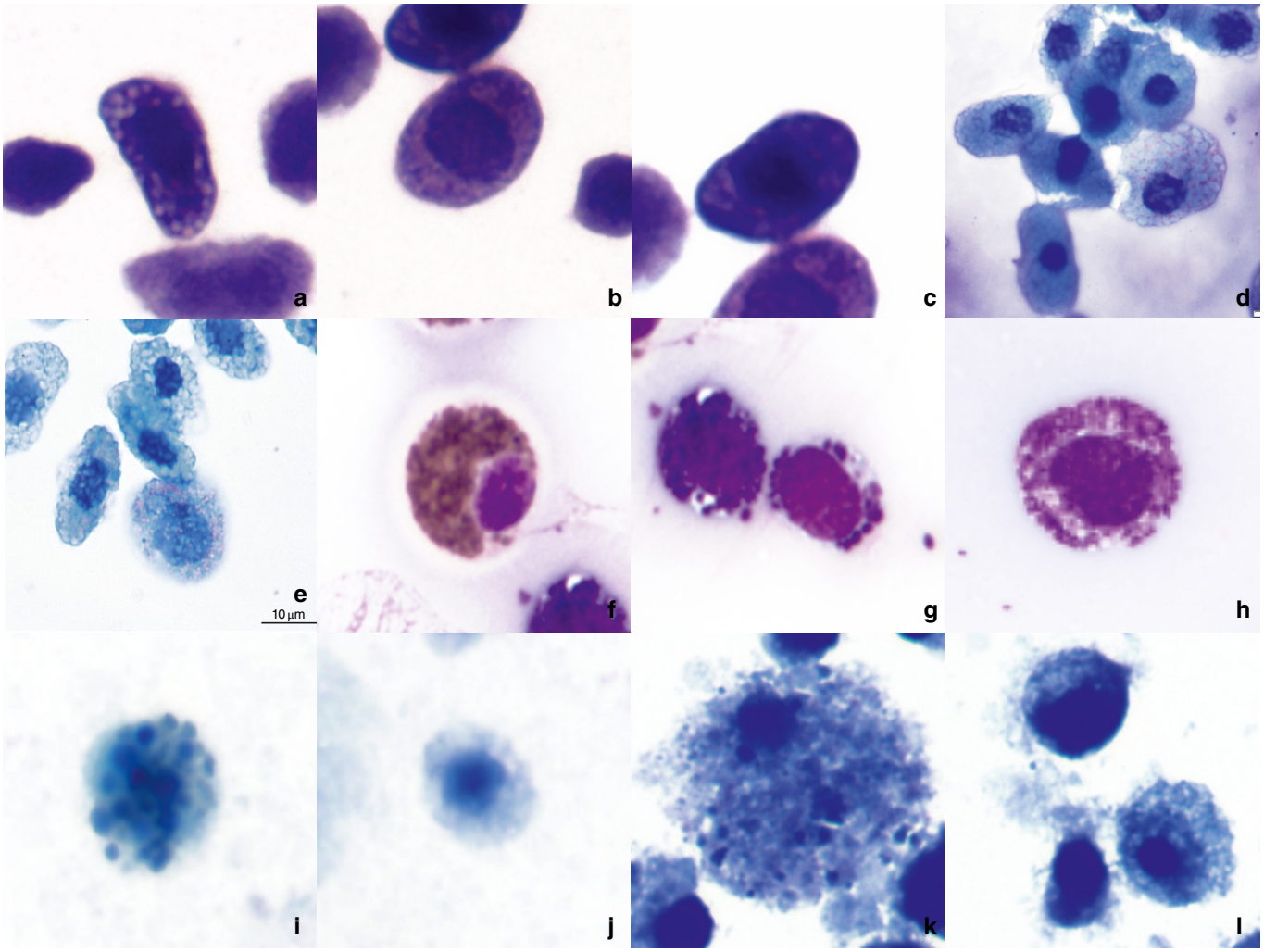


FIGURE 136.2 Hemocytes from selected invertebrates. All hemocytes are prepared using a formalin preservation technique and a Romanowsky stain, except for f-h. See text for approximate sizes. Apparently healthy blue crab (*Callinectes sapidus*): (a) Large granule hemocyte; (b) Small granule hemocyte; (c) Hyaline hemocyte (possible thrombocyte). Apparently healthy American horseshoe crab (*Limulus polyphemus*): (d) Amebocytes; (e) Amebocytes, noting slightly different granularity. Apparently healthy emperor scorpion (*Pandinus imperator*): (f) Unidentified granulocyte (possible heterophil); (g) Two cells with basophilic granules; (h) Larger granulocyte with basophilic granules. No formalin was used in the scorpion sample preparation, 100× objective, Wright–Giemsa stain. Courtesy of Dr. Nicole Stacy. Apparently healthy Pacific oyster (*Crassostrea gigas*): (i) Granulocyte; (j) Agranulocyte, aka hyalinocyte. Cell lineage is unknown. It should be noted that bivalves are reported to use cell-bound hemoglobin within nucleated cells similar to vertebrates. Diseased sand dollar (*Dendraster excentricus*): (k) Large, activated phagocyte; (l) Phagocytic coelomocytes with variable numbers of vacuoles. (Courtesy of Monterey Bay Aquarium.)

changes, with hyaline cells (possible thrombocytes) predominant during intermolt and small granular cells that phagocytize bacteria predominant through ecdysis, along with a moderate increase in all granulocyte concentrations. The differential cell counts shifted back to intermolt levels during the postmolt (shell hardening) period.[1] The highest mitotic activity is reported in the proecdysis/peeler stages (preparing to molt) in multiple species, which suggests need for increased hemocyte numbers during this vulnerable life stage.[24]

Arthropoda/Chelicerata—Horseshoe Crabs and Spiders

In contrast to the decapod crustaceans, there is a single type of hemocyte in the four extant species of horseshoe crabs; Atlantic, or American, horseshoe crab (*L. polyphemus*) found along the East Coast of North America, the mangrove horseshoe crab in Southeast Asia (*Carcinoscorpius rotundicauda*), the Indo-Pacific horseshoe crab (*Tachypleus gigas*), and the Japanese, or trident, horseshoe crab (*Tachypleus tridentatus*) found in

Southeast and East Asia. The cell is referred to as an amebocyte because it becomes motile and adherent when removed from the body or upon encountering foreign material. Inactivated amebocytes are large, approximately 20 µm in length, oval cells with cytoplasm that contains abundant large and small round granules (Figure 136.2). When exposed to bacterial endotoxin, the cells rapidly activate, markedly changing shape with numerous filopodia, and release granule contents.[26] This rapid response to endotoxin was the basis for the development of the Limulus Amebocyte Lysate assay in the 1960s, which is still a primary method for testing sterility of injectable drugs and implantable medical devices.[17]

In marked contrast to the horseshoe crab, spiders and tarantulas are reported to have multiple cell types, with varied nomenclature strategies proposed (Figure 136.2). Soares et al.[23] offered a comprehensive morphology and ultrastructure report of six cell types from the cardiac hemolymph from 16 Brazilian tarantula spiders (*Lasiodora sp.*). The cell names, sizes, and average abundance from the differential cell count are as follows: prohemocytes (10–15 µm; 8.1%) had a high N:C and homogenous cytoplasm filled with varied numbers of granules; granulocyte type I (25–30 µm; 35.2%) are elongate cells with a central lobed nucleus and cytoplasm filled with prominent granules; granulocyte type II resembles type I, but is larger (35–40 µm; 29.6%); spherules, or spherulocytes (15–25 µm; 11.1%), are round cells, with a round nucleus, a high N:C ratio, and large round cytoplasmic granules, postulated to be involved in coagulation; plasmatocytes (15–20 µm; 13%) were highly pleomorphic, from round to spindle-shaped and had no visible granules in the cytoplasm; oenocytoids (15–20 µm; 3%) are round cells with homogeneous cytoplasm. *Oenocytoids*, a term which may be unfamiliar, are cells present in low numbers in hemolymph of many insect species and their function is not known.[19] In contrast with the Brazilian tarantula (*Lasiodora sp.*), the wandering spider (*Cupiennius salei*) was reported to have a predominance of plasmatocytes (70–80%) with fewer granulocytes (20–30%) and only a few prohemocytes and cyanocytes.[16] Nomenclature for cells from spiders follows convention for other insects. More research, including exploration of cytochemistry and ultrastructural appearance, is warranted.

Mollusca/Bivalvia—Oysters, Clams

Marine bivalve hemocytes are characterized into two groups by light microscopy: granulocytes (cytoplasm contains abundant granules) and hyalinocytes (no visible granules) (Figure 136.2). Both phagocytic cell types aggregate and are motile using pseudopodia. A comparative study of hemocytes found in three Korean oysters (*Saccostrea kegaki*, *Ostrea circumpicta*, and *Hyotissa hyotis*) reported similar cell morphology and abundance. The hyalinocyte was most abundant and comprised approximately 60% of the cells followed by granulocytes (23–31%) and a third, much smaller (<10 µm), blast-like cell (3–5%).[14] Comparable hemocyte descriptions have been reported for the Pacific oyster (*C. gigas*) and authors suggest the blast-like cell may be a stem cell.[11]

Echinodermata/Echinoidea—Urchins and Starfish

The predominant hemocyte in the coelomic fluid of the purple sea urchin (*S. purpuratus*) are round phagocytes (small and large, approximately 30 and 50 µm, respectively). Rapidly motile round cells, termed vibratile cells (approximately 5 µm), move in a uniform direction by means of a single, long flagella. There are two types of spherule cells, or amebocytes (approximately 5 µm), one whose cytoplasmic vesicles contain the red pigment echinochrome A, with antibacterial properties, and the other amebocyte's vesicles lack this pigment.[22]

Hematopoiesis

Hematopoietic tissue of decapod crustaceans is found adjacent to, or enveloping, the ophthalmic artery (dorsal median artery) and on the dorsal and/or lateral sides of the cardiac stomach. The tissue comprises lobules, covered with fibrous connective tissue, with hemocyte precursors at different developmental stages within these connective tissue layers. Another hematopoietic tissue is situated between the stomach muscles and the anterior part of the hematopoietic tissue near the brain, initially termed the "cephalic gland" and later renamed the "anterior proliferation center". Early studies suggest that mesodermal cell masses on either side of the aorta may be the origin of hematopoietic tissue during embryonic and larval development in crayfish; however, the specific origins are not yet known.[24]

Giemsa-stained histologic sections of embryonic horseshoe crabs (*L. polyphemus*) showed the earliest appearance of hemocytes occurred at the 18th of 21-stage development. While no obvious hemopoietic tissue was found, the hemocytes were observed in the hemocoel cavities, usually in close association with connective tissue.[10] In contrast to the xiphosurans, hematopoietic sites are known for fellow chelicerates, e.g., scorpions and spiders. Scorpion hematopoiesis occurs in supraneural and lymphoid glands, while hematopoietic tissue in spiders is found in the heart wall.[16]

The site of hemopoiesis in bivalves remains unclear; however, immunohistochemistry has demonstrated hemocyte progenitor cells in the tubules and underlying hemolymph vessels of specialized regions of gill epithelium in the oyster (*C. gigas*).[20] A 2019 study induced coelomocyte proliferation in the echinoid purple sea urchin (*S. purpuratus*) after challenge with *Vibrio diazotrophicus* and by coelomic fluid depletion. Newly formed coelomocytes were detected both in the axial organ, a core of spongy tissue within the axial sinus of the hemal vascular system, and the pharynx, suggesting these are sites of hemopoiesis.[12]

HEMATOLOGIC RESPONSE TO DISEASE

Physiologic

There is little known regarding invertebrate response to physiologic stress.

Inflammation and Intoxication

A challenge study in lobsters showed that individuals in the treatment group in late-stage infection with gaffkemia (*Aerococcus viridans* var. *homari*) had immature hemocytes, suggesting a similar left-shift as observed in vertebrates. The total hemocyte count showed an initial increase at day 1 post inoculation with *A. viridans* (mean $26.6 \times 10^3/\mu L$). Then counts decreased to ranges comparable to the control group by day 4 ($16.5 \times 10^3/\mu L$).[3]

Mean total hemocyte concentrations from Pacific oysters (*C. gigas*) at an oil-spill site (345.2 hemocytes/μL) compared to those at an unpolluted reference location (401.2 hemocytes/μL) showed no significant difference. However, the proportion of granulocytes found in oil-spill oysters was half that of oysters sampled at the control site, suggesting the highly phagocytic granulocytes may be the cells most affected by petroleum intoxication. Oil-spill oysters had a slightly higher percent of blast-like cells (14.7% vs 10.7%) than control oysters.[11] Conversely, a report on parasitic infestation showed that mean total hemocyte concentrations approximately doubled in Pacific oysters (*C. gigas*) with the paramyxean protozoa (*Marteiliodes chungmuensis*) compared to the control group (410 hemocytes/μL and 210 hemocytes/μL, respectively). Differential cell counts differed as well, with infested oysters having increased granulocyte numbers.[6]

Spiders exposed to azadirachtin, a neem-tree extract used to control mosquitos, include reports for hemocyte counts because of the cell's role in phagocytosis of foreign material. Wolf spiders (*Schizocosa episina*) fed 10 ppm azadirachtin-treated prey showed a significant decrease in total hemocyte concentrations ($10.7 \times 10^3/\mu L$) compared to the control group (mean $37.3 \times 10^3/\mu L$).[21]

A 2019 study[5] of adult sea urchins (*Paracentrotus lividus*) challenged with bacterial lipopolysaccharide showed significantly higher total coelomocyte concentrations, and higher relative counts of phagocytic cells and red spherule cells, compared to control urchins. The test group of 12 urchins received an intracoelomic injection of *Escherichia coli* lipopolysaccharide suspended in a salt solution of similar composition to natural coelomic fluid, while the control group of 12 received a sham injection of the salt solution. Serial coelomic fluid samples were collected at 1, 3, 6, and 24 hours postinjection. Total coelomocyte counts were determined with a Burker hemocytometer. Differential coelomocyte counts from smears classified the cells as phagocytic cells, colorless spherule cells, red spherule cells, and vibratile cells. Results showed a significant rise in total cell concentrations 1 hour post-treatment ($10.4 \pm 0.74 \times 10^3/\mu L$; $p < 0.01$), compared to the control group ($8.1 \pm 0.65 \times 10^3/\mu L$) with peak levels at hour 3 ($11.6 \pm 0.65 \times 10^3/\mu L$), returning to the same level as the control urchins by 6 hours. The relative differential by cell type showed a rapid, significant increase in phagocytic cells and red spherule cells ($p < 0.001$), approximately twice that in controls. There was no significant difference in colorless spherule cells or vibratile cells compared with controls. The findings demonstrate the cellular defense mechanisms of specific coelomocytes in response to bacterial challenge.

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SECTION X

Quality Management and Laboratory Techniques

CHAPTER 137

Quality Management of Hematology Techniques

MARTINA STIRN and KATHLEEN P. FREEMAN

Extra-Analytical Factors
Analytical Factors of Special Importance in Veterinary Hematology
  Quality Goals (Performance Specifications)
    Milan Hierarchy
    ASVCP Guidelines: Allowable Total Error Hematology
    Biological Variation
  Nonstatistical Quality Control
    Manual Slide Evaluation
    Plausibility Checks in Veterinary Hematology
    Standard Operating Procedures and Documentation
    Personnel
  Statistical Quality Control
    Quality Control Material (QCM)
    QC Validation
    Repeat Patient Testing
    External Quality Assessment and Comparative Testing
Case Example

Acronyms and Abbreviations

ASVCP, American Society of Veterinary Clinical Pathology; CHCM, cellular hemoglobin concentration mean; CLIA, Clinical Laboratory Improvement Amendments; CV, coefficient of variation; ECVCP, European College of Veterinary Clinical Pathology; EFLM, European Federation of Clinical Chemistry and Laboratory Medicine; EQA, external quality assessment; ICSH, International Council for Standardization in Hematology; MPV, mean platelet volume; PDW, platelet distribution width; P_{ed}, probability of error detection; P_{fr}, probability of false rejection; POC, point of care; PT, proficiency testing; QA, quality assurance; QC, quality control; QCM, quality control material; RDW, red cell distribution width; RPT, repeat patient testing; SD, standard deviation; SOP, standard operating procedure; TE, total error; TE_a, total allowable error; TE_{obs}, total error observed; WHO, World Health Organization.

Many automated and manual techniques are used in veterinary hematology and quality management is an integral part of ensuring accurate and precise results. Different species with different hematologic properties complicate veterinary hematology and further increase the importance of comprehensive quality management. In recent years, the American Society of Veterinary Clinical Pathology (ASVCP) published several guidelines on quality management and control, providing tools to the veterinary community to achieve and improve quality in veterinary hematology.[2,21,50,11] Importantly, quality management does not only comprise analytical aspects, but also preanalytical and postanalytical factors.

EXTRA-ANALYTICAL FACTORS

Clinical, preanalytical, analytical, and postanalytical phases represent the "brain-to-brain loop"; a continuum of concepts relevant for laboratory testing starting when the clinician first makes a decision to order laboratory testing, through ordering, collection, identification, transportation, specimen preparation, analysis, reporting, interpretation, to taking appropriate actions for the patient via further monitoring, additional testing, and/or treatment.[38] Plebani has presented a paradigm for human laboratory testing, known as the "five rights" for patients, which provides valuable insight for veterinary patients. The five rights include: (1) appropriateness, (2) personalized and patient-centered service, (3) focus on outcomes, (4) value, and (5) diagnostic partnership. This paradigm addresses clinical and preanalytical factors such as ensuring that the right test is selected for the right patient, at the right time (with correct patient preparation), with the right specimen collection and handling, and the right transportation. The preanalytical stage within the laboratory includes the correct pretreatment

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(if needed), sample separation, aliquoting, sorting, and routing through the laboratory. In the postanalytical phase, the "five rights" comprise the right turnaround time, data validation, units, reference intervals/decision limits, and interpretive comments to aid the clinician. In the clinical (postanalytical) phase, the "five rights" involve the right acknowledgment (e.g., timely review of laboratory results), interpretation, utilization (diagnosis/therapy), follow-up, documentation of results in the medical record, and actions initiated. These rights emphasize the joint responsibility of the laboratory and clinicians for the right/wrong utilization of laboratory information.

Preanalytical aspects of hematologic testing are related to animal status (e.g., fasting, physical exercise, drug intake), sampling process (e.g., animal handling, excitement, order of draw), and the sample itself (e.g., anticoagulant, tube fill, storage, labeling). Information concerning important preanalytical requirements should be provided to clients in writing to ensure appropriate sample quality.[50]

Although, the influence of fasting status is less pronounced for hematologic analyses than for biochemistry or hormone analyses, lipemic samples should be avoided, since turbidity will interfere with photometric hemoglobin (Hgb) measurement. In addition, lipids may form small droplets in vitro that cause spurious increases in platelet counts, mean cellular hemoglobin (MCH), mean cellular hemoglobin concentration (MCHC), and WBC counts (Figure 137.1).[54,55] In addition to analytical interferences, fasting status may also lead to in vivo variations in hematologic values. While severe food restriction for more than 48 hours has been shown to cause bone marrow and lymphoid depletion in rats,[30] less severe fasting for shorter time periods results in no or variable mild hematologic alterations in rats and monkeys.[34,56] While these alterations are clinically insignificant, they may be important in toxicology studies, requiring careful consideration of the fasting status.

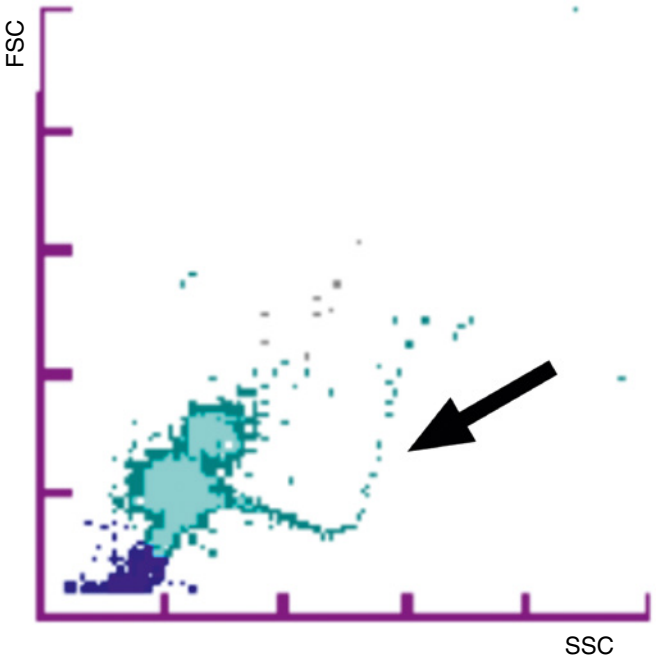


FIGURE 137.1 Sysmex XT-2000iV WBC/BASO scattergram of a canine blood sample (*x*-axis displays the side scatter; *y*-axis displays the forward scatter). Lipid droplets form a sigmoid curve on the scattergram (black arrow). (Source: Courtesy of Clare Doyle, SYNLAB VPG Hitchin.)

Before and during sampling, excitement should be avoided to reduce the presence of epinephrine-induced leukocytosis and spurious alterations in the WBC differential, due to shifting of neutrophils and lymphocytes from the marginal to the central pool.[44]

Hematologic analyses require K_2-EDTA- or K_3-EDTA-anticoagulated, citrated, or heparinized whole blood. Heparinized whole blood is only recommended for some birds, reptiles, and fish, since their blood may hemolyze when collected into EDTA (corvids, crows, jays, ravens, magpies, ostrich, brown kingfisher, pigeon, toucan, some species of ducks, cranes, hornbills and galliformes, chelonians, stingrays, and some bony fish).[26,10] In mammalians and other reptiles, birds, and fish, heparinized whole blood should not be used for hematology, since platelets and WBCs frequently clump and blood film staining quality is poor. Citrated whole blood is mainly used for coagulation assays and in rare cases with EDTA-induced platelet and WBC aggregates for hematology.[26] In other situations, citrated whole blood is not recommended, since lower platelet counts due to platelet aggregation commonly occur.[45] Except for the conditions above, EDTA is the anticoagulant of choice for hematological assays. It exists as spray-dried K_2-EDTA and liquid K_3-EDTA; statistically significant differences comparing tubes with spray-dried K_2-EDTA tubes and liquid K_3-EDTA tubes were considered to be clinically insignificant and due to the dilutional effect of the liquid anticoagulant.[48] Both types of salts can be used for hematologic analyses. Tubes filled in accordance with manufacturer-recommended volumes are more important. According to the Clinical and Laboratory Standards Institute (CLSI), the draw volume should be no more than 10% below the manufacturer-recommended volume and more pronounced underfilling of the tubes has the potential to affect accuracy[13] (e.g., decrease of spun PCV due to shrinking of RBC artifactually lowers mean cell volume (MCV)).[55] Comparable information is lacking for samples from veterinary species, but an effort should be made to achieve the recommended blood/anticoagulant ratio by filling to the line indicated on blood tubes. Specimen tubes should be labeled with a unique identifier that allows the laboratory to assess tube fill and sample quality.

Before sample shipment, blood films should be made in the clinic and protected from condensation, refrigeration, and extreme temperatures during transport to the laboratory.[50] Morphologic changes occurring as soon as 3 hours after blood draw are well described in humans.[57] Changes include nuclear swelling, decreased chromatin clumping, and cytoplasmic vacuolization in WBCs. In RBCs, crenation, sphering, fragmentation, and disappearance of nucleated RBCs may occur over time. Morphological changes in canine neutrophils during

storage have recently been described; when stored at room temperature, Döhle-body-like inclusions were observed after only 4 hours.[5] Hematologic sample stability has been assessed for different species and different analyzers.[8,6,39,23,12] Increased hematocrit (Hct) and MCV and decreased MCHC due to RBC swelling are commonly observed using different analyzers in multiple species and occur in as little as 6 hours after sampling. Inconsistent alterations, likely depending on species and hematology analyzer, are reported for platelet, WBC, and differential cell counts.[8,6,23,12] Most of the available information has been obtained using samples from healthy animals; but, one study in dogs showed that discrepancies due to sample storage were more pronounced in clinically ill dogs.[8] Laboratories should therefore provide guidance to clinicians regarding sample stability valid for their respective analyzer and careful evaluation of blood smears and scattergrams is needed to ensure accuracy of automated results.

Postanalytical procedures must provide legible and accurate results, transmitted in a timely manner to clients. Report formatting must be easily understood and, depending on the clients' expectations, explanatory comments might be needed. For example, an online survey showed important differences between clinicians' and clinical pathologists' understanding of the clinical relevance of RBC morphology terms.[32] Results should be released with validated or verified reference intervals. Details about reference intervals should be made available to clients upon request.[2]

The use of sophisticated laboratory information systems is the main driver reducing postanalytical errors. However, especially in veterinary hematology where many analyses are still performed manually, procedures should be in place to avoid manual data entry errors. Such procedures include double-verification procedures, medical validation of results by clinical pathologists, result review criteria, and δ checks. In human laboratories, efforts are made to implement automatic validation of test results to reduce subjectivity, time, and the need for a physician in the laboratory.[25]

Specimens should be archived under appropriate conditions or disposed of safely, according to biosafety regulations.[2] Except for stained hematology slides, hematologic samples can only be stored for a limited amount of time. Procedures for storage and disposal should be defined according to laboratory policies and accreditation requirements.[2] It is the responsibility of the laboratory management to define biosafety risks; all laboratory personnel must be educated of these risks and specimens must be handled and discarded according to state and national biosafety regulations.

ANALYTICAL FACTORS OF SPECIAL IMPORTANCE IN VETERINARY HEMATOLOGY

Point-of-care (POC) and large hematology analyzers used in reference laboratories have undergone major technical improvements in the last decades and large number of different methods are applied in veterinary hematology. Therefore, quality management of the analytical phase is of immense importance. It comprises general aspects applicable to all areas of veterinary clinical pathology and procedures specific to hematology. Automated hematology instruments must be validated prior to use with analytical quality requirements established as part of the instrument validation study.[21] Quality control (QC) procedures, statistical and nonstatistical, should be defined for all methods. Statistical QC refers to the analysis of control materials and interpretation of internal and external QC results.[21] Nonstatistical QC in hematology includes quality planning activities, the use of standard operating procedures (SOPs), definition of blood film and pathologist review criteria, δ checks (comparison of serial patient test results), correlation of Hgb concentration and Hct, correlation of automated hematology results and blood film findings, training and standardization among laboratory personnel, assessment of staining quality, and monitoring of increased MCHC. Additional specific QC procedures, such as calibration of pipettes and balances and monitoring of cell-counting reagent quality, are required for nonmammalian hematology.[50]

Quality Goals (Performance Specifications)

Historically, quality improvement in laboratory medicine focused mainly on analytical aspects, such as assay standardization, automation, information technology, and personnel training.[28] Introduction and monitoring of quality indicators, such as analytical performance specifications, were important milestones. In a conference of the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM), held in 2014 in Milan, it was agreed that "for patient care, optimizing the quality of the total (preanalytical/analytical/postanalytical) examination process is the ultimate goal and, therefore, it would be desirable to go beyond setting analytical performance specifications and to establish examination performance specifications."[42] In veterinary hematology, only a few specific quality goals for the analytical phase are available, but more general goals and principles can be transferred from human medicine.

Milan Hierarchy

The "Milan hierarchy" is a consensus agreement defined during the 1st Strategic Conference of the EFLM in Milan in 2014. It describes three models to set analytical performance specifications (Table 137.1).[42]

1. The effect of analytical performance on clinical outcomes.
2. Components of biological variation.
3. State of the art, which is the highest level of analytical performance technically achievable.

TABLE 137.1 Summary of Milan hierarchy models for quality goals. Advantages and disadvantages are adapted to veterinary hematology.

Model	Key points	Advantages	Disadvantages
Model 1 Effect of analytical performance on clinical outcomes	Outcome studies investigate the impact of the test's analytical performance on clinical decisions or outcomes	Highly relevant to patients, since it addresses the influence of analytical performance on clinical outcomes	Can only be used if a strong link between the test and clinical outcome exists Actual test method may have different measurement quality than the method used in outcome studies Outcome studies may not be readily available
Model 2 Biological variation	Attempts to minimize the ratio of analytical noise of the biological signal	May be used for quality goals, as well as in analysis of patient results	The clinical relevance of biological variation data needs to be assessed Biological variation data are not available for all species
Model 3 State of the art	The highest achievable analytical performance based on current technology and methods	Information on state-of-the-art analytical performance is available for most hematology analyzers	Can only be used if a strong link between the state-of-the-art performance and clinical outcome exists State-of-the-art performance is not achievable by all laboratories using different methods

Source: Based on Sandberg et al.[42]

ASVCP Guidelines: Allowable Total Error Hematology

The ASVCP has released allowable total error recommendations as analytical quality specifications based on:

1. Clinical expert opinion on what is clinically desirable for dogs, cats, and horses.
2. Instrument performance derived from external quality assessment (EQA)/proficiency testing (PT) data.
3. CLSI guidelines.

The ASVCP guidelines provide an excellent reference for laboratory personnel and clinicians to objectively determine suitability of their analyzers and result interpretation (see Chapter 138).

Biological Variation

Biological variation data can be used to set analytical quality specifications. A database containing numerous analytes has been maintained on the Westgard website[37] and will now be expanded by the EFLM.[19,52] Since information on biological variation in animals is very limited, the human database was considered a useful tool to estimate biologic variation across species. However, concern has recently been raised about the data quality and the EFLM has created a task force to develop a critical appraisal list for evaluation of biological variation data.[1] This list focuses on details about the population and samples, preanalytical procedures, test method, estimates of analytical variation, outlier handling, and statistical analyses.[1] Implementation of these criteria will likely change human biological variation data substantially. A similar guideline for conducting biologic variation studies in animals is available[22] and should enable high-quality data collection for veterinary species. Available biological variation data in animals are maintained on the veterinary biological variation website.[3]

Nonstatistical Quality Control

Sample quality assessment of hematologic samples is a vital nonstatistical quality control tool. Lipemia, hemolysis, and clotted samples are the main interfering factors in hematologic samples. In accordance with ASVCP guidelines, any sign of clotting should result in a request for a fresh sample. Hematology results should not be reported for clotted samples. Evaluation for hemolysis and lipemia can be easily conducted if PCV is obtained for each patient. Hematology analyzers generally measure RBC counts, MCV, and Hgb concentration and calculate Hct, MCH, and MCHC from those measurements. Abnormal RBC indices, especially increased MCHC, therefore, can be used as a flag for detection of errors among measured RBC indices.[55] Common causes for

elevated MCHC include lipemia and Heinz bodies (spectral interferences in the Hgb assay), as well as in vitro and in vivo hemolysis (measured Hgb concentration includes Hgb from RBCs and free Hgb in plasma). Elevated MCHC results should always trigger assessment of plasma color and RBC morphology. Less common causes for elevated MCHC include extreme leukocytosis (spectral interferences in the Hgb assay) and true increases in MCHC due to eccentrocytes, pyknocytes, or spherocytes.[44] ADVIA analyzers provide a calculated and a measured MCHC, called cellular hemoglobin concentration mean (CHCM). Differences between MCHC and CHCM should prompt investigations regarding interfering substances; in addition, the CHCM can be used to calculate accurate Hgb concentrations. Sysmex analyzers offer a comparable calculation, called measured RBC Hgb content (RBC-He). In addition to an impedance RBC count used for calculation of MCHC, Sysmex analyzers provide an optical RBC count (RBC-O), which together with the RBC-He is used for the calculation of Hgb-O (optical). δ-RBC and δ-Hgb values (i.e., difference between RBC count or Hgb concentration of the impedance channel/photometric measurement and values derived from optical channel) can be used to distinguish underlying causes of elevated MCHC.[7] Increased MCHCs due to hemolysis require distinction between in vivo and in vitro hemolysis for clinical interpretation. RBC count and PCV correctly define the RBC mass available for oxygen transport when hemolysis occurs in the animal, whereas Hgb concentration provides information about available oxygen transport capacity in samples with in vitro hemolysis. RBC indices should be invalidated accordingly in these instances.

If undetected, clotted samples lead to erroneous hematology results. Therefore, macroscopic assessment of all samples is mandatory. If any doubts remain after macroscopic assessment or in cases of unexplained cytopenias, samples should be checked for small clots using a wooden stick to stir the anticoagulated blood in the submission tube. Small, red to tan, fibrin clots adhere to the wood and can be found upon gross inspection. Clotted samples should not be used for hematologic analyses.

RBC aggregation induces error in hematology results and may indicate important clinical information, e.g., immune-mediated hemolytic anemia (IMHA). RBC aggregation can be distinguished from rouleaux formation by a 1:9 dilution with saline. RBC aggregates due to immune-mediated mechanisms will persist and are visible macroscopically and/or microscopically, while stacking due to increased protein concentration in rouleaux formation will disperse. The agglutinin may be a cold-sensitive antibody and agglutination may interfere with electronic or optical RBC evaluation at room temperature. Small RBC aggregates are counted as a single large particle. If aggregates are larger than the upper threshold/gate, they are fully neglected by the hematology analyzer. This leads to spuriously low RBC counts, abnormally high MCV and MCHC, and erroneous Hct, while Hgb concentration is unaffected [55].

Manual Slide Evaluation

Manual blood smear review is the thorough microscopic analysis of a stained blood smear.[16] It is an integral part of veterinary hematology to ensure accuracy of automated hematology results and to detect morphological changes relevant for diagnosis.[16] For human hematology, the International Society of Laboratory Hematology (ISLH) has released criteria for action following automated CBC and WBC differential analysis.[4] Criteria include the age and sex, whether the request is for an initial or serial CBC, and whether there are significant differences between current and previous results. They are based on set thresholds and presence or absence of flags and aim to have a false negative rate of <5%.[15] Different studies in human medicine have, however, shown that institution-specific adaptations are needed.[16,15,24]

Comparable detailed guidelines are currently lacking in veterinary medicine. The ASVCP states that criteria should be established for microscopic blood film review and suggests confirmation of automated differential cell counts at predetermined thresholds.[50] Defined criteria should decrease the number of manual blood smear reviews as much as possible, for economic reasons and best use of technical and pathology staff. Veterinary experts' opinions concerning blood film reviews are diverse: some institutions review all blood films, whereas others have criteria triggering blood film review and/or manual WBC differentiation. The suitability of different approaches will depend on the patient population (i.e., sick patients, preanesthetic, or geriatric screenings), the available hematology analyzer (i.e., laser- or impedance-based instruments), and the knowledge base of laboratory personnel. In all instances, laboratory processes should ensure that accurate, automated WBC differentials are released and are not overwritten by manual WBC differentials with poor imprecision unless proven to be in error. Laser-based instruments provide, besides numerical results, sophisticated graphical result displays (cytograms); studies in animals demonstrating their usefulness as criteria for manual blood smear review are limited. For the ADVIA 120, cytogram sensitivity for left-shift detection was between 80 and 97% based on indistinct separation of polymorphonuclear and mononuclear cells and a shortened population of polymorphonuclear cells in the baso cytograms across the species studied. Specificities between 18 and 49% were poor.[43] For the Sysmex XT-2000iV, left shift, toxic changes, nucleated RBCs, and immature cells cause obvious changes in the cytograms.[29] However, no peer-reviewed information about cytogram sensitivity and specificity in the detection of morphological WBC changes is available. Considering the limited information currently available and the diversity of veterinary hematology, criteria for manual blood smear review should aim for a high sensitivity for the detection of morphologic abnormalities and inaccurate results. With further study and information, each laboratory will be able to reduce the number of slides reviewed and will increase analytical specificity. With a laser-based instrument, a safe approach would be

to release automatic results if they are within reference intervals and if cytograms are unremarkable. A blood smear should be reviewed when results are outside reference intervals or when abnormal cytograms are detected. See "Case Example" section in the following text. In addition to manual blood smear review, each laboratory should define criteria for pathologist's review. These criteria depend on knowledge base and training of technical staff and should be based on numerical and morphological results. Pathologist's review criteria should ensure that the clinical pathologist can confirm unusual and clinically highly relevant results. Interpretive comments should be provided to clinicians to ensure excellent communication that is required for correct diagnostic implementation in diagnosis and therapeutic choice.

With manual blood film review, plausibility of automated WBC, platelet, reticulocyte, and WBC differential counts should be assessed. Total WBC count (cells/μL) can be estimated at 100× magnification by multiplying the average number of WBCs present in 10 fields (total WBC number ÷ 10) × 100 to 150.[26] Accuracy of the WBC differential can either be estimated during overall slide review or 100 or 200 consecutive WBCs can be identified and counted at 400× or 500× total magnification. Considering the inherent imprecision of manual WBC differentials,[27] it is not advisable to define an exact cutoff for differences accepted between automated and manual WBC differentials. Rather, the manual WBC differential and cytogram review should confirm if WBCs were correctly classified by the hematology analyzer. Sysmex analyzers allow manual regating. Thus, if the analyzer was unable to separate cell populations, WBC differentials may be corrected by manual regating, providing highly precise WBC differential results (Figure 137.2).

Platelet counts can be estimated by multiplying the average number of platelets per field at 1000× (oil) × 15. This number may vary dependent on the microscope objectives. A recent study in dogs has shown that the variability of the platelet estimate is high, operator-dependent, and influenced by the area of the smear.[36] Plausibility assessment of the platelet count should, therefore, include evaluation of cytograms and histograms, platelet size, and plateletcrit. Neither automated nor manual platelet counts will be accurate if platelet aggregates are present.

Every laboratory should have procedures defining platelet aggregate assessment and reporting. Platelet aggregate presence must be clearly conveyed to the clinician on the report. Invalidating platelet counts in all samples with platelet aggregates will, especially in cats with platelet aggregates in up to 71% of samples, be frustrating for clinicians. When platelet counts are within reference intervals, platelet aggregates are clinically insignificant and laboratory procedures should focus on cases with automated counts indicating thrombocytopenia. Blood film review is required in all thrombocytopenic samples to assess platelet aggregates. An arbitrary five-tiered classification system (0–5) was introduced by Tvedten et al.,[47] scoring the number and size of platelet aggregates in the feathered edge and monolayer area. While 1+ and 2+ platelet aggregation appeared to have no significant effect on platelet count, 3+ and 4+ aggregation predicted a marked effect.

Modern laser-based hematology analyzers have flags for platelet clumps and, depending on number and size of platelet clumps, they might be visible in cytograms and histograms. These flags and graphical result displays negate the need for blood film assessment in clearly defined cases. It should be noted that the ADVIA platelet aggregate flag was less sensitive than manual blood film inspection.[47] Preanalytically, Iloprost, a synthetic analog of prostaglandin I2 can be added to EDTA tubes prior to blood sampling, to prevent platelet aggregation.[40,47]

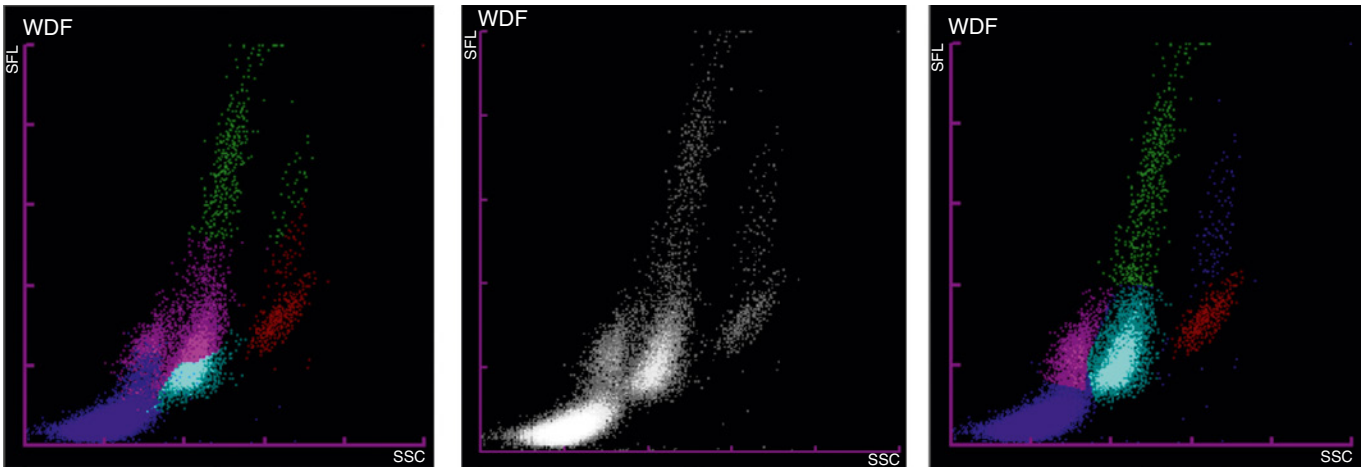


FIGURE 137.2 Sysmex XN WDF scattergrams of a feline blood sample with a WBC count of 10.3 × 10^3/μL and nine nucleated RBCs per 100 WBCs. The *y*-axis displays side fluorescence, *x*-axis displays side scatter. Lymphocytes are pink, neutrophils and basophils are light blue, monocytes are green, and eosinophils are red. The left scattergram represents the original gating of the Sysmex XN. It clearly indicates the instrument was not able to correctly classify WBCs. The middle scattergram is displayed in black and white to better visualize the nicely separated cell clusters. The right scattergram shows the separation attained after manual gating. A correct automated WBC differential with high precision was achieved.

Laser-based hematology instruments measure absolute reticulocyte counts utilizing a fluorescent dye staining RNA content. Pseudoreticulocytosis has been reported in humans and a dog with lymphoid leukemia due to misclassification of leukemic cells or particles thereof as reticulocytes[33] and accuracy of reticulocyte counts should be ensured (see Chapter 65). In addition to cytogram evaluation, blood films can be assessed for the degree of polychromasia. A semiquantitative assessment scheme for polychromasia has been published for dogs, cats, and horses,[26] and a study in dogs has shown a good agreement between polychromasia and automated reticulocyte percentages.[14] As a less-subjective alternative, a manual polychromatophilic cell count also correlates well with automated reticulocyte counts in dogs.[14] Due to high imprecision, manual reticulocyte counts using brilliant cresyl or new methylene blue-stained blood films may not be recommended for most mammalian species. Modern hematology analyzers are very sensitive and detect reticulocytes with little RNA content, which may stain similarly to adult RBCs on Romanowsky-stained blood films. A positive bias for automated counts compared to manual counts or estimates is therefore expected.

Plausibility Checks in Veterinary Hematology

Automated hematology analyzers either calculate Hct from the RBC count and MCV or determine Hct from the RBC pulse height. The PCV (aka microHct or spun Hct) is determined by high-speed centrifugation and is the most accurate and reproducible method. The World Health Organization (WHO) has released a recommended method for PCV determination by centrifugation[53] in which they consider sources of error including nonstandardized centrifugation time and force, inadequate mixing of the sample and filling of the capillary tube, and inaccurate reading. A recent study with veterinary students and clinicians showed: 25% of students made errors associated with inadequate mixing, 23% of the students read results incorrectly, and 91% of clinicians filled the tubes incorrectly according to recommended WHO standards (between two-thirds and three-quarters full).[9] The maximum absolute error in this study was 0.20 L/L (20%), emphasizing the importance of training and standardization. For every species, each laboratory needs to assess the optimal packing time.[53] This can be done using a well-mixed normal and a polycythemic blood sample. Two capillary tubes from each specimen are filled and centrifuged (between 10,000 and 15,000 g) for 2 minutes. The PCV is measured and recorded. With freshly filled capillary tubes, the process is repeated with increasing centrifugation time by 30 seconds until the PCV does not change with further increases in centrifugation time. The optimal packing time is equivalent to the second time interval at which there is no further reduction in PCV. PCV should periodically be measured in duplicate and results should not differ by more than 0.005 L/L (0.5%).[53] See Chapter 138 for information on total allowable error (TE_a).

Hgb concentration is approximately one-third of Hct in most species and this ratio was originally adopted from humans. Agreement within three units is considered acceptable.[26] In the author's experience, up to four units variation may be acceptable in cats. A study in cattle implies that a relationship of Hgb (g/dL) = 0.3 × PCV + 3 may provide a better estimate than the conventional estimate of one-third.[46] In birds, Hgb (g/dL) = 0.3 × PCV appeared to be a reasonable estimate of Hgb concentration in eight orders; however, in Phoenicopteriformes, a different relationship was found.[51] The "rule of three" is only an estimate and may vary in different species. If systematic deviations are observed for certain species, breeds, or strains, the laboratory should consider determining specific formulas to decrease the need for spun PCVs.

In general, the "rule of three" can be used in veterinary medicine as a plausibility check of automated Hgb concentration and Hct in most samples submitted. If the Hct is outside this range, then a spun PCV should be performed. If Hct differs more than three units, spun PCV should be reported and MCV and MCHC should be invalidated or manually calculated.

Standard Operating Procedures and Documentation

Documentation problems are the most common deficiencies identified during on-site inspections.[41] Documentation of processes is required by any certification standard, such as the ISO/IEC 15189, and the ASVCP also highlights the importance of documentation and provides detailed recommendations about SOPs.[21] All procedures performed in the laboratory should be included in SOPs, which can be organized as paper copies, electronically, or, preferably, both. They should be easily accessible by technicians, managers, and assistants as needed. A procedure for revision and document control should be in place. The outline of SOPs varies from laboratory to laboratory. A SOP detailing the correct format will ensure that all SOPs in an institution follow the same outline and that no information is forgotten. The ASVCP also provides an example for an SOP outline which can be modified for the laboratory accordingly (Table 137.2.).[21]

Personnel

Personnel training is vital in veterinary hematology. Laboratory personnel should be qualified and knowledgeable regarding cell identification, including species variations. Technologists must have more general and non-species-specific knowledge, such as use and maintenance of laboratory equipment, repair or replacement of inadequate equipment, and problem-solving procedures. According to the ASVCP quality assurance guidelines,[21] veterinary laboratory personnel should also understand linearity differences in animal samples, interfering substances, expected physiologic ranges and reference intervals, common problems encountered with veterinary samples, and appropriate use of species-specific criteria. Documentation of all available

TABLE 137.2 ASVCP Suggested General Standard Operating Procedure Outline.

- Purpose of the procedure.
- Principles of the assay.
- Details on sample management (e.g., patient preparation, specimen collection and handling, minimal volume, acceptable sample type).
- Operational aspects (e.g., interferences, reportable range, analytical sensitivity and specificity).
- Details on reagents (e.g., storage location and conditions, preparation, open shelf-life, manufacturer information).
- Details on equipment and supplies (e.g., necessary equipment, location of supplies).
- Calibration and quality control procedures.
- Step-by-step instruction of the process.
- Reference intervals for each appropriate species.
- Details on interpretation and reporting, including recommended actions for critical values, repeat criteria, review criteria, literature references, and documentation (e.g., name, date, and signature of author and review).
- Appendices may include logs or worksheets, package inserts, and quick reference guides.

relevant internal and external trainings should be accessible to all personnel, including a checklist with SOPs and demonstrated competencies.

Comparative smear evaluation to ensure standardization in blood film evaluation is a tool that documents competency of laboratory personnel. A prerequisite is the standardization of nomenclature and grading of morphological features. The International Council for Standardization in Hematology (ICSH) has released recommendations for such standardization in humans.[35] While a semiquantitative evaluation scheme, including grading number and severity of degenerative changes in neutrophils, is widely accepted and used in the veterinary field (see Chapter 45), a semiquantitative scheme for RBC morphology which grades anisocytosis, polychromasia, hypochromia, poikilocytosis, codocytes, spherocytes, echinocytes, and other shapes for dogs, cats, horses, and cattle, has not been widely adopted.[26] Grading of morphological features should provide the clinicians with clinically useful information. The ICSH therefore implemented a two-tiered grading system (2+ and 3+), except for schistocytes, where even the observation of small numbers is considered to be significant and 1+ may also be assigned.[35] In veterinary hematology, most laboratories use a three-tiered grading scheme (1+/few/mild/slight, 2+/moderate, and 3+/marked/many). Standard operating procedures may define poikilocyte numbers per field to aid in appropriate semiquantitative classification.

Comparative smear evaluation performed by all hematology technicians in the laboratory should be organized by the laboratory management. Comparative smear evaluation should be done on a regular basis (e.g., once per month) and include different species as well as different pathologic changes that are encountered in the laboratory. All laboratory personnel reading routinely smears should participate and results should be documented and discussed within the team.

Statistical Quality Control

Statistical quality control is an integral part of laboratory quality systems in veterinary hematology. It refers to the analysis of control materials and interpretation of the results with pre-established acceptance and rejection criteria.[21]

Quality Control Material (QCM)

QCM measurement monitors the entire test system and is used to monitor routine analytical performance.[31] The list of requirements for high quality control materials is long and includes: ethical origin, sufficient volume, homogenous, stable, pathogen free, cost effective, and QCM must span medical decision limits.[18] The latter is especially challenging across multiple species and disease states. QCM must be handled according to manufacturer instructions, labeled with the date received and opened, and discarded when expired. Each laboratory should also verify reagent stability over the maximum run length. Commercially available QCM for impedance and flow cytometry hematology instruments cannot be used interchangeably and the specific hematology analyzer must be considered when choosing an appropriate QCM. Discussion with the manufacturer's technical support is beneficial. Commercially available QCM used for hematology instruments are usually derived and optimized for the analysis of human specimens. Target values (e.g., MCV) may therefore not always be in the relevant range for veterinary species. In addition, a recent study has shown that quality control with human-based QCM resulted in a lower observed total error (TE_{obs}) compared to TE_{obs} calculated from meta-analysis where species-specific material was used.[17]

Due to cellular instability, the use of whole animal blood as the sole QCM is not advisable; but it may be used for assessment of within-run, same-day imprecision. Periodic repeat patient testing (RPT) may overcome some of the disadvantages of human-based QCM, including clinically relevant concentrations, patient matrix effects, and commutability.[49,20] Used together, stabilized commercially available QCM of known concentrations and repeated measurement of patient samples can be combined to answer the breadth of QA questions relevant to the diagnostic laboratory.

Selection of the type, number, and frequency of QCM is part of the QC validation process. For hematology, a minimum of two control materials with different target values and a maximum run length of 24 hours are generally recommended.[2,31]

QC Validation

QC validation consists of the selection of "rules" for the statistical monitoring of method performance.[21] While many laboratories still use the manufacturer's ranges as "rules," the use of Westgard rules, or similar statistics based on QC validation, is recommended. For determining candidate QC rules, an analytical quality requirement (TE_a or clinical decision interval) needs to be defined for each test, coefficient of variation (CV) needs to be estimated from replication studies, and bias needs to be assessed and compared to manufacturer target values for QCMs in method comparison studies or proficiency testing schemes. For example, normalized OPSpecs Charts or the computerized EZ RUNS or EZ RULES 3 calculators can be used to choose appropriate Westgard rules with a high probability of error detection (usually >90%) and a low probability of false rejection (<5%) (https://www.westgard.com/essay31.htm).

Repeat Patient Testing

Repeat patient testing is a statistical QC method using patient data. Advantages of RPT include low-cost, species-specific matrix relevant to the laboratory, and absence of deterioration that may affect commercial QCMs during transport and storage. Disadvantages include the availability of suitable samples for RPT, patient sample instability, and subjectivity in setting RPT control limits. Studies in veterinary hematology using canine samples for RPT showed that RPT-QC can achieve similar performance to commercial QCM-QC and that it is possible to detect deviations from stable performance based on desired quality requirements.[49,20]

Sample aging under defined conditions results in an expected degree of variation between baseline and repeat measurements over time. Acceptable time periods depend on the analyte, but 24 hours is acceptable for most analytes. Variations exceeding the expected variation indicate a problem with the test system.[20] Results of RPT-QC, like the results of commercial QCM, are interpreted with predetermined control limits. Control limits are determined by measuring a set of pilot data; based on these results, control limits are defined. According to Westgard, RPT data from at least 20 days should be collected. Differences between the original and repeat results are calculated and plotted on a control chart. Control limits containing about 95% of the data are graphically estimated.[20] Flatland et al. suggested that an additional 20 days of data should be evaluated to ensure that the initial estimate of control limits is accurate.[20] These control limits will be used to determine if future daily RPT-QC results are acceptable or not. In contrast to QCM-QC, where manufacturer target values can be used to calculate bias, RPT-QC bias is set to zero during QC validation. If RPT is applied in a laboratory, participation in proficiency testing schemes and comparative testing is therefore of immense importance in order to monitor bias.

External Quality Assessment and Comparative Testing

The ASVCP has released guidelines on proficiency testing/external quality assessment and comparative testing for reference and in-clinic laboratories[11] (see Chapter 138). ASVCP recommends that commercial and in-clinic laboratories participate at least quarterly in a proficiency testing program,[11] so that the accuracy of the laboratory's analyzers can be monitored on a regular, preventative basis. Prevention of misdiagnosis is imperative.

Comparative testing compares the results of two or more different instruments or methods, usually carried out independently and internally within a laboratory or network of laboratories. In-clinic laboratories may compare their results with those obtained by duplicate sample submission to a reference laboratory. Comparative testing is an important QC tool, especially for laboratories with POC analyzers, since most EQA programs do not have a sufficient number of participants with POC analyzers, resulting in weak peer groups which may produce erroneous proficiency testing comparisons. POC testing sites, therefore, often compare their test results to those from a reference laboratory by measuring a patient sample on their POC analyzer and sending it out to the reference laboratory. A lack of comparability between single samples may, however, only be due to imprecision and no conclusions about bias (systematic errors) can be drawn. Bias assessment requires more comprehensive comparisons with multiple samples over a range of results, measured repetitively. The same acceptance criteria used in proficiency schemes can be applied (usually within two or three standard deviations or within TE_a). However, interpretation of result comparability can be difficult if different methods and instruments are compared, which are not expected to be identical. If bias is expected due to different instruments or methods, reference intervals can be used to define clinical comparability of results. Placement within reference intervals (divided in quartiles) or the degree of variation above or below the reference intervals is compared; results are acceptable if they fall within the same area.[11] However, if the reference intervals have not been established according to recommended ASVCP guidelines, the results may be erroneously partitioned.

POC owners should be aware that comparative testing does not replace regular internal QC, nor does it replace PT. It is an additional statistical QC tool, which provides assessment of accuracy and precision, and should be considered to ensure that patient's results are comparable between the two laboratories. Comparative testing may be used if: unexpected results are obtained in patient samples, internal QC or PT results are unacceptable, analyzers have undergone major maintenance or repair, or if no PT program is available.

The CLSI recommends classifying unacceptable results as clerical errors, methodological problems, equipment problems, technical errors, problems with

EQA testing material, problems with evaluation of results, and no explanation. This should aid in the identification of the root cause.[11]

CASE EXAMPLE

History: Nine-year-old female boxer dog that presented for bloody diarrhea and vomiting.

Assessment of numerical results reveals an appropriate "rule of three" relationship for hemoglobin concentration and hematocrit. Besides the severe anemia, a severe microcytosis and hypochromia are striking. However, all results except Hgb concentration are flagged by the analyzer (Figure 137.3).

In this case example, impedance and laser light scatter do not accurately measure RBC, reticulocyte, and platelet counts. Manual regating, an option with Sysmex analyzers, cannot be accomplished, since RBCs,

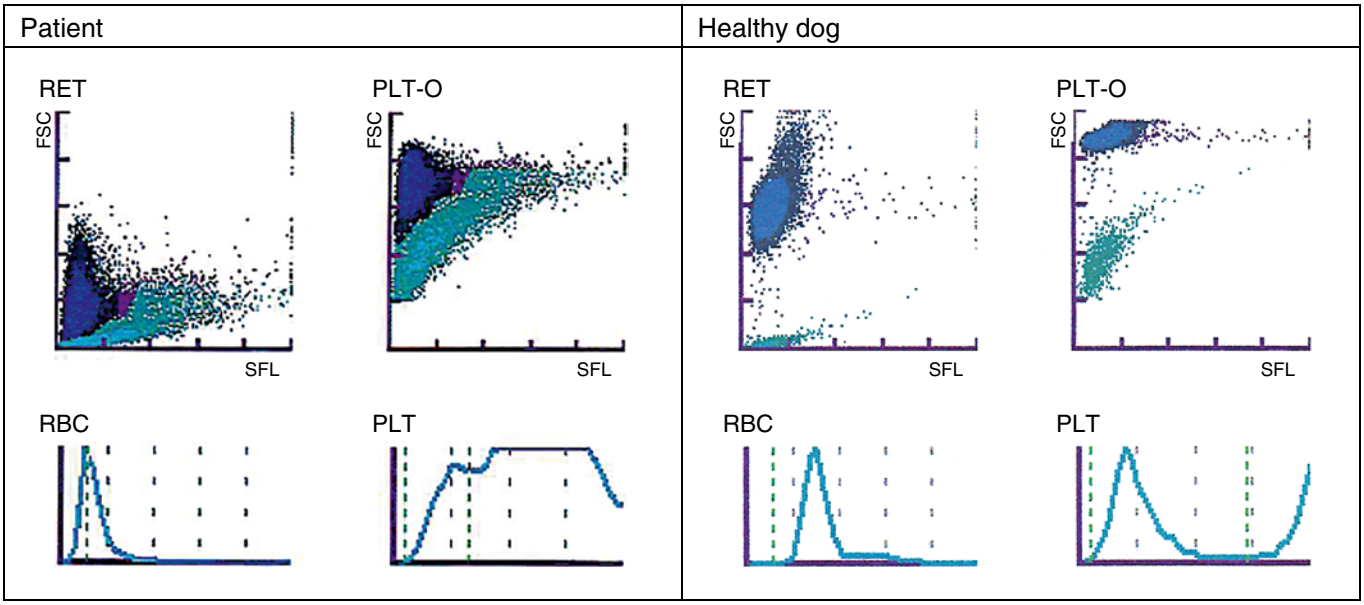


FIGURE 137.3 Cytograms and histograms. On both cytograms, forward scatter (FSC) on the *y*-axis reflects cell size, side fluorescence light (SFL) is depicted on the x-axis. The blue population shows RBCs, magenta-staining events are reticulocytes, and the lighter blue-green population represents platelets. In the patient, smaller RBCs and reticulocytes are shifted downward into the platelet population. Since platelets and RBCs, as well as platelets and reticulocytes, are not clearly separated, optical counts are not reliable and should not be released. The patient RBC histogram (patient, lower left) shows a distinct peak, shifted to the left compared to the RBC histogram for a healthy dog, reflecting the presence of small RBCs; however, the PLT histogram (patient, lower right) with lack of a well-defined peak, as seen in the healthy dog, indicates that platelets and RBCs cannot be distinguished by impedance technology due to the small RBC size. Impedance RBC and platelet counts are therefore not reliable and should not be released.

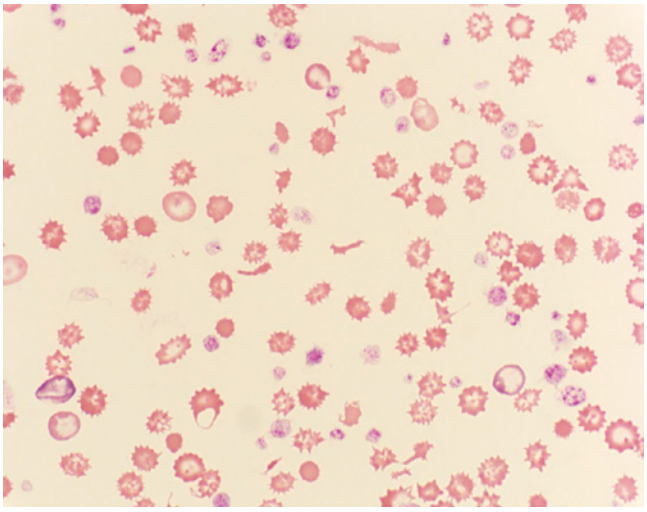


FIGURE 137.4 Blood film findings. Blood film examination confirms a severe microcytic, hypochromic anemia with only slight polychromasia; poikilocytosis is marked with presence of schistocytes, keratocytes, acanthocytes, and echinocytes. Platelet numbers are mildly increased. This is a typical case of iron deficiency anemia and was caused by chronic gastrointestinal bleeding from an intestinal adenocarcinoma.

TABLE 137.3 Hematology results.

Measurand	Result	Reference interval
RBC count (impedance)	1.7	6.1–8.1 × 10^6/μL
RBC count (optical)	–	6.1–8.1 × 10^6/μL
Hemoglobin	2.0	14.4–19.1 g/dL
Hematocrit	7	42–55%
MCV	41	64–73 fL
MCHC	29	34–36 g/dL
Reticulocyte count	43.9	10^3/μL
PLT count (impedance)	353	130–394 × 10^3/μL
PLT count (optical)	–	130–394 × 10^3/μL

platelets, and reticulocytes are overlapping in both channels. Hemoglobin concentration and PCV must be used to assess patients RBC mass; platelet counts can only be estimated on the blood film. Blood film examination is also required to evaluate RBC morphology and estimate regeneration (Figure 137.4).

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CHAPTER 138

Total Error and Proficiency Testing

STEN WESTGARD and KATHLEEN P. FREEMAN

Why You Should Perform Proficiency Testing?
How Proficiency Testing Is Conducted and Judged
Graphs and Statistics in Your Proficiency Testing Report
Proficiency Testing Is Built on a Foundation of Allowable Total Error
Why Are There Different Allowable Total Error Goals?
Allowable Total Error Goals for Veterinary Hematology
The Challenge of the Future: Evolving Goals and Changing Practices

Acronyms and Abbreviations

ASVCP, American Society for Veterinary Clinical Pathology; %Bias, percent bias; CAP, College of American Pathologists; CLIA, Clinical Laboratory Improvement Amendments; CV, coefficient of variation; CVI or CV index, coefficient of variation index; EQA, external quality assessment; ISO, International Organization for Standardization; PT, proficiency testing; SD, standard deviation; QA/QC, quality assurance and quality control; TE_a, allowable total error; TE_{obs}, observed total error.

There is an apocryphal story about reaching your goals, numerously called the "Texas Sharpshooter Fallacy" or the "Story of the Master Archer," from the Torah, or even a fragment found in the texts of Cicero. It goes something like this: a warrior, desiring to master the bow and arrow, seeks out a mentor. As he wanders, the people he encounters point him in the right direction, and he begins to see arrows stuck into the bull's-eyes of targets, growing more numerous and numerous. Sometimes it's a bull's-eye in the trunk of a tree, or the side of a barn, or in a high branch, or the top of a steeple. Sometimes the targets are large, sometimes small. But always there is an arrow in the bull's-eye of each target.

Finally, he reaches the man responsible for all these bull's-eyes. He asks, admiringly, how the master archer has been able to be so accurate.

> "Oh, it's simple. I just shoot the arrow first, and **paint the target around the arrow** afterward."

This, then, is our conundrum with external quality assessment (EQA) in laboratory testing. Are we building the target first, and actually hitting a bull's-eye? Or are we shooting first, and merely painting the bull's-eye around wherever the arrow lands?[24]

WHY YOU *SHOULD* PERFORM PROFICIENCY TESTING?

Unlike many other industries and professions, laboratories never assume they are correct. Medical laboratories assure the quality of their results through continuous assessment activities. Therefore, not only do laboratories need to check that they are internally consistent, through the implementation of internal quality control (IQC), they also need to participate in EQA to confirm they are in alignment with the rest of the testing world. External quality assessment [EQA or proficiency testing (PT)] not only comprises interlaboratory comparisons focused primarily on the analytical quality of testing, but may also include all phases of the testing cycle, including interpretation of results.

Only by successfully completing both IQC and PT can laboratories assure their customers, clinicians, clients, and patients that they are delivering precise, accurate results.

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IQC is not sufficient because it can only tell us if we are producing a *consistent* result. In order to know if we are obtaining the *right* result, we must have some comparison against an external value, preferably a reference value. Note that PT can tell us two things, depending on how it is structured: (a) It can tell us if we're getting the same result as other labs and instruments, if all we do is compare to a consensus value. That essentially confirms that we all deliver a similar result, even if it may still be the wrong result. Ideally, of course, we want more than that. (b) We want our PT program to tell us if we are reporting the right result, which requires that the PT program has some ability to determine what the correct value of each survey specimen is. This is a challenging task even in the human-based testing world with routine biochemistry analytes. When we enter the world of veterinary testing, with different species, the challenge is orders of magnitude higher.

While there is practical utility in performing proficiency testing, most medical testing laboratories working with human specimens participate in surveys because they must, not because they volunteer. Nearly every type of regulation and accreditation in the world requires proficiency testing as part of laboratory operation. Some important organizations mandating or recommending proficiency testing participation include the International Organization for Standardization (ISO), the global standard for quality. Clinical Laboratory Improvement Amendments (CLIA) regulations are required in the USA and CLSI recommendations incorporate global input from human laboratory experts.[16,5,3,6]

While no formal federal or international regulatory mandates exist for veterinary laboratories, American Society for Veterinary Clinical Pathology (ASVCP) has issued the following recommendations for EQA/PT:[2]

> "4.4.1 ASVCP recommends that both commercial and in-clinic laboratories participate in a PT program. . ..
> 4.4.2 EQA/PT participation should be used in addition to daily internal (meaning internal to the laboratory) quality assurance and quality control (QA/QC)
> 4.4.3 EQA/PT program participation for hematology and biochemistry testing is considered to be of highest importance."

HOW PROFICIENCY TESTING IS CONDUCTED AND JUDGED

While EQA programs vary considerably from country to country and program to program, there is essentially one common process: the program prepares specimens, sends them out to all participant laboratories, who then run these specimens as if they were actual patient samples, and those results are recorded and reported back to the survey organizers, who tabulate the data and perform a number of different calculations and assessments of the results. Participant laboratories can access the results in a variety of ways.

The ASVCP-recommended frequency of PT is quarterly or four times per year for in-clinic veterinary equipment regardless of waived status.[8] However, PT for all analysis in reference laboratories is not standardized and can be highly variable. In US human medical laboratories, PT frequency is largely regulatory driven. Regulated analytes must be tested four times per year, typically with five samples per challenge. Nonregulated analytes can be tested less frequently. The mandated judgment of PT is that the laboratory must pass with a score of 80%. That is, four out of five of the results must be considered acceptable and one result can be unacceptable. The first time a PT survey fails is bad, but a consecutive survey failure on the same analyte typically brings more dramatic consequences. Different PT programs enforce different success rates.

The consequences of failing a PT survey also vary. In countries like the USA and Germany, the CLIA regulations and the Rilibak regulations not only mandate the frequency and judgment criteria of PT, they also mandate significant consequences of failure, which can include loss of reimbursement or even the laboratory's operational license. Other PT programs are only educational and failure results in letters and warnings, but no actual operation or financial repercussions will occur. Eventually, if enough PT failures accumulate, any lab with ISO 15189 accreditation will find those failures are serious nonconformances that can jeopardize the entire laboratory's accreditation.

Historically, PT have had exceptionally low failure rates. In 1987, less than 1% of laboratories failed their College of American Pathologists (CAP) survey.[12] As the CLIA regulations came into effect, in 1994, that failure rate fluctuated between 1.3% and 5.6%.[22] Between 2007 and 2011, the CAP survey recorded failures rates between 3.0% and 3.74%.[15] Even the latest update proposed by CLIA in the USA was constructed with minimizing the failure rate of laboratories to only 1–2%.[5,23] Therefore, we expect that the goals of PT are attainable and only few laboratories would fail proficiency testing.

GRAPHS AND STATISTICS IN YOUR PROFICIENCY TESTING REPORT

Every PT report will generally provide the laboratory's mean in comparison to the reference mean and/or a peer group mean, dependent upon the program. These details may be included in a box plot, histogram, or Youden plot, which provide a visual display of the participating laboratory's results in the PT program.[2,21]

There are multiple statistics that can be displayed in a PT report, but the most common is the standard deviation index (SDI), which is simply:

$$\text{SDI} = (\text{reported value} - \text{expected/assigned value})/\text{group SD}.$$

This helps the laboratory understand how far away each value is from the expected or target value. SDI describes

the systematic error or bias of a method in terms of the variation of the group.

For example, an SDI of 0.0 indicates the lab mean is exactly the same as the group mean. An SDI of 2.0 indicates the lab mean is 2 group SDs higher or lower than the group mean, which indicates the laboratory is bordering on having an accuracy problem. Any SDI greater than 2.0 should be investigated by the laboratory.

Another statistic that may be included in the report is the coefficient of variation index (CVI or CV index) This is a comparison of the laboratory CV to the group CV using the equation:

$$\text{CVI} = \text{Lab CV}/\text{Group CV}.$$

A CVI of 1.0 indicates the laboratory CV is the same as the group CV. A CVI <1.0 suggests the laboratory's imprecision is better (less) than that observed for the group. A CVI >1.0 suggests the laboratory's imprecision is worse (higher) than that observed for the group.

Both the SDI and the CVI are crude statistics. They can certainly identify when laboratories are significantly different than the group. But if a laboratory has acceptable CVI and SDI, that is no guarantee they are providing clinically appropriate results. These are more appropriately thought of as negative indicators. They can tell you when you're doing badly, but can't give you assurance that you're doing well. A more demanding assessment would be needed for that.

PROFICIENCY TESTING IS BUILT ON A FOUNDATION OF ALLOWABLE TOTAL ERROR

For quantitative tests, in order to definitively determine whether a single PT result is acceptable or unacceptable, there must be a quantitative value that can be set around the "expected value," "consensus value," or "true value" (depending on which type of PT program you are participating in). It is unreasonable to expect, and impossible to achieve, the exact result in your PT challenge as the expected value of the survey due to both random and systematic errors. In every reported result both types of error are present. Some difference is inevitable, and indeed allowable. This is where the term "total error" is derived. The observed total error (TE_{obs}) represents the combination of random and systematic errors that is found in a single test result. There must be some amount of total error that is acceptable; that doesn't change the clinician's interpretation of the patient's health, doesn't alter the diagnosis, and doesn't impact the patient's treatment. This is what is called analytical allowable total error (TE_a) and is defined as an analytical performance specification that sets a limit for combined imprecision (random error) and bias (inaccuracy or systematic error) that can be present in a single measurement or single test result without confounding the clinical usefulness. Total error is calculated according to CLIA and ASVCP guidelines as 2CV + bias% (or 2SD + bias/units).[11] Implicitly, when PT surveys set goals for acceptability, they are defining analytical allowable total errors. The goals set by PT cannot, by definition, be more difficult to achieve than the clinically important quality of the results.

Once we accept the concept of TE_a, then we can compare our TE_{obs} against the TE_a. When the TE_{obs} is smaller than the TE_a, the PT result is a success. If this is applied to a routine testing result, the reported results can be used clinically with confidence. If the TE_{obs} is larger than the allowable total error, the laboratory has failed part of the PT challenge, and the results being reported may be generating false positives, false negatives, or other clinically significant errors.

WHY ARE THERE DIFFERENT ALLOWABLE TOTAL ERROR GOALS?

While the concept of TE_a is a simple one, the implementation of TE_a in the real world has been contentious and variable. Looking at different PT programs around the world, you will find that the TE_a goals for even routine biochemistry measurands, which have been tested for decades, are not standardized. In Germany, the Rilibak goals for albumin at 20%; while in the USA, CLIA sets them at 10% and ASVCP at 15%; while in China, they are set at just 6%. That's more than a threefold difference in performance specifications, and there is no known dramatic difference in clinical use in these three countries. The reasons behind the TE_a differences in the PT programs of different countries are the subject of an ongoing effort of a European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) "Task and Finish" group dedicated to sorting out the best way to set performance specifications. They categorize the different kinds of acceptance criteria as regulatory, statistical, or clinical.[1]

Regulatory goals, e.g., the German Rilibak and US CLIA PT goals, are set primarily to identify poorly performing laboratories. They are often wider and based on "state of the art." Statistical acceptance criteria may also be based on "state of the art," but in a purely mathematical way, typically ±3 SD of the group of laboratories in the survey. This is a crude way of keeping all laboratories "in the herd" since 3 SDs are approximately 99% of the normal distribution. This type of approach was initially used commonly with laboratory testing, but in more modern versions of CLIA PT acceptance criteria, these group SD goals have been eliminated as much as possible.[23] This hierarchy of EQA/PT acceptance criteria is strikingly like the new hierarchy of analytical performance specifications that was ratified at the first Strategic Consensus Conference of the European Federation of Clinical Chemistry and Laboratory Medicine in 2014.[20] This created a three-tiered arrangement of performance specifications, simplified as clinical, biological, and state of the art.

Clinical limits are based on changes in test results that change the clinical decisions, diagnoses, and treatments. Kristensen and Meijer note: "These limits are desirable but may be difficult to implement because very few

clinical decisions are based solely on one particular test."[1] It is recognized that very few analytes have studies that can help define the goals at this level. Troponin and HbA1c are examples where clinical performance specifications have been determined in human medicine.

Biological performance specifications are derived from known biological variation for analytes. These are considered very evidence-based, since they are dependent not upon the regulators or state of the art, but on the scientific evidence of variation within the body. The drawback of these goals is that often variation is very small and the goals are difficult or impossible to achieve. A database of such goals was started in 1999 by Ricós et al. and updated biennially until 2014, reaching a total of more than 350 performance specifications.[18,4] At the 2014 EFLM conference, various shortcomings with the database were noted,[4] at which time the EFLM took over the task. As of this writing, however, no new update has been posted.

Obviously, for veterinary testing, the biologic variation approach poses a special challenge. While human-based testing can focus on numerous analytes and just one species, veterinary laboratories have multiple species with possibly quite different biological variation. Biologic variation data for multiple species have been collected in an online resource, http://vetbiologicalvariation.org. There is a healthy and vigorous discussion in the literature about the best way to collect and utilize this data to set performance specifications.[10,13]

Finally, the third type of performance specification in the Milan Hierarchy is the state of the art. In concept, this must be the highest technically achievable analytical performance of the moment. It does not mean every method's performance is acceptable.

ALLOWABLE TOTAL ERROR GOALS FOR VETERINARY HEMATOLOGY

Given the controversy and strong debate among the single set of well-studied human measurands, it's not hard to imagine that setting performance specifications for veterinary measurands is orders of magnitude more complex. Veterinary laboratory medicine is not focused on just one species, but multiple species (e.g., dogs, cats, horses, and livestock); and adding further complexity, even within species, there are different breeds, reproductive status, management, and other factors.

An additional complicating factor is veterinary hematology quality control material, which can be difficult to produce and may have a very short shelf-life. The ideal commutable IQC material for veterinary hematology is species-specific fresh whole blood with EDTA. Unfortunately, this is stable for a maximum of 72 hours for Hb and RBC, and significantly less (24 hours) for total and differential white blood cell and platelet counts. A control material that has a stability of 24 hours is not usable unless generated within the laboratory. Therefore, commutability is sacrificed for the more stable, commercially prepared control material. Vendors provide IQC material which is also often used as PT material, provided that different control lots are used. Out of practicality, we frequently find ourselves using controls and EQA/PT materials that are generated for the human medical market, then making a leap of faith that if our analyzer is acceptable for humans, it must also be acceptable for other species. When species-specific concerns must be addressed within the laboratory, whole blood from patient samples may be used to assess species-specific imprecision (CV) of the assay.[9] The instability and matrix effects of PT hematology materials, for any species, can be problematic. It may be difficult to separate out the matrix effects and degradation from any real method or instrument problems.[14]

ASVCP guidelines have summarized hematology TE_a in domestic species; though there are significant species differences (Tables 138.1 and 138.2). Of note are the unique challenges for feline samples, particularly due to tendencies of feline blood to clot quickly, clumps on blood smears, the presence of both punctate and aggregate reticulocytes, and higher imprecision for reticulocytes in cats. They also noted that reticulocyte counts in horses are "rarely clinically relevant."[17]

From a strict metrological perspective, we should calculate out all ATEs for each species, each breed, and at each key decision level. And then perform EQA/PT on each type. However, in practical terms, we will need to find a consensus goal for each analyte. One approach is to use the most stringent TE_a among the species commonly tested in the veterinary laboratory.[7] Thus, we may gravitate toward using a single performance specification, allowable total error, which is closer to a human goal than any particular animal goal.

THE CHALLENGE OF THE FUTURE: EVOLVING GOALS AND CHANGING PRACTICES

In 2016, a review of 10 veterinary in-clinic hematology analyzers using canine EDTA blood samples found that "these instruments often fail to meet quality requirements." At least 60% of analyzers reasonably determined RBC, WBC, Hct, and Hgb, when assessed by the ASVCP quality goal criteria. Platelets were less reliably measured, with 80% deemed suitable for low platelet counts, but only 30% for high platelet counts. Automated differential leukocyte counts were generally considered unsuitable for clinical use. Fewer than 50% of analyzers were able to meet requirements for statistical quality control for any measurand.[19]

This is a sobering conclusion about the current state of the art of veterinary hematology and underscores the critical need for better quality and more vigilant proficiency testing. EQA/PT, through an evolution of goal-setting, can gradually, incrementally pressure the improvement of hematology method performance by manufacturers of these instruments and drive better results for better care. Our current level of quality is not enough. Compliance to current practices is not adequate for the needs of our veterinary patients.

TABLE 138.1 Comparison of current hematology goals across veterinary and human testing. Note the variation of allowable total error between countries and organizations.

Hematology analyte	Total allowable error goal (TE_a%)	TE_a source
Hemoglobin	**±10%**	**ASVCP**
	±7%	CLIA, WSLH, AAB, CAP
	±6%	ESFEQA, Rilibak
	±5%	MIN SPANISH EQA
	±4 g/L < 100 g/L	IQMH*
	±5% ≥ 100 g/L	
	±5 ≤ 100	RCPA 2010
	±5% > 100	
	±4.19%	BV DES
	±3.87%	EuBIVAS 2018**
Hematocrit	**±10%**	**ASVCP**
	±6%	CLIA, WSLH, AAB, CAP
	±12%	ESFEQA
	±0.02 L/L < 0.20 L/L	IQMH*
	±7% ≥ 0.20 L/L	
	±8%	MIN SPANISH EQA
	±9%	Rilibak
	±0.04 ≤ 20	RCPA 2010
	±20% > 0.2	
	±3.97%	BV DES
	±3.86%	EuBIVAS 2018**
	±2.5%	BV DES
	±5%	MIN SPANISH EQA
	±9%	ESFEQA
Mean cell volume	**±7%**	**ASVCP**
	±2.42%	BV DES
	±7%	MIN SPANISH EQA
	±10%	ESFEQA
	±5 ≤ 50	RCPA 2010
	±10% > 50	
	±5 fL < 50 fL	IQMH*
	±5% ≥ 50 fL	
	±1.60%	EuBIVAS 2018**
Platelets	**±20% (ref labs)**	**ASVCP**
	±25% (in-clinic labs)	
	±25%	CLIA, WSLH, AAB, CAP
	±13.4%	BV DES
	±15%	ESFEQA
	±25 ≤ 100	RCPA 2010
	±25% > 100	
	±10 × 10^9/L < 20 × 10^9/L	IQMH*
	±15% ≥ 20 × 10^9/L	
	±8.51%	EuBIVAS 2018**

(*Continued*)

TABLE 138.1 (Continued)

Hematology analyte	Total allowable error goal (TE_a%)	TE_a source
RBC	**±10%**	**ASVCP**
	±6%	CLIA, WSLH, AAB, CAP
	±4%	MIN SPANISH EQA
	±4.4%	BV DES
	±0.3 × 10^{12}/L ≤ 4 × 10^{12}/L	ESFEQA
	±8% > 4 × 10^{12}/L	
	±0.2 ≤ 2	RCPA 2010
	± 10% > 2	
	±0.2 × 10^{12}/L < 2 × 10^{12}/L	IQMH*
	±10% ≥ 2 × 10^{12}/L	
	±8%	Rilibak
	±3.84%	EuBIVAS 2018**
WBC	**±15% (ref labs)**	**ASVCP**
	±20% (in-clinic labs)	
	±15%	CLIA, AAB, CAP
	±9%	MIN SPANISH EQA
	±17.6%	BV DES
	±0.7 × 10^9/L ≤ 4 × 10^9/L	ESFEQA
	±18% > 4 × 10^9/L	
	±0.5 × 10^9/L < 5 × 10^9/L	IQMH*
	±10% ≤ 5 × 10^9/L	
	±0.5 ≤ 5	RCPA 2010
	±10% > 5	
	±18%	Rilibak
	±11.5%	EFLM 2018**
Neutrophil count	**±15%**	**ASVCP**
	±23.35%	BV DES
	±1 ≤ 10	RCPA 2010
	±10% > 10	
Lymphocyte count	**±15%**	**ASVCP**
	±0.5 × 10^9/L < 4 × 10^9/L	IQMH*
	±15% ≥ 4 × 10^9/L	
	±2 ≤ 10	RCPA 2010
	±20% > 10	
	±17.6%	BV DES
	±13.74%	EuBIVAS 2018**
Monocyte count	**±60% within RI**	**ASVCP**
	±50% high value	
	low values not clinically relevant	
	±0.5 × 10^9/L < 4 × 10^9/L	IQMH*
	±15% ≥ 4 × 10^9/L	
	±3 ≤ 10	RCPA 2010
	±30% > 10	
	±27.9%	BV DES

TABLE 138.1 (Continued)

Hematology analyte	Total allowable error goal (TE_a%)	TE_a source
Eosinophil count	**±90% low value**	**ASVCP**
	±50% within R	
	±50% high value	
	±0.5 × 10^9/L < 4 × 10^9/L	IQMH*
	±15% ≥ 4 × 10^9/L	
	±3 ≤ 10	RCPA 2010
	±30% > 10	
	±37.1%	BV DES
Basophil count	**No recommendation**	**ASVCP**
	±0.5 × 10^9/L < 4 × 10^9/L	IQMH*
	±15% ≥ 4 × 10^9/L	
	±3 ≤ 10	RCPA 2010
	±30% > 10	
	±38.5%	BV DES
(Automated)reticulocyte count	**±20% (canine only)**	**ASVCP**
	±33% (instrument group mean)	IQMH*
	±35% (%)	MIN SPANISH EQA
	±16.5%	BV DES
	±14.38%	EuBIVAS 2018**

CLIA: US Clinical Laboratory Improvement Amendments regulations; WSLH: Wisconsin State Laboratory of Hygiene; AAB: PT program of the American Association of Bioanalysts; RCPA: Royal College of Pathologists of Australasia;
CAP: PT program of the College of American Pathologists; MCV, mean cell volume.
Rilibak are the German EQA performance specifications. Note these are the interlaboratory comparison figures. The Rilibak do state within-laboratory specifications that are smaller, but these are supposed to be used for root-mean-square deviation (RMSD) calculations, which is an alternative approach to allowable total error.
Min Spanish EQA are the minimum consensus performance specifications of multiple Spanish EQA surveys.
* IQMH is the PT program of the Institute for Quality Management in Healthcare. www.IQMH.org. These data are used here with permission. 2018. Copyright Institute for Quality Management in Healthcare (IQMH). All rights reserved. No part of the material protected by this copyright may be reproduced without written permission from IQMH. Please contact infor@iqmh.org for the most current data pertaining to IQMH allowable performance limits.
BV DES represents the desirable performance specifications of the Biologic Variation Database;
ESFEQA: European Society for External Quality Assessment.
** EFLM 2018: https://www.ncbi.nlm.nih.gov/pubmed/29605821" Coşkun A, Carobene A, Kilercik M, Serteser M, Sandberg S, Aarsand AK, Fernandez-Calle P, Jonker N, Bartlett WA, Díaz-Garzón J, Huet S, Kızıltaş C, Dalgakıran I, Ugur E, Unsal I; European Biological Variation Study of the EFLM Working Group on Biological Variation. Within-subject and between-subject biological variation estimates of 21 hematological parameters in 30 healthy subjects. Clin Chem Lab Med. 2018 Apr 2. pii: /j/cclm.ahead-of-print/cclm-2017-1155/cclm-2017-1155.xml. doi: 10.1515/cclm-2017-1155. Note that this paper only calculated desirable imprecision and desirable bias, but desirable total allowable error has been calculated from the data.
Source: Based on Nabity et al.[17]

TABLE 138.2 Recommendations for allowable total errors (%) in veterinary hematology. ASVCP standards are applied across common species (dog, cat, horse, cow); biologic variation standards are for dog and cat, respectively; expert opinion is for dogs and cats. The CLIA values are those applied for human testing in the USA.

Parameter	TE_a ASVCP (38) (%)	TE_a (%) derived from biological variation (38, 68)—dog			TE_a (%) derived from biological variation (70)—cat			TE_a (%) based on expert opinion (58)[6]	CLIA[1] value
		Optimum	Desirable	Minimal	Optimum	Desirable	Minimal		
RBC	10	3.4	6.9	10.3	4.2	8.4	12.6	13.3	6%
Hgb	10	3.5	6.9	10.4	4	8	12.1	11	7%
Hct/PCV	10	3.5	7	10.5	4.1	8.1	12.2	12.8	6%
MCV	7	1.3	2.6	3.9	2.3	4.6	6.9	–	–
MCHC	10	1.4	2.9	4.3	0.5	1	1.5	–	–
Reticulocytes	20	–	–	–	20.9	41.8	62.7	–	–
WBC	15/20[2]	10.9	21.9	32.8	11.3	22.7	34	16.6	15%

(*Continued*)

TABLE 138.2 (Continued)

Parameter	TE_a ASVCP (38) (%)	TE_a (%) derived from biological variation (38, 68)—dog			TE_a (%) derived from biological variation (70)—cat			TE_a (%) based on expert opinion (58)[6]	CLIA[1] value
		Optimum	Desirable	Minimal	Optimum	Desirable	Minimal		
PLT (reference laboratory)	20/25[3]	8.8	16.7	25.1	9.1	18.2	27.3	13.3	25%
Neutrophils	15	14.4	28.7	43.1	14.5	29.1	43.6	20	±3SD
Lymphocytes	15	–	–	–	15	30.1	45.1	25	±3SD
Monocytes	60/50[4]	–	–	–	15.2	30.3	45.5	20	±3SD
Eosinophils	90/50[5]	–	–	–	14.6	29.2	43.7	25	±3SD
Basophils	NP	–	–	–	–	–	–	–	±3SD

[1]Clinical Laboratory Improvement Amendment Act (human standard applied in the USA); [2]15% for reference laboratories and 20% for in-house laboratories; [3]20% for reference laboratories and 25% for in-house laboratories; [4]60% within reference interval, 50% above reference interval; [5]90% below reference interval, 50% within and above reference interval; [6]Expert opinion at normal values reported; NP indicates that no recommendation is possible.
Source: Dr. Martina Stirn and Dr. Kathleen P. Freeman.

While veterinary hematology EQA/PT has a long way to go to catch up to the other laboratory testing fields, remember the oft-told saying about quality: It's a journey with no end. There are always improvements to be made. There will always be advances in technology, updated needs for diagnosis, and changes in treatment. The net result of these evolving changes will be a need to update and modify the acceptance criteria and performance specifications for EQA/PT. Just as soon as we get comfortable with one allowable total error, it may be time to retire the old goal and embrace a new goal.

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CHAPTER 139

Quantitative Diagnostic Test Validation

BENTE FLATLAND

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Analytical Performance Validation
  Analytical Properties of Quantitative Tests
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    Imprecision
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    Linearity and Reportable Range
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  Judging Method Analytical Performance
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    Method Decision Chart
Diagnostic Performance Validation
  Sensitivity and Specificity
  Predictive Values
  Receiver Operating Characteristic Curves
  Likelihood Ratios
  Error Grids

Acronyms and Abbreviations

%Bias, percent bias; CLIA, Clinical Laboratory Improvement Amendments; CLSI, Clinical and Laboratory Standards Institute; CV, coefficient of variation; ISO, International Organization for Standardization; NLR, negative likelihood ratio; NPV, negative predictive value; PLR, positive likelihood ratio; PPV, positive predictive value; ROC, receiver operating characteristic; SD, standard deviation; SN, sensitivity; SP, specificity; STARD, Standards for the Reporting of Diagnostic Accuracy; TE_a, allowable total error; TE_{obs}, observed total error; UM, uncertainty of measurement.

INTRODUCTION

Diagnostic laboratory test performance can be divided into analytical performance, diagnostic performance, and clinical utility. The latter refers to how much a diagnostic test improves patient health outcomes relative to the current best alternative.[5] This chapter focuses on analytical and diagnostic performance and provides an overview of the study designs and statistical tools used in their assessment. Analytical performance assessment characterizes an assay's measurement performance through the study of analytical properties such as imprecision, bias, linearity, etc. Validation of analytical performance is the process of proving whether an assay can perform such that it is suitable for its intended clinical purpose.[45] The term verification is most often used in the context of proving particular analytical performance claims (e.g., verifying a manufacturer-reported linear range). Diagnostic performance addresses how successful an assay is at diagnosing a particular disease condition in a given patient population. Analytical performance must be validated before diagnostic performance can be assessed.

ANALYTICAL PERFORMANCE VALIDATION

Analytical Properties of Quantitative Tests

Quantitative measurement procedures possess properties that can be scientifically studied, in order to determine if an assay is performing as required (Table 139.1). Analytical performance validation is an integral part of the instrument and test kit manufacturing process. Factors that laboratories may consider in selecting an analytical instrument or method are given in Table 139.2.[11] Individual laboratories should carry out some assessment of analytical performance prior to implementing a new assay for routine clinical use. At a minimum, manufacturer analytical performance claims should be verified. Validation of assays developed internally by a

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TABLE 139.1 Analytical properties of quantitative measurement procedures. Any or all of these properties may be assessed as part of analytical performance evaluation.

Property	Definition	Formula, if applicable
Bias	Represents trueness (systematic error) of a measurement. Bias is expressed as mean difference (in measurand units) or as %Bias (in units of percent). Bias is subcategorized as constant bias (constant throughout measurand concentration range) and proportional bias (changes with measurand concentration). Bias can be minimized using calibration procedures.	$\%\text{Bias} = \frac{\text{Mean}_{\text{test method}} - \text{Mean}_{\text{target}}}{\text{Mean}_{\text{target}}} \times 100,$ where "target" is mean from a comparative method, a peer group mean, or the known concentration of a control or reference material.[33] %Bias may be a positive or negative value.
Detection limit	Represents analytical sensitivity and is the smallest measurand concentration that an assay can distinguish from a blank specimen with a stated statistical probability.[17]	Limit of blank: $\text{LoB} = \text{mean}_{\text{blank}} + 1.65\ \text{SD}$ Limit of detection: $\text{LoD} = \text{LoB} + 1.65\ \text{SD}_{\text{spiked}}$
Imprecision	Represents random error. Expressed as the SD (measurand units) or the CV (%) of repeatability data. Conditions for studying repeatability should be clearly defined (see text). The clinical impact of imprecision on patient data can be minimized by performing replicate measurements and reporting the average result.	$\text{CV} = \frac{\text{SD}}{\text{Mean}} \times 100$
Interference	Analytical specificity—refers to analytical error caused by substances in the sample matrix, other than the measurand, that alter the results.	$\%\text{Change} = \frac{\text{Mean}_{\text{spiked with interferent}}}{\text{Mean}_{\text{equal volume of diluent added}}} \times 100$ Percent change refers to the change in measured concentration resulting from addition of interferent. Mean refers to the average of duplicate measurements.
Linearity (linear range)	Concentration range over which (predicted) known values for a specimen and measured values have a linear mathematical relationship. The linear range is delimited by the lowest and highest concentrations at which linearity exists.[43]	
Recovery	A method's ability to detect known quantities of measurand.	$\%\text{Recovery} = \frac{\text{Mean}_{\text{A}} - \text{Mean}_{\text{B}}}{\text{Measurand concentration added to A}} \times 100$ Mean: average of duplicate measurements. A is the aliquot spiked with measurand. B is the aliquot containing diluent (same as volume of measurand added to aliquot A). Means and added concentration are in measurand units.
Reportable range[a]	Range of measurand concentrations reported by an instrument or method.	
Total error	Observed total error (TE_{obs}) is the sum of random and systematic error, often used in clinical laboratories.	(1) TE_{obs} = Mean difference + 2SD (2) TE_{obs} = % Bias + 2CV (1) TE_{obs} in measurand units; (2) TE_{obs} in units of %. Absolute values for mean difference and %Bias should be used. The constant 2 is a Z-factor[b].[37]
Measurement uncertainty (MU, aka uncertainty of measurement, UM)	A metrological model for understanding measurement accuracy. Addresses dispersion of values and acknowledges that a quantity's "true" value is unknowable.[30] In clinical laboratories, MU is expressed as SD or multiples thereof.	Expanded MU = Measured result ± 2SD[37,27]

[a] Terminology and definitions vary. Related terms include working range, analytical measurement range, and clinically reportable range.[43]
[b] The Z-factor 2 is rounded from 1.96 and refers to bidirectional change and 95% statistical probability.[37]

TABLE 139.2 Considerations for choosing a laboratory method.

Consideration	Explanation or example(s)
Assay availability	Commercial assay versus developed internally
Instrument or test kit reputation	Anecdotal versus trade publications versus performance assessment as published in peer-reviewed medical literature
Acquisition options	Lease versus purchase versus loan
Service contract availability and details	
Instrument or test kit size ("footprint")	
Required environmental conditions	Temperature, humidity, water quality
Specimen type and volume	Whole blood versus serum versus plasma; acceptable anticoagulants
Turnaround time and specimen throughput capacity	
Test menu	Individual tests versus panels
Ease of instrument and software use	Patient and control data displays; patient and control data storage capacity and ease of retrieval; available flags and error notifications
Instrument connectivity	Ability to connect to electronic patient health record
Instrument maintenance	How often, how complex
Reagent and control material cost and shelf-life	Consider closed- and open-container shelf-life
Spent reagent disposal considerations	Toxicity or biohazard, if applicable
Operator training availability; technical support availability	

Source: Based on Flatland and Vap.[11]

laboratory is likely to be more exhaustive than for commercial assays. However, validation beyond verification of manufacturer's claims should be undertaken even for commercial assays if conditions of the laboratory's intended use differ from that validated by the manufacturer (e.g., different specimen type, different species, different reagent type, or other change). In human medicine, Clinical Laboratory Improvement Amendments (CLIA, the federal law governing performance of medical diagnostic laboratories) states in Section 493 that any laboratory modifying a Federal Drug Administration (FDA)-approved test system, developing a system not subject to FDA approval, or using a test system for which no manufacturer performance claims are available must evaluate, as applicable, accuracy, precision, analytical sensitivity, analytical specificity/interfering substances, reportable range, reference intervals, and "any other performance characteristic required for test performance."[1]

Quality Goals

Prior to beginning analytical performance verification or validation studies, the laboratory should set an analytical quality goal for the measurand in question. Although metrologists favor setting separate goals for imprecision and bias, medical laboratorians often think in terms of total measurement error (i.e., the combined effects of imprecision and bias upon a measured result).[49,48] Whether the total error model (favored by Westgard et al.) or uncertainty of measurement (a metrological model promoted in International Organization for Standardization (ISO) 15189[2]) is the better approach to understanding and assessing error in the clinical laboratory is under active debate[31,48,12] (see Chapter 138).

Historically, analytical quality goals have been derived from various sources, including regulatory requirements, expert consensus, biological variation data, "state of the art" (i.e., the highest level of analytical performance that is technically achievable), and patient outcome studies.[9,36] The American Society for Veterinary Clinical Pathology (ASVCP) endorses use of allowable total error (TE_a, an expert consensus-based goal) in two published guidelines,[14,28] and it is becoming more common for biological variation-based quality goals to be incorporated into veterinary assay validation and statistical quality control (QC).[21] In the US currently, veterinary diagnostic laboratories are not subject to regulatory oversight, and individual laboratories should choose quality goals defensible for their circumstances based on published recommendations, scientific evidence, finances, and logistics.

Validation Studies

Analytical properties defined in Table 139.1 are assessed using specially designed scientific studies. Analytical performance assessment typically begins during or immediately after installation of an analytical method in the laboratory. With more complex systems (e.g., a wet chemistry analyzer), at least some verification of manufacturer performance claims will likely be carried out by a field engineer installing the equipment. This should be documented in writing. Once a method is successfully installed, laboratory staff should engage in a familiarization period during which operators are trained to properly perform testing. The familiarization period typically includes learning the test principle and intended use; instrument operation, including specimen requirements, use of reagents, controls, and any calibrators; necessary chemical and/or biosafety precautions; reviewing manufacturer instructions; and writing standard operating procedures tailored to the laboratory.[17] Generating preliminary control data and quality control validation are also components of installing a new laboratory method.[42] Once operators are familiar with the test system and stable operation can be

documented via quality control procedures, verification of additional performance claims and further analytical performance validation can begin.

For any analytical performance validation, the laboratory must first choose whether to use commercial control material and/or patient specimens (e.g., pooled plasma). Advantages of commercial materials include that these are readily available, stable specimens with known target values. Disadvantages include that these may have a different matrix from veterinary patient specimens and may not be available at measurand concentrations of medical interest to veterinarians. Advantages of patient specimens are that they can provide a species-specific evaluation of assay performance and are hopefully available at concentrations of medical interest. Disadvantages include lack of specimen stability and, potentially, specimen availability. Analytical performance validation of assays involving unstable specimens (e.g., fresh whole blood) or labile measurands (e.g., blood gases, ammonium) may necessarily be limited by logistical concerns.

Imprecision

Precision is the closeness of agreement between independent measurement results obtained under specified conditions.[38] Imprecision (random error), the opposite of precision, is represented by standard deviation (SD), in measurand units or coefficient of variation (CV) in units of percent.[46] Observed SD and CV values can be compared to manufacturer performance claims and/or quality goals for imprecision, if available. SD and CV values can additionally be used in the calculation of TE_{obs} (see Table 139.1).

When reporting imprecision study results, it is important that the circumstances be clearly stated because measurement conditions will impact the results. Historically, experiments assessing imprecision have referred to calculation of "within-run" and "between-run" SD and CV.[17,46] Current Clinical and Laboratory Standards Institute (CLSI) guidelines in human diagnostic laboratory medicine recognize that more tightly controlled "repeatability" conditions (same operator, equipment, and laboratory) are expected to show greater precision than less tightly controlled "within-laboratory" conditions (different operators or equipment).[38,6]

Imprecision should be studied at measurand concentrations of medical interest (i.e., that reflect concentrations likely to occur in the anticipated patient population) and that span most or all of reportable value range for the instrument in question. Imprecision study designs can vary, depending upon the evaluator's needs or intended use of the imprecision data. In general, study designs targeted to instrument manufacturers and assay developers (who establish performance claims) are more complex than those aimed at diagnostic laboratories (who simply need to verify manufacturer claims).

A simple estimate of repeatability can be obtained by performing ≥20 replicate measurements of a specimen and calculating mean, SD, and CV of the resulting data.[17,46] There is precedent in the veterinary literature for crude precision assessment of in-clinic equipment using three to five replicate measurements.[34,35] CLSI guideline EP15 for human diagnostic laboratories recommends assessment of both "repeatability" (formerly known as within-run imprecision) and "within-laboratory" imprecision (formerly known as total imprecision). Repeatability assesses imprecision when all measurements are made under identical conditions. Within-laboratory imprecision employs less-rigid conditions (e.g., different operator and/or different runs) and a longer timeline (days), but still limits assessment to the same laboratory, equipment, and reagent lot.[6] Both repeatability and within-laboratory SD and CV can be calculated from data generated by measuring two concentrations of material in triplicate (in one run), once daily, for 5 days. The EP15 guideline provides formulae and a worksheet to facilitate calculations.[6]

When analyzing precision, it is important to look at average measurand concentration (i.e., mean of the dataset) in addition to SD and CV. Dispersion of precision study data (i.e., SD or CV) can be constant throughout the range of measured values or vary with measurand concentration; this relationship is sometimes referred to as an assay's precision profile.[26] Since CV is SD expressed as a percentage of the mean, if SD is constant throughout a range of measured values (i.e., independent of concentration), CV will vary inversely with measurand concentration. If SD varies with measurand concentration, then CV will be more constant through the range of measured values. An assay's precision profile can help determine whether more than one quality goal is needed to monitor performance at different measurand concentrations.[26] The precision profile also impacts choice of regression model during method comparison data analysis. When SD is constant throughout the range of measured values, unweighted regression models may be used; when SD varies with measurand concentration, weighted regression procedures are more appropriate.[26]

Bias

Bias of an assay or instrument is assessed relative to a target that is chosen to represent the true measurand concentration, which, in reality, is unknowable. Observed bias can be compared to a quality goal for bias, if available, or used in calculation of TE_{obs} (see Table 139.1).

The target in bias assessment can be a peer group mean from external quality assessment (EQA) program data, results from another analytical method, or can be the known, manufacturer-reported results for an assayed control material or certified reference material.[10] In veterinary laboratory medicine, it may not be possible to obtain species-specific commercial materials for use in bias assessment, and bias is often studied using a combination of commercial (often human-derived) assayed control materials and species-specific method comparison data.[8,15,34,35] It is important that method comparison studies involve adequate specimens ($n \geq 40$) that are independent and have concentrations spanning most or all of the reportable ranges of the methods involved.[18]

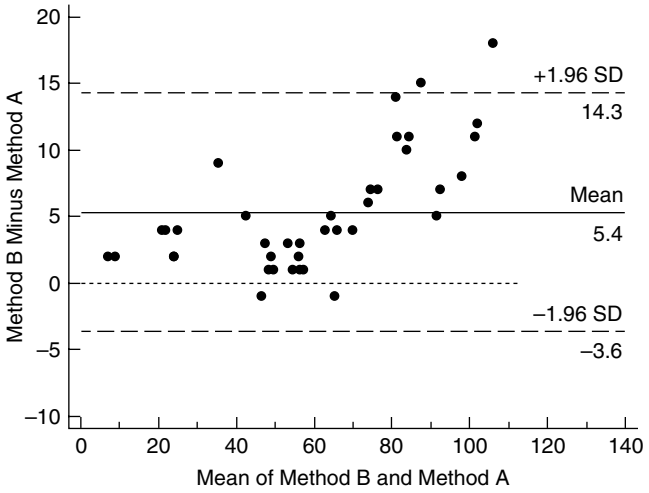


FIGURE 139.1 Bland–Altman difference plot for two fictitious instruments, Method A (reference method) and Method B (method undergoing performance assessment). The difference between the methods is plotted on the *y*-axis, and the mean of both methods is plotted on the *x*-axis. The small-dashed line parallel to the *x*-axis corresponds to zero (no difference between methods). The solid line parallel to the *x*-axis is the mean difference (5.4 units), and the larger dashed lines represent ±1.96 standard deviations around the mean difference (from −3.6 to 14.3 units). Virtually all differences are greater than zero, indicating constant systematic error (results of Method B are numerically greater than those from Method A throughout the range of measured values). Proportional bias is evident from the upward trend in the data points (as measurand concentration increases, differences become numerically larger).

Bias (systematic error) is divided into constant and proportional bias, each of which can be assessed individually. Method comparison statistical analysis includes descriptive statistics for each method's data set, difference plot analysis, and regression analysis. Difference plots (Figure 139.1) are used for graphical assessment of data and may be interpreted using either Bland–Altman limits of agreement (defined as ±1.96 SD from the mean difference) or limits based on the combined imprecision of both methods.[3,18,17] Regression analysis provides statistical evidence of constant bias if the 95% confidence interval for the y-intercept does not include the value zero and statistical evidence of proportional bias if the 95% confidence interval for the slope does not include the value 1. The regression model should be chosen based on the range, correlation coefficient, and precision profile of the data sets.[18,26] Passing–Bablok and Deming regression models are often used, as assumptions for least squares regression may not be met by clinical laboratory methods.[26]

Linearity and Reportable Range

An assay's reportable range is determined by the manufacturer and should reflect the concentration range within which measured values meet declared quality goals for imprecision and bias. The upper limit is often identical to an upper linearity limit. The lower limit may correspond to the lower linearity limit or may be based on another quantity such as a detection limit.[26] Verification of linear range is important if the laboratory uses two-point calibration, during which an instrument's calibration is assessed based on two measurand concentrations (high and low) only.[43]

Verification of linear range can be assessed using serial dilutions of a concentrated patient specimen or specimen pool; use of at least four concentrations is recommended to establish linear range; calibration verification can be done using as little as two concentrations.[43] A suitable diluent (physiologic saline, zero calibrator) should be used, and the anticipated concentrations of each dilution should be calculated using the dilution factors.[17] Measured values are compared to expected values using scatter plots and linear regression.[17,43] A 95% confidence interval for the slope that includes the value 1.0 provides statistical evidence of linearity. Regression residuals can be used to guide analysis and provide additional evidence supporting linearity ("runs test").[26] In regression, a residual (calculated by statistical software) is the difference between an observed (measured) value and the corresponding value predicted by the regression model. Residuals can be plotted on a graph showing residuals on the *y*-axis and the observed value, or average of the observed and predicted values, on the *x*-axis. A line parallel to the *x*-axis is drawn at zero, and distribution of residuals around the zero line is evaluated. If data are linear, residuals should be randomly distributed around the zero line.[24] Nonlinearity is indicated by deviation of points away from zero (known as a "run," or sequence of points, above or below the zero line).[26]

Detection Limit

The detection limit is the smallest measurand concentration that an assay can distinguish from a blank specimen with a stated statistical probability.[17] Verification of a detection limit is most crucial for assays expected to measure low concentrations, such as hormone assays and drug tests. Various detection limits are defined, and two are presented here. Limit of blank (LoB) is the highest measurement result likely to be observed with a stated probability using a blank specimen. Limit of detection (LoD) is the smallest quantity of measurand that can be detected with a stated probability.[40]

To determine LoB, a suitable blank specimen (e.g., plasma, serum, or physiologic saline to determine LoB of a hematology instrument) is measured in replicate ($n \geq 20$ is suggested).[17,40] Data are used to calculate mean and SD. LoB is calculated using the formula in Table 139.1. The constant 1.65 is a Z factor for estimating LoB given a one-tailed distribution (positive direction) with 95% probability. LoD is determined via replicate measurements of both blank and spiked specimens; these should have a similar matrix. Data are used to calculate mean and SD of each specimen type, and LoD is calculated per the formula in Table 139.1.[40]

Recovery and Interference

Recovery and interference address analytical specificity. Recovery studies are used to estimate proportional systematic error, and interference studies are used to estimate proportional error induced by substances in the sample matrix other than the measurand of interest.[41] In a recovery study, paired specimen aliquots containing, respectively, added measurand or an equal volume of diluent (e.g., water, serum, plasma, physiologic saline, or calibration blank) are measured in duplicate. Recovery is calculated by averaging results for each duplicate pair and calculating the percent recovery (Table 139.1).

An interferent study follows the same design, except that interferent (known quantity of hemoglobin, bilirubin, lipid, or other substance of interest) is added to the spiked aliquot rather than measurand. The percent change in concentration as a result of interferent addition is calculated as presented in Table 139.1. Alternatively, concentration change can be expressed in measurand units as a mathematical difference between the spiked and diluted aliquots.[41] Interference may be positive (causing increased measurand concentration) or negative (causing decreased measurand concentration). More bias is expected with increased quantity of interferent. The mathematical relationship between interferent quantity and measured results may be linear or nonlinear. A graphical depiction of interference is called an interferograph, which depicts interferent concentration on the *x*-axis and percent change in measurand concentration following interferent addition on the *y*-axis.[16]

Judging Method Analytical Performance

Observed Total Error

Observed total error (TE_{obs}) combines observed bias and imprecision into one quantity calculated with a stated statistical probability (Table 139.1). TE_{obs} can be compared to a predetermined quality goal; such goals are often given in units of percent. If the value for TE_{obs} is numerically smaller than the quality goal, then the assay performs within acceptable limits. If the value for TE_{obs} is numerically larger than the quality goal, further refinement of the assay (e.g., instrument adjustments, operator training, reagent changes) is needed.[23] If improving assay performance is not possible, then the laboratory must decide whether an alternate assay is needed, or whether accepting the existing assay and a more lax quality goal is justifiable.[23]

Sigma Metric

Six sigma (σ) is a statistically based quality control system adopted from manufacturing. The sigma metric is a unitless indicator of assay performance. In clinical laboratory medicine, σ ranges from 0 to 6.[20] The metric is calculated as $\sigma = \frac{(TE_a - \%Bias)}{CV}$, where TE_a is allowable total error and %Bias and CV are calculated as presented in Table 139.1. Assays with $\sigma = 5$ or 6 are stable, high-performing methods with minimal analytical error. Assays with $\sigma \leq 3$ are considered poor performers.[20] Sigma-based assay selection, control rule selection, and quality control

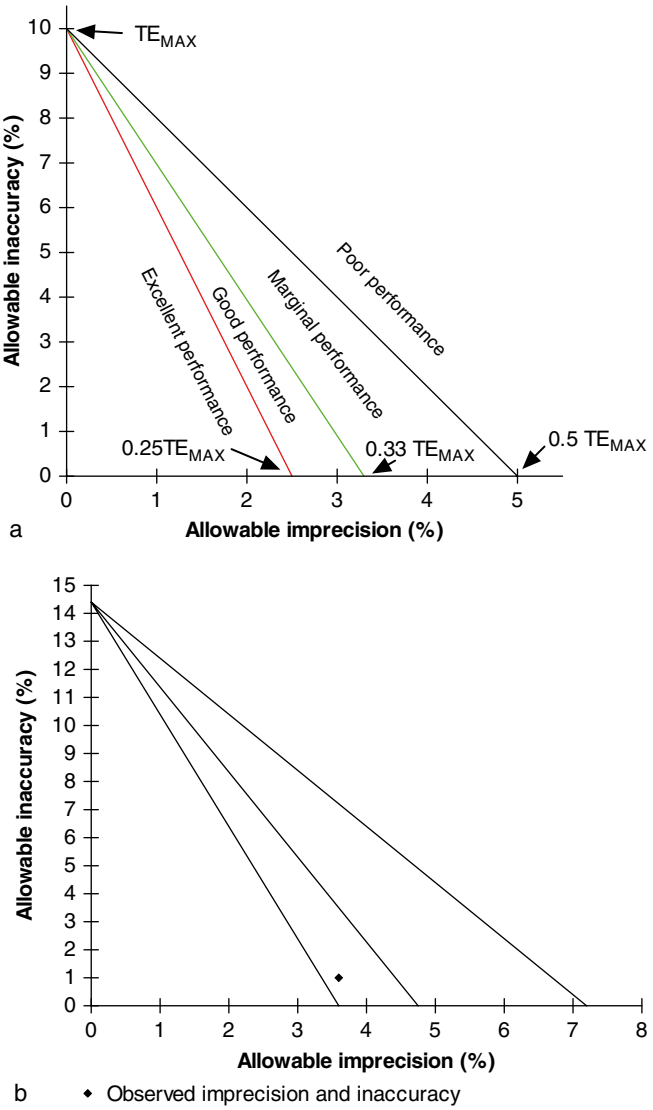


FIGURE 139.2 MEDx charts for assessing analytical performance. (a) MEDx chart for a test with a maximum allowable total analytical error (TE_{MAX}) of 10%. The lines represent different currently accepted total error criteria: line marked ($0.5 \cdot TE_{MAX}$) represents the total error criterion TE_{MAX} = inaccuracy (bias) + 2SD; line marked ($0.33 \cdot TE_{MAX}$) represents the total error criterion TE_{MAX} = inaccuracy (bias) + 3SD; line marked ($0.25 \cdot TE_{MAX}$) represents the total error criterion TE_{MAX} = inaccuracy (bias) + 4SD. TE_{MAX} can be calculated from biological variation data or be derived from another source (e.g., TE_a based on expert consensus, or a regulatory requirement). By plotting the observed inaccuracy and imprecision of a test into its MEDx chart, the analytical performance of the assay can be classified as poor, marginal, good, or excellent; (b) MEDx chart for total WBC count with TE_{MAX} 14.4%. Inaccuracy and imprecision were estimated for WBCs on an automated hematology analyzer as 1% and 3.6%, respectively, and using the MEDx chart, it is concluded that the test has good analytical performance.

are strongly advocated by Westgard QC, and multiple resources regarding six sigma implementation in the clinical laboratory are available via that organization.[47,49]

Method Decision Chart

The method decision (MEDx) chart (Figure 139.2) is a graphical tool designed to facilitate assessment of assay analytical performance in light of a quality goal. MEDx charts plot allowable bias and imprecision (in units of %) on the *y* and *x* axes, respectively. The *y*-axis is scaled to TE_a, and the *x*-axis scaled to half of the *y*-axis. Lines drawn from the maximum *y*-axis value to various points on the *x*-axis divide the chart into zones indicating excellent, good, marginal, and poor analytical performance. Lines demarcating these zones represent TE_a = %Bias + 2SD, %Bias + 3SD, and %Bias + 4SD. To use the chart, an "operating point" representing an assay's observed %Bias and CV (Table 139.1) are plotted on the chart. The zone in which the operating point falls indicates method performance.[44] MEDx charts are closely related to operating specifications (OPSpecs) charts, a graphical tool used for quality control validation that plots lines representing various control rules relative to the assay's operating point.[39]

DIAGNOSTIC PERFORMANCE VALIDATION

Once analytical performance of a test is deemed satisfactory for the intended purpose, evaluation of diagnostic performance can begin. In its simplest version, diagnostic test performance validation compares results from a test under evaluation, i.e., index test, to another test deemed the "gold standard" for disease diagnosis (known as single-test accuracy studies).[25] Importantly, both the index and gold-standard tests should be applied to the same study population suspected to have the target disease of interest on the basis of clinical signs which represent predetermined inclusion criteria. All study subjects should be tested using the gold-standard test, irrespective of their index test result; i.e., the two tests should be applied and interpreted independently.[25] The study population is subsequently divided into diseased and nondiseased individuals based on a negative gold-standard test result, and diagnostic accuracy of the index text is calculated.[25].

Diagnostic accuracy studies can be impacted by bias, and careful study design is crucial.[50] Study inclusion and exclusion criteria, stage and signs of disease, and the population studied must be carefully defined. Detailed data reporting that includes clear descriptions of the study design and results is necessary to help the reader understand context, since indices of test performance can vary with disease prevalence, disease severity, diagnostic setting, and other patient characteristics. Authors of diagnostic accuracy studies should follow STARD (Standards for the Reporting of Diagnostic Accuracy) guidelines and checklists for study design and data reporting.[4]

Sensitivity and Specificity

Diagnostic test performance data can be summarized in a two-by-two table as follows:

	Gold-standard test POSITIVE	**Gold-standard test NEGATIVE**	**Total**
Index test POSITIVE	True positives (TP)	False positives (FP)	TP + FP
Index test NEGATIVE	False negatives (FN)	True negatives (TN)	FN + TN
Total	TP + FN	FP + TN	TP + FN + FP + TN

Sensitivity (SN) is the probability of a positive test result in a diseased individual (sometimes referred to as true positive rate, although there is no time factor). Specificity (SP) is the probability of a negative test result in a nondiseased individual (true negative rate).[7] SN and SP are calculated from the table using these formulae and often expressed in units of percent:

$$SN = \frac{TP}{TP + FN}; SP = \frac{TN}{FP + TN}.$$

Ninety-five percent confidence intervals for SN and SP can be calculated using statistical procedures for binomial distribution.[25] Overall diagnostic accuracy of a test is defined as the proportion of correct classifications out of all classifications and is calculated as:

$$\text{Accuracy} = \frac{(TN + TP)}{TN + TP + FN + FP}.$$

SN and SP vary with the cutoff value used to define disease and have an inverse relationship; as SN increases for a given test, SP decreases, and vice versa. An inherent limitation of using a single decision threshold to determine diseased versus nondiseased status is that determination of disease presence or absence is dichotomous (binary) and interpretation of disease status does not factor in magnitude of the abnormal result.[13] Use of receiver operating characteristic (ROC) curve analysis to tailor cut-point selection for a given test and testing circumstances and the use of likelihood ratios (LRs) to estimate post-test probability of disease mitigate this limitation.[13]

Predictive Values

Positive predictive value (PPV) is the probability that an individual having the target disease will test positive. Negative predictive value (NPV) is the probability that an individual without the target disease will test negative.[7] PPV and NPV are impacted by SN and SP.

Importantly, PPV and NPV are also affected by the prior probability or prevalence of disease; PPV and NPV will vary as disease prevalence varies. Single NPV and PPV values calculated for a given prevalence, SN, and SP will not be uniformly applicable to all possible clinical situations.[7] PPV and NPV can be calculated from the two-by-two table provided in the earlier text if the study population's disease prevalence is the same as the animal population to which the test will be applied. In that case, PPV and NPV are calculated as:[17]

$$\text{PPV} = \frac{\text{TP}}{\text{TP} + \text{FP}}; \text{NPV} = \frac{\text{TN}}{\text{TN} + \text{FN}}.$$

Otherwise, PPV and NPV are calculated using the formulae:[7]

$$\text{PPV} = \frac{\text{SN} \times \text{prevalence}}{(\text{SN} \times \text{prevalence}) + \left[(1 - \text{SP}) \times (1 - \text{prevalence})\right]};$$

$$\text{NPV} = \frac{\text{SP} \times (1 - \text{prevalence})}{\left[\text{SP} \times (1 - \text{prevalence})\right] + \left[(1 - \text{SN}) \times \text{prevalence}\right]}.$$

PPV is greatest when prevalence is high; NPV is greatest when prevalence is low. Calculating PPV and NPV for a range of possible prevalences to facilitate clinical use of PV has been recommended.[7]

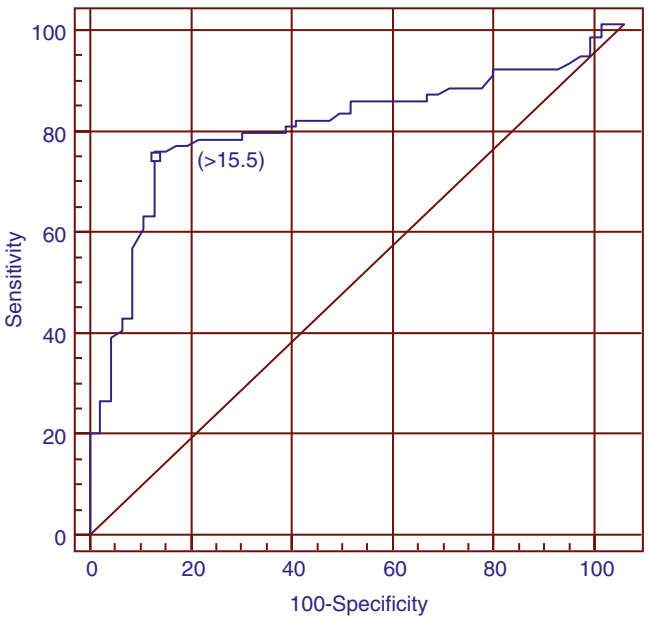


FIGURE 139.3 Receiver operating characteristic (ROC) curve. Tests with poor performance approach the diagonal line. Tests with good performance trend upward and leftward. The highest sensitivity and specificity are represented in the point at the upper left corner. The cutoff value that gives this point in this plot is 15.5. For screening diagnostics, sensitivity should be maximized. (**Sources:** Gardner and Greiner;[13] Linnet et al.[25])

Receiver Operating Characteristic Curves

ROC curves plot the true positive rate (SN) on the *y*-axis and the false positive rate (1-SP) on the *x*-axis.[13] The curve is constructed by plotting SN and (1-SP) pairs (operating points) for each possible cutoff value defining diseased and nondiseased for a given test (Figure 139.3).[13,25]

In general, the closer the plotted curve of SN and (1-SP) data pairs is to the upper left-hand corner of the graph, the better the test is at discriminating diseased and nondiseased individuals. This upper left corner represents the maximum combined SN and SP for that test.[13,25] The formal metric evaluated is area under the curve (AUC). AUC ranges from 0.5 (probability of a positive or negative test result equivalent to a coin toss) to 1 (perfect test performance, i.e., 100% SN and 100% SP).[13] ROC curves are commonly used to assess overall diagnostic accuracy of a given index test. They may also be used to refine decision thresholds for disease diagnosis and to compare accuracy of multiple tests used in the same clinical context[13] (see Chapter 140).

Likelihood Ratios

Positive or negative likelihood ratios, for dichotomous test results, reflect the probability of obtaining a positive or negative result in a diseased individual divided by the probability of obtaining a positive or negative result in a nondiseased individual. Mathematically, likelihood ratios represent slopes of the ROC curve and are calculated as:

$$\text{Positive LR} = \frac{\text{SN}}{(1 - \text{SP})}; \text{Negative LR} = \frac{(1 - \text{SN})}{\text{SP}}.$$

Positive LR (PLR) ranges from 1 to infinity; negative LR (NLR) ranges from 0 to 1.[13] For example, a PLR of 4.0 means that a positive test result is four times as likely to occur in a diseased versus a nondiseased individual. A LR of 1.0 means that a test result does not change the pretest probability of disease (i.e., it provides no useful information).[13] Guidelines for LR interpretation are that tests with satisfactory diagnostic performance should have a PLR of at least 2.0 and a NLR close to zero.[17]

Error Grids

An error grid is a graphical tool comparing the clinical impact of results from an index test (*y*-axis) to those from a comparative method (*x*-axis) using paired results from the same set of patient samples. The Parkes consensus error grid for evaluation of human glucometers is a common example.[32] CLSI guideline EP27 provides instructions for error grid construction and recommends $n \geq 100$ patient samples for error grid analysis.[29] As for method comparison studies assessing analytical performance, data used in error grid analysis should span a range of medically important concentrations and include data at or near commonly used medical decision thresholds. The error grid divides

paired test results into zones (labeled A, B, and C) predicting the likelihood of causing patient harm. Results falling into Zone A have minimal potential to cause patient harm, whereas results falling into Zone C are highly likely to cause patient harm. Zone B results represent moderate potential for patient harm.

The main challenge of error grid analysis is building the grid and establishing the zone boundaries, which are based on medical decision thresholds for the measurand (e.g., blood glucose), patient population (e.g., patients receiving insulin), and disease (e.g., Type 1 vs. Type 2 diabetes) in question.[32] Species is an additional consideration for veterinary measurands. To facilitate risk assessment and the setting of zone boundaries, medical decision thresholds must have clear clinical consequences (e.g., administer dextrose vs. administer insulin). Once constructed, an error grid can be used repeatedly to compare the clinical performance of two assays, assuming decision thresholds and their medical implications have not changed.[32] Error grid use is not common in veterinary method comparison studies, but has been reported with glucose and cortisol testing.[19,22]

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CHAPTER 140

Reference Intervals and Decision Limits

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Acronyms and Abbreviations

ASVCP, American Society for Veterinary Clinical Pathology; AUC, area under the curve; CI, confidence interval; CLSI, Clinical and Laboratory Standards Institute; CV_i, intraindividual variation; CV_g, interindividual variation; ECVCP/ESVCP, European College of Veterinary Clinical Pathology/European Society of Veterinary Clinical Pathology; IFCC, International Federation of Clinical Chemistry and Laboratory Medicine; RCV, reference change value; RI, reference interval; ROC, receiver operating characteristic; STARD, Standards for Reporting of Diagnostic Accuracy Studies.

INTRODUCTION

The diagnostic workup of veterinary patients is a decision-making process. Preliminary differential diagnoses are established based on a thorough history and physical examination. Subsequently, decisions that rule in or rule out the differential diagnoses are based on interpretation of laboratory tests including hematology and other clinical pathological data. For these decision-making processes to be consistent, established decision criteria are essential. These criteria may be in the form of decision limits or population-based and subject-based reference intervals (RIs). Optimally, these criteria are objectively tailored to the specific clinical situation as with decision limits or to the demographic or individuality of the specific patient as with various types of RIs. This is necessary to limit false decisions, decreasing the risk of false diagnoses. Creatinine is a perfect example of an analyte for which different types of decision criteria may be most relevant in specific clinical scenarios. Although population-based creatinine RIs are most frequently applied, breed-specific RIs are known to be more representative in certain breeds, such as greyhounds.[44] For monitoring renal function in an individual feline patient, subject-based RIs are more sensitive.[40] Finally, creatinine decision limits are used for staging renal failure in cats and dogs using the International Renal Interest Society guidelines.[23] Establishing any of these decision criteria requires an investment of time and effort and should be approached with an awareness of the objectives and procedures for determining each of these references. In addition, knowledge of biological variation is paramount for the exploration of reference values.

Decision limits categorize patients as "condition present" or "condition absent"; therefore, they are dependent on the clinical objective and only applicable for specific clinical situations.[36] Because decision limits are not relevant for every analyte or in every clinical situation, population-based RIs are more commonly used. Reference intervals describe the reference value distribution obtained from a reference sample group of healthy subjects representative of the patient population.[36] A measured value outside the RI is unlikely to

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originate from a healthy subject and will thus signify a diseased patient. As such, population-based RIs are independent of the clinical objective and more globally applicable; however, they must be representative of the specific population for which they will be used.

A joint guideline from the Clinical and Laboratory Standards Institute (CLSI) and International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) "Defining, Establishing, and Verifying RIs in the Clinical Laboratory" was updated and released in 2008.[9] Working groups from the American Society for Veterinary Clinical Pathology (ASVCP) and the European College of Veterinary Clinical Pathology (ECVCP) published a similar joint guideline for veterinary species.[15] The guideline is a welcome opportunity to standardize how RIs are established and communicated in veterinary medicine and should be applied as widely as possible.

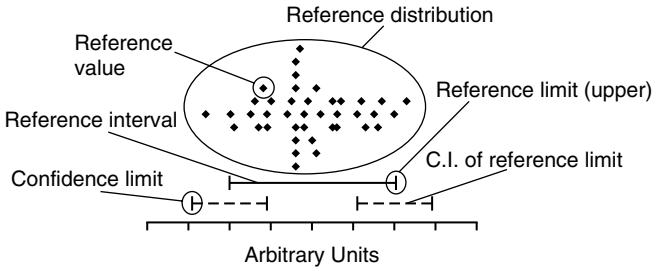


FIGURE 140.1 Nomenclature used in the establishment of RIs. A reference population (e.g., healthy dogs) is defined. Reference values (diamonds) are determined from a reference sample group. These values characterize the reference distribution from which reference limits are calculated that delineate the RI. Reference limits have a certain degree of variability that can be expressed by a confidence interval (CI), which is delineated by confidence limits. (**Sources:** CLSI[9] and Friedrichs et al.[15])

ESTABLISHMENT OF PROSPECTIVE REFERENCE INTERVALS

Several options exist for RI generation including de novo establishment (prospective), establishment using existing databases (retrospective), and transference from another source. Step-by-step guidelines for establishing de novo RIs for veterinary species and definitions of terms have been published by the ASVCP and closely align with the CLSI document revised in 2008 (Tables 140.1 and Figure 140.1).[9,15] A checklist of recommended elements for researchers, clinicians, authors, reviewers, and editors has been generated by the ASVCP Quality Assurance and Laboratory Standards Committee. The checklist is intended to aid in complete reporting of the process and results associated with RI development. Transparent reporting of the strengths and limitations of the RI enable the user to better interpret clinical data. The list is not intended to be used to bar manuscripts from publication by reviewers (Appendix 1). The benefits of available, less than ideal data should be weighed against the need for near-perfect data. Although the choice of statistical procedures stimulates much discussion, the number one priority in de novo RI establishment is to minimize unintended variability during reference subject selection and the collection and analysis of reference

TABLE 140.1 Outline of the protocol for obtaining reference values and establishing RIs.

1.	Establish a list of analytical interferences and sources of biological variability from the literature.
2.	Define the reference population, establish selection, exclusion, and partition criteria, and design a questionnaire to reveal these criteria in potential reference subjects.
3.	Create a consent form for participation in the RI study and have the owner of the reference subject complete the questionnaire and sign the consent form. Categorize the potential reference subjects as healthy based on history, physical examination, and clinical pathology data.
4.	Decide on the appropriate number of reference subjects with consideration of desired confidence limits and the homogeneity of the intended reference population.
5.	Select subjects (or data in retrospective studies) from the reference sample group based on the inclusion criteria and health assessments.
6.	Prepare the selected reference subjects for specimen collection consistent with the routine practice for that analyte. Collect and handle biological specimens in accordance with routine practices for patient specimens.
7.	Analyze specimens using well-defined methodology and consistent with routine practices for patients.
8.	Review the reference data for possible errors and prepare a histogram to visually evaluate the distribution of data.
9.	Inspect the reference data for possible outliers. Use statistical methods appropriate to data distribution to identify and eliminate verified outliers. Repeat this process until no outliers remain.
10.	Replot the reference data on a histogram and determine distribution, usually by a goodness-of-fit test. If data are not Gaussian, attempt transformation to a Gaussian distribution.
11.	Calculate the upper and lower reference limits, and the reference limit confidence intervals, using parametric, nonparametric, or robust methods depending on the distribution of reference data and size of the reference sample.
12.	If reference sample size is sufficient, determine need for partitioning if there is evidence of clinically important differences in the analyte across subgroups.
13.	Document the details of these procedures for future reference by clinicians and laboratorians, who may transfer the RI.

Sources: CLSI[9] and Friedrichs et al.[15]

samples. Identifying an adequate number of healthy reference subjects that are representative of the patient population and analyzing high-quality samples with well-controlled methods are significant achievements. Postsampling statistical procedures cannot correct for poor quality samples from unscreened reference subjects analyzed by methods that are unmonitored or unvalidated. If reference values are of high quality, the choice of statistical procedure is of little consequence providing rules based on data distribution are followed.

A well-conducted RI study should establish selection and exclusion criteria that define the reference population and document how health is determined. Exclusion criteria may include selected physiologic states (e.g., pregnancy) and extreme physiologic conditions (e.g., recent intense exercise). All subjects with evidence of disease should be excluded. When relevant physiologic information pertaining to the analyte of interest is available, identification of reference subjects preferably occurs by a direct a priori or prospective method, where criteria for selection, exclusion, and partitioning are decided before selection of reference subjects. When the effects of various partitioning factors (sex, age, reproductive status, etc.) are unknown, as may occur for a new analyte, a direct a posteriori or retrospective sampling method may be used in which selection, exclusion, and partitioning criteria are applied after the results are reviewed. To be representative of the laboratory's patient results and to model preanalytical variability, sample collection and handling should conform to the laboratory's established protocols.

Statistical analysis begins with graphic presentation of the reference values, most commonly as a frequency histogram. Gaussian and right-skewed distributions are most common. If the history and health of the population are well-documented, reference samples are of high quality and analytical methods are validated, the emphasis should be on retaining rather than deleting apparent outliers.[9] However, when health status is unavailable or is difficult to document, as occurs in free-ranging wildlife, outlier status should be objectively interrogated and any suspect values eliminated. Because additional outliers may be revealed upon elimination of far outliers, this process should be repeated in a population with unknown health status until all identified outliers are removed. Statistical procedures for outlier detection include distribution-dependent and distribution-independent methods and procedures that detect outliers at one or at both extremities.[21,25] For outlier procedures that are distribution-dependent, reference data must be Gaussian or transformed to Gaussian prior to application. Distribution-independent methods can be applied to data of any distribution.

Once outliers are addressed, the frequency histogram should be revised and data distribution verified as Gaussian or non-Gaussian, most commonly through a goodness-of-fit test. The Shapiro–Wilk is a powerful normality test for reference distributions, although the power for all such tests is low when sample sizes are small.[31,37] Whenever the reference value distribution is Gaussian or can be transformed to fit a Gaussian distribution, the RI can be determined by parametric methods.[9,12] Several transformation functions can be applied; however, the Box Cox procedure efficiently transforms many reference value distributions to normality.[18]

Alternatively, because assumptions of normality are easily violated,[31] the IFCC and ASVCP recommend nonparametric statistics whenever reference sample sizes of at least 120 are attained. The nonparametric method is a ranking method that commonly represents the central 95% of the healthy population and is determined as follows: n reference values are ranked and an interval is then delineated by ranks r_1 (2.5th) and r_2 (97.5th), with $r_1 = 0.025(n + 1)$ and $r_2 = 0.975(n + 1)$. In theory, a RI can be determined by nonparametric methods with as few as 39 samples; as this is the lowest value of n where r_1 and r_2 will reach integer values, namely, $r_1 = 1$ and $r_2 = 39$. However, at this power, no outlying values can be removed.[15]

An iterative (robust) method has been described that offers a relevant alternative to the nonparametric approach when $n < 120$. In short, it works by initially locating the median and spread of observations and then, through an iterative process, actual observations are down weighted according to their distance from the central tendency. For detailed instructions on computation, which can be performed in common spreadsheets, original literature can be consulted.[22] The robust method performs well with small sample sizes (n = 20–40), unintended inclusion of outliers, and without prior transformation in right-skewed distributions typical of reference data. All three methods (parametric, nonparametric, and robust) can be calculated, and the resulting RIs compared; if they are similar, the choice of method is inconsequential.[22] If they differ, the desired interval may depend on the analyte and the clinical question. Selection of the narrowest interval minimizes overlooking possible pathologic changes, but may increase the false positive rate.

Confidence intervals (CIs) of the upper and lower reference limits should be determined in order to reflect the precision of the limits. These confidence intervals will be narrower with larger reference sample sizes and with more homogeneous reference sample groups.[6] Confidence limits can be calculated using parametric, nonparametric, and bootstrap methods based on data distribution and the size of the sample.

The question of sample size pervades all RI discussions. A reference sample size of 120 is often touted as an "optimal" number, although n = 120 is better described as "sufficient," as this number permits the nonparametric calculation of the 2.5th and 97.5th percentile limits with nonparametric 90% CIs around these limits. Statistically speaking, the optimal reference sample size is a function of the reference value distribution and the desired tolerance and confidence of the RI.[41] The question of sample size differs whether one is determining RIs for homogeneous populations (e.g., dogs of the greyhound breed) or for heterogeneous populations (e.g., dogs of all breeds). The former requires fewer and the latter more reference subjects in order to be representative of the relevant patient population. In general, more is better. An example of misrepresentation of RIs due to small sample sizes is provided in the veterinary literature.[6]

ESTABLISHMENT OF INDIRECT REFERENCE INTERVALS BY DATA MINING

One solution to the frequent problem of small reference sample sizes is to harvest results from laboratory databases, a procedure called data mining.[27] In addition to very large sample sizes that permit creation of subgroup RIs specific to age and sex classes, advantages include harmonization to the laboratory's patient demographics, preanalytical procedures, and analytic methods and faster completion of the task with less inconvenience to subjects. The possibility of including results from unhealthy subjects can be minimized by using biological and statistical filters to exclude results likely originating from unhealthy individuals and by selecting databases comprised primarily of samples collected from patients during wellness checks, blood donor screenings, or preoperative assessments for elective surgeries. Several intriguing procedures are described for establishing indirect RIs from laboratory archives, some of which are available as open source software.[2,11,28]

TRANSFERENCE AND VERIFICATION OF REFERENCE INTERVALS

An alternative to establishing de novo RIs is transference and verification of a preexisting RI. Transference may occur when a newer model replaces an older analyzer or new methods or analyzers are introduced. The laboratory may choose to adopt RIs from the manufacturer or from another laboratory. When choosing a RI for transference, collection and processing protocols, analytical methodology, quality performance, and population demographics are important considerations. RI verification is more often successful when these factors are closely aligned between the donor and recipient laboratories.

A straightforward RI verification procedure compares 20 reference values, collected from the laboratory's healthy patient population, with the donor RI. Although previously described as a validation procedure, the term verification is preferred for the simplified process described here; validation implies a more comprehensive assessment.[9,35] Age and sex distribution of the sample group should mirror that of the laboratory's patient population. The donor RI is accepted if two or fewer reference values fall outside the donor RI and rejected if five or more fall outside the donor RI. If three or four fall outside the donor RI, the procedure can be repeated with an additional 20 reference values or the RI can be rejected. If verification again fails, then the donor RI is rejected (Figure 140.2a). Distribution of the 20 test samples within the donor RI should be considered. Values that are clustered toward the upper or lower limit or around the mean indicate that the donor RI is not entirely representative of the patient population and a de novo RI should be developed. When no verification samples from the receiving laboratory's patient population exceed transferred RI, the RI may be too wide. Although this scenario is not addressed in the CLSI guidelines, establishing de novo RI is advised. In an example of transference and verification of RIs from one coagulation analyzer to a different coagulation analyzer from the same manufacturer, rejection of the donor RI was correctly anticipated given the differences in preanalytical and analytical methods.[17] In another example using a larger sample group, 30 of 36 equine biochemical and hematologic RIs from a large reference laboratory were transferred and verified for Friesian horses, whereas six were not.[39] For these six analytes, de novo RIs were determined and reported.

An indirect or data-mining approach to verification has been described. This approach requires that the majority of samples in the laboratory's database be from healthy individuals so that the health-based distribution can clearly be identified.[35] The most useful parameter for bias assessment using this indirect approach is to compare the midpoint of the mined data (mean, median, or mode depending on data distribution) to the corresponding midpoint of the proposed RI.[26] The midpoint is resistant to results from unhealthy subjects and has less uncertainty. In addition, the expected flagging rate for the proposed RI, i.e., the rate of results above and below the respective reference limits, can serve as an indicator of comparability.[26] Laboratory RIs should be reverified, by direct or indirect methods, every 3–5 years as recommended in ASVCP guidelines.[15]

If the donor RI is rejected, any discrepancy may be due to systematic bias between methods, which can be quantified through a method comparison study (Figure 140.2b).[24] Although method comparison studies often include samples from unhealthy subjects whose values exceed the reference limits, this is discouraged when comparing methods for RI transference so as not to overestimate the strength of the correlation.[35] If systematic bias is identified and the *y*-intercept of the regression line is small compared to the RI, then both the upper and the lower donor reference limits can be transformed by the regression equation (Figure 140.2b). If the analyte has a narrow range of values, such as with electrolytes, the mean difference between methods may be sufficient to adjust the transferred reference limits. The transformed RI subsequently should be verified with samples from 20 healthy subjects as previously described (Figure 140.2c). If verification is unsuccessful, additional reference values must be obtained to establish the RI de novo.

REFERENCE INTERVALS FOR SUBGROUPS OR INDIVIDUALS

Subgroup Reference Intervals (Partitioning)

The suitability of a particular population for RI generation assumes homogeneity in that population. If homogeneity is violated in a clinically important manner, the subgroup (partition) should be identified and separate RIs created. Clusters of outlying values within healthy populations suggest heterogeneity due to breed, sex, age, or other characteristics and should prompt further investigation and documentation. Several examples of veterinary subgrouping exist, including measures of red blood cell mass in Friesian horses.[39] Most often, the discrepancy

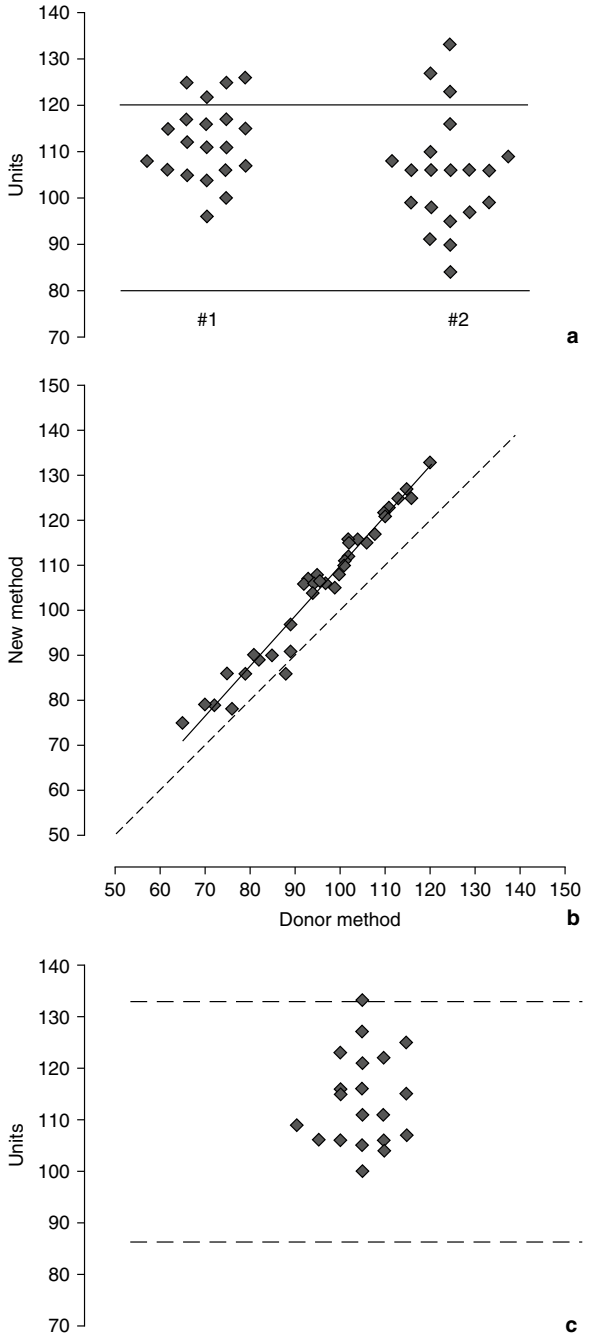


FIGURE 140.2 Transferred RIs (black lines) are verified by comparison of reference values from the laboratory's healthy patient population (diamonds, $n = 20$). If one or two values fall outside the RI, the donor RI is accepted. However, if three or four values fall outside (a, #1), the process must be repeated using the same acceptance criteria (a, #2). In this second assessment, three values fell outside the donor RI; therefore, the RI is rejected; (b) A method comparison study can determine the presence and magnitude of systematic bias, and if the *y*-intercept is small compared to the RI, the regression equation can be used to transform the donor reference limits to fit local methodology. In this case, the linear regression analysis result was $Y = 1.11X - 1.3$, $r^2 = 0.96$ with a *y*-intercept of 1.3 that is small compared to the donor RI of 80–120; the reference limits were transformed by the regression equation [1.11 × 80 – 1.3; 1.11 × 120 – 1.3] to the new interval of 87.5–140.9; (c) The transformed RI is then verified as described, in this case with success as fewer than two reference values fall outside. The dotted line in (b) represents the line of agreement between methods ($Y = X$).

is unidirectional; outlying observations are either systematically higher or lower than established limits. Therefore, the subgroup will have a statistically different mean or median than the general population. Differences in variation causing bidirectional discrepancy are infrequent and will not be described here.

The clinical significance of a subgroup discrepancy should be assessed before creating partitioned RIs. One criterion for partitioning RI is if the difference between subgroup means exceeds 25% of the combined RI width; a second criterion is if more than 4% of individuals in the subgroup fall outside the established RI.[20,30] Additional factors to consider include ease of identifying subgroup members, reference sample size for each subgroup, reference value distribution of both the subgroup and the combined group, and prevalence of subgroup members within the larger population. Reference interval partitioning is commonly based on phenotypically recognized subgroups, but may include genotype if this information is available and clinically relevant.

Subject-Based Reference Intervals

Biological variation describes the expected variation of a blood value across a group of individuals as well as the variation within an individual. Factors that affect biological variation include, but are not limited to, age, sex, breed, pregnancy, and production states as well as season and geographic location. The width of a population-based RI is mainly determined by the analyte's combined analytical, intraindividual (within-individual, CV_i) and interindividual (or within-group, CV_g) variation.[1,5,14] Analyte variability within an individual (CV_i) has a similar or narrower width than that within the group (CV_g). Validated biological variation data in veterinary species are expanding. Updated procedures and guidelines for generating and reporting biologic variation data can help standardize how biological variation is determined and communicated and should be applied as universally as possible.[1,5,14] A cross-disciplinary committee of professionals with expertise in this topic established the website, vetbiologicalvariation.org.[40]

An objective measure, for determining whether biological variability within an individual mirrors the variability within the group or whether it is narrower, is the index of individuality defined as the ratio CV_i/CV_g.[5] If CV_i is much smaller than CV_g, there is marked analyte individuality, indicating that a population-based RI would be insensitive in detecting pathological changes in an individual patient. The inverse of the index of individuality, CV_g/CV_i, is a more intuitive ratio in that an analyte with a low inverse ratio has low individuality and an analyte with a high inverse ratio has high individuality.[7] An index of individuality <0.6, or an inverse ratio >1.67, is generally accepted as a criterion for defining high individuality. In contrast, an index of individuality of >1.4, or an inverse ratio of <0.7, indicates low individuality. The prevailing clinical decision may influence the appropriate decision criterion when an index falls between these or, alternatively, analyte individuality can be increased by partitioning the RI into subgroups.[7] In theory, when an inverse ratio is high,

subject-based references are optimal for detecting pathologic changes in an individual, and when the inverse ratio is low, population-based RI suffice for clinical decision-making. In practice, clinical decisions are made using the available RI, and information on biological variability and analyte individuality are used to determine the sensitivity of that RI. When insufficient numbers of individuals are available to create a representative population-based RI, such as occurs with zoological or endangered species, subject-based references may be a suitable alternative.

For a subject-based approach, data on biological and analytical variability are used to determine a homeostatic set point for an individual patient, and a reference change value (RCV) is calculated that signifies a clinically significant difference in a single blood result or between serial blood results for that patient.[5,7] The homeostatic set point is an individual's average measurable quantity of an analyte that is maintained by dynamic physiologic control during health; CV_i represents variation around this set point, and CV_g represents variation between homeostatic set points across individuals. The RCV is defined as the amount of change needed between two results from the same individual to be considered different within a stated probability, taking into account analytical error (CV_a) and expected variation within an individual (CV_i).[5,7]

Several prerequisites central to the generation of population-based references are equally important for establishing subject-based references. These include meticulous control of preanalytical variation, selection of healthy reference subjects, identification and removal of outliers, and utilization of statistical models relevant to data distribution.[5] Minimizing analytical variability (CV_a) is of even greater importance for biological variation studies; therefore, when possible, samples should be analyzed in duplicate within one analyzer run.[5,14] Review of published biological variation parameters in animals reveals differences for the same analyte across studies, documenting the need for a universal and rigorous approach.[40]

DECISION LIMITS (THRESHOLDS)

Unlike RIs that are descriptive of the distribution of blood values in healthy subjects, a decision limit is a predetermined test threshold that distinguishes between two subgroups of a target population.[15,36] Decision limits have several purposes, including distinguishing a particular condition in patients presenting with analogous signs of disease, defining a critical value (flag) for clinical action, establishing prognosis by staging or grading, or assessing disease risk at a population level. Examples of these include a decision limit for pancreatic lipase immunoreactivity for the diagnosis of pancreatitis in dogs presenting with acute abdomen,[42] an empirical critical value for neutropenia to suspend chemotherapeutic drug administration,[32] a mitotic index that predicts a high-grade mast cell tumor,[29] and nonesterified fatty acid concentrations for herd-level risk of periparturient disease.[33]

The clinical scenario and the disease spectrum within the target population are integral components to the contextual selection and use of a decision limit. Establishing the clinical usefulness of a test decision limit, however, requires not only selecting a limit and determining diagnostic accuracy of the test but also exploring diagnostic efficacy.[8] The latter is a property not of the test, but of the circumstances in which the decision limit is applied. Relevant factors include the prevalence or probability of disease, possible clinical outcomes and their relative values, risks to patients of incorrect information, and costs and benefits of various treatment options. Guidelines for designing and interpreting prospective studies to establish decision limits and to assess diagnostic accuracy are addressed by CLSI and the ASVCP and are summarized in the following text.[8,15]

The process begins with defining the clinical question and the target population to which the decision limit will apply.[10] In addition, the assay's role in decision-making for the relevant condition should clearly be stated. A new test may be investigated for triage, replacement, or as an add-on to existing tests for the same condition.[10] The target population must be well defined with regard to the nature, duration, and severity of the condition as well as to age-range, sex, and other relevant findings using inclusion and exclusion criteria. As for RI determination, study subjects should be representative of the target population and selected prospectively to include statistically relevant numbers of both condition-positive and condition-negative individuals to avoid selection bias.

Test subjects should include individuals with the disease of interest, with look-a-like diseases, and with conditions that might otherwise be difficult to categorize in order to represent the condition's spectrum encountered in clinical practice; i.e., not restricted to healthy subjects or subjects with clinically apparent disease. As an example, the sample group selected to assess the discriminatory ability of natriuretic peptide assays for cardiac disease included dogs presenting with respiratory signs that had either primary cardiac disease, with and without heart failure, or primary respiratory disease.[4] Selecting subjects without knowledge of test results also minimizes selection bias that can affect the choice of a decision limit and estimates of diagnostic accuracy. Having an equal number of subjects in each category minimizes the uncertainty of diagnostic accuracy estimates, but is not always possible.[8]

The true clinical status of the subjects should be established by accepted and unbiased diagnostic criteria, independent of the test under investigation or a chemically related analyte. For tests evaluating disease severity or prognosis, standardized staging, grading, or scoring schemes and common outcome assessments should be used. Tests with subjective assessments should be determined by consensus.[8] Verification or definitive diagnosis may require long-term follow-up

including documented response to treatment or disease progression or histopathologic evaluation through biopsy or necropsy. This can minimize censoring of difficult to categorize subjects or subjects with indeterminate test results and maintain adequate sample size.

Testing should occur across the anticipated spectrum of disease for which the test is intended and at the same time that comparative testing is performed. If multiple tests are being compared, all tests should be performed on the same sample, at the same time, and in all subjects. If sample stability permits, batch analysis minimizes between-run analytical variation. Protocols for categorizing unexpected or indeterminate test results should be established in advance.[10] Excluding subjects with such results leads to bias favoring test performance. Sample collection should comply with protocols for patient preparation and sample handling for the analyte.

Analytical requirements for accuracy and precision are equally applicable to decision limits and RIs. A major challenge for universal application of a decision limit is ensuring precision and minimizing bias across laboratories. A major prerequisite to cross-laboratory precision is calibration material traceability to recognized standards, such as the CRM 470 standard for human serum proteins established by the IFCC.[3] However, such programs are rare, if not absent, in veterinary clinical laboratory medicine. As such, decision limits in veterinary medicine are most often derived for assays that are performed at a central laboratory or by the same methodology at multiple laboratories. It is important to remember that decision limits, like RIs, are determined in a defined population and may not perform equally in populations with demographic and pathophysiologic differences. Creation of universal calibration standards for acute phase proteins in veterinary species is under discussion by the International Society of Animal Clinical Pathology.[13]

Receiver operating characteristic (ROC) curves are integral to assessment of diagnostic assays.[8] Applications of ROC curves include establishing decision limits, determining whether a test performs better than chance, assessing diagnostic accuracy, and evaluating whether two tests for the same process have equivalent diagnostic potential.[8] To prevent overestimation of diagnostic performance, separate studies for selecting decision limits and establishing diagnostic accuracy should be performed. More detailed descriptions of ROC curve construction and interpretation can be found in published resources.[8,16,19]

Selection of a decision limit to distinguish between condition-positive and condition-negative status entails a trade-off between sensitivity and specificity. The purpose of the test in any specific clinical scenario dictates whether a decision limit with high sensitivity (rule in), high specificity (rule out), or balanced test performance is desired. The ROC curve graphically represents the entire theoretical range of diagnostic test performance for the selected subject group at multiple decision limits. The true positive fraction (sensitivity) is plotted on the *y*-axis, and the false positive fraction (1-specificity) is plotted on the *x*-axis with perfect test performance located in the upper left corner.[19] Because sensitivity and 1-specificity are independently plotted, the ROC curve is independent of disease prevalence.

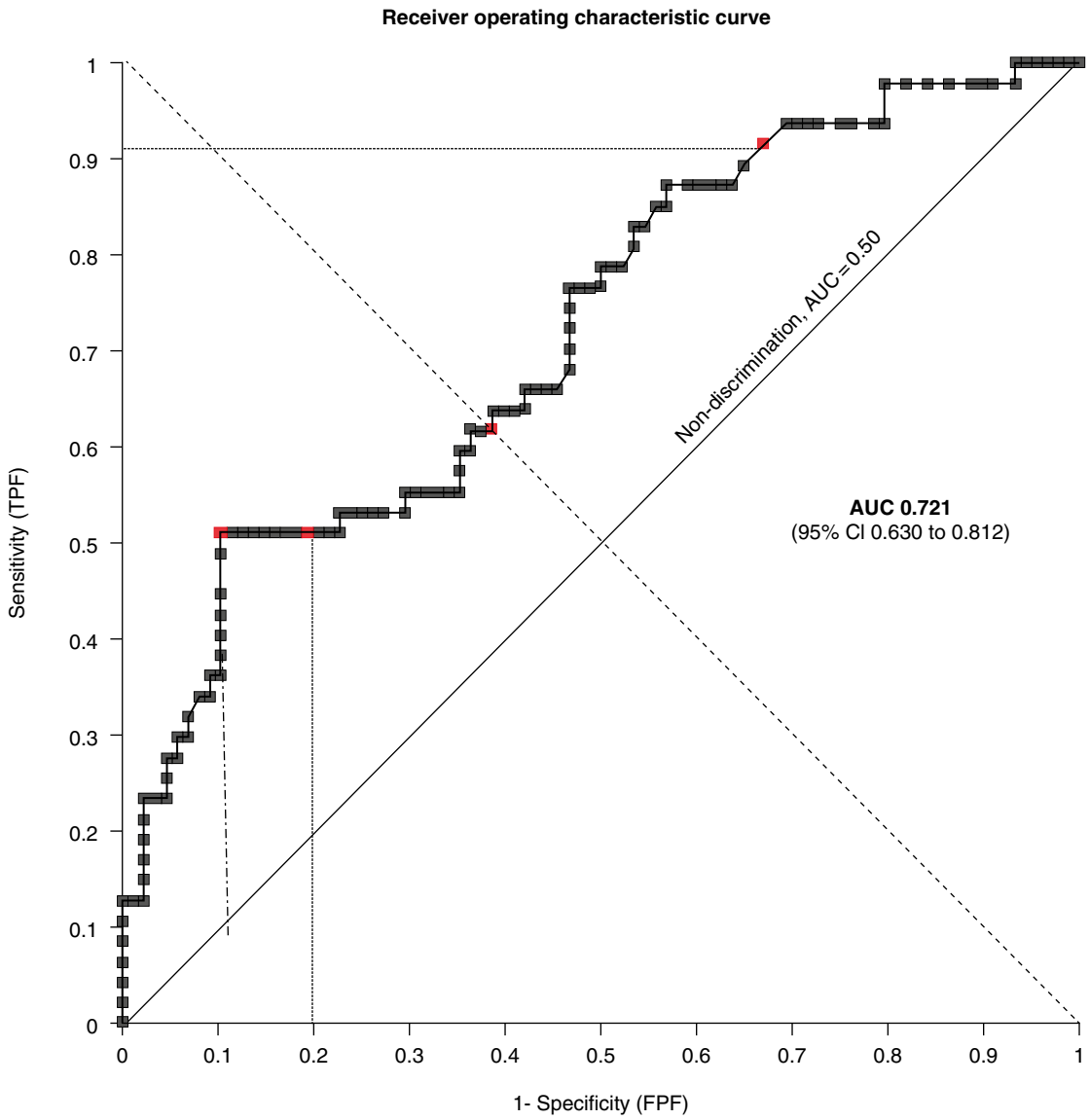
Using a ROC curve, the decision limit can be selected in several ways (Figure 140.3). One is to select a balanced decision limit where sensitivity equals specificity. This point is located where a line drawn from the lower right to the upper left corner intersects the test curve. A second method maximizes the summation of sensitivity and specificity (highest test accuracy), which occurs at the point of maximal vertical distance of a line drawn from the nondiscrimination line to the test curve. This method also maximizes the Youden's index.[43] A third method selects a decision limit based on a desired sensitivity or specificity based on the test's purpose. For example, if a highly sensitive decision limit for sodium:potassium ratio (NA:K) is desired to screen patients for hypoadrenocorticism, then a decision limit of <27 should be selected in order to capture all such patients for subsequent adrenocorticotropic hormone stimulation testing. On the other hand, if a more specific decision limit is desired, then a Na:K of <15 can be chosen.[38] As this example demonstrates, multiple decision limits can be selected for the same subject cohort by modifying the clinical question.

Selecting a decision limit by these methods does not account for factors contributing to diagnostic efficacy as previously described. To address this limitation, some authors suggest using a test index called, "number needed to misdiagnose."[19] This index is the number of cases correctly diagnosed before a single incorrect diagnosis is made. The closer the test is to the gold standard, the higher the index. Although mathematically complicated, the equation can be adapted to account for pretest probability and the cost of true positive and false positive test results.

The area under the curve (AUC) on a ROC plot is an estimate of a test's discriminatory ability (diagnostic accuracy) as well as representative of the average sensitivity over all specificities, and, conversely, the average specificity over all sensitivities.[8] Although theoretically starting at zero (100% misclassification), the applied accuracy interval is from 0.5 (nondiscrimination) to 1.0 (perfect accuracy). Ninety-five percent CIs should be reported with the AUC; if the CI includes 0.5, then the test may provide no useful information. The AUC also can be used to statistically compare the accuracy of different tests; in general, the higher the AUC, the more discriminatory the test.

PRESENTATION OF REFERENCE INTERVALS AND DECISION LIMITS

Reference intervals typically are printed on laboratory reports along with patient results. This only should be done when the provided RIs are applicable to the patient demographic. In addition, identification of results that fall above and below the RI often is denoted. Subgroup RIs should thoroughly be described and



Test >	(Sensitivity)		(Specificity)	FP proportion	FN proportion	Youden's index
242	0.915	0.915	0.330	0.670	0.085	0.244
368		0.617	0.614	0.386	0.383	0.231
524		0.511	0.795	0.205	0.489	0.306
571		0.511	0.898	0.102	0.489	0.408

FIGURE 140.3 Decision limits that satisfy specific clinical purposes can be selected from a ROC curve. The following decision limits are displayed in red and defined from left to right as: (1) A decision limit of >571 units maximizes the sum of sensitivity and specificity (maximum Youden's index), and is located on the curve at the maximal vertical distance from the line of nondiscrimination. This is also the closest point of the curve to the upper left corner of the graph. (2) Optimization at a chosen specificity of approximately 80% (approximately 20% FPF) yields a decision limit of >524 units. (3) The decision limit of >368 units, at which sensitivity and specificity are equivalent, is located at the intersection of the curve with a line drawn from the lower right to the upper left corner (dashed line) of the graph. (4) Optimization at a chosen sensitivity of approximately 90% yields a decision limit of >242 units. The AUC is an overall measure of a test's discriminatory ability (diagnostic accuracy). Created with Analyse-it® version 5.10.8 and a series of 138 test results from 47 condition-positive and 88 condition-negative dogs. AUC: area under the curve. CI: confidence intervals. FPF: false positive fraction. TPF: true positive fraction.

communicated to users. Reference intervals that represent a proportion of reference values other than the typical 95% coverage also should be prominently displayed.[9] If decision limits are available for certain analytes, these should be reported separately and not admixed with RIs. If multiple decision limits are utilized for different clinical situations, a brief summary of recommendations as well as the source of the limits should be included in the report. Additional details of the RI or decision limit study, such as the inclusion and exclusion criteria and the number of subjects, should be provided upon request in a written or an electronic report. The ASVCP RI guidelines provide a comprehensive list of information that should be included in summary documents and in publications of RI.[15] A more recently generated checklist is also available for authors and reviewers (Appendix 1). A recent study reports compliance for each of 35 such items in RI publications from nondomestic species.[34] A checklist of information for inclusion in reports of diagnostic accuracy studies is addressed in the updated Standards for Reporting of Diagnostic Accuracy Studies (STARD) guidelines.[10]

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Author Checklist for Manuscripts Reporting Reference Intervals

Reference Interval Checklist prepared by QALS Committee, April 2020[a]

METHODS

1.a) Reference population – inclusion and exclusion criteria ☐ Yes ☐ No 1.1, 1.2

1.b) Population geographic location ☐ Yes ☐ No 1.1

1.c) Partitioning factors, if applicable ☐ Yes ☐ No 1.2, Section 12

2.a) Sample size; number of reference individuals ☐ Yes ☐ No 1.2.3

2.b) Justification of sample size ☐ Yes ☐ No Section 1.2

3.a) Animal acquisition ☐ Yes ☐ No 5.1

3.b) Animal source ☐ Yes ☐ No 1.2.2–1.2.4

3.c) IACUC approval (or equivalent) ☐ Yes ☐ No

4.a) Laboratory specimen collection protocols ☐ Yes ☐ No Section 5

4.b) Lab specimen handling procedures ☐ Yes ☐ No Section 5

4.c) Analytical methods ☐ Yes ☐ No Sections 6 and 7*

5.a) Data inspection and outlier tests (Tukey's, Dixon), eg, histograms, box plots ☐ Yes ☐ No Section 8

5.b) Handling outlier values (strategy used to review and decide if values are removed). For wildlife especially, outlier detection should be repeated until outliers are identified and considered for removal. ☐ Yes ☐ No Section 9

5.c) State the threshold for interpreting the *P*-value of the test for normal distribution. For each measurand, provide the *P*-value of the normality test and conclusion about the distribution (Gaussian or Non-Gaussian) ☐ Yes ☐ No Section 10**

6.a) Reference intervals or limits ☐ Yes ☐ No Section 11***

6.b) Confidence intervals around the upper and lower reference limits; include the formula/method used (e.g., parametric, robust) ☐ Yes ☐ No Section 11

6.c) If partitioning included, report the steps (5.a -6.b) for each subgroup ☐ Yes ☐ No Section 12

6.d) Confirm steps 5.a – 6.b were recalculated for all measurands after outliers removed from original population data ☐ Yes ☐ No

6.e) Statement confirming methods follow ASVCP guidelines ☐ Yes ☐ No

RESULTS

7.a) Report the study period, including animal recruitment ☐ Yes ☐ No

7.b) Final number of reference individuals, after outlier removal ☐ Yes ☐ No

7.c) Number of samples excluded for technical issues or outliers ☐ Yes ☐ No

7.d) Clinical and demographic characteristics of reference population ☐ Yes ☐ No

7.e) Distribution results: normality test used, p-value, threshold used for interpretation of p-value, and conclusion about distribution (Gaussian or Non-Gaussian) ☐ Yes ☐ No**

7.f) Computational method (e.g., parametric, robust) and reference intervals for each measurand (the method is based on sample size) ☐ Yes ☐ No

7.g) Confidence intervals (CI) around the lower and upper limits of the reference interval, or the reason why the CI could not be reported ☐ Yes ☐ No****

7.h) Statistical differences, or other means used for partitioning ☐ Yes ☐ No

7.i) Reference intervals for partitioned groups ☐ Yes ☐ No

7.j) For low sample number groups, report descriptive statistics only ☐ Yes ☐ No

7.k) N<10, present all data ☐ Yes ☐ No

DISCUSSION

8.a) Limitations for reference intervals/limits/values ☐ Yes ☐ No

8.b) Unique value of the reference intervals, e.g., for unusual species or unique disease challenge ☐ Yes ☐ No

8.c) Practical application of the reference intervals, and where additional data are needed ☐ Yes ☐ No

REFERENCES

9.a) Citations for reference interval guidelines or review articles ☐ Yes ☐ No

9.b) Citations for methodology validation in the studied species, if applicable ☐ Yes ☐ No

[a]https://cdn.ymaws.com/www.asvcp.org/resource/resmgr/qals/reference-interval-checklist.pdf, accessed September 2021

*If analyses performed by author(s), include a statement about the analyst(s) proficiency, and quality control/quality assurance procedures to ensure the instruments were performing properly at the time of the study. If analyses performed by a reference laboratory, an attempt should be made to obtain QA/QC information.

**Normality, or distribution, tests are designed for large populations. Smaller population sizes are often obtained in veterinary medicine and it has been demonstrated that a threshold p-value of 0.3, instead of 0.05, more accurately characterizes the results (e.g., Shapiro-Wilks, D'Agostino-Pearson, etc.). If the tests' p-value <0.3, the measurand would be judged as having a Non-Gaussian distribution.

***The ASVCP Quality Assurance and Laboratory Standards committee developed and Excel template to create a reference interval reporting table (https://www.asvcp.org/page/QALS_Guidelines, accessed September 2021). Formats are provided to report hematology and chemistry reference intervals (where $n \geq 20$) or descriptive statistics ($n < 20$).

****Comparing the width of the confidence intervals (CI) around the lower and upper reference interval (RI) with the width of the RI × 0.2 may aid in evaluating the level of precision; when the CI width > 0.2 RI width, then collecting additional reference samples may be warranted.[34]

CHAPTER 141

Bone Marrow Evaluation

NATALI B. BAUER and KENDAL E. HARR

Bone Marrow Collection in Dogs and Cats
Indications and Contraindications
Collection of a Bone Marrow Aspirate versus Core Biopsy
Techniques and Locations of Bone Marrow Collection
Bone Marrow Collection in Horses
Bone Marrow Collection in Ruminants
  Bone Marrow Collection in Cattle
  Bone Marrow Collection in Sheep
  Bone Marrow Collection in Llamas and Camels
Bone Marrow Collection and Processing in Laboratory Animals
  Sampling Procedures
  Flow Cytometry Staining Techniques
  Materials for Push Smear Bone Marrow Cytological Preparation
  Cytologic Cytospin Slides from Flow Cytometric Samples
  Processing and Staining for Histologic Preparations
Slide Preparation and Staining of Clinical Bone Marrow Samples
  Slide Preparation
  Staining
Evaluation of Bone Marrow Smears in Laboratory Animals
Evaluation of Bone Marrow Smears in Patients
Use of Hematology Analyzers for Bone Marrow Analysis

Acronyms and Abbreviations

BM, bone marrow; BSA, bovine serum albumin; CD, cluster of differentiation; CD71, transferrin receptor; CD45, leukocyte common antigen; CLL, chronic lymphocytic leukemia; $DiOC_6$, dihexyloxacarbocyanine; EDTA, ethylenediaminetetraacetic acid; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; FL, fluorescence; FSC, forward scatter; MDS, myelodysplastic syndrome; MGG, May–Grünwald–Giemsa stain; M:E ratio, myeloid:erythroid ratio; nm, nanometer; LDS, laser dye styryl; PBS, phosphate-buffered saline; PE, phycoerythrin; SSC, side scatter.

BONE MARROW COLLECTION IN DOGS AND CATS

Indications and Contraindications

Bone marrow (BM) examination is indicated to investigate hematopoietic disease and blood smear abnormalities. Bone marrow collection should be performed in animals with suspected lymphoproliferative or myeloproliferative disease and neoplasia, myelodysplastic syndrome (MDS), staging of diagnosed lymphoma, histiocytic neoplasia, multiple myeloma, metastatic carcinoma (especially mammary carcinoma), and other sarcoma. Unexplained hypercalcemia, hyperproteinemia (gammopathy), and fever of unknown origin also may raise the suspicion of paraneoplastic syndrome and warrant BM evaluation. Diagnostic workup of unexplained, persistent cytopenias including nonregenerative anemia, neutropenia especially in the absence of left shift and toxic change, as well as bicytopenia or pancytopenia requires assessment of production. Abnormal peripheral blood cell populations, such as blasts, rubricytes in the absence of regeneration, and atypical morphology of WBCs, RBCs, or platelets, also necessitate BM evaluation.

Contraindications for BM aspiration include severe coagulopathies. Thrombocytopenia, however, is not a contraindication for BM collection because significant

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hemorrhage is rarely a problem. The risk of BM infection is negligible.[22] The most important contraindication, however, is performing a BM aspirate that is unnecessary.[8]

Collection of a Bone Marrow Aspirate versus Core Biopsy

The advantage of aspiration is that single cells are evenly spread on a slide enabling excellent cell morphology evaluation and lineage identification.[23] During BM aspiration and smear preparation, BM tissue architecture is somewhat disrupted. Tissue architecture is maintained by core biopsy allowing the proportions and location of hematopoietic cell populations and stromal cells to be evaluated.

Indications for BM core biopsy include:[23] (1) a BM aspiration which is repeatedly not successful (punctio sicca); (2) nondiagnostic BM cytology (e.g., BM aplasia or hypoplasia); (3) focal abnormalities (e.g., myelofibrosis, granulomatous inflammation, multiple myeloma, BM necrosis, metastatic carcinoma); (4) metastatic neoplasia. Some clinicians routinely collect both samples because the need for BM core biopsy frequently cannot be predicted at the time of BM aspiration.

Techniques and Locations of Bone Marrow Collection

Common collection sites in dogs and cats include the anterior greater tubercle of the humerus and the iliac crest (Figure 141.1). In toy breeds and cats with a small iliac crest, the femoral trochanteric fossa can be used. Use of the sternum risks penetrating the thoracic cavity and injuring intrathoracic organs and should only be used as a last resort or during necropsy. Bone marrow aspiration and core biopsies are painful and heavy sedation with analgesia or general anesthesia is typically used.

The sedated or anesthetized dog is positioned in lateral recumbency. The skin over the collection site is shaved and aseptically prepared. Approximately 1–2 mL of a 2% lidocaine solution is used for local anesthesia of all tissue layers, especially the well-innervated periosteum. A stab incision with an 11-scalpel blade may be made to decrease skin contamination and interference during needle rotation. A 13–19 gauge × 2.5 cm Klima-Rosegger, Rosenthal, or Illinois sternal needle with an interlocking stylet is used for BM aspiration with smaller sizes used in cats and larger sizes in most dogs.[4] The flat portion of the humeral greater tubercle or iliac wing is palpated. The needle is inserted with slow rotating movements either at a 90° angle (perpendicular) to the humerus or at a 45° angle, parallel to the long axis of the ilium. After removal of the stylet, a 6–12 mL syringe typically containing EDTA is attached to the needle.

The syringe can be prepared using a 5–10% EDTA anticoagulant solution, created by mixing 0.5 mL of sterile saline into a 7 mL EDTA tube.[5] This solution is drawn into the collection syringe, and some EDTA may be expelled dependent upon the animal's size and expected BM yield. Anticoagulant choice should be based on planned diagnostic protocol. Heparin is the anticoagulant of choice for some flow cytometric protocols. However, heparin anticoagulated marrow should not be used for microscopic cytologic examination, as it causes significant staining and morphologic artifacts.

Marrow is aspirated into the syringe by applying quick negative pressure until about approximately 1 mL or more of marrow is collected (dependent upon the size of the animal). The syringe contents are expelled into a Petri dish and should contain a mixture of blood, floating fat globules, and granular white-gray BM particles (Figure 141.2).

A microhematocrit tube or thin glass pipette is used to pick up several BM particles and only a small amount of blood, thereby concentrating the marrow particles (Figure 141.3). This material is transferred to a glass slide and spread using a gentle squash smear technique.[4]



FIGURE 141.1 Demonstration of iliac crest site for BM collection in a dog.

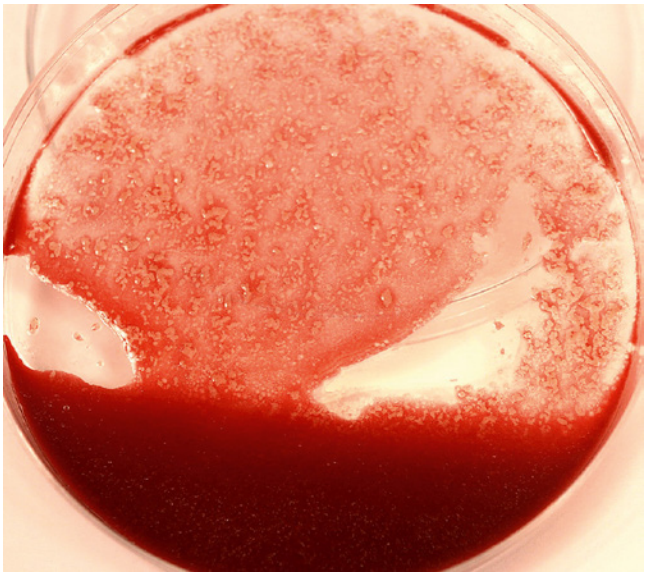


FIGURE 141.2 Bone marrow aspirate biopsy material expelled into a Petri dish displays numerous glistening fatty particles which can be collected by microhematocrit tube or pipette. (Source: Photo courtesy of University of Florida Clinical Pathology Collection.)

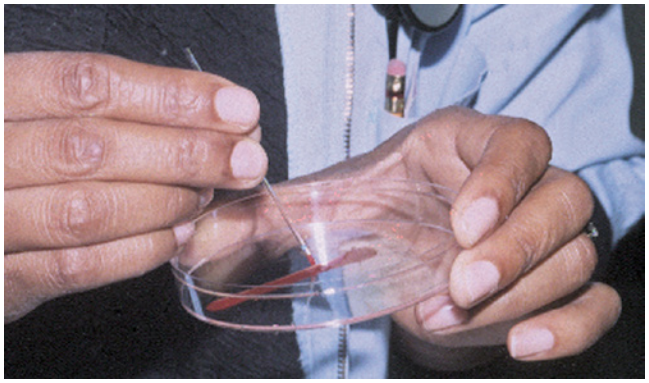


FIGURE 141.3 Particle collection. Particles are picked up using a microhematocrit tube to be expelled onto a glass slide. A squash preparation is then made from the droplet. (Source: Photo courtesy of Rose Raskin, University of Florida.)

At least six to eight quality BM smears should be prepared which can then be used for special stains as needed for neoplastic diagnosis. After BM collection, gauze is applied with firm pressure to the aspiration site for a few minutes to stop any bleeding.

Core biopsy specimens may be taken at 1 cm or more distant to the previous needle placement or at an alternate site for better representation of the patient's bone marrow tissue. Core biopsies should not be performed in close proximity to the aspirate site, as artifactual hypocellularity of the core sample may be induced. The core biopsy is performed with an 11–13 gauge Jamshidi needle, a cylindrical needle with a tapered distal end and beveled cutting tip. Once the Jamshidi needle is seated into the cortical bone, the stylet is removed; then the needle advanced by rotation as far as possible. The needle may be withdrawn slightly and then readvanced at a slightly different angle, or the needle can be rotated/stirred to break off the sample and ensure it remains in the needle core. Once the needle is withdrawn, the stylet is introduced into the needle in a retrograde fashion, so the core sample can be gently collected and transferred to a tissue cassette. Rolling the core sample on a glass slide prior to placement in formalin can provide additional cytologic samples. The core is placed in neutral, buffered 10% formalin for a minimum of 2 hours. The core sample should ideally be at least 1 cm long and contain 10 trabeculae microscopically.[4] The fixed marrow core must be decalcified prior to histologic processing. The core may be transferred to 70% ethanol for transport, which has the benefit of decreasing potential toxicity and formalin fumes.

BONE MARROW COLLECTION IN HORSES

The sternum is the most commonly used site for BM collection in adult horses, with the rib at the costochondral junction also possible.[29,31] In foals, the iliac wing is also a possibility and precludes penetration of the chest and pericardium. The sternum is not covered by significant muscle mass and the cortical bone is relatively thin. Disadvantages include an awkward angle for the procedure in the standing, sedated horse and proximity to the heart, though at some distance from the sternum in the adult horse. The tuber coxae are only used for obtaining marrow in foals and young horses, as thick cortices can make access difficult.[29] The rib contains active marrow throughout life and is an easy site to reach, but has several disadvantages including thick cortices which can be difficult to penetrate in adult horses. The rounded rib surface can make it difficult to seat the needle in the bone and increases the risk of slipping off the rib and puncturing the pleural cavity, potentially resulting in pneumothorax.

BM aspiration and core biopsies are performed in standing, sedated patients. The skin over the site should be clipped and aseptically prepared. All tissue layers, including the periosteum, are anesthetized with a 2% lidocaine solution. A 15 gauge × 5 cm Jamshidi BM biopsy needle (also called a Janus® needle) is preferred for marrow aspiration. A stab incision is made in the skin and the needle is inserted until it reaches the bone. The bone is penetrated using a slow rotational movement until the needle is firmly seated in the bone. For the sternebrae, the needle is then advanced 1–2 cm further into the marrow cavity. If the iliac wings are used, a technique similar to that described for the dog can be used. BM aspiration and sample preparation are similar to that described for the dog and cat with size appropriate instrumentation. An 11 gauge × 10 cm Jamshidi biopsy needle is used to obtain core biopsy specimens with similar sampling and preparation as described for the dog and cat.

BONE MARROW COLLECTION IN RUMINANTS

Bone Marrow Collection in Cattle

Bone marrow aspiration smears and core biopsy specimens from adult cattle have been obtained from the sternum (preferred) and the rib using a Turkel® trephine needle (Trephine Instruments Inc., USA).[14,36] Advantages of the sites are similar for that described in the horse. The iliac crest is unsatisfactory because of bovine pelvic anatomy. The adjustable depth control of the Turkel trephine needle helps prevent penetration of the pleural cavity, but does not eliminate the risk.

Cattle should be sedated before BM aspiration. Intravenous xylazine provides adequate sedation. A 15 gauge × 5 cm Jamshidi BM biopsy needle may be used for marrow aspiration and an 11 gauge × 10 cm Jamshidi biopsy needle can be used to obtain core specimens. The aspiration site is the 3rd or 4th sternebra. This site can be located by counting the ribs starting with the last one; however, the location on the sternum coincides with an imaginary line joining the olecranon. With the ox in a standing position, a small area over the sternum is shaved and aseptically prepared, and all layers of tissue, including the periosteum, are anesthetized with a 2% lidocaine solution. A stab incision is made in the skin and the Jamshidi needle is inserted (with stylet in place) until it reaches the bone. The bone is penetrated using a slow rotational movement until the needle is firmly seated in the bone. The

needle should be advanced approximately 1–1.5 cm. One can frequently sense when the needle enters the marrow cavity because of a sudden reduction in resistance.

In calves, the proximal humerus is an excellent site for marrow collection. Calves can be restrained in lateral recumbency and the method described for dogs and cats can be followed. A 15 gauge × 5 cm Jamshidi or Illinois sternal biopsy needle with the needle guard removed can be used for aspiration biopsy and an 11 gauge × 10 cm Jamshidi needle can be used for core biopsies.

Bone Marrow Collection in Sheep

The sternum is the preferred site for BM collection in sheep.[9] Sheep are restrained in a sitting position by an assistant who holds a foreleg in each hand. The 3rd or 4th sternebra is located by following the rib to its articulation. The site is aseptically prepared and anesthetized as described for cattle. A 15 gauge × 5 cm Jamshidi or Illinois sternal biopsy needle with the needle guard removed can be used for aspiration. For BM aspiration, the depth of penetration from the skin surface varies between 2.5 and 3.0 cm and bone penetration is approximately at 0.5 cm. An 11 gauge × 10 cm Jamshidi needle can be used for core biopsies.

Bone Marrow Collection in Llamas and Camels

The sternum is the preferred site for bone marrow collection in llamas.[1] Bone marrow collection has been described from the dorsal rib of dromedary camels.[25] Adult llamas and camels are sedated with intravenous xylazine and can be standing, placed in a chute, or in left lateral recumbency. Generally, a good marrow sample from camelids may be obtained from the midline of the sternum at the level of the elbow's caudal aspect when positioned naturally.[17,18,20] In llamas, the 3rd or 4th sternebra is located by following the rib to its articulation. In dromedary camels, a site over the 5th through 8th dorsal rib was aseptically prepared and anesthetized as described for cattle. The site is aseptically prepared, anesthetized with 2% lidocaine, and an 11–16-gauge bone marrow biopsy needle is used to obtain samples. Bone marrow samples are most frequently aspirated into EDTA, but may be collected with no anticoagulant if bone marrow smears are prepared immediately. Slides are air-dried and stained with appropriate stains.

BONE MARROW COLLECTION AND PROCESSING IN LABORATORY ANIMALS

In recent years, toxicological clinical pathology generally uses flow cytometric analysis for quantitative BM evaluation, and cytologic preparations to assess morphologic changes and abnormalities in maturation or proliferation. Quantitative BM evaluation includes total nucleated cell counts, differential cell counts, and a myeloid:erythroid (M:E) ratio. Flow cytometry counts more than 10,000 cells, thus providing a much faster and accurate measurement of cell populations than microscopic assessment.

Sampling Procedures

Following humane euthanasia of small rodents (mouse and hamster), dislocate the hind limb from the pelvis, strip away the muscle, and cut the femur at the edges of the stifle and coxofemoral joints (Figure 141.4). Split the femoral cylinder open lengthwise with a scalpel or small electrical saw, use a small brush dipped in hypotonic fetal bovine serum (two parts serum:one part distilled water), brush cells out of the femur and onto a glass slide, air-dry slides, and stain them with a Romanowsky stain.[19,30]

Bone marrow collection from rats and chinchillas does not have to be terminal, but should be done under general anesthesia. Marrow can be collected from the iliac crest, trochanteric fossa of the proximal femur, or tibial crest. The sternum may be used; however, accidental perforation of the chest is possible. The periosteal bone and skin are blocked with 2% lidocaine and a stab incision is made through skin. An 18–20 gauge, 1 inch biopsy needle is rotated through the periosteum into the bone marrow cavity similarly to that described for the dog. Marrow is then aspirated by applying negative pressure with a 3 cc syringe, with a similar volume to marrow ratio as that in the dog. The sample is smeared or brushed onto a slide and stained as mentioned in the earlier text.[2,31]

Terminal collection in the dog and cynomolgus monkey may use the fifth rib which is removed at necropsy and trimmed of muscular tissue. A 1 cm piece of rib is cut from the distal end articulating above the costal cartilage (Figure 141.5). The BM tissue is flushed, approximately five times, with 2 mL phosphate-buffered saline (PBS) containing 50% fetal bovine serum using a needle and syringe (Figure 141.6). The BM cell suspension is

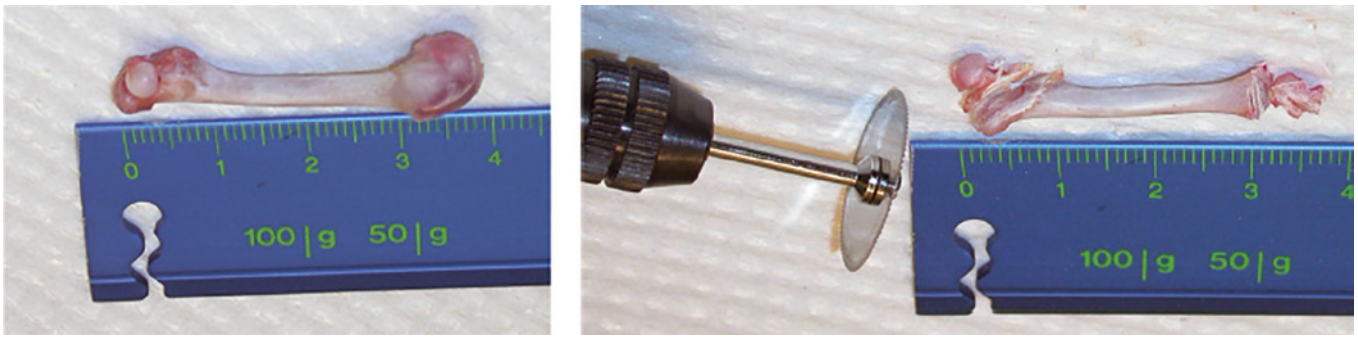


FIGURE 141.4 BM sampling procedure from the femur in rodents.

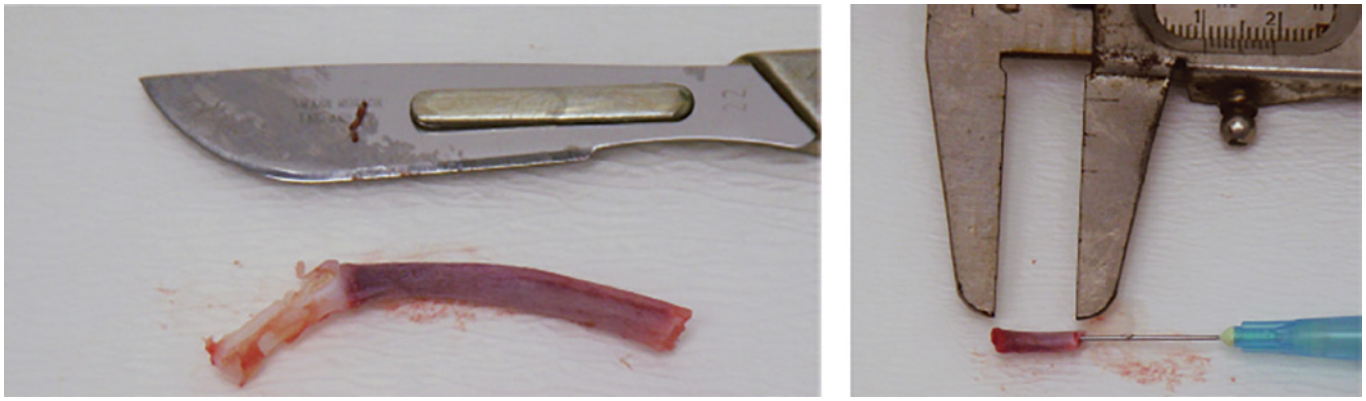


FIGURE 141.5 Terminal bone marrow sampling procedure from the rib in dog or monkey.



FIGURE 141.6 The flushing of BM from the piece of rib (dog and monkey) or from the femur of rodents.

washed and resuspended in cold PBS containing 0.5% bovine serum albumin (BSA). The nucleated cell count is determined using a hematology analyzer and the cell concentration is adjusted to 10^7 cells/mL (Figures 141.5 and 141.6).

Flow Cytometry Staining Techniques

Saad et al. described a method for rat BM differential cell counts using a combination of two monoclonal antibodies for the differential expression of leukocyte common antigen (CD45) and transferrin receptor (CD71).[30] This was coupled with the side scatter for cellular complexity and nucleic acid staining using LDS-751 (Thermo Fisher Scientific, Carlsbad, CA, USA) for separation of nucleated cells from mature red blood cells (Figure 141.7). A similar approach can also be used for monkey and mouse BM analysis. Briefly, BM cells are labeled with fluorescein isothiocyanate (FITC)-conjugated anti-CD45 and phycoerythrin (PE)-conjugated anti-CD71 monoclonal antibodies. The tube is incubated in an ice bath in the dark for 20 minutes, then washed with PBS containing 0.5% BSA. The cell pellet is resuspended in 0.4 mL cell fixative (1% formaldehyde). Twenty µL of LDS-751 staining solution is added at least 20 minutes before sample analysis. The LDS-751 staining solution is prepared by diluting 20 µL stock solution (1 mg/mL methanol) with 3 mL PBS. Flow cytometric analysis is done using a 488 nm excitation wavelength from an argon-ion laser. Data can be collected from 10,000–20,000 cells from each sample in list mode to save forward scattered (FSC) values and side scatter (SSC) in a linear scale, and FL1 (green), FL2 (orange), and FL3 (red) in a logarithmic scale. A cytogram of CD45 versus SSC can be plotted for all LDS-751-positive cells (nucleated cells) on a cytogram of LDS 751 versus SSC. Standardized gates define the percentages of erythroid, lymphoid, and myeloid cells. Dog BM analysis is based on cellular complexity and CD45 expression in combination with LDS-751 staining (Figures 141.7 and 141.8).

Other flow cytometry approaches for BM analysis of laboratory animals are described in the literature. For rodents, Martin et al.[16] described a flow cytometric method based on membrane potential using 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) staining (Figure 141.9). A combination of 2′,7′-dichlorofluorescein-diacetate staining (indirect myeloperoxidase staining) and monoclonal antibodies for lymphocyte subpopulations (OX-52 for T-lymphocyte and OX-4 for B-lymphocyte) was used by Criswell et al.[5] for the rat BM differential analysis (Figure 141.10). Flow cytometric methods using cellular

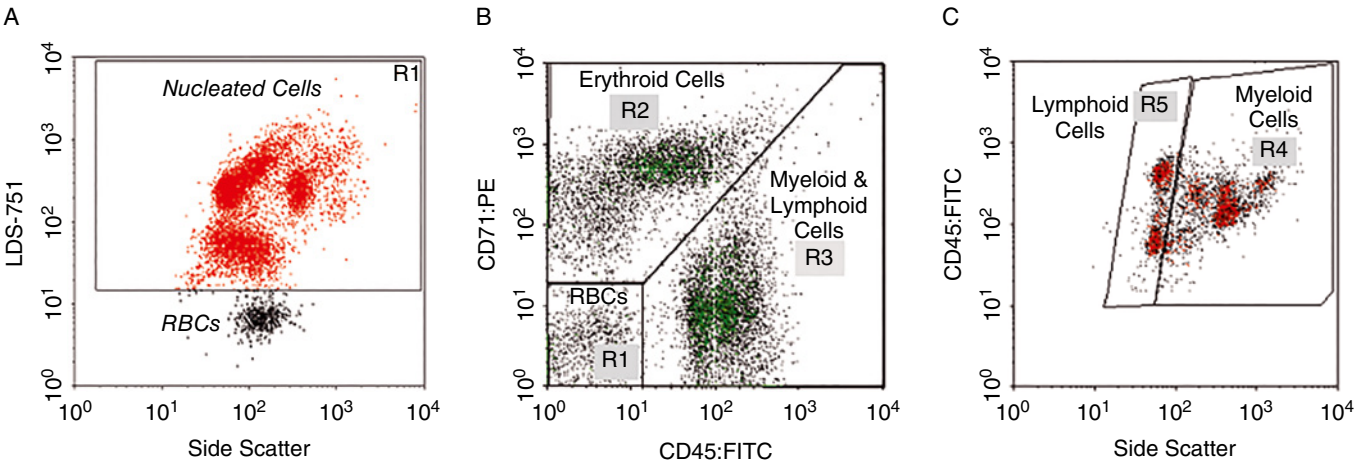


FIGURE 141.7 Rat BM differential analysis using combination of CD45:FITC, CD71:PE, and LDS-751 staining. Cytogram C represents events in gate R3 of cytogram B.

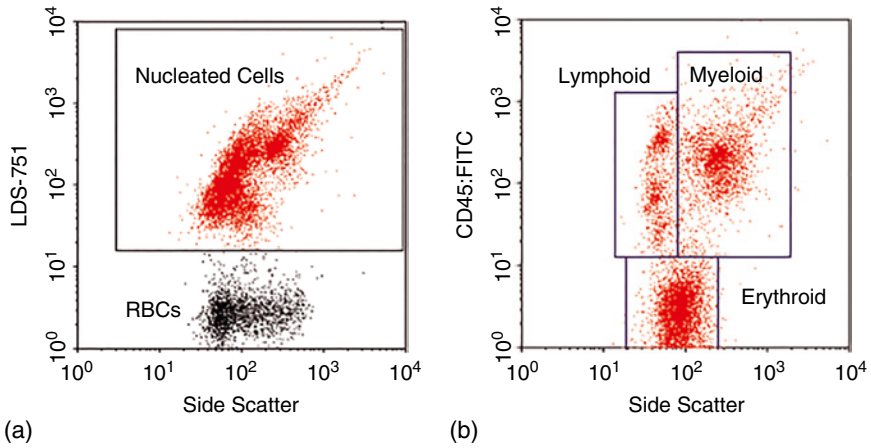


FIGURE 141.8 Dog BM differential analysis using combination of CD45:FITC, cellular complexity (SSC), and LDS-751 staining. Cytogram B represents events in nucleated cells gate (cytogram A).

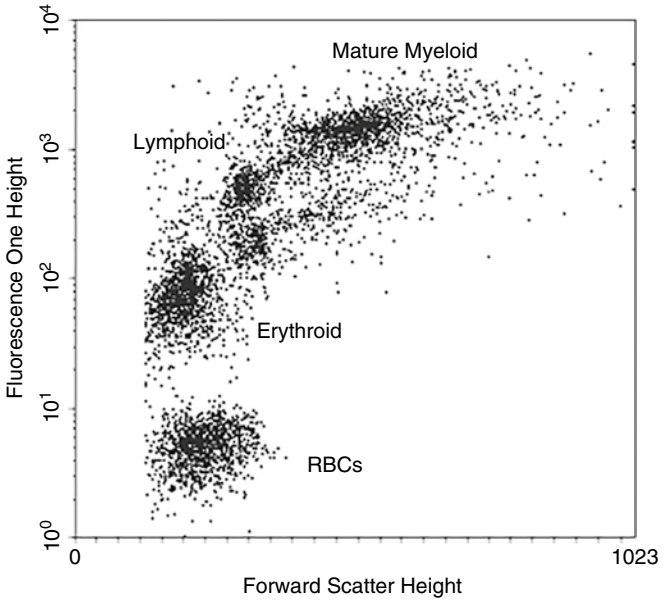


FIGURE 141.9 Rat BM differential analysis using 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) staining.

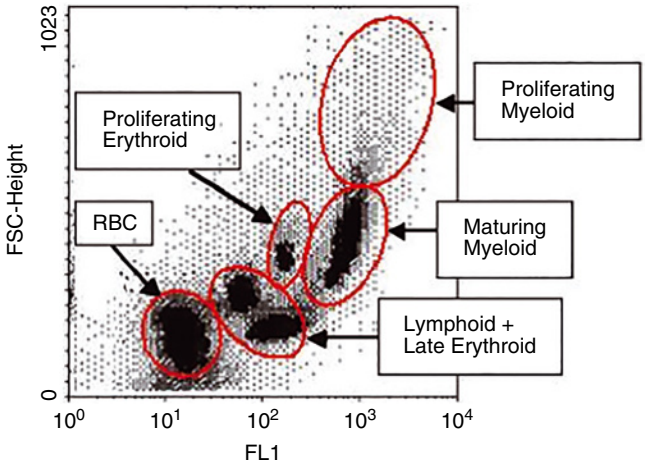


FIGURE 141.10 Rat BM differential analysis using 2′,7′-dichlorofluorescein-diacetate staining (indirect myeloperoxidase staining).

complexity and light scatter properties in combination with CD71 or CD45 expressions have been described for rat[23] and dog[26] BM evaluation. More recent work by Zhao et al.[41] described a method for rat BM analysis using the combination of CD3, CD45R, CD71, CD11b/c, and the DNA dye DRAQ5.

Materials for Push Smear Bone Marrow Cytological Preparation

Reagents for push/compression smear cytological preparation include: immunological grade bovine albumin, 22%, polymerized (Median Diagnostics), several pieces of 4 × 4 cm square parafilm, bone cutter for sampling of longitudinally sliced femur, scissors, paintbrush no. 0 or 1 regardless of species, and glass slides. The following procedure may be used:

1. Place a drop of bovine albumin on several pieces of parafilm square.
2. For rodent femur preparations, cut the femur lengthwise, using sharp tools.
3. Pick out as much BM as possible with the brush; place on parafilm next to the drop of bovine albumin fluid.
4. Dip brush in albumin fluid and mix into the BM to make a uniform solution.
5. Place a drop of BM suspension on four to five slides.
6. Place another slide gently on top of the slide containing the sample and spread slides apart.
7. Air dry.

Cytologic Cytospin Slides from Flow Cytometric Samples

For cytospin slides, take 100 µL of the BM suspension prepared for flow cytometry and add 400 µL PBS containing 0.5% BSA. From this diluted cell suspension, 100 µL is placed in the funnel of a cytocentrifuge. The specimen is centrifuged at 50 g for 3 minutes and the slide is removed and air dried.

Processing and Staining for Histologic Preparations

Histologic processing must create sections that are 4 µm or less, which is thinner than many tissues. Recommended stains include standard H&E, Giemsa (to improve assessment of the erythroid line and potential mast cells), and periodic acid–Schiff (to improve assessment of the granulocytic lines and other proteinaceous material). Rodent BM requires slightly longer staining time with Giemsa staining for the best differentiation between nucleated RBCs and lymphocytes. The Giemsa stain needs to be freshly prepared just before use and care should be taken to maintain a pH of 6.8 for the phosphate buffer. Optional stains include Prussian blue for the evaluation of iron stores, reticulin to assess reticulin fibrosis, and trichrome to assess collagen fibrosis. Increased trichrome staining, indicating type 1 collagen, in humans is associated with more severe disease and a poorer prognosis.[13]

SLIDE PREPARATION AND STAINING OF CLINICAL BONE MARROW SAMPLES

Slide Preparation

Several techniques for preparing BM smears in a clinical veterinary setting have been described. The most commonly used techniques are:

1. Preparation of a wedge-type smear (no anticoagulant needed).
2. Drop-squash or particle-squash preparations (require EDTA as anticoagulant).

For preparation of a wedge-type smear, several drops of the aspirated BM are ejected onto a microscope slide and a spreader slide is backed into the area containing concentrated BM particles and a wedge-type smear is prepared. Generally, up to 10–12 slides should be made.[23]

For the pull or squash preparation, concentrated BM particles from the hematocrit tube are placed on a slide and gently spread by a second longitudinally placed slide or coverslip.[24] The slide is subsequently tilted, so excess blood is drained away. EDTA-anticoagulated BM can be placed in a Petri dish for collection of BM particles. Marrow particles, seen as white to clear specks in the red marrow, can be picked up with a microhematocrit capillary tube using capillary action and are carefully transferred onto a second slide so that squash preparations can be made.[24]

Staining

After preparation, BM smears are quickly air-dried. Routine staining is performed with Romanowsky-type stains (May–Grünwald–Giemsa stain, Wright stain). It is important to remember that BM smears require a longer staining time because they are thicker and the material is often unevenly distributed. Aqueous Romanowsky stains like Diff-Quik®, however, are less reliable because they do not stain chromatin structure as clearly as methanolic Romanowsky stains like May–Grünwald–Giemsa or Wright. Additional stains may be needed so that unstained slides should be saved. Cytochemical staining methods are discussed in Chapter 144.

EVALUATION OF BONE MARROW SMEARS IN LABORATORY ANIMALS

Cytological examination of a bone marrow smear is not routinely recommended in laboratory animals, but rather considered on a case-by-case basis.[3,28]

Indications for bone marrow cytological examination include:

- Evaluation of early hematopoietic precursors (maturation and potential drug-induced changes of morphology).
- Unexplained increases or decreases of cell counts in the peripheral blood.
- Differentiation between lymphoid and erythroid cells.
- Changes in erythrocyte indices such as increased mean cell volume suggestive of dyserythropoiesis.
- Atypical leukocyte morphology in the peripheral blood.

Conditions in which bone marrow cytology may be not necessary include:

- Decreased bone marrow cellularity due to reduction of all hematopoietic cell lineages proportional with a degree of restricted food intake (especially in rats, to a lesser extent in dogs).
- Changes in peripheral lymphocyte count, as there is no correlation with bone marrow lymphopoiesis.
- Regenerative changes in bone marrow or peripheral blood.
- Dysfunction of platelets or granulocytes.

Evaluation is performed similarly to clinical samples; however, use of interpretative terms such as hypocellularity or hyperplasia is discouraged. Findings should be reported in relation to a control group and interpretation of treatment-related change is done using hematologic and histopathologic data.

EVALUATION OF BONE MARROW SMEARS IN PATIENTS

Unlike evaluation of BM in laboratory animals, clinical evaluation of BM is qualitative. Thus, only the proportions of cellular populations in relation to each other, their maturation, and cellular morphology are reported. A 200–500 cell differential may be performed in order to better define cell proportions. For interpretation of these cytological findings, clinical signs and hematological results of the patient must be considered, as they indicate the actual demand for RBCs, WBCs, and platelets in the blood. For instance, BM with normal cellularity and a M:E ratio within the reference interval would be considered normal for a nonanemic animal, but hyporegenerative for an animal with a severe anemia and no reticulocytosis.

Criteria to be evaluated in BM smears include: (1) estimation of cellularity; (2) assessment of megakaryopoiesis; (3) evaluation of BM iron (not in cats); (4) M:E ratio; (5) maturation of cellular lineages (megakaryopoiesis, erythropoiesis, granulopoiesis, lymphopoiesis); (6) presence of dysplastic changes; (7) evaluation of other cell types (plasma cells, mast cells, macrophages, osteoclasts, osteoblasts); and (8) evidence of neoplastic cells or parasites.

While cellularity, megakaryopoiesis, stainable BM iron, and the presence of atypical cell clusters are evaluated by assessing several BM particles at 100× magnification, M:E ratio, maturation, and cellular morphology are assessed at 500× to 1000× magnification. As in people, cellular lineages are evaluated quantitatively and qualitatively, i.e., their numbers are classified as decreased, normal, or increased.[15] Cellularity is estimated in BM spicules by comparing the proportion of cells to fat tissue (cell-to-fat ratio). Normal cellularity ranges between 25% and 75% cells[10,34] and is age-dependent. BM spicules of growing animals contain approximately 75% cells, those of adults contain 50%, and marrow spicules of older animals 25% cells. As recommended previously for people, cellularity is reported as acellular, decreased, normal, increased, and markedly increased.[15]

The number of hemosiderophages in BM spicules is indicative of the amount of stored BM iron. Stainable BM iron is increased in chronic inflammation and immune-mediated anemia; however, it is decreased in iron deficiency and in newborn or young animals.[35] However, hemosiderophages are unequally distributed and their number may be under or overestimated. In cats, BM iron is typically not readily apparent.

Megakaryopoiesis assessment can also be difficult due to uneven distribution throughout the marrow and should be evaluated on low power fields where platelet precursors can be easily identified. The megakaryocyte percentage is highly dependent on the smear preparation technique and quality of BM films.[24] Therefore, megakaryopoiesis is routinely only semi-quantitatively assessed. Generally, large BM spicules should contain three to seven megakaryocytes[34] and >70% of megakaryocytes are mature.[10] Promegakaryocyte percentage >50% suggests regeneration[35] or a maturation defect. Megakaryopoiesis quantification can be performed by counting the number of megakaryocytes in 70–100 low power fields (100× magnification) on three slides.[23,24] Mean counts of 2.76 megakaryocytes/low power field and 1.55 megakaryocytes/low power field were obtained in one study, using drop-squash and particle-squash preparations, respectively.[24] Counting megakaryocytes in 20–25 spicule-containing fields achieves an acceptable variance of 20%.[21]

Proportions and maturation of erythroid, myeloid, and lymphoid cellular lineages are examined at 500× to 1000× magnification. M:E ratio determination should be accomplished using a 500–1000 cell differential count. Because particles have a greater granulocyte percentage, 50% of cells should be classified from BM particles and 50% from the smear's interparticle regions.[35] A differential cell count is indicated to document slight to moderate increases in blast cell counts that may differentiate myelodysplastic or low-grade leukemic conditions from benign conditions. Alternatively, a subjective M:E ratio

can be estimated during cytologic BM smear evaluation.[35] The normal M:E ranges from approximately 0.7 to 2.5 in healthy dogs,[20] 1.2 to 2.2 in healthy cats, 0.9 to 3.8 in healthy horses, 0.3 to 1.9 in healthy cows, and 0.8 to 1.7 in healthy sheep.[12]

M:E ratio interpretation requires consideration of BM cellularity and cytopenias in the peripheral blood. A normal M:E in the context of a hypercellular marrow indicates an equal increase in both myelopoiesis and erythropoiesis, which may or may not be expected based on changes in the peripheral blood. An increased M:E may be due to either a decrease in erythropoiesis or an increase in granulopoiesis; whereas a decreased M:E suggests either a granulopoietic hypoplasia or an erythropoietic hyperplasia. A M:E index of >75:1 suggests pure red cell aplasia due to immune-mediated destruction of early erythroid precursor cells.[39] In contrast, pure white cell aplasia is rare.[40]

The maturity of cells in the erythroid and myeloid series should also be evaluated. Similar to human interpretation, maturation is assessed and reported as normal or abnormal and cellular morphology should be evaluated, commented on, and described if abnormal.[15] Normally, >85% of cells in the granulocytic (i.e., metamyelocyte, band, and segmented cells) and erythroid (i.e., rubricyte and metarubricyte) lineages should be in the mature nonproliferative pool. Immaturity in these lineages along with marked dysplasia are caused by leukemias or myelodysplastic syndromes. Immaturity and maturation arrest of cellular lineages accompanied with mild to moderate dysplasia might be due to an immune-mediated hemolytic anemia or neutropenia in which precursor cells are destroyed at a particular stage of maturation.

Small mature lymphocytes are normally <10% of cells in dogs.[8,10] Normal lymphocyte proportion can be higher in the cats, up to 16%[26] and rarely up to 28%.[7] Increased lymphopoiesis of up to 56% lymphocytes[37] can be observed in immune-mediated diseases and is generally accompanied by mild lymphocytosis in the peripheral blood ($<10^9$/L).[39] Differentiation from chronic lymphocytic leukemia (CLL) might be difficult in some cases. However, CLL results in a diffuse BM infiltration and a progressive moderate to severe lymphocytosis in the peripheral blood. Atypical lymphocytes with nucleoli, uniform indented nuclei, or azurophilic intracytoplasmic granules may be seen. CLL is characterized by a monomorphic cellular population, generally T-lymphocytes; while in reactive lymphocytosis, a mixed cellular population mainly comprised of B-lymphocytes is seen. Thus, immunophenotyping by flow cytometry may be helpful to differentiate neoplastic from reactive lymphocytosis[39] (see Chapters 64 and 69–72).

Plasma cells are present in low numbers (<5%).[8,10] Mildly to moderately increased plasma cell numbers indicate chronic inflammatory processes. Markedly increased number of plasma cells (>30%) or atypia including pale blue cytoplasm, fine chromatin pattern, or high numbers of flame cells, i.e., plasma cells with an eosinophilic cytoplasmic rim, suggest multiple myeloma. Definitive diagnosis includes presence of additional criteria such as monoclonal gammopathy, hypercalcemia, anemia, Bence Jones proteinuria, and/or osteolysis in long bones or vertebrae (see Chapter 70).

Low numbers of macrophages are normally found in the BM. Increased numbers of macrophages are observed in chronic infectious diseases, such as leishmaniasis, ehrlichiosis/anaplasmosis, or systemic mycosis. Macrophages with cytophagia indicate immune-mediated cell destruction. Increased macrophage proliferation (>3%)[38] with concurrent bi- or pancytopenia in the periphery are suggestive of hemophagocytic syndrome, aka hemophagocytic lymphohistiocytosis).[11] Hemophagocytic syndrome is a hyperinflammatory immunopathy which can be due to a primary neoplasia in the macrophage line or secondary to underlying disease including severe inflammation, infections such as ehrlichiosis, anaplasmosis, leishmaniasis, or a paraneoplastic syndrome. Macrophage proportions >30% and cellular atypia such as marked macrocytosis, macrokaryosis, and multinucleation are suggestive of histiocytic neoplasia[39] (see Chapters 18, 49, and 74).

Osteoblasts and osteoclasts are rarely found in the BM. They may be observed in young, growing animals. In adult animals, they are indicative of a disturbed calcium:phosphate ratio or neoplasia.

BM may be evaluated for staging of lymphoma or may reveal metastasis of other tumors. Neoplastic plasma cells, mast cells, or histiocytic cells may be observed. Uncommonly, nonhematopoietic tumor cells are found, such as metastatic carcinoma.[34]

USE OF HEMATOLOGY ANALYZERS FOR BONE MARROW ANALYSIS

Cytological examination of BM is time-consuming, labor-intensive, and requires a highly experienced examiner. Thus, hematology analyzers have been used in laboratory animals (dogs and rats)[6,27,32] and patients (dogs)[2,33] to perform a preliminary BM differential count, i.e., the classification of granulocytic and lymphoid/erythroid cells and determination of the M:E ratio. In patients, typical cytograms are indicative of some forms of leukemia. In the ADVIA 2120™ (Siemens), leukocytes are classified based on their peroxidase content and their cellular volume. Especially, the information about the cellular peroxidase concentration might be helpful to classify the origin of neoplastic cells (lymphoid versus myeloid/monocytic).

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CHAPTER 142

Flow Cytometry

UNITY JEFFERY

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Acronyms and Abbreviations

APC, allophycocyanin; CHCM, corpuscular hemoglobin concentration mean; CHr, reticulocyte cellular hemoglobin content; CV, coefficient of variation; MCVr, mean cell volume reticulocyte; MPV, mean platelet volume; PCT, plateletcrit; PLT-I, impedance platelet count; PLT-O, optical platelet count; FITC, fluorescein isothiocyanate; FMO, fluorescence minus one; FRET, fluorescence resonance energy transfer; FSC, forward scatter; IMHA, immune-mediated hemolytic anemia; MFI, median fluorescence intensity; PE, R-phycoerythrin; LED, light-emitting diode; PMT, photomultiplier tubes; RBC-optical, RBC optical count; RDW, red cell distribution width; RET-H*e*, reticulocyte hemoglobin equivalent; SSC, side scatter; V, volume.

Flow cytometry measures multiple parameters across a large number of cells. This has many valuable applications in clinical hematology, as diagnosis and prognostication of hematologic disorders frequently rely on determination of relative or absolute frequencies of specific cell types. When combined with ex vivo stimulation of cells, flow cytometry can detect functional hematologic disorders and provide experimental insights into the hematopoietic system. Modern flow cytometers achieve high resolution, allowing measurement of microparticle generation to be included in these functional assessments. Flow cytometry also has the potential to guide therapeutic decisions, e.g., by revealing personalized therapeutic targets or providing early detection of therapeutic failure.

As flow cytometry takes on an increasingly important role in veterinary hematology, this has been matched by a requirement for increasingly complex assays. Successful development of flow cytometry assays requires knowledge of flow cytometer mechanics and the properties of the various cellular markers available. Additionally, to ensure patient safety, it is necessary that assays are adequately validated and quality assurance procedures are in place to monitor long-term assay performance. Hematology instruments that utilize flow cytometry are discussed here, with information regarding manual methods and more general hematology quality assurance provided in Chapter 137.

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INSTRUMENTATION AND BASIC PRINCIPLES OF LIGHT SCATTER AND FLUORESCENCE

Most flow cytometers use hydrodynamic focusing to force cells into single file as they flow past a focused laser. This is achieved by a combination of the instrument's fluidics and the flow cell. The sheath fluid (e.g., blood bank saline) and the sample are moved separately through the instrument toward the transparent quartz glass flow cell by air pressure, syringe, or peristaltic pump. The sample is then injected into the sheath fluid. The pressure of the sample fluid is higher than the sheath fluid, creating laminar flow in which the sheath fluid and sample move parallel to each other without mixing. This produces a central core stream of sample, surrounded by sheath fluid. If instrument settings are optimal, as the diameter of the flow cell narrows, the cells are organized into single file with each cell aligned in the center of the core stream. If this goal is achieved, illumination of each cell is consistent as it passes through one or more lasers focused in the center of the core stream.

The instrument optics includes lasers that illuminate cells, filters, and detectors that collect the resulting light scatter and fluorescent signals. Lasers emit light of a specific wavelength, and the number and wavelengths of the lasers available vary between flow cytometers. The site at which each laser is focused in the flow cell is referred to as the interrogation point. In colinear flow cytometers, multiple lasers are focused at the same interrogation point. Each laser in a parallel flow cytometer has a different interrogation point, so cells are illuminated on multiple occasions, and thus generate temporally separate signals for each laser that must be collated to link all the data generated by a single cell.[56]

When cells (or particles) move through the laser beam, they create light scatter. Low-angle or forward scatter (FSC) (i.e., 2°–20°) reflects cell size, although FSC is also influenced by other properties such as refractive index. Side scatter (SSC) refers to light that is deflected at high angles. This is influenced by the internal complexity of the cell (e.g., cytoplasmic granules) (Figure 142.1). Very small objects, such as circulating microparticles, are an exception to this general rule. For these particles, FSC or SSC may provide information about size, and the parameter that provides the best resolution varies between different flow cytometers (Figure 142.2).[67]

If specific targets on or within the cell have been stained with fluorescent labels, such as fluorochrome-conjugated antibodies, fluorescent signals are also emitted as cells move through a laser beam of the appropriate excitation wavelength. This excitation wavelength is absorbed by the fluorochrome and causes excitation of an electron. As the electron returns to its resting or ground state, it emits energy in the form of a light photon of lower energy and, therefore, longer wavelength than the excitation wavelength.

Fluorescent signals, FSC, and SSC are routed to detectors. Typically, this involves dichromatic mirrors that direct light to filters that restrict the wavelengths that reach each detector. Band-pass filters allow a narrow range of wavelengths to pass; short-pass filters allow only wavelengths of light below a certain threshold to pass, and long-pass filters allow only wavelengths above a certain threshold to pass. Filters can thus be constructed to allow only wavelengths that correspond to the peak emission wavelengths of specific fluorochromes to reach a particular detector. This combination of a filter and detector is often referred to as the channel for detection of certain fluorochrome (e.g., the fluorescein isothiocyanate (FITC) channel or green channel).

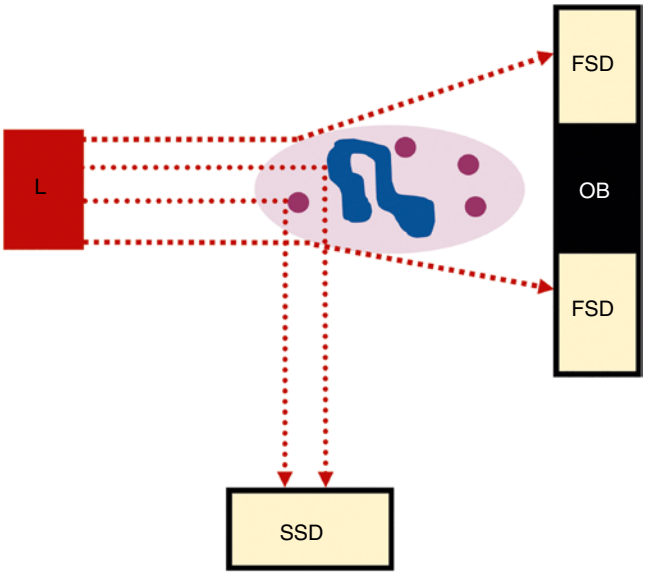


FIGURE 142.1 Cell size and complexity influence light scatter. When a laser (L) illuminates cells, this creates low-angle forward scatter, which reflects cell size. This light reaches the forward scatter detector (FSD), which incorporates an obscuration bar (OB) that blocks light coming directly from the laser source without deflection. Features of the cell, such as granules or membrane irregularities, create high-angle side scatter and these photons reach the side scatter detector (SSD), providing a measure of complexity.

Detectors convert light photons to electrons. There are several types of detector, but photomultiplier tubes (PMT) and photodiodes are used most commonly.[78] Photodiodes are light-sensitive semiconductors that are typically reserved for FSC, because they do not produce internal amplification of the signal and so can only be used for brighter signals.[78] Avalanche photodiodes are used in some newer flow cytometers and have a higher quantum efficiency in the infrared range than PMTs, used to detect fluorescence and side scatter in most instruments.[81]

Within the PMT vacuum tube, received light photons hit a photocathode. This results in emission of electrons, which in turn hit a series of dynodes that each emit increasing numbers of electrons. This creates a greatly amplified electric current by the time the photoanode of the PMT is reached. This electric current passes to a transimpedance amplifier, where it is converted to a voltage pulse, which is proportional to the photon signal originally received by the detector.[78] This pulse is further processed with subtraction of nonspecific noise by the baseline restorer and, in some cases, by an additional programable amplification step.[78]

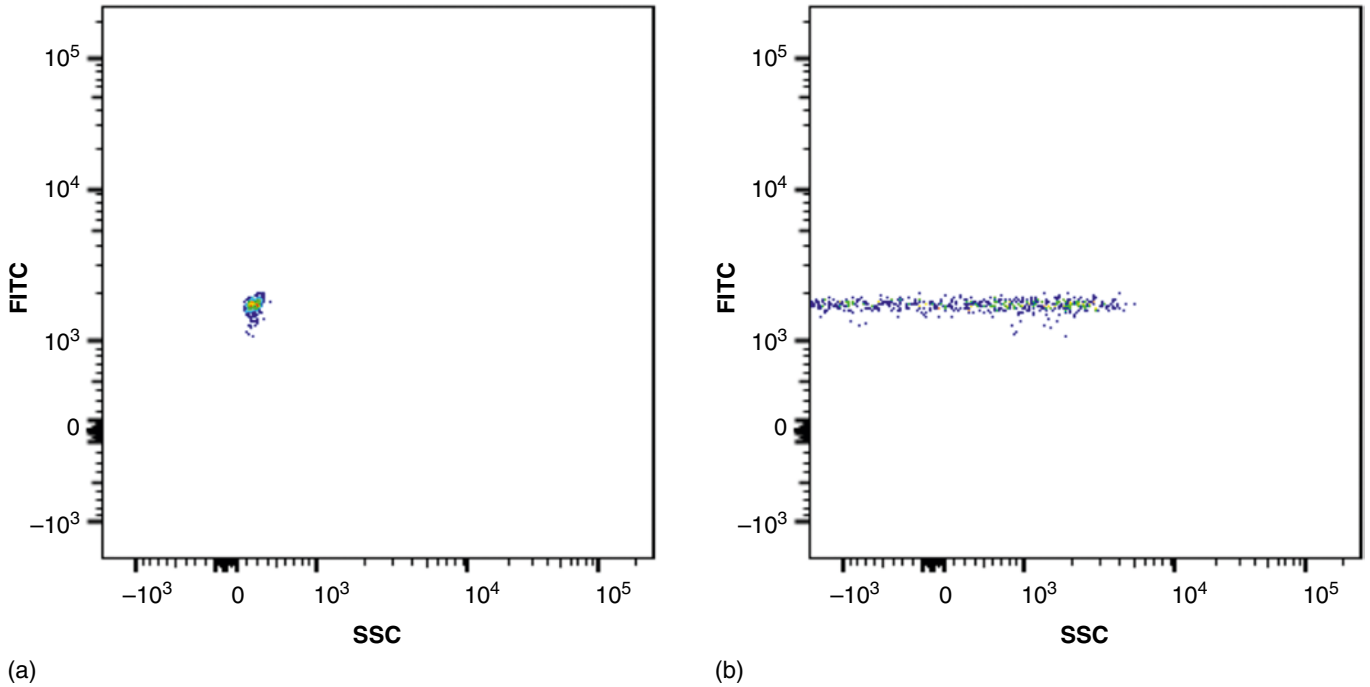


FIGURE 142.2 Side scatter provides better resolution than forward scatter for small particles on some instruments. Polystyrene sizing beads of 0.5 μM diameter were analyzed using a Fortessa X-20 (BD Biosciences). (a) When side scatter is plotted against fluorescence, a tight population is produced, reflecting the uniform diameter and fluorescence of the beads; (b) By contrast, forward scatter is highly variable, producing a dispersed population which does not accurately represent the size characteristics of the beads.

The combination of fluidics, optics, and electronics means that each time a cell passes through the laser interrogation point and generates photon signals, multiple voltage pulses are generated that reflect its FSC, SSC, and fluorescence. The peak of each pulse, referred to as the pulse height, reflects the maximum photon signal received from the cell by the relevant detector.[56] This maximum emission generally occurs when the cell is maximally illuminated in the center of the laser interrogation point.[56] The pulse area is a measure of the total amount of light emitted or scattered by the cell.[27,56] The pulse width reflects the duration of the pulse[56] and thus is influenced by the size of the cell.

For analysis, these continuous data are converted into more manageable discrete digital data by grouping similar values into categories, termed bins. The more bins used, the better the ability to distinguish differences in the signals generated by different cells (i.e., the better the resolution). These binned data are provided to the end user as a flow cytometry standard (FCS) file, which can be analyzed using flow cytometry software. Flow cytometry data analysis typically involves setting gates that group cells or particles based on FSC, SSC, and/or fluorescence properties. Populations can be compared using the percentage of cells classified as positive for a marker or combination of markers, or their median fluorescence intensity (MFI) for markers of interest.

Principles of Cell Sorting

Commonly available flow cytometers fulfill the requirements for many clinical and experimental hematology applications. There are more specialized flow cytometers available, including imaging flow cytometers (which allow concurrent microscopic imaging of individual cells) and cell sorters. Flow cytometric cell sorters typically use electrostatic focusing to separate and collect cells with specified flow cytometry characteristics for use in downstream applications, such as culture or further experimental analysis.

In most sorters, this is achieved by breaking the flow stream up into a series of single-cell droplets generated after cells pass through the laser interrogation point.[3] The time between a cell passing through the laser and being released in a droplet (termed the drop delay) should be constant.[3] This ensures that, when pulses detected by the electronics classify a cell as meeting the user's requirements, an electric pulse is applied to the droplet containing the same cell as it breaks off from the flow stream. This creates an electronic charge on the droplet, and as it subsequently moves through a strong electric field, it is deflected into a collection tube. Droplets that contain cells that do not meet the user's specifications are not charged and so move through the electric field without deflection into a centrally located waste channel. The success of cell sorting depends on correctly linking flow cytometry findings of one cell to a droplet containing the same cell, and consistently generating droplets that contain only single cells.[3] The conditions needed to achieve this are beyond the scope of this chapter, but references with more detail are provided.[3,15]

SAMPLE PREPARATION AND PROCESSING

The aim of processing flow cytometry samples is to achieve a single-cell suspension labeled for the markers of interest. General suggestions for achieving this are

discussed here, but specific samples and markers will have their own individual requirements. Detailed review of relevant literature before designing a protocol avoids producing inaccurate results due to preanalytical errors. For example, review of published guidelines regarding centrifugation conditions for microparticle studies is invaluable for investigators measuring microparticle concentrations for the first time.[38-40] Similarly, performing optimization experiments to confirm that a protocol consistently and efficiently generates reliable results before analyzing clinical or experimental samples can ensure high-quality data and allows removal or refinement of protocol steps to avoid wasting time or resources.

Preferred material varies with specimen and assay type. For immunophenotyping of peripheral blood and bone marrow aspirates, samples anticoagulated with EDTA are frequently used. This has the advantage that cellular morphology is well-preserved allowing concurrent cytologic assessment.[30] Flow cytometric evaluation of platelets can be performed on whole blood, platelet-rich plasma, or preparations that have been further purified (e.g., by gel filtration), with sodium citrate commonly used as the anticoagulant for functional assays. Platelet-rich plasma is collected by slow, room temperature centrifugation (e.g., 170 g for 10 minutes with no brake).[2] For veterinary patients where acceptable venipuncture volume may be limited, this slow centrifugation results in a low volume of platelet-rich plasma that is challenging to separate from blood. Mixing blood in a 1:1 or 1:2 ratio with a buffer such as buffered saline-glucose-citrate increases fluid volume, and platelet concentration is typically adequate for functional assays or further purification.[2]

Delay between sample processing and analysis leads to increased dead and dying cells. Nonviable cells display nonspecific antibody binding and altered marker expression,[31,80] release DNA that increases cell clumping,[3] and generate debris.[3,17] Delay in analysis may also alter responsiveness in functional assays,[10] alter autologous antibody binding in immunological assays,[43] and result in greater in vitro generation of microparticles.[39] For immunophenotyping of unfixed specimens, storage of up to 24–48 hours is typically considered acceptable. However, the maximum delay varies with sample type and marker of interest. For example, immediate analysis has been recommended for high-grade lymphomas, while blood for B-cell clonality screening may tolerate longer storage.[30] Heparinized whole blood from healthy dogs stored for 12–16 hours did not significantly alter expression of lymphocyte markers CD3, CD4, CD8, and CD21.[18] Immunophenotyping after 24 hours storage also did not alter clinical interpretation for six canine lymph node aspirates (three normal, three with lymphoma) stored in a buffer containing fetal bovine serum and EDTA.[22]

Addition of autologous serum, protein supplementation (e.g., fetal bovine serum or bovine serum albumin), or use of tissue-culture-specialized flow transport media may help preserve viability during transport and storage.[30] Potential undesirable effects of such agents should be considered; e.g., avoiding media that contains phenol red is recommended due to its contribution to background fluorescence.[30] Cell fixation at collection may prolong sample stability.[71] However, the reported success of immediately postcollection fixation protocols varies, and there are also marker-specific effects of fixation.[71] Frozen, cryopreserved peripheral blood mononuclear cells can also be used for flow cytometry, but cryopreservation may influence findings for some cell types.[6] Users should survey the literature and where necessary perform validation studies to determine the effects of storage and transport, particularly for functional assays and microparticle quantification. Even small details can have a major impact on such studies; e.g., microparticle quantification samples must be transported upright with minimal agitation until centrifugation to avoid in vitro microparticle generation.[39]

Many flow cytometry protocols involve keeping cells on ice during transport and processing. This may increase viability and reduce changes such as internalization of surface receptors and bound antibodies. However, this should be confirmed for the cell type and assay performed, as in some cases cooling cells may be unnecessary or undesirable.[30] Notably, cooling of platelets is generally avoided, as it can induce activation.[47]

Cells are usually washed and stained in a buffer that contains a source of protein (e.g., bovine serum albumin), which promotes viability and helps to block nonspecific antibody binding. The metabolic inhibitor, sodium azide, is commonly incorporated into buffers to prevent bacterial contamination and inhibit capping and internalization or loss of cross-linked antibody–antigen complexes. However, addition of sodium azide can reduce cell viability and negatively affect staining for some markers.[95]

Buffer formulation can be adjusted to reduce cell clumping. Large cell clumps can clog the flow cytometer, and smaller aggregates could generate data that reflects multiple rather than individual cells. Clumping can be reduced by use of calcium- and magnesium-free buffers, addition of EDTA, and if dead cells are in large numbers, DNase. Mesh filters can also be used before analysis to remove larger clumps.[3] Buffers can also be a source of debris, which is problematic when trying to separate small particles such as platelets or microparticles from background noise.[17] Filtering of buffers immediately before use is recommended for these samples.[17] Specific additives are required for some functional assays. For example, in plasma-based platelet activation assays, Gly-Pro-Arg-Pro amide is added to inhibit fibrin polymerization.[47]

For WBC flow cytometry, RBCs are usually removed during processing.[84] This removes a source of nonspecific binding and autofluorescence and ensures that events collected are relevant to the experimental or clinical question. Density gradients or RBC lysis by ammonium chloride or commercial lysing agents are described. Lysis has been recommended for immunophenotyping due to concerns about selective WBC loss with density

gradients.[84] The processing stage during which lysis is performed is variable, with some protocols requiring bulk RBC lysis as an initial step and others including a lysis step for each individual tube after staining.[84] Guidelines regarding timing of lysis steps for specific applications are available.[84] For example, stain–lyse–wash protocols are suggested as providing the best signal discrimination, but are recommended against when cell loss must be minimized.[84]

Cells are labeled by incubation with antibodies or other markers (e.g., nucleic acid dyes). This can be performed in tubes or a 96-well plate. Cell number added to each tube or well should be consistent, with a target of 10^6 cells per tube commonly suggested.[80] If labeling of intracellular markers is performed, fixation and permeabilization are necessary.[84] Fixation before or after staining can also be performed when staining surface markers, allowing greater flexibility in the timing of analysis, and reducing biosafety concerns.[30,84] However, fixation can alter staining properties and surface marker display, and should therefore be validated.[30,84] Fixation is also unsuitable if viable cells are required for downstream applications, e.g., culture of sorted cells.

Direct staining, in which the sample is incubated with primary antibodies conjugated to fluorochromes, is common. Two-step or indirect staining can also be performed by incubating cells with a primary antibody that is either unlabeled or tagged with biotin. Then, a secondary antibody that binds the primary antibody and is tagged with a fluorochrome; or for biotin-tagged primary antibodies, a fluorochrome bound to streptavidin. To avoid quenching of the fluorochromes, incubations are performed in the dark. This is particularly important for more unstable dyes, such as some tandem dyes.

Protocols often include multiple wash steps both before and after staining. However, washing is best minimized or avoided in some settings. This includes low cellularity samples and microparticle assays, as cells or microparticles will be lost during washing.[30] Platelets aggregate upon centrifugation, and washing therefore requires addition of inhibitors (e.g., prostaglandin I2). If washing is not performed, unbound antibody can be quenched by adding large volumes of buffer or fixative.[63]

Manually setting up flow cytometry assays carries a risk of technical error, particularly when large numbers of antibodies are added to a single tube, or large numbers of tubes with different combinations of antibodies are required. Use of commercial antibody cocktails reduces the risk of errors in antibody addition, but unfortunately these are currently not widely available for veterinary applications.[30] Users can make and store their own antibody cocktails; but it is important to confirm that this does not compromise assay performance due to aggregation or differences in storage requirements for different components of the cocktail.[30] Even if long-term storage of antibody cocktails is not possible, assay setups that combine larger volumes of antibody into a master-mix that is split between multiple tubes are preferable to repeatedly adding small volumes of antibody to different tubes, as this reduces pipetting error and risk of cross-contamination of antibodies. There is a move toward automating the staining process, which not only reduces the risk of error but increases traceability and has the potential to increase throughput.[26]

CHOICE OF ANTIBODY AND FLUOROCHROME

Antibody Selection

The ideal antibody for flow cytometry binds the target of interest with high-affinity and minimal off-target binding. When sourcing commercial antibodies, two basic considerations help with initial selection. First, purchase an antibody sold commercially as suitable for flow cytometry. Flow cytometry requires antibodies to bind epitopes in their native or near-native conformation rather than the denatured proteins typically used in western blotting. Flow cytometry may use different fixation techniques than immunohistochemistry and often omits antigen-retrieval steps. Therefore, an antibody that performs well in other techniques may not be suitable for flow cytometry. Second, source an antibody sold as cross-reactive with the species of interest, including for proteins that are generally well-conserved between species, as the epitope against which an antibody was raised may be outside evolutionary well-conserved regions.

Even when using an antibody sold as suitable for the application and species, investigators or laboratories should verify performance. There is variability between manufacturers in the assiduousness of antibody performance testing and differences in performance of antibodies sold by different manufacturers as reactive against the same target. Performance may also vary with different protocols and conjugates.[34] Investigator-performed validation often relies on obtaining expected staining patterns in cells known to be positive and negative for the marker of interest.[62] While suggestive of antibody binding to the target of interest, this does not directly confirm that the antibody is bound to the marker of interest. Furthermore, this approach may not be adequate to confirm acceptable performance in all samples. For example, staining may follow the expected pattern in cells from a healthy individual, but differences in the ratios of the protein of interest versus other antigens may result in off-target binding in some patient samples.[89] For clinical samples, correlation of flow cytometry results with cytology, histology, and clinical characteristics can help confirm performance.[62]

Use of alternative techniques, such as microscopy or western blotting, may provide additional evidence that the antibody is specific for the antigen of interest. These techniques have the advantage that they are widely available; but, as already discussed, antibodies may perform differently in different assays. More rigorous approaches to antibody validation have been recommended, but may be challenging to employ in a clinical setting.[89] These include genetic strategies in which expression of the protein of interest is eliminated; orthogonal strategies that compare results to an antibody-independent method,

such as mass spectrometry, and use of tagged proteins or independent antibodies binding the same antigen at a different epitope.[89] In addition to validation at initial purchase, it is important to confirm stable performance at each lot change.[23] When developing new multiantibody panels, confirming performance is similar using the antibody alone and in combination with other antibodies is necessary due to the possibility of steric hindrances and other interferences.[23]

To achieve good antibody performance, the staining protocol must be optimized. Undesirable binding may arise because an antibody recognizes multiple similar epitopes or recognizes an epitope shared by multiple antigens (i.e., specific but unwanted epitope recognition).[29] Alternatively, undesirable binding can be the result of nonspecific, weak electrostatic interactions or binding of the Fc portion of the antibody to Fc receptors on antigen-presenting cells.[29] Weak, nonspecific interactions can be minimized by antibody titration.[29] Several protocols are available. One of the simplest optimization techniques is to stain the same cell population with increasingly dilute antibody solutions. Then, calculate the ratio of median fluorescent intensity for the positive and negative cells.[13] The highest ratio provides the best balance between specific staining and background noise.[13] As discussed, dead and dying cells can be a significant source of nonspecific binding. Use of viability stains to identify and exclude dead cells from analysis helps to avoid misinterpretation of dead cells as cells with aberrant marker expression.[37] Fc blocking reagents can overcome nonspecific binding by Fc receptors, but endogenous plasma immunoglobulins perform the same function and render Fc blocking unnecessary for most blood-based assays.[37]

Fluorochrome Selection

There are several considerations when choosing fluorochromes. The laser must be capable of exciting the fluorochrome at the appropriate wavelength, and the filter must be able to detect the emitted wavelength. The next consideration is the extent to which each potential fluorochrome will distinguish positive versus negative cells for the marker of interest. This is influenced by several factors, including fluorochrome brightness, marker expression, and spectral overlap.[29]

Spectral overlap occurs when light emitted by multiple fluorochromes reaches the same detector.[29] To demonstrate this, consider selecting a second fluorochrome to use in a two-color flow cytometry experiment with a FITC-labeled antibody and a flow cytometer with 530/30, 585/40, and 675/25 filters. FITC has a 525nm emission peak, meaning it is most likely to emit photons of 525 nm, which can be detected using a 530/30 band-pass filter. However, FITC also emits lesser numbers of photons at longer wavelengths that could be detected by other filters. The probability of FITC emitting photons in the range detected using a 585/40 nm band-pass filter is greater than for those detected by a 675/25 filter. In other words, the FITC signal will spill over or bleed into the 585/40 filter. Selecting allophycocyanin (APC), which has an emission peak of 660 nm, as the second fluorochrome would therefore be preferable to R-phycoerythrin (PE), with its emission peak of 578 nm.

Compensation is a mathematical process which corrects for dye signal spill over into another detector, allowing combinations of fluorochromes with some spectral overlap (Figure 142.3).[49] However, while compensation brings the average fluorescence for the spillover channel to zero, it does not remove measurement

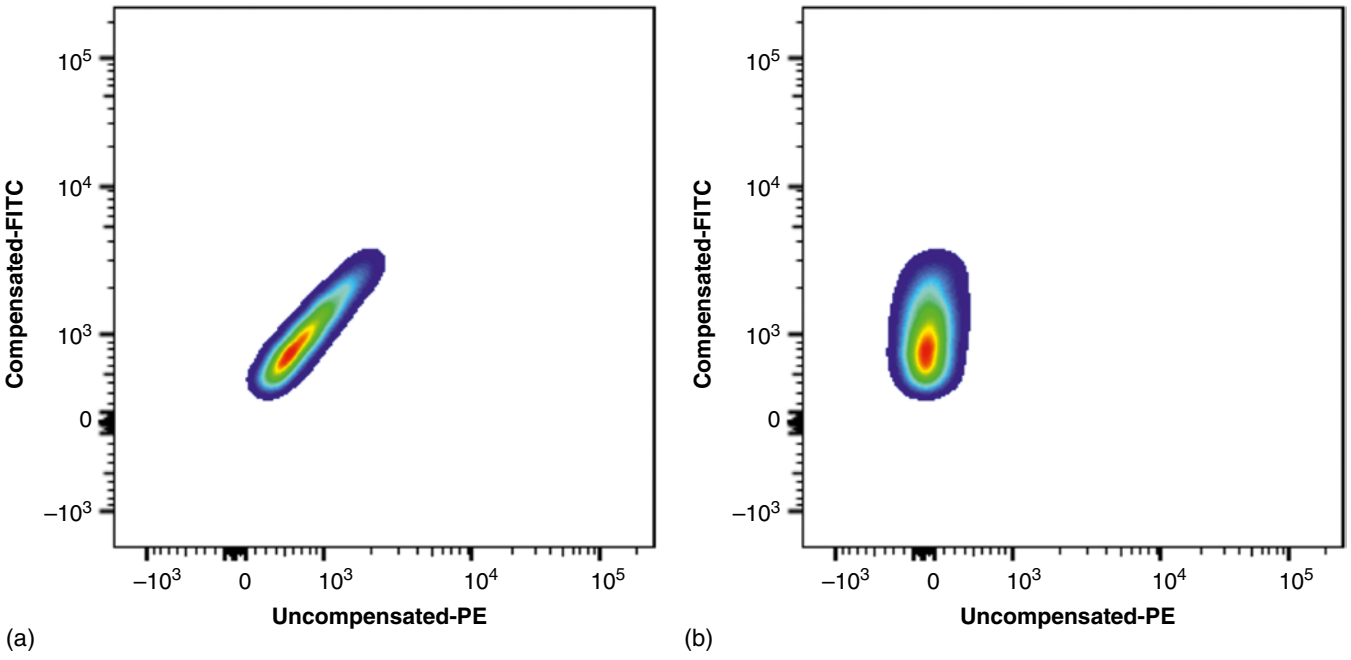


FIGURE 142.3 Compensation is a mathematical operation that corrects for spectral overlap between fluorochromes. Cells are shown stained with FITC alone. (a) Before compensation, there is spillover of the FITC signal into the PE detector; (b) Compensation corrects this spillover and brings the median fluorescence intensity for PE to zero.

error.[49] This creates data spreading, which reduces the ability to distinguish dim events from background noise in the compensated channel.[49] Therefore, combining fluorochromes to minimize spectral overlap improves sensitivity for markers expressed at low concentrations.[29] This can be challenging when constructing large multicolor panels, but semiconductor quantum dots can improve fluorochrome separation.[11] These manufactured nanocrystals have narrow emission spectra, so multiple quantum dots can be combined with minimal spectral overlap.[11] Additionally, quantum dots can be excited by a wide range of wavelengths, allowing multiple quantum dots to be interrogated using a single laser.[11]

Tandem dyes are another example of manufactured dyes that expand the number of fluorochromes that can be combined in a multicolor panel. These are covalently bound pairs of fluorochromes that display fluorescence resonance energy transfer (FRET). When excited by an appropriate wavelength, the donor fluorochrome transfers energy to the acceptor fluorochrome, resulting in emission of light. This can create an increased difference between excitation and emission wavelength, termed the Stokes shift. For example, PE has an excitation peak of 496 nm and an emission peak of 578 nm, but in the tandem dye PE-Cy7, the emission peak is shifted to 785 nm. Tandem dyes, therefore, facilitate construction of multicolor panels in which the same laser is used to excite multiple fluorochromes without excessive emission spectral overlap.

There are specific considerations when using tandem dyes. The efficiency of FRET is influenced by the ratio of donor to acceptor molecules, which may vary between lots.[58] FRET efficiency is also reduced if there is degradation of the tandem dye. This may occur during storage, particularly if conditions are not ideal (e.g., exposure to light, freezing),[58] or during incubation with cells.[42] If the donor fluorochrome emits energy without excitation of the acceptor, this could create misleading results. Consider an experiment using both PE- and PE-Cy7-conjugated antibodies. Unrecognized degradation of the PE-Cy7 fluorochrome would lead to cells binding this antibody being misclassified as positive for the marker detected by the PE-conjugated antibody.[49] Additionally, if the PE and PE-Cy7 antibodies are bound in close proximity to the same cell, FRET may occur between the two conjugates, resulting in quenching of the PE signal by its interaction with PE-Cy7.[58]

When deciding which fluorochrome to use for each marker of interest, the marker expression must be considered. Markers expressed at low concentration should be labeled using the brightest fluorochromes for adequate sensitivity.[49,58] Brightness depends on the specific fluorochrome's signal strength and the instrument's efficiency of detection. However, brightness is also influenced by the nonspecific signal generated by negative cells. This background noise is the result of both sample (e.g., autofluorescence) and instrument (e.g., electronic noise) properties.[58] The instrument-specific stain index provides a measure of brightness[49]:

(Median positive – median negative)/2*SD negative.

For detection of low-expression markers in multicolor panels, it is also necessary to consider the impact of other fluorochromes.[57] Sensitivity is reduced if the detector for a low-expression marker is also receiving signals from other fluorochromes.[57] This is influenced by instrument features (e.g., alignment, filters) and can be quantified by construction of a spillover spreading matrix.[57] The details of this technique for panel optimization are beyond the scope of this chapter, but are found in the reference provided.[57]

Non-Antibody-Based Probes

There are a number of non-antibody-based fluorescent probes. These include dyes used to distinguish live and dead cells (e.g., nucleic acid dyes, amine dyes, and vital dyes), nucleic acid probes to assess gene expression and phosphatidylserine-binding molecules for detection of platelet activation and apoptotic cells (e.g., lactadherin, annexin V). An advantage for veterinary studies is that these probes are generally not species-specific. However, users must be familiar with the properties of the specific probe. For example, annexin V binding to phosphatidylserine requires continuous exposure to calcium.

COMPENSATION AND GATING CONTROLS

Inclusion of controls is vital to ensure appropriate compensation and gating. Gating is the process of selecting cells of interest and relies on gating controls which allow the user to set boundaries that define cells as positive or negative for a specific marker.

Unstained Cells

An unstained, but otherwise identically processed sample allows autofluorescence assessment. In single-color experiments, unstained samples can often be used to define positive versus negative cell populations (i.e., as a gating control). However, when two or more fluorochromes are combined, an unstained control is not a suitable gating control because it will not reflect the background introduced by other fluorochromes.

Single-Stained Controls

For experiments using more than one fluorochrome, compensation controls are required.[50] These are stained with a single fluorochrome to allow quantification of the spillover signal from this fluorochrome into the detectors used for other markers.[50] To allow calculation of compensation, both a positive and a negative population must be present. In most situations, either cells or compensation beads can be used.[50]

Compensation beads can be purchased from numerous manufacturers and are typically a mix of polystyrene microspheres that capture antibodies (producing the positive population) and uncoated beads that do not (producing

the negative population). An advantage of compensation beads is that they can generate bright signals for fluorochromes that would produce only dim signals for cells which have rare- or low-expression markers.[50] Furthermore, beads consistently produce a clear separation between the positive and negative population. However, beads cannot usually be used for non-antibody-conjugated fluorochromes (e.g., nucleic acid dyes) and may not be suitable when autofluorescence is prominent (e.g., due to use of a violet laser or cell properties).[30]

Regardless of whether cells or beads are used, compensation controls should be handled identically to test samples to ensure any effects of the protocol (e.g., fixation, permeabilization) on the fluorochrome are taken into account.[50] It is also important that both the positive and negative populations should have similar autofluorescence (e.g., beads cannot be used as the negative control for cells or vice versa).[30]

Fluorescence Minus One (FMO) Controls

Fluorescence minus one (FMO) controls are prepared by staining cells with all but one of the antibodies used in a panel.[29] These controls allow the background signal generated by autofluorescence, spectral overlap, and data spreading revealed by compensation to be defined.[29] FMO controls are therefore required for multicolor studies, as unstained or single-stained controls would not reveal the full extent of spectral overlap and data spreading.[29] FMO controls are useful to define negative and positive gates, but FMO gates do not include the contribution from nonspecific binding of the omitted antibody.[50] Therefore, antibody titration and blocking steps must be optimized when using this approach.

Isotype Controls

Isotype controls are antibodies of the same class which are conjugated to the same fluorochrome as the assay antibody, but they do not recognize any antigens present in the sample.[29] In theory, isotype controls allow determination of nonspecific binding.[29] In practice, their use has fallen out of favor, with many authors advising against isotype controls for quantitative flow cytometry experiments detecting cell surface markers.[29,80] This reflects the difficulty in manufacturing an isotype control that perfectly matches the nonspecific binding characteristics of the assay antibody. For example, more cells may stain with an isotype control than a test antibody.[29] For immunophenotyping, gating based on naturally occurring negative populations within the sample has been proposed as an alternative to isotype controls.[80]

Other Controls

A number of other controls can be used for gating including gating controls that exploit marker-binding properties. For example, in platelet studies using annexin V to label exposed phosphatidylserine, a calcium-free buffer can be used as a gating control. As discussed in the earlier text, annexin V requires continuous exposure to calcium in order to maintain binding to phosphatidylserine. Any fluorescence of the calcium-free control therefore represents nonspecific binding, providing a threshold to distinguish positive versus negative populations. Cells known to be negative for the marker of interest can also provide an internal, negative control for gating.[29] However, nonspecific binding and autofluorescence can vary between different cells types.[29] Consequently, the internal negative population must be the same cell type as the positive population.[29] Differently treated cells can also provide biological gating controls.[50] For example, unstimulated cells could be used to define the negative population for a marker that is upregulated upon stimulation.[50] When determining if biological controls are suitable for gating, it is important to consider if experimental conditions may alter cellular properties, such as autofluorescence or nonspecific binding. There are also nonbiological controls that are useful in specific experiments, such as the sizing beads used for setting microparticle gates.[12]

Samples split into multiple aliquots and analyzed on multiple occasions can determine if assay performance is consistent over time.[50] For immunophenotyping, these often take the form of frozen cryopreserved peripheral blood mononuclear cells.[50] Such controls both monitor the consistency of analyzer performance and allow error detection in assay setup.

SAMPLE ANALYSIS

Instrument Setup

Several settings need to be defined by the user for experimental analysis. These include the number of events collected per tube or well. The more events collected, the greater the statistical power of the data analysis, but also the longer the acquisition time and the greater the risk of tubes running dry and air being introduced into the fluidics. For many analyzers, the default is to collect 10,000 events in a gate defined by the user, but this must be considerably increased for analysis of rare events.

Only events that reflect cells (or particles) of interest contribute to the statistical power of the analysis. To keep data file sizes manageable, the user can exclude events generated by cellular debris or electronic noise by defining the minimum signal that triggers data collection and storage, termed the threshold. Events below the threshold are ignored. The threshold should be set conservatively to ensure that useful data are not lost.[78] Typically, FSC is used to define the threshold, as in most instances both cells positive and negative for fluorescence are of interest.[97] Exceptions include DNA measurement of the cell cycle, or detection of very small objects such as bacteria. Sizing beads are commonly used for setting the threshold in microparticle studies.

Many flow cytometers require the user to adjust the PMT voltages to ensure that photon signal amplification received is optimal. The higher the voltages applied to

the PMT dynodes, the greater the number of electrons released and, thus, the greater the current generated in response to the photon signal received.[96] Therefore, if the PMT voltage is too low, cells generating dimmer signals will be missed. Whereas if the voltage is too high, excessive background noise will be generated. One commonly used approach to determine a baseline appropriate voltage is to assay a calibration bead that produces dim fluorescence (e.g., the peak 2 bead of the SPHERO Rainbow Calibration Particles) at progressively increasing voltages.[50] The coefficient of variation (CV) for the beads' fluorescence decreases as the voltages increase, reflecting improved signal to noise ratio.[50] Eventually, a plateau is reached beyond which increasing the voltage further does not improve the signal generated for the dim bead.[50] The voltage at which the plateau is reached provides an indication of the optimal voltage for that PMT on that instrument with the current optical configuration, but may change if alterations are made to lasers, filters, etc.[50] This baseline is a useful starting point for adjusting voltages for individual experiments.[50] If bright events for a fully stained sample containing cells that reflect the full range of expected fluorescence for the experiment are off scale, the PMT voltage can be reduced.[50] If cells, known to be negative events, are producing strong fluorescence signals, this is likely due to nonspecific staining (e.g., due to failure to adequately titrate antibodies), rather than voltage setup.[50]

The flow rate can also be adjusted. A slow sample flow rate increases data collection time, but improves hydrodynamic focusing, creating a narrow core stream.[98] This reduces coincident events, when two cells or particles pass through the laser interrogation point simultaneously. At slow flow rates, cells also move consistently through the center of the laser interrogation point, producing more constant illumination than at higher flow rates. This means slower flow rates produce better data resolution, which is particularly important when analyzing rare events and small particles. Flow rate should be kept constant throughout analysis, as gates set for data acquired at one flow rate may not be suitable for data acquired at another.

Data Collection and Analysis

Flow cytometry studies generally involve collection of FSC, SSC, and fluorescence data. This can be in the form of pulse height, pulse area, and/or pulse width. Pulse area (which depends on pulse height and width) is most commonly used for displaying and gating cells, but can be problematic for platelets and small particles. If the laser beam's diameter is similar to that of the cell or particle, pulse width is linearly related to particle diameter for most parameters.[27] This is not the case if the cell or particle of interest is smaller than the diameter of the laser beam, and so pulse height provides better resolution of particles and platelets than pulse area.[40]

Coincident events can be generated when two individual cells pass through the laser beam simultaneously, or when aggregates (often referred to as doublets) form between two or more cells. Signals from these pairs of cells cannot be separated by the electronics. If they are not recognized as an artifact, results may be misleading.[36] Coincident events and doublets have similar pulse height to individual cells, but take longer to pass through the laser, increasing pulse width and area. This means that when FSC or SSC area or width is plotted against height, doublets and coincident events lie away from the linear distribution of the main population of individual cells. Linear distribution assessment cannot be used for small particles, as the relationship between particle diameter and pulse width is not linear. Instead, coincident and swarm events are identified by a lack of linearity when samples are diluted.[35] Use of light scatter properties for doublet identification may also be unsuitable if these properties are altered by experimental conditions.[9]

Automated flow cytometry data analysis is an area of active research.[74] Currently, most flow cytometry analyses rely on manual gating.[74] This is usually performed on data displayed as one-parameter histograms or two-parameter plots. Most commercial flow cytometry software also allow construction of more complex data displays of three or more markers, but these are challenging to use for manual gating.

Gating often follows a hierarchal or sequential approach, in which a subpopulation of cells is selected by an initial gate, and then broken down into smaller subpopulations by subsequent gates. This is referred to as parent–child gating, as the later "child" gate contains only cells from the earlier "parent" gate. Combination Boolean gating can identify complex subpopulations, using AND, NOT, and OR gates.[97] Back gating can be used to verify that placement of earlier gates has not led to loss of cells of interest.[97] This is particularly useful if population characteristics change due to experimental conditions. For example, a platelet gate set based on resting platelets may exclude a significant proportion of stimulated platelets, and if this is not recognized, display of activation markers may be underestimated.

Flow cytometry results can often be reported as the percentage of cells positive for a particular marker or combination of markers. Absolute concentrations, rather than percentages, are reported by flow-cytometry-based hematology instruments and in a number of other flow cytometry applications including microparticle quantification, CD34+ enumeration, and WBC contamination assessment in leukoreduced blood products. For volumetric flow cytometers, the sample volume is recorded and subpopulation concentrations can be calculated directly.[21] In other systems, concentrations can be calculated based on counting beads added at a known concentration to each flow cytometry sample[66] or derived from CBC data and flow cytometry percentages collected concurrently.[14,21]

For markers that show a staining continuum rather than discrete positive and negative populations, it may be necessary to report staining intensity. This can involve broad categories such as dim versus bright, as is frequently reported for immunophenotyping markers whose staining intensity has prognostic significance.[14] The European Canine Lymphoma Consensus Network

has recommended that where possible this classification expression should be based on comparing the staining intensity of neoplastic cells to that of the nearest normal hemic population.[14] When more subtle changes in marker expression have clinical or experimental consequences, MFI or indices derived from MFI comparisons (e.g., ZAP-70 expression index for human chronic lymphocytic leukemia) between cell populations may be reported quantitatively.[53] MFI is sensitive to small changes in sample processing and instrument performance, and therefore close attention to quality control is particularly important.[53]

QUALITY ASSURANCE

Flow cytometry shares many general quality control requirements with other assays (see Chapter 137). These include the need for staff training and competency assessment, standard operating procedures for sample handling and processing, instrument maintenance, reagent quality assessment (e.g., verifying lot-to-lot performance),[23] appropriate control selection, reporting, and quality control procedures. To ensure ongoing adequate performance, the laboratory must implement a quality control protocol incorporating both statistical and nonstatistical components.[80]

Flow cytometry also has specific requirements related to instrument quality control. During the initial setup phase and after maintenance or alterations to configuration, extensive instrument validation is required.[80] These validation procedures are typically performed by a manufacturer's representative and are not discussed here, but references are provided for readers requiring more detailed information.[64,80,96] Proficiency testing is desirable for clinical assays, but can be challenging for flow cytometry assays that use unfixed cells.[70] Frozen, cryopreserved peripheral blood mononuclear cells or stabilized samples[70] may provide suitable alternatives to fresh specimens for proficiency testing (see Chapter 138). Flow cytometers and hematology instruments incorporating flow cytometry require regular cleaning to maintain resolution, and thus tight CV for quality control and sample particles or cells.[84] Statistical quality control requirements vary between instruments and applications. For research flow cytometers, there are numerous software and quality control material options, which often involve manufacturer-specific indices and protocols. These generally include PMT standardization, checks on optical alignment, laser power and fluidics, assessment of sensitivity and linearity, and compensation checks.

A brightly fluorescent commercial quality control bead can be used to monitor PMTs. Either the voltage required to obtain a specific MFI or the MFI achieved for a set voltage can be measured for each PMT. MFI or voltage can be compared to predefined targets and plotted against time in a Levey–Jennings plot for early detection of performance shifts.[61,64] At the time of writing, veterinary guidelines that define acceptable performance are not available, but published targets include a <5% change in voltage to achieve a MFI located between the 10th and 90th percentiles of at least 20 replicates[64] or ≤15% change in MFI for a specific voltage.[33] Failure to achieve a target may be due to faulty PMT, loss of laser power, a dirty filter, or improper optical alignment.[33,64]

Optical alignment refers to the focusing of lasers on the core stream center, the detectors, and filters which may scatter light.[74] This can be assessed using alignment particles, which have very uniform size and fluorescence, and therefore a small CV when analyzed on a correctly aligned flow cytometer.[74] Adjusting alignment to maximize peak height and minimize CV allows the user to set-up flow cytometers that require daily alignment (e.g., most cell sorters), while monitoring shifts in CV for standardized beads alerts users to problems in instruments with fixed alignment.[64,74]

Linearity is necessary for correct compensation, and is commonly assessed using combinations of multiple quality control beads of known, differing intensities.[74,84] Fluorescence sensitivity is the ability of the flow cytometer to resolve dim particles from background noise or unstained particles.[74] This can be assessed directly using a combination of unstained and dimly stained particles.[74,84] Alternatively, detector performance can be summarized using mathematically derived Q and B statistics based on light-emitting diodes (LEDs) or fluorescent particles.[74] Q is the number of photoelectrons generated in the detector per fluorochrome molecule, and B is the number of fluorochromes that would produce the amount of background light detected.[28]

APPLICATIONS IN VETERINARY CLINICAL LABORATORIES

Hematology Instruments

Counting blood cells automatically began in the 1930s with a photoelectric method using dark-field optics. In the 1950s and 1960s, electrical impedance technology was developed, which exploits the poor electrical conductivity of cells. In impedance instruments, cells are suspended in an electrolyte solution and pass through a small aperture between two electrodes, creating changes in conductance that are proportional to cell size. By comparing the voltage peak created by each cell to voltage threshold established for particles of known size, cells could be classified by cell type and indices such as mean cell volume (MCV). Alternatively, cells can be identified using floating thresholds, where a valley is identified between two distributions in order to identify the threshold which best distinguishes different cell types in the individual sample.

In the mid-1970s, the Ortho Diagnostics Systems entered the multiparameter hematology instrument market and launched a cell-counting instrument which was based on laser light scatter and utilized the concept of hydrodynamic focusing. Numerous hematology instruments are now available which incorporate flow

cytometry, either as the sole means of analysis or in combination with impedance technology. These include reference laboratory analyzers (e.g., ADVIA 120 and ADVIA 2120i, Siemens Medical Solutions; Sysmex XN-V series, Sysmex Corporation) and benchtop instruments suitable for in-clinic use (e.g., ProCyte Dx manufactured by Sysmex and distributed by Idexx; Element HT5, Heska Corporation). The American Society for Veterinary Clinical Pathology has released quality assurance guidelines for point-of-care testing, which include specific recommendations for statistical and nonstatistical quality control of benchtop hematology instruments.[19] Flow cytometry can enumerate RBCs, WBCs, and platelets in peripheral blood, and provide descriptive indices. Some instruments can also provide valuable information for other samples, including bone marrow, body cavity effusions, and cerebrospinal fluid.

RBC Measurands

ADVIA hematology instruments rely exclusively on light scatter for measurement of RBC count and MCV. To ensure light scatter reliably reflects cell volume rather than cell shape, cells are converted to spheres by treatment with sodium dodecyl sulfate and fixed with glutaraldehyde.[32] FSC (2°–3°) generated by these sphered cells is proportional to cell volume (V) and SSC (5°–15°) to hemoglobin concentration (HG).[32] These are displayed to the user as individual histograms and as a RBC V/HG cytogram, in which the FSC for each RBC is plotted on the *y*-axis and SSC on the *x*-axis (Figure 142.4).[32] This plot allows rapid recognition of shifts away from a normocytic, normochromic RBC population, such as microcytic hypochromic RBCs in iron deficiency, or abnormal subpopulations including RBC aggregates in an agglutinating sample.[32] Several indices are calculated from the light scatter measurements, including MCV, red cell distribution width (RDW), and corpuscular hemoglobin concentration mean (CHCM). ADVIA systems also provide a colorimetric HG measurement after lysing RBCs, from which mean corpuscular hemoglobin concentration (MCHC) is calculated.[8] Discrepancy between MCHC and CHCM can provide insights into potential interferences. For example, MCHC but not CHCM will be falsely increased by lipemia, severe hemolysis, or Heinz bodies.

The Sysmex XT-2000iV combines flow cytometric and impedance technology and thus provides both an optical (RBC-O) and impedance (RBC) RBC concentration.[59] Comparison of these two measures can identify inaccuracies in quantification, as has been described for a dog with a discrepancy between RBC-O and RBC because leukemic cells or cell fragments falsely elevated RBC-O.[59]

Reticulocyte Measurands

Flow cytometry detects both cell volume and RNA content. The latter can be measured using fluorescent stains (e.g., polymethine), optical light scatter, or absorption created by dyes such as new methylene blue. Flow cytometry offers considerable advantages over manual reticulocyte counts, notably increased precision. Manual canine reticulocyte counts performed by a single technician are reported to have a CV of 19–23%[1] as compared to 1–6.1% for automated reticulocyte counts reported by the Sysmex XT-2000iV.[8] However, automated reticulocyte counts are susceptible to interferences including false increases due to Howell–Jolly bodies, neoplastic WBC fragments,[59] or intra-RBC parasites such as *Babesia* spp.[65] There are also species-specific considerations, as illustrated by the good correlation between manual counts of feline aggregate reticulocytes and automated reticulocyte counts by the Sysmex XT-2000iV.[44] It is also necessary to use instrument-appropriate reference intervals and criteria for defining anemia as regenerative, because automated counts are typically higher than manual counts in normal animals.[54] Further information on manual reticulocyte counts is provided in Chapter 137.

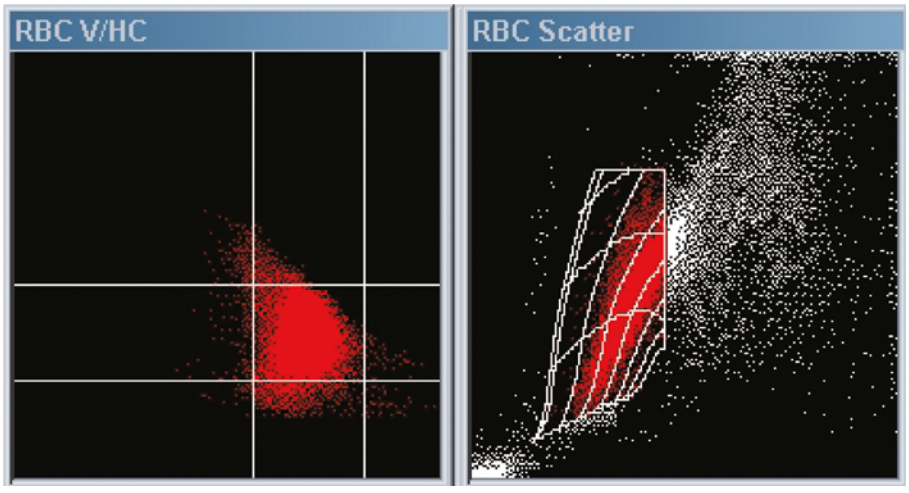


FIGURE 142.4 Red blood cell scatter cytogram and corresponding RBC V/HG cytogram of a normal dog. The RBC scatter cytogram displays high-angle light scatter (5°–15°) on the *x*-axis and low-angle light scatter (2°–3°) on the *y*-axis. The RBC V/HG cytogram displays hemoglobin concentration on the *x*-axis and RBC volume on the *y*-axis.

Flow cytometry can provide further characterization of reticulocytes including reticulocyte volume and hemoglobin concentration, such as the mean cell volume reticulocyte (MCVr), reticulocyte cellular hemoglobin content (CHr), and corpuscular hemoglobin concentration mean reticulocyte (CHCMr) reported by ADVIA analyzers or the reticulocyte hemoglobin equivalent (RET-H*e*) provided by the ProCyte.[20,73] Reduced CHr has been reported in dogs with absolute iron deficiency, but similar reductions occur in other conditions, including inflammatory disease and portosystemic shunts.[51,69,73,79]

WBC parameters

The ADVIA hematology systems use two methods for flow cytometric WBC assessment, the peroxidase channel and the basophil or lobularity channel.[7] Both the peroxidase and basophil channel provide a total WBC count, with the basophil channel usually reported. The instrument will flag discrepancies between the two counts, providing internal quality control. For the peroxidase channel, WBCs are treated with 4-chloro-1-naphthol, which is converted to a dark precipitate by those cells with endogenous peroxidase activity.[72] Cells are gated based on FSC (i.e., size) and peroxidase-staining intensity, producing a five-part differential consisting of neutrophils, eosinophils, lymphocytes, monocytes, and large unstained cells (Figure 142.5).[7,54,82] Feline eosinophils lack peroxidase activity, and are instead identified by light-scatter and oxazine-750 staining in the reticulocyte channel.[7,54] The instrument provides a PEROX cytogram with FSC on the *y*-axis and peroxidase staining on the *x*-axis.[82] This allows the user to screen for inaccuracies in the WBC differential, such as reduced peroxidase activity in immature neutrophils resulting in merging of the neutrophil population into the monocyte gate when a left shift is present.[82]

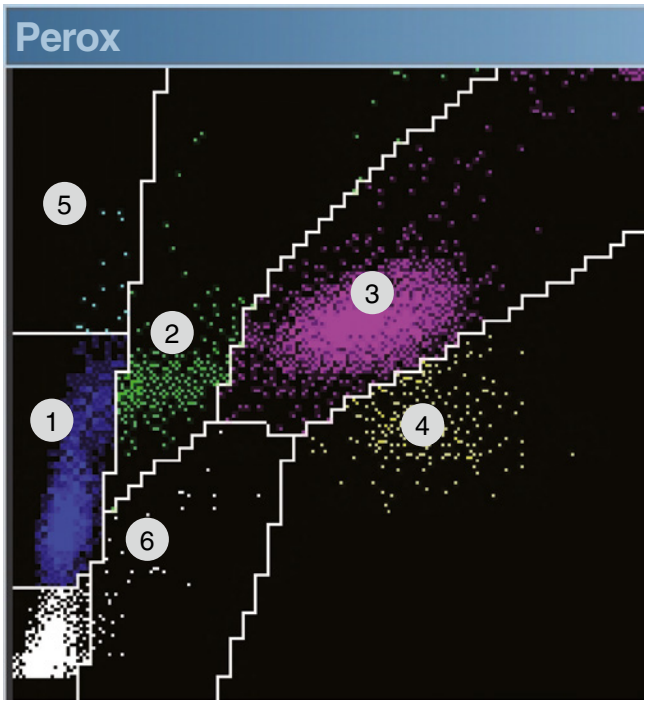


FIGURE 142.5 Peroxidase (PEROX) cytogram of a healthy dog. Cells are gated using light absorption, proportional to peroxidase staining, on the *x*-axis and cell size on the *y*-axis. Gate 1: lymphocytes; gate 2: monocytes; gate 3: neutrophils; gate 4: eosinophils; gate 5: large unstained cells; gate 6: platelet clumps.

In the basophil/lobularity channel, WBCs are lysed and the cytoplasm removed by treatment with phthalic acid and surfactant.[82] The bare nuclei are then gated on FSC (i.e., size) and SSC (i.e., nuclear density/lobularity), allowing cells to be classified as mononuclear (i.e., lymphocytes, monocytes), polymorphonuclear (i.e., neutrophils, eosinophils), or lysis-resistant.[82] In rabbits and humans, basophils are lysis-resistant, allowing differentiation of basophils from other WBCs by the ADVIA systems.[46] Canine and feline basophils are lysed, resulting in unreliable basophil counts in these species.[46] The ADVIA systems provide a BASO cytogram with FSC on the *y*-axis and SSC on the *x*-axis, and the predrawn gates allow the user to rapidly identify changes that may have clinical significance. Examples include increased lysis-resistant cells in leukemia, a lack of separation between mononuclear and polymorphonuclear populations, or shortening of the polymorphonuclear population, consistent with a left shift.[82]

The Sysmex XT-2000iV also identifies basophils by lysis resistance, as displayed on the WBC/BASO channel cytogram, with the same limitations as the ADVIA 120/2120 in veterinary species.[45] The Sysmex XT-2000iV's second WBC channel separates WBCs into neutrophils, lymphocytes, monocytes, and eosinophils based on SSC (i.e., complexity) and staining with polymethine, which binds nucleic acids and cytoplasmic organelles (Figure 142.6).[45] The ProCyte DX is a benchtop instrument that also provides a WBC differential based on SSC and fluorescent measurement of nucleic acid content.[45] Both instruments provide error flags and the Sysmex XT-2000iV will block reporting of the differential cell count if cell populations are not clearly separated. However, visual inspection of the provided scattergram appears more sensitive to potential errors.[45,87] For example, in a study analyzing 106 feline blood samples analyzed on the ProCyte DX, 14 samples had overlapping neutrophil and lymphocyte populations resulting in lymphocyte concentration overestimation and underestimation of neutrophil counts, but only nine samples were flagged by the instrument.[87] Using this methodology, lack of separation of neutrophils and lymphocytes is common in samples with a left shift and toxic change, due to increased RNA content in neutrophils.[87]

Platelet Measurands

In-clinic instruments generally rely on impedance for platelet counts and assessment of platelet indices, such as mean platelet volume (MPV). The Sysmex XT-2000iV generates both impedance (PLT-I) and optical platelet counts (PLT-O), and the agreement between these two

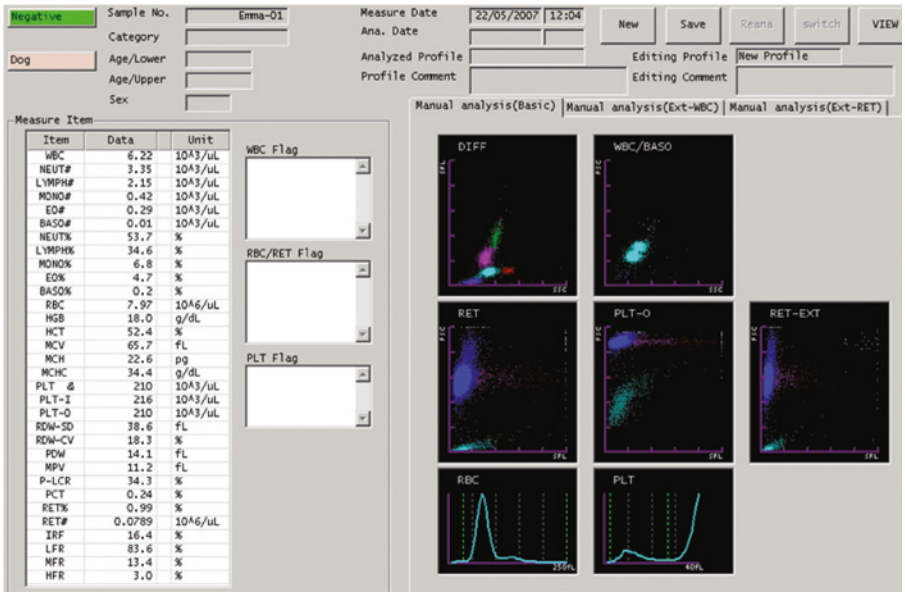


FIGURE 142.6 Routinely available data reported by the Sysmex XT-2000iV (canine sample). On the left, numerical data; and on the right, the graphical report including plots of the WBC SSC (i.e., complexity) against side fluorescence (DIFF) and FSC against side fluorescence for reticulocytes (RETIC) and platelets (PLT-O).

indices is a useful indicator of potential interferences.[77] For the Sysmex XT-2000iV, optical characterization of platelets relies on FSC to measure size, and fluorescent measurement of RNA content.[85] These data are displayed as a cytogram with FSC on the *y*-axis and fluorescence on the *x*-axis.[85] RNA measurement allows user-specified gates to be constructed for identification of immature platelets with increased RNA content, termed reticulated platelets.[60] The Sysmex XT-2000iV can provide optical platelet indices including MPV and the calculated value, plateletcrit (PCT),[60,77] but does not consistently report these; e.g., if the impedance platelet histogram is flagged as abnormal.[77,86] A potential source of confusion when reviewing the veterinary literature is the use of "plateletcrit" to both describe the calculated value from optical systems and the direct measurement by quantitative buffy coat analyzers.[86,88]

The ADVIA hematology analyzers use flow cytometry alone for determining platelet count.[8] Data collection and reporting are similar to RBCs, with a number of graphical displays generated from light scatter by sphered platelets. This includes individual histograms and the PLT scatter plot, in which SSC is plotted on the *x*-axis and FSC on the *y*-axis.

Provision of scatter plots or cytograms by both instruments allows the user to rapidly identify abnormities such as large platelets or potential interferences such as RBC ghosts.[85] The ADVIA systems report several optical platelet measurements, including MPV and the calculated PCT. Evidence regarding the clinical utility of platelet indices in veterinary species is limited, but the PCT (determined by an ADVIA 120) increases above a predefined threshold earlier than platelet count in dogs recovering from immune-mediated thrombocytopenia.[75] This suggests PCT may be valuable for monitoring platelets in patients with increased MPV.[75] The ADVIA 2120 has been reported to underestimate PCT of healthy Cavalier King Charles Spaniels compared to a quantitative buffy coat analyzer, particularly those with the most severe breed-associated macrothrombocytopenia.[88] This, at least in part, likely reflects exclusion of large platelets from the platelet gate by the ADVIA 2120 and other instruments.[88]

Immunophenotyping

Immunophenotyping is the process of identifying cells based on antigen display. In veterinary medicine, immunophenotyping is most commonly applied to the classification of lymphoproliferative or myeloproliferative disorders based on lineage-specific markers (see Chapters 63 and 64). These classifications provide prognostic information[4,16,76,94] and have the potential to guide therapy, e.g., by distinguishing mediastinal lymphoma from lymphocyte-rich thymoma.[41] Immunophenotyping can also be used to evaluate lymphocyte alterations in primary or acquired immune deficiencies, such as X-linked severe combined immunodeficiency[25] or feline immunodeficiency virus,[52] and immune-mediated diseases such as immune-mediated hemolytic anemia (IMHA)[83] (see Chapters 35–38). Immunophenotyping also has applications in transfusion medicine, such as detection of platelet surface antigens.[48]

Functional Assays

Flow cytometry is used in a number of WBC and platelet functional assays (see Chapters 78 and 79). One major advantage of flow cytometry in platelet functional assessment is the concurrent assessment of platelet activation, using markers such as phosphatidylserine

exteriorization or P-selectin expression and microparticle release in response to stimuli. This allows recognition of subtle platelet function defects, such as reduced microparticle release in canine Scott syndrome.[10]

Investigation of Immune-Mediated Disease

Flow cytometry can be used to detect increased antibody binding to RBCs, neutrophils, or platelets (see Chapters 35–38 and 80). Increased antibody binding may be consistent with primary or secondary immune-mediated disease, but sensitivity and specificity of each assay and the effect of time delay between sample collection and analysis should be considered.[93] Initial studies suggested that detection of RBC-bound immunoglobulins by flow cytometry had a high sensitivity for IMHA in dogs and horses. When sick animals were included as controls, there was a lower specificity than direct Coombs testing.[68,92] In two larger studies, 18 of 54 dogs[24] and 5 of 22 dogs[55] with clinical findings consistent with IMHA were negative for RBC-bound antibodies, suggesting diagnostic sensitivity may be lower than initial estimates when in routine use. Publications regarding detection of neutrophil-bound IgG describe only a small number of cases, but do suggest that this may be helpful in diagnosis of immune-mediated neutropenia.[90,91] Direct assays for detection of platelet-surface-associated IgG are reported to be 30–77% sensitive for primary immune-mediated thrombocytopenia in dogs[5,93] with a specificity of 76%.[5]

CONCLUSION

High-quality flow cytometry data require stringent quality assessment with attention to assay validation as well as instrument monitoring and maintenance. When quality assurance is in place, flow cytometry has the potential to improve diagnosis and treatment of clinical patients, as well as provide experimental insights that will lead to improvements in future care.

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CHAPTER 143

Testing for Immune-Mediated Hematologic Disease

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Coombs Test
  Methodology
  Diagnostic Significance
  Alternate Technologies for Antiglobulin Tests
Antineutrophil Antibody Tests
Antiplatelet Antibody Tests
Antinuclear Antibody Test
Investigation of Drug-Induced Autoantibodies
  Method of Antibody Detection
  Drug-Induced Hematologic Disease in Veterinary Medicine

Acronyms and Abbreviations

ANAs, antinuclear antibodies; DAT, direct antiglobulin test; dsDNA, double-stranded DNA; ELISA, enzyme-linked immunosorbent assay; FeLV, feline leukemia virus; FIP, feline infectious peritonitis; FITC, fluorescein isothiocyanate; FSC, forward scatter; hnRNPs, heterogeneous nuclear ribonucleoproteins; IAT, indirect antiglobulin test; ID, immunodiffusion; IIF, indirect immunofluorescence; IMHA, immune-mediated hemolytic anemia; IMN, immune-mediated neutropenia; IMT, immune-mediated thrombocytopenia; ITP, immune thrombocytopenia; Jo-1, histidyl tRNA, synthetase; LE, lupus erythematosus cell; LIA, line immunoassay; MCTD, mixed connective tissue diseases; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PM–Scl, polymyositis–scleroderma antigen; PSAIg, platelet-surface-associated immunoglobulins; RBC, red blood cell; RBMX, RNA-binding motif protein, X chromosome; RNP, ribonucleoproteins; ROC, receiver operating characteristic; Scl-70, topoisomerase I antigen; SLE, systemic lupus erythematosus; Sm, Smith antigen; SS-A/Ro, Sjögren's-syndrome-related antigen A; SS-B/La, Sjögren's-syndrome-related antigen B; SSC, side scatter.

Immune-mediated hematologic diseases such as immune-mediated hemolytic anemia (IMHA), immune-mediated thrombocytopenia (IMT), immune-mediated neutropenia (IMN), and disorders such as lupus erythematosus have been previously described in veterinary medicine (see Chapters 35–37, 43, 52, and 80). Testing typically involves detection of antibody directed against cellular antigens. This chapter will describe specific tests available for the diseases mentioned in the preceding text.

COOMBS TEST

The Coombs test, also known as the antiglobulin test, is used to detect antibody and complement on the surface of RBCs in IMHA diagnosis. Antibodies directed against the RBC surface cause premature RBC destruction. These are often incomplete antibodies that sensitize or coat RBC, but fail to agglutinate them without addition of potentiators to alter the RBC zeta potential or without the addition of antiglobulin reagent. Most incomplete antibodies are IgG and are thus detected by anti-IgG serum, as contained in antiglobulin reagent. IgM antibodies also can be incomplete. These incomplete antibodies produce antibody-dependent complement activation; thus, antiglobulin reagents are also designed to react with complement components on the RBC surface, with the main components detected being C4 and C3. Polyspecific reagents for veterinary use typically

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contain anti-IgG, anti-IgM, and anti-C3. Monospecific reagents are also available.

Methodology

Two forms of the Coombs test are used in veterinary medicine: the direct antiglobulin test (DAT) and the indirect antiglobulin test (IAT). The DAT, that detects immunoglobulin and/or complement bound directly to RBC, is used to evaluate IMHA patients (see Table 143.1). In this procedure, the addition of antiglobulin reagent to RBC with bound immunoglobulin or complement causes agglutination. The IAT detects unbound antibodies against RBCs in the patient's serum, and is used in crossmatching or antibody screening. The antiglobulin test can also be performed using microdilution (microtiter) plates, immunochromatography strips, gel columns, and capillary tubes.[70] Microdilution plates allow for a greater number of antiglobulin reagent dilutions and use smaller volumes of reagent and RBC per well. The use of multiple dilutions increases test sensitivity.[10]

A comparison of four different direct Coombs test methods in anemic and nonanemic dogs showed comparable and consistent results, even after storage of chilled blood samples for 1 week.[10] Using polyclonal antiglobulins, the immunochromatographic strip, capillary, and gel microcolumn methods were compared to a standard microtiter DAT. Transfusion did not cause false DAT+ results, and dogs treated with immunosuppressive drugs still remained DAT-positive for days to weeks.

Diagnostic Significance

The Coombs test is most commonly used in IMHA diagnosis in veterinary medicine. The antiglobulin test, as an aid in IMHA diagnosis in dogs, was first described by Miller et al. in 1954.[48] Direct antiglobulin test positive hemolytic anemia was first described in cats in 1973 by Scott et al.[60] Several studies which used the antiglobulin test to detect IMHA in horses have been described.[43,47]

Sensitivity of the DAT in IMHA detection can be variable, with sensitivity as low as 48% reported in dogs.[32] However, the test remains a useful diagnostic tool, especially when combined with clinical signs of hemolytic anemia, spherocytosis, and persistent autoagglutination. False negative test results may be caused by incomplete RBC washing, insufficient RBC-bound antibody, elution of Ig or C3, or prozone effect.[52,71]

Interestingly, false positive results in the absence of IMHA can also occur. Dogs with infections, inflammatory disorders, and myeloproliferative and lymphoproliferative diseases have been reported to have a high incidence of C3b bound to RBC.[62] Hemolytic disease in these dogs was minimal or absent. A positive DAT needs to be interpreted with caution with concurrent conditions. In another study, 16 of 20 anemic cats were DAT-positive. Eleven of the 16 were feline leukemia virus (FeLV)-positive, and the remaining five positive cats had inflammatory disease or hemoplasmosis. The prevalence of positive antiglobulin tests in 89 healthy cats was recently documented.[1] A direct, microtitration antiglobulin test at 37, 23, and 4 °C was used. At 37 °C, only four cats were positive at a low dilution (1:2), and two cats were transiently positive at a high dilution (≥1:2048). At 4 °C, 91% of the cats had invalid results, and agglutination was present in the negative control well. Optimization and validation of the DAT to minimize analytical error are required prior to use in IMHA diagnosis.

Alternate Technologies for Antiglobulin Tests

Most of the alternate DAT technology has been designed to either increase test sensitivity or decrease the subjectivity associated with agglutination assessment. Enzyme-linked antiglobulin tests appear to enhance sensitivity, but are difficult to perform and decrease the diagnostic specificity for IMHA.[4,33] Flow cytometry has been shown to be a sensitive diagnostic technique for IMHA in dogs and horses, with some decrease in specificity.[56,75] Flow cytometry has been used to determine RBC-bound immunoglobulin isotypes in dogs with IMHA. Results suggest that IMHA dogs with two or more immunoglobulin isotypes have a more severe degree of anemia, spherocytosis, and autoagglutination.[29] The test necessitates a flow cytometer in the laboratory (see Chapter 142).

TABLE 143.1 Direct antiglobulin test (DAT) tube technique.

1.	Place one to two drops of a 2–5% RBC suspension in a 10 or 12 × 75 mm test tube.
2.	Wash three to four times with normal saline or with phosphate-buffered saline (PBS). Thoroughly decant the last wash residual in order to avoid dilution or neutralization of the antiglobulin reagent.
3.	Immediately add antiglobulin reagent in amounts and as diluted per manufacturer's guidelines. Standard dilutions for canine antiglobulin are 1:2, 1:4, and 1:8. Prepare a negative control tube by adding saline instead of reagent to a tube.
4.	Incubate tubes according to manufacturer's directions.
5.	Centrifuge, gently dislodge RBC button, and record agglutination reactions observed as the cells come off the button. If no agglutination is apparent, a small amount of the tube contents can be placed on a slide and examined microscopically.
6.	If available, add IgG-sensitized RBCs (known as "check" cells) to nonreactive (unagglutinated) tests. Centrifuge and examine for agglutination.
7.	If a positive DAT is obtained, monospecific reagents (if available for the species) can be used to determine whether immunoglobulin or complement (or both) are present. Freshly washed cells must be used for this step.

ANTINEUTROPHIL ANTIBODY TESTS

IMN has been described in dogs, cats, and horses.[9,17,46,79] The disease is frequently diagnosed by exclusion of other diseases known to cause neutropenia, supportive bone marrow findings, and by response to corticosteroids. Direct diagnosis involves detection of antineutrophil antibody; however, antineutrophil antibody tests are not widely available. Previous techniques for detection have included leukoagglutination,[69,79] indirect immunofluorescence of bone marrow aspirates,[46] and flow cytometry.[31,73] An indirect leukoagglutination test used to determine antineutrophil antibodies in two dogs demonstrated that serum from the patients but not from a control dog caused agglutination of the patient's neutrophils following incubation with Coombs reagent.[46] A leukoagglutination method described failed to identify any antineutrophil antibody in 55 feline neutropenic patients.[13] An indirect flow cytometry assay and leukoagglutination testing were used to detect IMN in a 3-day-old foal with neonatal isoerythrolysis.[79]

Direct and indirect flow cytometry are described as the most sensitive tests for antineutrophil antibodies. Canine patient serum was incubated with paraformaldehyde-fixed neutrophils and subsequently incubated with fluorescein-conjugated rabbit anti-dog IgG.[73] Indirect flow cytometry demonstrated antibody in five of six dogs with a clinical diagnosis of IMN and lack of antibody detection in six of six dogs with neutropenia associated with other diseases.[72]

ANTIPLATELET ANTIBODY TESTS

Immune-mediated thrombocytopenia has been described in dogs[40], horses,[45] and rarely in cats.[34] To be consistent with the International Working Group recommendations for standardization of terminology and a recent veterinary review, IMT is now referred to as immune thrombocytopenia (ITP).[37,58] ITP may be primary (idiopathic), or secondary to primarily tick-borne infectious agents, neoplasia, or drug administration.[40] In dogs with primary ITP, the platelet-reactive antibodies are of an IgG class directed against platelet surface glycoprotein GPIb or GPIIb and/or GPIIIa (CD41/CD61).[41,42,61] In secondary ITP, platelet destruction can be caused by immune-complex binding to platelet Fc receptors, exposure of new antigens after membrane damage, nonspecific adsorption of foreign antigens (i.e., drugs), or newly generated antigens produced during the course of infectious or neoplastic diseases.[40] Methods to detect platelet antibodies in ITP patients include the direct immunofluorescent test for megakaryocytes; however, it requires a bone marrow aspirate and has variable sensitivity.[40] Bone marrow evaluation has merit in these cases for increased megakaryocytopoiesis has been reported in dogs with ITP that also have platelet-bound antibodies.[18]

Indirect methods to detect serum antibodies that have binding specificities for platelets (platelet-bindable antibodies) and direct methods to detect platelet-surface antibody (platelet-surface-associated immunoglobulins (PSAIg)) include enzyme-linked immunosorbent, immunoradiometric, and immunofluorescent (microscopy or flow cytometry) labeling techniques.[35,61,77] Indirect assays to detect antiplatelet antibodies in canine serum are not often performed because levels of circulating platelet-bindable antibodies are typically low resulting in low diagnostic sensitivity.[35,42]

Direct assays using flow cytometry to detect PSAIg are currently the most common methods for dog, horse, and cat blood samples.[18,34,45,77] Direct immunofluorescent assays for ITP have high diagnostic sensitivity (90–95%) and low diagnostic specificity.[42] Although direct PSAIg flow cytometry assays are common in human medicine, finding veterinary diagnostic laboratories that perform this assay is difficult. One of the first direct flow cytometry assays for PSAIg detection in veterinary species used a monoclonal antibody specific for canine IgG.[38] More information about this diagnostic assay is available at http://www.ksvdl.org/laboratories/clinical-immunology/#platlet. Recent modified assays use polyclonal antibodies to canine immunoglobulins[3] (Figure 143.1). In one report, often platelets with PSAIgG are smaller in size based on forward scatter (FSC).[77] Furthermore, dogs considered to have primary immune-mediated thrombocytopenia have significantly lower platelet concentrations and low mean platelet volumes than dogs with thrombocytopenia due to other diseases.[3] It is not known whether the formation of smaller platelets is a consequence of activation and microparticle formation during the assay or an in vivo phenomenon. There is recent interest in studying platelet microparticles as risk factors for prothrombotic conditions in patients with ITP or other diseases.[15] Not all laboratories use CD61 to identify platelets. CD61 expression can be greatly reduced in patients with ITP,[3,78] which may indicate CD61 modulation in these patients.

Direct labeling techniques identify the presence of surface-associated immunoglobulins, but do not identify autoantibodies specific to platelet glycoproteins. Diagnostic sensitivity and specificity of any method for PSAIg detection should reflect its ability to detect immunoglobulins on the platelet surface in patients with ITP.[30,59] Enhanced diagnostic sensitivity for primary ITP diagnosis in dogs can be achieved when results of direct/indirect IgG and IgM flow cytometry assays were combined with increased reticulated platelet results.[3] However, this combination decreased diagnostic specificity.[3] Use of a receiver operating characteristic (ROC) curve to optimize sensitivity and specificity is needed (see Chapters 80 and 140). Primary ITP diagnosis, in both human and veterinary medicine, is often based on clinical impression, exclusion of known causes of thrombocytopenia, and response to treatment.[30,61,67] For this reason, the American Society of Hematology defined PSAIg detection inappropriate and unnecessary in primary ITP patients.[23,30] Nonetheless, the PSAIg assay could be useful as a screening test for primary ITP due to its high sensitivity.[30,68] A negative result in a patient not treated with glucocorticoids rules out an immune-mediated mechanism of the

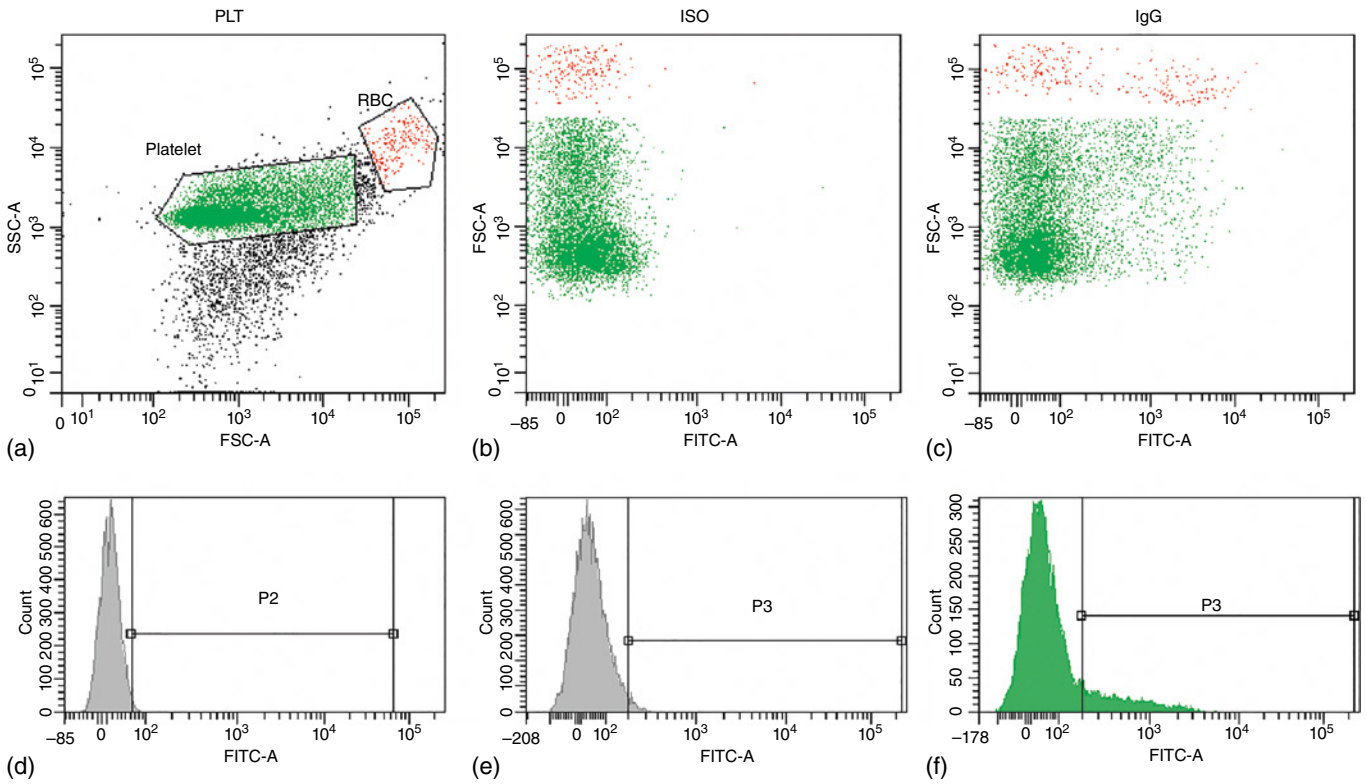


FIGURE 143.1 Results of the direct immunofluorescent test to detect PSAIgG on canine platelets using biexponential digital flow cytometric methods (LSRFortessa X-20, Becton Dickinson, San Jose). (a), (b), and (c) represent dot plots of cells from platelet-rich plasma (PRP). (a) Scatter plot of the complexity (SSC-A) and size (FSC-A) of platelets (green), and contaminating RBCs (red). Two distinct populations of platelets of different sizes are noted; (b) Size versus fluorescent plot of PRP incubated with an isotype control antibody (FITC-labeled mouse IgG); (c) Size versus fluorescence plot of PRP incubated with a monoclonal antibody specific to canine IgG. (d), (e), and (f) represent histograms of the gated platelet population. (d) Unlabeled platelets; (e) Platelets incubated with isotype control in which vertical line is placed where <1% of the cells are positive to the right; (f) Platelets binding to anti-canine IgG are shifted to the right of the vertical line (15% of the gated platelet population). (**Source:** Kansas State University, Clinical Immunology Diagnostic Laboratory.)

platelet destruction; whereas, positive results must be confirmed by more specific assays, such as the monoclonal antibody-specific immunomobilization of platelet antigen assay, only available in human medicine.[8,55,68] Other testing including the measurement of thrombopoietin levels or evaluation of the function of regulatory T and B cells have been pursued in human medicine, but are not available as diagnostic tests in animals.[37]

A major disadvantage of PSAIg assays is poor sample stability that can lead to false positive results.[76,77] Platelets can be activated by improper blood collection, poor sample handling (i.e., unnecessary agitation of the tube), low storage temperature, prolonged time to sample preparation (storage time), and centrifugation steps, with subsequent exposure of IgG, typically contained within the alpha granules, on the platelet surface.[14,39,51,76] Platelet shape changes can be evaluated by flow cytometry as an indicator of activation. When activated, the platelet population (FSC versus side scatter (SSC) plots) spreads from the normal, well-defined oval shape to an elongated and narrower scatter plot (Figure 143.2).[51]

Flow cytometry methods using whole blood samples have been proposed as alternative methods to minimize in vitro platelet activation that occurs during the preparation of platelet-rich plasma.[65] However, prolonged contact with leukocytes before platelet separation can trigger platelet activation.[67] Because the principal sources of platelet activation occur in a preanalytical phase, strict preanalytical quality control is required. Positive results must be interpreted with caution in the context of clinical and supporting laboratory findings.

ANTINUCLEAR ANTIBODY TEST

Antinuclear antibodies (ANAs) are a heterogeneous population of antibodies reacting against self nuclear components.[36,74] ANAs do not appear to play a prominent role in the pathogenesis of disease, but their identification is used as one of several clinical and laboratory criteria for diagnosis and monitoring of autoimmune disorders in both human and veterinary medicine[6,7,36] (see Chapter 52).

The indirect immunofluorescence ANA (IIF-ANA) test is considered the gold standard screening test for detection of circulating ANAs in both human and veterinary medicine, and it is often referred to as the "ANA-test."[7,28,36] Briefly, serial serum dilutions are applied to a commercially available glass slide, which contains nuclear-rich tissue substrates fixed in separate wells. Typically, substrates are composed of either rat liver sections or human monolayer epithelial cells (e.g., Hep-2, HeLa, Vero,

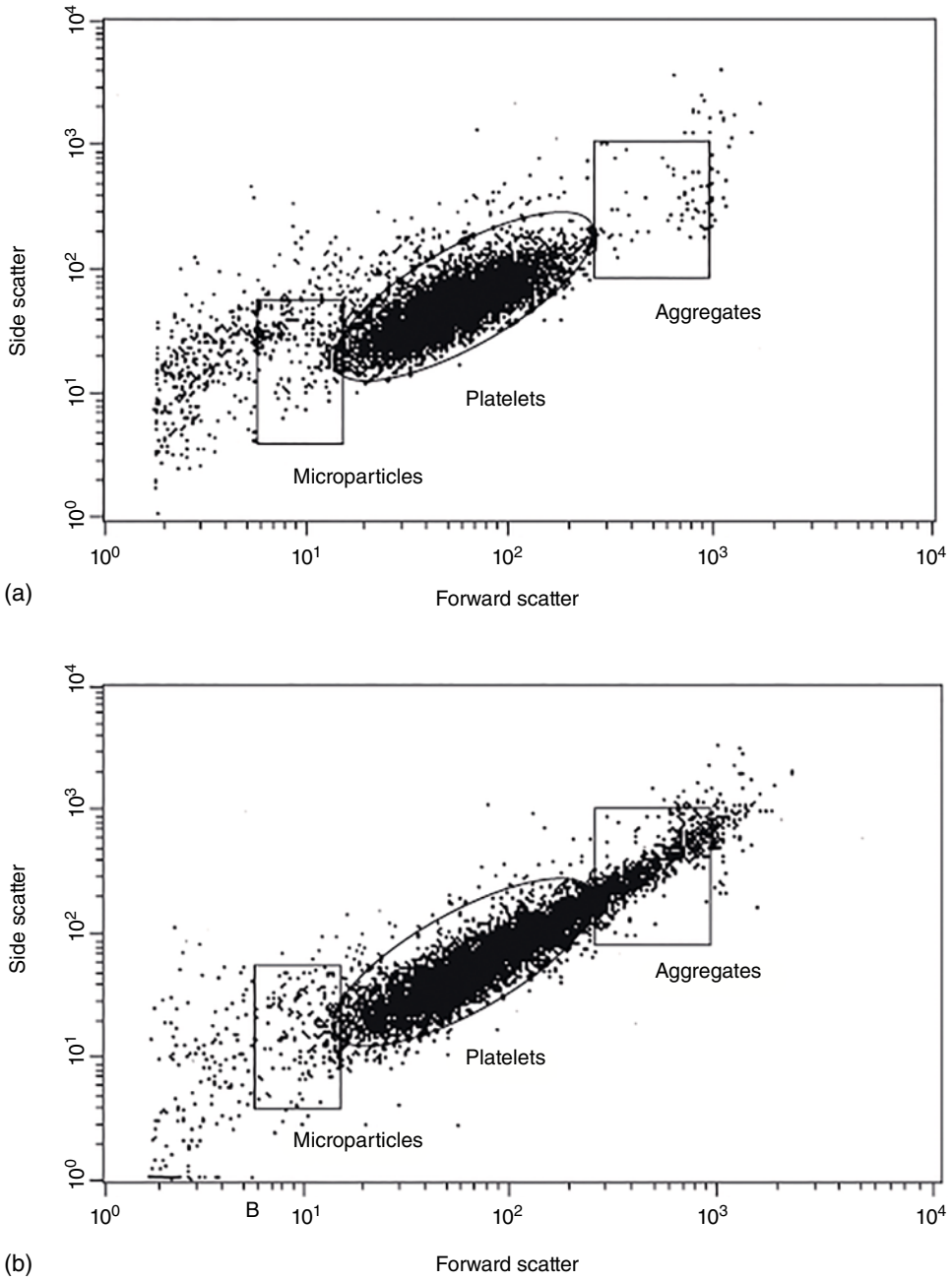


FIGURE 143.2 Forward-angle versus side-angle light scatter plots. (a) The scatter of nonactivated platelets is a characteristic well-defined oval shape; (b) Platelets activated with 20 ng/mL phorbol myristate acetate. The platelet population spreads to form an elongated and narrower scatter plot. Platelet fragments (microparticles) and platelet aggregates are produced upon activation and are discernible as two distinct populations with a lower and higher forward scatter, respectively. (**Source:** Moritz et al.[51])

Hep-2000).[26,28,5-7] After a 30-minute incubation, the slide is rinsed with phosphate-buffered saline (PBS) and secondary anti-species-specific antibody, typically conjugated with fluorescein isothiocyanate (FITC), is applied to the wells. After a 30-minute incubation, the slide is washed with PBS to remove unbound antibody, and analyzed using fluorescence microscopy. Positive and negative controls for veterinary species are usually not commercially available; serum aliquots from patients that previously tested negative and positive are typically stored by laboratories for this purpose. A negative ANA reaction is identified by a lack of fluorescence in the substrate, which may appear reddish-orange because of the counterstain. Samples are considered positive when a clearly discernible fluorescent pattern in the nucleus is present.[28,36,54]

In human medicine, the pattern of nuclear fluorescence in the IIF-ANA test suggests the specific antibody type. Speckled staining is the most frequently identified pattern in humans, but is not specific for a particular autoimmune disease. The speckled pattern

is seen when antibodies to non-DNA components, collectively called extractable nuclear antigens, are present. These include ribonucleoproteins (RNP), topoisomerase I antigen (Scl-70), Smith antigen (Sm), and Sjögren's-syndrome-related antigens A and B (SS-A/Ro, SS-B/La), which are associated with mixed connective tissue diseases and systemic lupus erythematosus (SLE), scleroderma and SLE, and Sjögren's syndrome sicca complex and SLE, respectively. A homogeneous or peripheral nuclear staining usually indicates the presence of antibodies directed against chromatin constituents, such as double-stranded DNA (dsDNA) or histones. In people, the presence of these autoantibodies is characteristic of SLE or drug-induced lupus. Centromere and nucleolar staining are more often associated with scleroderma, polymyositis, and other connective tissue diseases.[7,36]

Because the IIF-ANA test has low specificity, positive human samples are often further analyzed for the identification of the specific autoreactive antibodies, which can be linked more directly to determined autoimmune disorders.[36] Specific ANAs can be identified with ELISA, immunoblot, human protein arrays, immunodiffusion (ID), and radioimmunoassay using either purified extracts or recombinant antigens.[5,6,36,74] In some human medical laboratories, a panel for the simultaneous detection of a variety of specific ANAs has replaced the IIF-ANA test.[5] The application of ANAs in veterinary medicine is mostly restricted to the use of the IIF-ANA test as an aid in SLE diagnosis.[7,28] Historically, the lupus erythematosus cell test (LE test) was used to diagnose this disorder, but is uncommonly used due to a high incidence of false-negative and false-positive results (see Chapter 52). SLE is rarely reported in cats and horses, and lack of comparative data makes the IIF-ANA titer interpretation difficult.[22,26,66] Antinuclear antibody titers in cats diagnosed with SLE are usually low (below 1:40) and overlap with the titers of healthy subjects. Furthermore, titers as high as 1:100 can transiently be found in both FeLV- and feline infectious peritonitis (FIP)-positive cats.[26,66]

In dogs, a positive IIF-ANA titer is expected in 97–100% of SLE cases, but the sensitivity of immunofluorescence varies between laboratories and substrate employed.[11,26] Antinuclear antibodies can also be produced in many inflammatory and infectious diseases, and a positive immunofluorescence result must be interpreted in the context of clinical signs and laboratory data.[28,64] Therefore, a positive IIF-ANA test is listed in the revised American Rheumatism Association diagnostic criteria for people adapted for dogs, but should not be considered the sole criterion for canine SLE diagnosis.[12,28]

Positive IIF-ANAs have been described in dogs with non-autoimmune disorders such as atopic dermatitis, onychodystrophy, and black hair follicular dysplasia, and in subjects seropositive for *Bartonella vinsonii* subsp. *berkhoffii*, *Ehrlichia canis*, or *Leishmania infantum*.[24,26,53,64] Dogs with nonprimary immune-mediated disorders tend to have low positive titers (91% ≤ 1:256), but positive titers up to 1:4000 occasionally can occur.[11,24,28,50,64]

By contrast, higher immunofluorescence titers were found to be clearly correlated with signs of autoimmune diseases (1:100 on Hep-2)[28] and titers ≥1:640 were considered to be a good predictor of immune-mediated diseases in dogs.[63] Positive titers can also be found in healthy dogs, especially when cryopreserved sections of rodent liver are employed.[26,28] The appropriate threshold titer of positivity must be determined in each laboratory based on a control population and substrate type. In general, high titer cutoffs (e.g., 1:100) are required with rat liver substrates to decrease the false-positive rate.[28] Hansson et al. found the Hep-2 substrate to be superior to rat liver sections not only for its high specificity (no false positives at 1:25 dilution in 100 healthy dogs), but also because it provides an easier and more consistent pattern discernment.[28]

Using Hep-2 slides, two major patterns are recognizable in dogs: the homogeneous pattern, characterized by an intense fluorescence staining of the chromosomal area in mitotic cells; and the more commonly occurring speckled pattern, chromosome-negative (Figure 143.3).[7,28] An association between homogeneous immunofluorescence pattern and SLE has been reported to occur in dogs, but the speckled immunofluorescence pattern does not appear to be associated with a specific canine autoimmune disease, as observed in people.[7,27]

The identification of specific ANAs is not routinely done in veterinary medicine.[7] In dogs, specific ANAs have been analyzed by using double immunodiffusion technique of Ouchterlony, ELISA, human protein arrays, and line immunoassay (LIA), which is a type of immunoblot that uses recombinant, synthetic, or natural antigens attached in separate lines on a strip.[6,7,36,74] Anti-dsDNA antibodies can be confirmed with the *Crithidia luciliae* indirect immunofluorescence test.[7]

Some ANAs have been identified in both human and canine patients, such as antibodies directed against histones, RNP A, RNP C, RNP-70K, Sm, SS-B, SS-A (both Ro-60 and Ro-52), Scl-70, polymyositis–scleroderma antigen (PM–Scl), histidyl tRNA, synthetase (Jo-1), and proliferating cell nuclear antigen (PCNA).[7,11,49] Other autoantigens, such as the anti-type 1 (anti heterogeneous nuclear ribonucleoproteins (hnRNPs) G, encoded by the gene RBMX) and anti-type 2, are confined to dogs and are not detected by tests for human ANAs.[7,27,49,74]

The significance of most of these canine autoantibodies is still unclear. Previous literature reports that in dogs with SLE, ANAs are rarely directed against dsDNA, but are mostly against nuclear proteins such as histones and RNP.[11,27,49,54,74] In a subsequent study, antibodies against dsDNA were found in a quarter of IIF-ANA-positive dogs with unknown clinical diagnosis, while antibodies against histones were found less often.[7] In previous studies, an association between immunofluorescence patterns observed using the IIF-ANA test and specific ANAs was not identified, even though reacting bands were exclusively found in speckled patterns.[27,74] More recently, the homogenous pattern has been most frequently associated with anti-dsDNA and antinucleosome

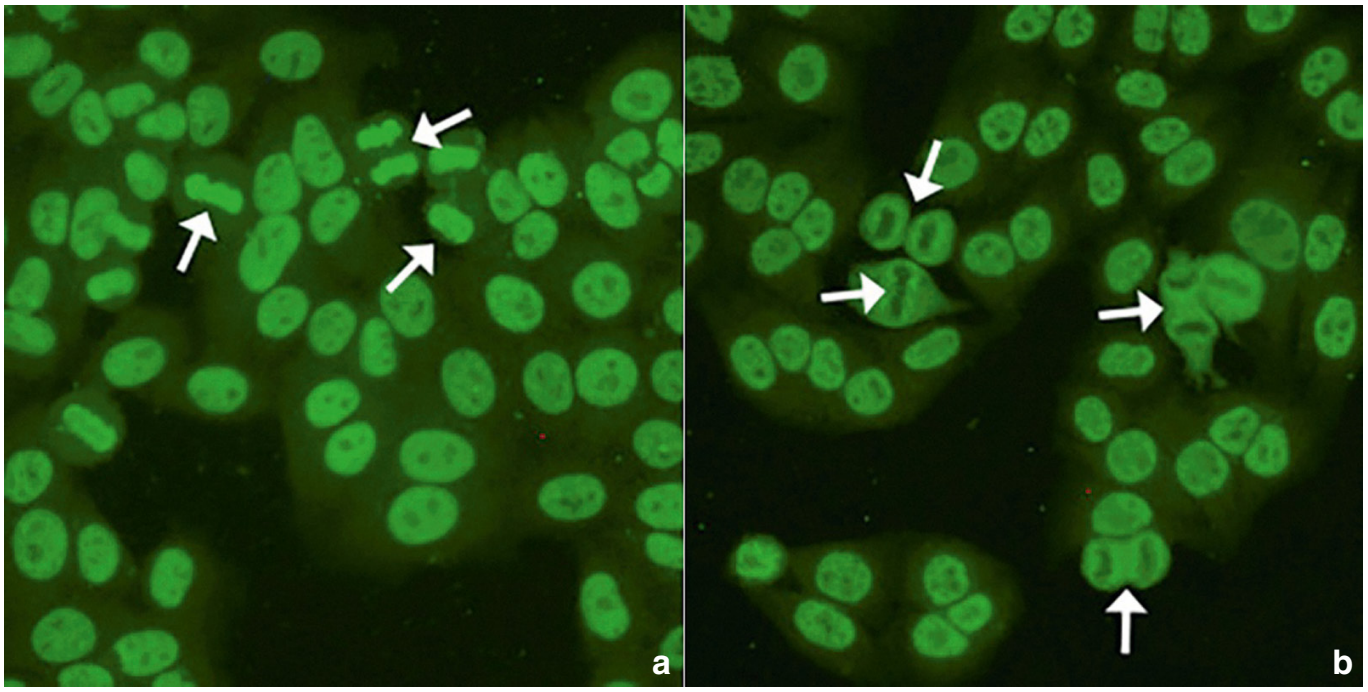


FIGURE 143.3 Antinuclear antibody patterns of canine sera showing ANA positivity in the indirect immunofluorescence test (IIF-ANA) using human Hep-2 cells as substrate. Canine serum samples reacted with FITC-conjugated anti-canine IgG to produce (a) homogeneous pattern. Arrows are pointing at the fluorescent chromosomal regions in mitotic cells; (b) Speckled pattern. Arrows are pointing at the negative staining of the chromosomal regions in fluorescent mitotic cells. (Source: Reproduced from Bremer HD et al.[7], Elsevier Publishing.)

antibodies, while dogs with the speckled pattern have high concentrations of anti-RNP and/or anti-Sm antibodies.[7]

The discrepancies between studies are most likely due to different detection methods, the lack of well-defined diagnostic criteria for the different canine autoimmune diseases, and differences in inclusion criteria for investigated populations.[7,27]

INVESTIGATION OF DRUG-INDUCED AUTOANTIBODIES

Drugs can induce the formation of antibodies, either against the drug itself, against intrinsic cellular antigens modified by the drug, or both (see Chapters 13 and 15). This has been most frequently described in humans with drug-induced IMHA.[2,20,21] Drug-dependent antibodies require that the drug be present. Drug-independent antibodies react similarly to true autoantibodies (i.e., no drug is needed to demonstrate the presence of antibody). Drugs can bind loosely or firmly to cell membranes and antibodies may be made to (1) the drug itself, necessitating drug binding for in vitro detection; (2) cell membrane components; or (3) part drug, part membrane components, producing an in vitro reaction typical of an immune complex mechanism.

Method of Antibody Detection

Drug-induced anti-RBC antibodies in humans are usually associated with a positive DAT. Further investigation of suspected hemolysis due to drug-dependent antibodies requires special methods,[19] e.g., drug-treated RBCs are prepared for detection of antibodies to penicillin. Washed, packed, group O RBCs are incubated with penicillin dissolved in buffer. The cells are then washed and a PBS cell suspension is prepared. These cells are mixed with patient serum, incubated, centrifuged, and examined for hemolysis or agglutination. Antibody-free serum and serum containing known antibody are used as negative and positive controls, respectively. The cells are also tested by an indirect antiglobulin technique. For demonstration of immune-complex formation involving drugs, the patient serum is incubated with a suspension of the drug in question. The mixture is then added to enzyme-treated group O RBCs, incubated, centrifuged, and examined as described for the drug-coated cells. Controls are used as indicated in the earlier text. Drug-independent antibodies react similarly to most IMHA cases, with a positive DAT test result.

Diagnosis of drug-induced immune-mediated granulocytopenia or thrombocytopenia is generally considered when there is a temporal association of the drug and the cytopenia, rather than by actual antibody detection in the presence of drug. An exception would be

heparin-induced thrombocytopenia, a condition described in human patients receiving unfractionated or low-molecular-weight heparin. Immunogenic complexes formed by heparin and platelet factor 4 cause antibody formation, and these anti-heparin/PF4 antibodies can be detected with commercially available immunoassays.[1,25]

Drug-Induced Hematologic Disease in Veterinary Medicine

Drug-induced immune hematologic disease has been poorly documented in veterinary medicine, though pancytopenia has been associated with penicillin and potentiated sulfonamide administration in horses.[16,44] Most reports show a temporal association of a drug with hematologic disease, but no actual testing for drug-associated antibody. The Coombs test was positive in horses with penicillin-induced hematolytic anemia, and their sera produced agglutination of penicillin-coated RBCs. In one horse, antipenicillin antibody titers decreased progressively throughout the observation period.[57]

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CHAPTER 144

Electrophoresis and Acute-Phase Proteins

ALESSIA GIORDANO

General Principles of Electrophoresis
Electrophoretic Methods in Veterinary Medicine
  Zone Electrophoresis
    Agarose Gel Electrophoresis
    Capillary Zone Electrophoresis
    Immunoelectrophoresis
    Isoenzyme Electrophoresis
Acute-Phase Protein Detection
  Fibrinogen (Factor Ia)
  Serum Amyloid A
  C-Reactive Protein
  α-1-Acid Glycoprotein
  Haptoglobin
  Negative Acute-Phase Proteins
Quality Assurance for Electrophoresis and Acute-Phase Protein Detection
  Preanalytical Phase
    Selection of Suitable Specimens
    Interfering Substances
Analytical Performance
Electrophoretogram Interpretation and Reporting
  Normal Electrophoretic Patterns in Domestic Animals
  Pathologic Electrophoretic Patterns
    Inflammation
    Polyclonal Gammopathy
    Monoclonal Gammopathy
    Nephrotic Syndrome
    β–γ Bridging

Acronyms and Abbreviations

AGE, agarose gel electrophoresis; AGP, α-1-acid glycoprotein; ALP, alkaline phosphatase; APP, acute-phase proteins; APR, acute-phase response; ASVCP, American Society for Veterinary Clinical Pathology; CAE, cellulose acetate electrophoresis; CZE, capillary zone electrophoresis; CK, creatine kinase; CRP, c-reactive protein; CV, coefficient of variation; EDTA, ethylenediaminetetraacetic acid; EPH, electrophoresis; ELISA, enzyme-linked immunosorbent assay; Hp, haptoglobin; HRE, high-resolution electrophoresis; LDH, lactate dehydrogenase; pigMAP, porcine major acute-phase protein; PON-1, paraoxonase-1; RBP, retinol-binding protein; RID, radial immunodiffusion; SAA, serum amyloid A; SDS, sodium dodecyl sulfate; SPE, serum protein electrophoresis; TIBC, total iron-binding capacity; TRF, transferrin; TTR, transthyretin.

GENERAL PRINCIPLES OF ELECTROPHORESIS

Electrophoresis (EPH) separates molecules such as nucleic acids, proteins, lipids, and carbohydrates in an aqueous solution based on their physical and chemical properties, such as size, weight, electrical charge, and shape, using an electric field that induces migration of charged molecules toward an electrode. The main components of an electrophoretic apparatus include: two metal electrodes of opposite charge (anode or positive electrode, cathode or negative electrode), connected by a conductive buffered electrolyte solution, and a power supply. The alkaline buffered electrolyte solution (pH 8.4–8.8) induces most of the proteins to be negatively charged; thus, the proteins migrate toward the positively charged anode. Electrophoretic conditions are characterized by electrical parameters (i.e., current, voltage, amperage, temperature) and factors related to buffers and support media, such as ionic strength, pH, viscosity, pore size, etc. Electrophoresis equipment must provide constant voltage, amperage, and temperature to minimize the variability of migration; thereby, optimizing

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result reproducibility. For example, high temperatures may alter the migration rate, possibly resulting in distorted bands and, consequently, peaks. Dissipation of heat generated by electrical resistance can be a major problem, especially in older units.

ELECTROPHORETIC METHODS IN VETERINARY MEDICINE

In veterinary clinical practice, EPH techniques are mainly used to evaluate the protein profile of body fluids, by separating proteins in fractions based on mobility, e.g., serum protein electrophoresis (SPE) or separating unique proteins based on their molecular weight, e.g., urine electrophoresis. Additional techniques used in research settings may have application to clinical settings in the near future.

Zone Electrophoresis

The sample is placed in a narrow zone or band in the stabilizing medium at a suitable distance from each electrode. Molecules are fixed in lanes on a stabilizing medium or support (paper, cellulose acetate, agarose, or polyacrylamide gel) to avoid random dispersion into solution, such that migration fractionates the sample revealing reproducible banding patterns.

Separated bands may be stained with Ponceau red, Amidoschwarz acid violet, Coomassie blue, etc., and measured by densitometry.[12] A densitometer is a spectrophotometer that measures light, aka optical density, transmitted through a semitransparent material, e.g., the electrophoretic gel or support medium. It creates an electrophoretogram, which is a diagram or graph of peaks of different height and width directly corresponding to the fractionated protein bands' optical density and width. Area under each peak is expressed as a percentage which represents each fraction's relative proportion. Percentage values are then converted to absolute values by multiplying by the total serum or plasma protein concentration, likely measured by the biuret method, though measurement by other methods is possible. The electrophoretogram data should be digitally stored, printed, and interpreted with an appropriate data acquisition system.

Historically, cellulose acetate has been used as a solid support medium to separate proteins based on their isoelectric point (pI). Briefly, serum samples are placed on the cellulose acetate strip and into an alkaline buffer, usually a barbital buffer. Once the electric field is applied at a constant voltage, proteins with a pI higher than the buffer pH (e.g., albumin and most of the globulins), being negatively charged, migrate toward the anode. Gamma-globulins, which have a pI lower than the buffer pH, migrate very slowly and essentially do not move. Compared to paper, which was previously used as an EPH support, cellulose acetate has a lower adsorption capacity and generates sharper electrophoretic bands. Cellulose acetate electrophoresis (CAE) usually generates five to six electrophoretic peaks, namely, albumin, α-1-globulin, α-2-globulin, β-globulin (in some species further divided in β-1- and β-2-globulin), and γ-globulin from most animals' serum. While inexpensive, CAE can be time-consuming and high-resolution separation comparable to current techniques is not possible.[16]

Agarose Gel Electrophoresis

Thin-layer agarose gel EPH has replaced CAE in most veterinary diagnostic laboratories due to clearer separation of protein fractions and sharper peaks. Agarose gel electrophoresis (AGE) and CAE share the same principle, but precasted, 0.5–1% agarose gels are used in an almost fully automated procedure. After the densitometric analysis, software evaluates the peaks automatically generating relative values. The number of EPH fractions is usually comparable to that obtained with the most advanced CAE equipment, but better differentiation between α-1- and α-2-globulins and between β-1- and β-2-globulins is observed.[2] Agarose gels with variable pore sizes are available, which can be used to separate other molecules useful in diagnostic veterinary medicine, such as urine proteins, lipoproteins, nucleic acids, and isoenzymes.

Sodium dodecyl sulfate (SDS) is an anionic detergent, meaning that its molecules have a net negative charge. Above 1 mM SDS concentration, most proteins are denatured by negative charges on the SDS molecule and, concurrently, are strongly attracted to the anode. Adding SDS to agarose gel electrophoresis (SDS-AGE) or polyacrylamide gel electrophoresis (SDS-PAGE) separates proteins mainly by molecular mass instead of charge. SDS-AGE may be used clinically for proteinuria characterization. SDS-PAGE provides increased resolution, but is time- and labor-consuming for routine applications, and is therefore used for research purposes.[12]

Capillary Zone Electrophoresis

Compared to the previously described techniques, the principle of molecular separation used in capillary zone electrophoresis (CZE) is quite different. Separation of peptides, proteins, nucleic acids, and other compounds occurs in a free solution by the simultaneous and opposite effect of an electrical field and electroendosmotic forces that develop inside a small capillary tube, usually a narrow-bore fused silica capillary tube. A strong, alkaline (pH = 10) borate buffer is typically used which results in most proteins being negatively charged. The applied potential electrical difference between the two ends of the capillary tube creates the electrophoretic force. It attracts the positively charged molecules toward the cathode and the negatively charged molecules toward the anode. Concurrently, the internal surface of the capillary tube is negatively charged. The positively charged cations close to the capillary wall are attracted to the negatively charged cathode, dragging the buffer solution in the same direction. This electroendosmotic

flow exceeds the force of the electrical field, leading to a net cathodal migration of the protein fractions (Figure 144.1). The migration of negatively charged proteins (e.g., albumin), which are electrically attracted to the anode, is slowed in correlation with their charge:mass ratio.[3]

CZE, a high-resolution electrophoretic technique, fractionates serum proteins in more than six peaks in most species. This modified technique has a high capillary surface to volume ratio, which efficiently dissipates excessive heat generated when a high voltage of about 10,000 V is applied, thus decreasing the Joule effect. Usually, equipment for CZE is more expensive than the older techniques, but a significantly faster throughput of 4–6 minutes versus 1 hour, small sample volumes, and the ability to process one sample at a time with complete automation result in significant cost savings.[28] Moreover, CZE does not require densitometric analysis, since a UV detector that measures 200–220 nm absorbance quantifies separated proteins. Therefore, there is also no need for special stains, reducing toxic waste and cost.

Immunoelectrophoresis

Immunoelectrophoresis combines electrophoresis and immunodiffusion. After electrophoretic separation, monoclonal or polyclonal antibodies are applied which bind a specific protein or set of proteins. This technique was first used to identify individual proteins migrating in the different globulin fractions.[1] Identification of immunoglobulin monoclonal components found in multiple myeloma is currently a commonly utilized diagnostic application.

There are several available immunoelectrophoretic techniques, which are uncommonly used for diagnostic purposes.[24] In the Grabar/Williams method (or immunofixation), after standard AGE, antibodies are added to a trough cut in the agar. Individual proteins bound to the added antibodies will diffuse toward one another and precipitin arcs will form. The immunosubtraction method may be used with capillary equipment. Immunoglobulins (usually identified by monoclonal peaks) are removed from the serum sample using antibody-coated beads specific for the target immunoglobulin. Then, CZE is repeated on the absorbed serum. If the coupled antibody removed the target protein, treated samples will show subtraction (i.e., the monoclonal peak will disappear). The limitation of immunoelectrophoresis in veterinary medicine is the relative lack of cross-reacting and validated antibodies for domestic animals at this time.[28]

Isoenzyme Electrophoresis

The total serum activity of clinically relevant enzymes, e.g., creatine kinase (CK), lactate dehydrogenase (LDH), and alkaline phosphatase (ALP), is the sum of different isoenzymes originating in different tissues. Specifically, there are three predominant CK isoenzymes deriving from skeletal muscle (CK-MM), heart (CK-MB), and brain (CK-BB), five LDH isoenzymes with different tissue specificity (LDH-1 to LDH-5), and at least three ALP isoenzymes including hepatic (L-ALP), bone (B-ALP), and intestinal (I-ALP). Isoenzyme electrophoresis identifies the isoenzyme(s) contributing to the total increased serum enzyme activity which can provide information regarding the underlying tissue-specific pathogenesis of disease (e.g., increased CK-MB in myocardial disease in certain mammals).[30]

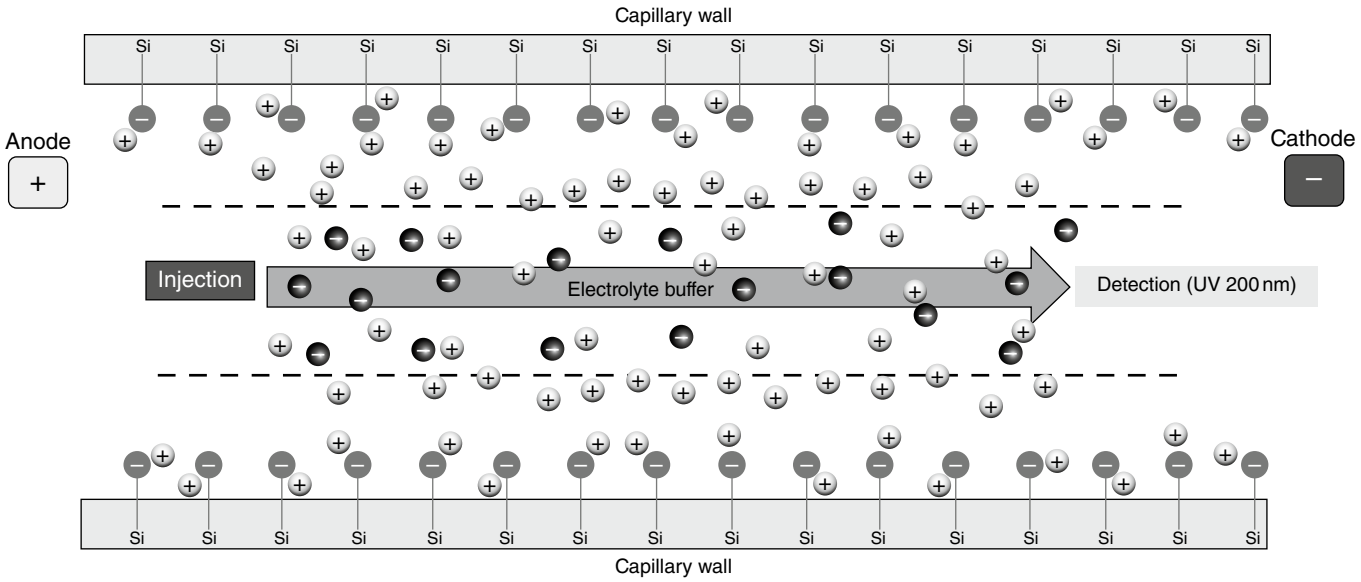


FIGURE 144.1 Capillary zone electrophoresis. The negatively charged silica surface of the capillary wall (Si) attracts positive ions from the buffer. An electrical double layer at the capillary wall–buffer interface is created, resulting in a net electroosmotic flow of the buffer toward the cathode. Protein migration velocity depends on the charge: negatively charged molecules will move more slowly than positively charged molecules.

ACUTE-PHASE PROTEIN DETECTION

The acute-phase proteins (APP) change their basal blood concentration during innate, acute inflammatory responses. The classic APP proteins and APP pattern vary between species and not all APPs have clinical relevance, especially across species (Table 144.1).[10,29,31] Several validation studies have expanded the use of APP in veterinary laboratories.[7,22,25,33,35] The following proteins represent the most common APPs used as diagnostic markers in domestic animals (Table 144.2).

Fibrinogen (Factor Ia)

Fibrinogen concentration measurement requires citrated plasma, as serum does not contain coagulation factors. It is mainly used in equids and cattle and is found in the beta region of the electrophoretogram. Heat precipitation is the simplest method to estimate fibrinogen concentration, but is not specific for fibrinogen. As fibrinogen concentration is second only to albumin, the heat precipitation method does approximate fibrinogen concentration. Briefly, the difference in total protein concentration, measured by refractometer, is calculated between two plasma samples, one at room temperature and one heated to 56–58 °C. However, this method is not sensitive enough to detect hypofibrinogenemia.[36] The Clauss modified thrombin clotting time evaluates the conversion of fibrinogen to fibrin and is measured in time to clot, not in concentration. A fibrinogen dysfunction or lower fibrinogen concentration results in a prolonged time to clot formation. Immunotesting is the most accurate assay to quantify fibrinogen if species-specific antibodies are available (see Chapter 88).

TABLE 144.1 Acute-phase proteins (APPs) in common domestic animals.

Species	Positive APPs	Negative APPs
	Major and moderate APPs	
Dog	CRP*; SAA; Fib; Hp	Alb; TRF; TTR; RBP; PON-1
Cat	SAA*; AGP; Hp	Alb; TRF; TTR; RBP; PON-1
Horse	SAA*; Fib; Hp	Alb; TRF; TTR; RBP; PON-1
Ruminants	Hp*; SAA	Alb; TRF; TTR; RBP; PON-1
Pigs	Hp; SAA; pigMAP*	Alb; TRF; TTR; RBP;
Birds	TRF*; Hp; AGP; SAA; Fib	Alb

AGP = α-1-acid glycoprotein; Alb = albumin; CRP = C-reactive protein; Fib = fibrinogen; Hp = haptoglobin; pigMAP = porcine major acute-phase protein; PON-1 = paraoxonase-1; RBP = retinol-binding protein; SAA = serum amyloid A; TRF = transferrin; TTR= transthyretin.
* Characteristic APP for the species.
Sources: Cray et al. [9]; Eckersall[12]; O'Reilly and Eckersall[29]; Petersen and Nielsen[31]; Rossi et al.[33]; Ruggerone et al.[35]

Serum Amyloid A

Serum amyloid A (SAA) is typically used to investigate inflammation in horses and cats, whereas its inconsistent increase during acute-phase response (APR) in dogs has limited the use of SAA as a marker in this species.[10,29] All analytical methods for SAA quantification that are commercially available and validated for use in veterinary species are antibody-based, including ELISA, turbidimetric immunoassay (TIA), and latex agglutination turbidimetric (LAT) immunoassay.[7,25] Most of these assays are designed for human SAA identification, using both monoclonal or polyclonal antibodies. However, several studies have validated ELISA, TIA, and LAT immunoassays in a variety of animal species, including cats, horses, dogs, and exotic species as well.[7,8,18] Many assays have been adapted for automated clinical chemistry analyzers, resulting in decreased time and cost and, therefore, in increased use in veterinary diagnostic laboratories. Recently, a monoclonal immunoturbidimetric SAA assay with an extremely broad working range has been validated for use in horses.[23]

C-Reactive Protein

C-reactive protein (CRP) is a major APP in dogs and people, and a minor APP in horses and pigs; whereas in cats and cattle, there is no evidence of a significant CRP concentration increase during inflammation.[10,31] For this reason, validation studies to transfer CRP assays used in human medicine were completed, mainly for use in canine species.[22,25,29]

Similar to other APPs, immunoassays such as ELISA, immunoturbidimetry, slide/capillary reversed passive latex agglutination tests, and time-resolved immunofluorometric assay (TR-IFMA) are used to measure CRP concentration in body fluids. The lack of cross-reactivity using human-based assays hampered the extensive use of CRP, but in recent years the identification of both cross-reacting antihuman polyclonal antibodies and canine-specific methods suitable for automation have enabled the clinical use of CRP in dogs.[22,32] A modified immunoturbidimetry assay for measurement of low or ultralow concentration CRP, referred to as high-sensitivity CRP (hsCRP) assay, has been validated using canine serum.[21]

α-1-Acid Glycoprotein

Unlike SAA and CRP measurement, a validated automated immunoassay for α-1-acid glycoprotein (AGP) detection in domestic species is still not available at this time. Species-specific antibodies are employed for AGP measurement in dogs, cattle, and cats, in which AGP reacts as a major APP. The radial immunodiffusion (RID) technique is the only validated method in veterinary medicine and is manually performed. RID is time-consuming and has increased imprecision in comparison to automated analyses.[28] A higher individual and analytical coefficient of variation was detected for AGP measurement with RID compared to other APPs in

TABLE 144.2 Main serum proteins of domestic animals and related peaks.

Electrophoretic zone	Proteins	Function
Prealbumin (some reptiles and birds)	Transthyretin	Hormones and vitamin transport
Albumin	Albumin (rare bisalbuminemia)	General transport, oncotic pressure maintenance
	Need to	
	Triglycerides	*Lipemic samples*
α-1-globulins	α-1-antitrypsin	Trypsin inhibitor
	α-1-acid glycoprotein	Acute-phase protein
	α-1-antichymotrypsin	Chymotrypsin inhibitor
	α-1-fetoprotein	Unknown
	α-1-lipoprotein	High-density lipoprotein
	Transthyretin (most mammals)	Hormones and vitamin transport
	Triglycerides	*Lipemic samples*
α-2-globulins	α-2-macroglobulin	Protease inhibitor
	Haptoglobin	Acute-phase protein
	Ceruloplasmin	Acute-phase protein
	Fibronectin	Wound healing
	Pre-β-lipoprotein	Very-low-density lipoproteins
	Retinol-binding protein	Binds vitamin A
	Vitamin D-binding protein	Binds vitamin D
β-1-globulins	Transferrin	Binds iron
	β-lipoprotein	Low-density lipoprotein
	Immunoglobulin A	Immunity
	Hemoglobin	*Hemolytic specimens*
β-2-globulins	Immunoglobulin M	Immunity
	C_3 complement	Host defense
	C_4 complement	Host defense
	Fibrinogen	Plasma specimens
γ-globulins	Immunoglobulin M	Immunity
	Immunoglobulin G	Immunity
	C-reactive protein	Acute-phase protein

Sources: Eckersall[12]; Harr[17]; Keren.[24]

dogs.[26] Immunoturbidimetry for feline and canine AGP measurement has been developed and validated on a research basis, but commercially available kits are still not accessible.[2]

Haptoglobin

Haptoglobin is used to monitor the APR mainly in ruminants, where its basal level is considerably lower than in inflammatory conditions.[9] Haptoglobin measurement may be performed using spectrophotometry, either automated or using multiwell ELISA plates, or by immunoassays. The first documented veterinary method employs detection of hemoglobin's peroxidase activity in the hemoglobin–haptoglobin complex and has been validated in a variety of domestic and wild ruminants.[11] Major limitations of this assay are significant interference with hemolysis such that hemolyzed samples should not be analyzed and a short shelf-life of reagents. Immunoreactive methods are typically minimally affected by hemolysis and use antisera against haptoglobin to produce a precipitated product that is measured by RID, nephelometry, or turbidimetry. However, antibodies against human haptoglobin used in commercially available kits may have decreased cross-reactivity in some animal species. Antibody lots must therefore be validated in each species to be investigated in the laboratory. In cows, a novel automated species-specific immunoturbidimetric assay has acceptable intra- and inter-assay coefficient of variation (CV)s, accuracy, and limit of quantification. Immunoassay using species-specific monoclonal antibodies has enhanced the specificity and sensitivity of haptoglobin detection in hemolyzed specimens.[4]

Negative Acute-Phase Proteins

Proteins that decrease in concentration during inflammation are defined as negative acute-phase proteins.[12] Albumin and transferrin (TRF) are considered major negative APPs in many species (except birds)[34] and can be measured by spectrophotometry on wet chemistry analyzers. Specifically, bromocresol green is commonly used for albumin measurement in animals, although this nonspecific dye-binding technique has low analytical sensitivity and specificity. The EPH absolute value has superior accuracy, and immunonephelometry is still considered to be the gold standard.[37]

Serum transferrin is measured as total iron-binding capacity (TIBC) by spectrophotometry using the saturation/precipitation method (see Chapter 27). Briefly, a known amount of excess oxidized, ferric ion (Fe^{3+}) is added to the sample to saturate all available transferrin sites and unbound Fe^{3+} is precipitated with magnesium carbonate. The amount of remaining iron bound to transferrin is then determined. The difference between the unsaturated iron-binding capacity (UIBC) and the initial amount of ferric iron is the TIBC. Lipemia and the presence of free iron in serum (e.g., during hemolysis and iron overload) may increase TIBC value. The accuracy of TIBC measurement as an index of iron deficiency is limited by the individual diurnal variation in plasma iron concentration.[19]

There are numerous other minor negative APPs including retinol-binding protein (RBP) and transthyretin (TTR), but assays for their measurement in domestic species have gained interest for research purposes only. Recently, paraoxonase-1 (PON-1), a high-density lipoprotein (HDL)-bound enzyme produced by the liver and involved in antioxidant processes, has been proposed as a negative APP based on its decreased activity during inflammation in people and animal species.[33,35] Spectrophotometry can be used to measure PON-1 activity and its ability to hydrolyze different substrates, most commonly paraoxon, phenyl acetate, 5-thiobutil butyrolactone, and 4-nitrophenyl acetate. The presence of several substrates, possible gene polymorphisms, and lack of control sera are current limitations when using PON-1 in clinical settings.[5]

QUALITY ASSURANCE FOR ELECTROPHORESIS AND ACUTE-PHASE PROTEIN DETECTION

Specific quality assurance (QA) guidelines for protein electrophoresis and acute-phase protein detection in veterinary laboratories are not available in the literature at this time. However, general recommendations about the preanalytical phase, method validation, reporting, reference intervals, etc., are defined in the American Society for Veterinary Clinical Pathology (ASVCP) Quality Control Guidelines and may also be applied to the methods described in this chapter.[14,15,39]

Preanalytical Phase

Selection of Suitable Specimens

Electrophoresis with AGE, CAE, and CZE is commonly performed on serum or plasma samples, but any body fluids containing an adequate protein concentration may be analyzed. Most EPH assays require small sample volumes (10–100 μL) allowing protein quantitation in small, volume-restricted animals. Conventional EPH techniques, such as AGE and CZE, have an analytical sensitivity of 0.25–0.50 g/L and bands of <1 g/L are visible but cannot be accurately quantified.[38] Specimens containing very low protein concentrations are therefore not suitable for EPH techniques used in clinical settings and require higher resolution techniques. Other biological fluids such as effusion, synovial fluid, and milk may have a sufficient protein content for accurate quantitation. Other interfering factors in these specimens may affect the analytical accuracy and specificity. Milk, some effusions, and synovial fluid may require hyaluronidase (or similar enzyme) addition to reduce the fluid viscosity. Low-protein fluids, e.g., urine and cerebrospinal fluid, may produce undetectable bands unless more sensitive techniques are employed (e.g., SDS-AGE/SDS-PAGE).[20]

APP measurement in veterinary medicine has been successfully performed in numerous body fluids, including milk, urine, synovial fluid, effusion, cerebrospinal fluid, and saliva. Saliva represents an optimal specimen when a noninvasive sampling approach is required. Method-specific reference intervals or decision thresholds must be established in the different fluids prior to clinical use, and after the analytical method has been analytically validated in that species.[6,14]

Interfering Substances

Fibrinogen in plasma samples migrates in the beta–gamma region and may interfere with EPH interpretation using AGE and CZE.[13] EDTA or heparinized plasma should be avoided if fibrinogen will interfere with needed diagnostic circulating protein evaluation in mammals. Plasma EPH is standardly used in avian and reptile species due to irreversible gelling of a percentage of serum samples.[27] Several methods to remove fibrinogen from plasma have been proposed, including ethanol precipitation and reptilase treatments.[13] Hemoglobin from hemolytic serum samples results in a pointed beta-1 peak; whereas high triglyceride levels may cause increased "albumin" and α-globulin bands. Bilirubin in icteric samples does not modify electropherograms using AGE, but increased albumin bandwidth due to increased bilirubin concentration has been reported using CZE.[3] In humans, intravenously administered iodinated, radio-opaque agents and antibiotics have been reported to generate abnormal spikes in albumin, α-1-, and β-globulin fractions using CZE.[3]

Lipemia, hemolysis, and icterus are not reported to affect immunoturbidimetric assays for CRP, SAA, and haptoglobin measurement.[4,22] Haptoglobin measurement by detection of peroxidase activity and colorimetric assays is decreased by free hemoglobin in hemolyzed samples, whereas lipemia and hyperbilirubinemia have no effect on Hp concentration.[11]

ANALYTICAL PERFORMANCE

A laboratory QA program should verify analytical precision and accuracy of all method components, including preanalytical and postanalytical factors, instruments, and reagents. Instruments must maintain constant temperature, voltage, and amperage to ensure result reproducibility. If instrument parameters are not constant, the laboratory should contact the manufacturer for repair or replacement of the instrument. Buffer pH must also be constant and, if stored, should be regularly checked with a quantitative pH meter. In contrast to human medicine, where specific standards and control materials are provided for this purpose, the peculiarity of electrophoretograms in different species may require the use of species-specific standards to assess EPH instruments' analytical performance. Nevertheless, a human-based QC material can be used as the standard which should be run as one lane on all gels to confirm that the equipment is performing within specifications. A species-specific control sample may be included with each run, to be used for statistical QC protocols.[28]

The inter- and intra-assay imprecision (coefficient of variation) of SPE reported in veterinary species of <3% for albumin and γ-globulin concentrations and <10% for the less represented fractions (e.g., α-1-globulins) are acceptable for diagnostic use. CZE is more reproducible (has a lower CV) than CAE and AGE.[16] Stored, frozen, patient samples may be used in repeatability experiments to verify that the assay's imprecision is acceptable. Accuracy assessment, without available species-specific standards of known concentration, may be achieved by serial dilution of normal and abnormal sera from species investigated by the laboratory. Dilutional effects must be considered for very low concentration serum proteins.[38]

Densitometry tracings should be evaluated to ensure that gates are correctly placed in protein troughs by the instrument's software. Protein fractions should be quantified in g/L after transformation of percentages using quantitative total protein concentration (using the biuret method as the established gold standard). As possible differences related to equipment settings and method may occur, laboratories should establish their own reference intervals.[15]

Multilevel standards for calibration and other QA are available for APP measurement.[25] Species-dependent, validated reagents should be employed, if available, to optimize results. The large variability in published reference intervals across veterinary laboratories requires establishment of internal reference intervals. Reference intervals reported in the literature should not be used by the laboratory or clinician, as their use may result in misdiagnosis. The known large variability in APP measurement begs for harmonization across laboratories.[25]

ELECTROPHORETOGRAM INTERPRETATION AND REPORTING

SPE identifies five to seven fractions, depending on the method. From the anodal to cathodal end, the peak order is albumin, α-globulin, β-globulin, and γ-globulin. Conventionally, the point halfway between the beginning and end of the curve corresponds to the limit between α-2- and β-globulins. The point halfway between the start of β-globulins and the curve's end is defined as the start of the γ-globulin region. This may be modified in pathologic electrophoretograms. Except for albumin, each peak represents numerous different proteins that can be further identified by other techniques. The precise composition of each peak is known in humans and one could speculate that the human data may be similar to many animal species, though likely not exact (Table 144.2).

SPE interpretation must be based on visual inspection of the electrophoretogram, the protein fraction absolute values, and possibly the gel banding pattern, especially to detect QA issues. The use of relative values (%) must be avoided, as it may lead to misdiagnoses.

Normal Electrophoretic Patterns in Domestic Animals

Albumin is the predominant fraction found in normal electrophoretograms. The number of globulin fractions varies with the species and method used, but should be standardized for the different species in the laboratory's clientele. Both α- and β-globulin regions may be subdivided into α-1- and α-2- or β-1- and β-2-globulins, respectively. This latter separation is less evident in ruminants and not possible when using plasma. In healthy dogs and cats, a further subfractioning of γ-globulins may be warranted. In most nonmammalian species, a band more anodal than albumin is often observed and therefore referred to as prealbumin.[27] This band has been shown to represent transthyretin as well as other possible α-globulins and is due to the varying migration of albumin in these species.[17] Examples of normal electropherograms are found in Figure 144.2.

Pathologic Electrophoretic Patterns

SPE has low diagnostic specificity for individual diseases. Therefore, confirmatory tests are required post hoc. Common electrophoretic abnormalities observed in diseased, domestic animals are provided (Table 144.3 and Figure 144.3).



FIGURE 144.2 Electrophoretograms from healthy animals. (a) Horse (AGE); (b) Cow (AGE); (c) Dog (AGE); (d) Cat (AGE); (e) Goat (AGE); (f) Rabbit (AGE); (g) Parrot (CZE); (h) Lion (AGE). From left to the right, peaks correspond to albumin, α-1-globulins, α-2-globulins, β-1-globulins, β-2-globulins, γ-globulins.

TABLE 144.3 Electrophoretic patterns and associated pathological conditions.

Electrophoretic pattern	Pathological condition
Increased α-1- and α-2-globulins, decreased albumin and transferrin	Acute inflammation
Increased α-2-, β-2-, and γ-globulins (C3 band)	Subacute inflammation
Increased α-1- and α-2-globulins, and polyclonal γ-globulin increase	Chronic inflammation, infectious diseases (e.g., feline infectious peritonitis; leishmaniasis in dogs)
Very low albumin, transferrin, and γ-globulin with increased α-2- and β-1-globulins	Nephrotic syndrome
Decreased albumin and total proteins with a polyclonal increase in γ-globulins ± β–γ bridging	Liver disease/cirrhosis
Decreased total protein, albumin, and γ-globulin	Mild protein loss
Polyclonal increase in γ-globulins with oligoclonal banding	Chronic infections, autoimmune diseases, lymphoproliferative processes
Hypogammaglobulinemia	Immune deficiency
Monoclonal gammopathy	Lymphoproliferative process involving B cells and plasma cells
Biclonal gammopathy	Possible presence of two clones of monoclonal immunoglobulins (neoplastic)
Oligoclonal gammopathy	Ehrlichiosis in dogs

Sources: Keren[24]; Moore and Avery[28]; Tate et al.[38]

Inflammation

APPs migrate in the α-1-, α-2-, or β-2-fractions (Table 144.2) and acute inflammation may produce a spike in these regions. Haptoglobin contributes to a significantly increased α-2-globulin concentration, especially in ruminants, due to its higher concentration compared to other characteristic APPs in many species. In chronic inflammation, albumin concentration may decrease and a polyclonal or apparent monoclonal gammopathy may appear.

Polyclonal Gammopathy

The γ-region is predominantly composed of IgG-type immunoglobulins. Polyclonal gammopathies are indicated by a broadly increased γ-band that may be caused by any sterile or septic inflammatory process including autoimmune disease, but are commonly caused by infectious disease (e.g., canine leishmaniasis, feline infectious peritonitis). A subfractioning of γ-globulins may be warranted in diseases causing hypergammaglobulinemia. A sharper, monoclonal-appearing peak may be observed in some infections (e.g., canine ehrlichiosis), referred as oligoclonal gammopathy. This must be differentiated from true monoclonal gammopathy.

Monoclonal Gammopathy

Monoclonal gammopathies are characterized by a homogenous formation of paraproteins that present as a sharp, well-defined single peak. By definition, the peak should have the same width or be narrower than the albumin peak. Despite the name, monoclonal components may also be observed in the β-fraction and, rarely, in the α-2-fraction.[28] Differential diagnosis for this appearance includes multiple myeloma, extramedullary plasma cell neoplasia, Mott cell neoplasia, other B cell lymphoma, and ehrlichiosis.

Nephrotic Syndrome

The relative changes in protein proportions found in nephrotic syndrome are the perfect example of why only absolute protein concentrations should be used. Urinary loss of albumin and small proteins results in hypoalbuminemia and proportional shift in the A:G ratio. Larger proteins migrating in the α-2- and β-regions (e.g., α-2-macroglobulin, lipoproteins, and many others) remain in circulation and their relative proportion (%) increases, generating apparent spikes on the electrophoretogram. Hypoalbuminemia should be noted and validated reference intervals for absolute protein concentrations then used to assess for abnormality in other banding regions to prevent incorrect interpretation.[28]

β-γ Bridging

A lack of demarcation between β-2- and γ-fraction, results from increased concentrations of IgA, IgM, and/or IgG and is usually associated with severe infections. In the presence of severe hypoalbuminemia, it is also the hallmark of chronic liver failure/cirrhosis due to hepatitis.[28]

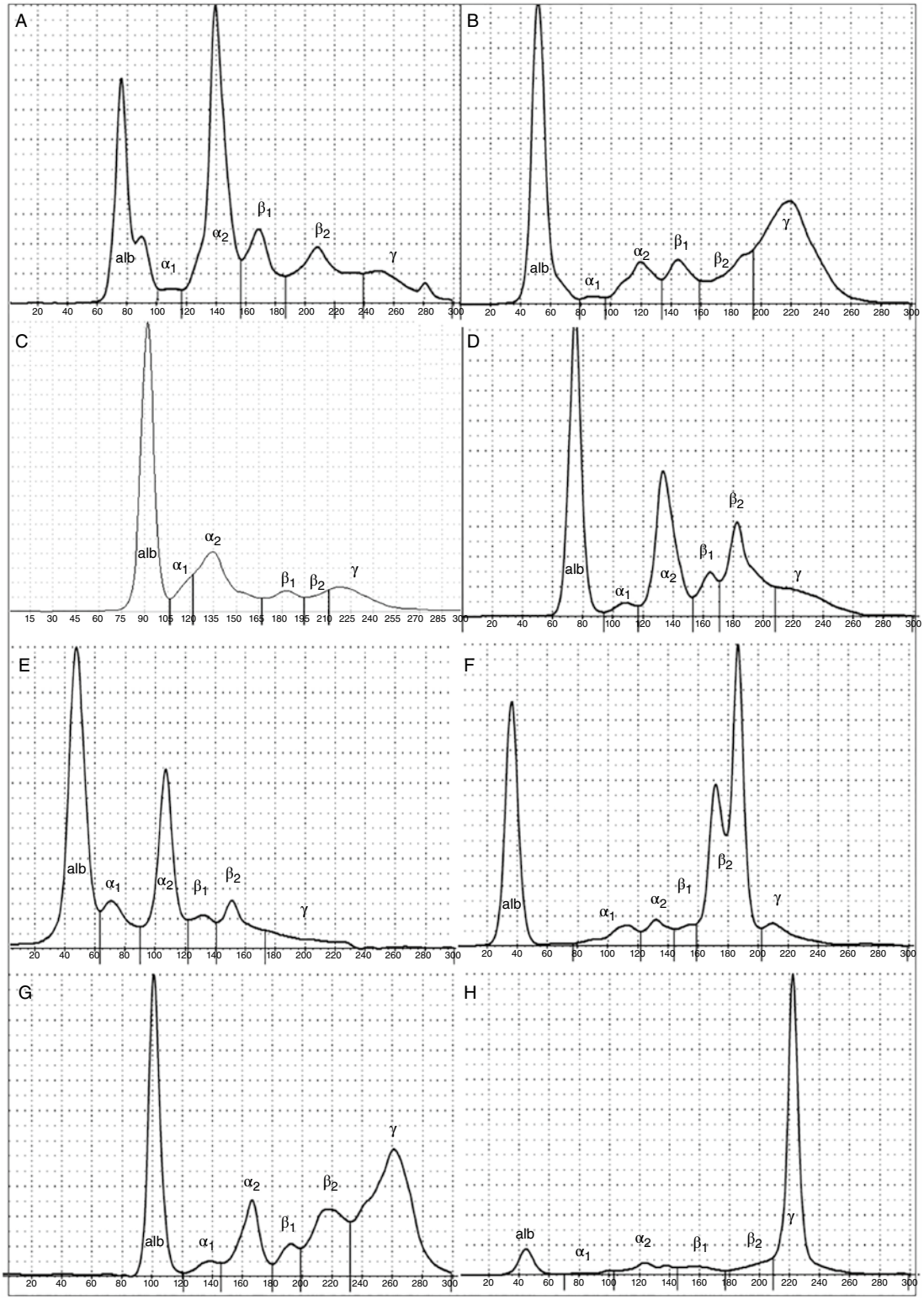


FIGURE 144.3 Electrophoretograms from diseased animals. (a) Horse with nephrotic syndrome (AGE), known absolute protein concentrations are required for interpretation of the graph; (b) Horse with chronic hepatitis (AGE) representing a polyclonal gammopathy; (c) Cow with acute inflammation (AGE), the increased α2 globulin concentration is likely Hp; (d) Dog with proteinuria (AGE), known absolute protein concentrations are required for interpretation of the graph; (e) Python with anorexia (AGE) with increased α2 globulin concentration which could be due to oviparity or acute phase proteins; (f) Cat with a plasma cell tumor (AGE) representing a possibly biclonal gammopathy; (g) Dog with leishmaniasis (AGE) representing a polyclonal gammopathy; (h) Cat with multiple myeloma (AGE) representing a monoclonal gammopathy. From left to the right, peaks correspond to albumin, α-1-globulins, α-2-globulins, β-1-globulins, β-2-globulins, and γ-globulins.

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CHAPTER 145

Molecular Diagnostic Techniques

ROBERT J. OSSIBOFF

Sampling and Preanalytical Considerations
Nucleic Acid Extraction
Tenets of PCR
  End-Point PCR
  Nested and Heminested PCR
  Reverse Transcription (RT)-PCR
  Real-Time (Quantitative) PCR
  Rolling Circle Amplification
Sequencing Modalities
In Situ Hybridization

Acronyms and Abbreviations

CT, cycle threshold; dNTP, deoxyribonucleotide triphosphate; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; FFPE, formalin-fixed paraffin-embedded; ISH, in situ hybridization; mRNA, messenger ribonucleic acid; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; RCA, rolling circle amplification; RNA, ribonucleic acid; RT-PCR, reverse transcription polymerase chain reaction.

The development and advances in molecular diagnostic techniques have rapidly accelerated the discovery, detection, and characterization of infectious agents in the last 35 years. Since the inception of PCR technology in the early 1980s,[17,19] the field of molecular diagnostics has evolved rapidly while the costs and time associated with running such tests have decreased dramatically. Projects that may have originally constituted doctoral dissertations can now be completed in short order by relative neophytes in the laboratory.

As molecular diagnostics will routinely guide the diagnosis and treatment of disease in animals, it is imperative that clinicians and pathologists understand the basic tenets of PCR and common variations of the technique. This chapter will provide an overview on the basics of PCR and advanced molecular diagnostics to serve as a primer appropriate for veterinary clinicians.

SAMPLING AND PREANALYTICAL CONSIDERATIONS

The most sensitive and specific diagnostic assays are still unable to compensate for poor sample collection and storage. As such, consideration should always be given to the ideal sample for testing, the appropriate materials needed for collection and submission, storage conditions before and during transit, and transit time. It is always recommended to refer to the diagnostic laboratory performing the testing for any specific sample recommendations. Certain laboratories may recommend the use of one swab type over another. Some laboratories may also recommend the use of nucleic acid extenders, such as RNAlater®, for certain assays, while others may wish to avoid the material. In general, maintaining samples frozen or on ice will not adversely affect nucleic acid extraction. However, allowing samples to warm to ambient or higher temperatures for extended periods of time may result in nucleic acid degradation. This is particularly true if RNA targets are desired, as RNAses are ubiquitous and will rapidly cleave RNA into small fragments that may decrease assay diagnostic sensitivity.

Preserved samples can pose unique challenges for molecular testing. In general, samples preserved fresh in ethanol will serve as an excellent source of nucleic acid. The same is not usually true for samples preserved in formalin. Formalin chemically cross-links proteins and nucleic acids by two distinct and extremely effective methods.[18] Cross-linking results in substantial DNA fragmentation, and the longer a sample is maintained in formalin, the more dramatic the effect can be. Formalin

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can also directly modify nucleic acids.[28] Formalin storage of samples for ≥72 hours will greatly reduce detection of PCR amplicons of 300–350 nucleotides or more. While such samples can still be useful for real-time PCR, their utility for end-point PCR is limited. However, molecular assays can be designed with formalin-fixed paraffin-embedded (FFPE) tissues in mind to maximize the diagnostic potential for some agents. Another processing technique that can have negative effects on downstream nucleic acid analysis is acid decalcification, as may be routinely performed on samples containing mineral or bone. If decalcification is required, chelating agents, such as EDTA, should be used. For blood samples, lithium heparin can have significant inhibitory downstream effects in molecular testing, and other common anticoagulants, such as EDTA, are recommended.[24] Unstained blood smears and cytologic impressions can also be a viable source of nucleic acids for molecular testing, depending on the desired testing.[3]

The last major preanalytical consideration, if applicable, is the selection of an appropriate diagnostic laboratory. There are many veterinary molecular diagnostic laboratories in both the academic and private sectors. Not all are created equal or have the same commitment to quality assurance and quality control. It is important to confirm that the laboratory you are utilizing runs appropriate controls, sequences samples for confirmation when appropriate, and can provide interpretation of results in complex cases. Moreover, not all tests will have the same sensitivity and specificity, and when dealing with veterinary species, it is always important to verify the test has been validated in the host in question. Establishing a professional relationship with the laboratories that you utilize for testing will go a long way in meaningful discourse on the findings and limitations in test results.

NUCLEIC ACID EXTRACTION

The extraction of nucleic acids from samples has been greatly facilitated by the ready availability of a variety of commercial extraction kits. Kits for manual and automated extractions of DNA, RNA, or both DNA and RNA, are available for a variety of input material, including blood, swabs, fresh tissues, FFPE tissues, fecal material, tissue culture cells, and other organic material. While extraction biology can vary widely, column-based extraction methods are the most commonly available.[23] Briefly, in column-based extractions, samples are lysed, nucleic acids are bound to packed silica columns, washed with various (often proprietary) formulations of salts and detergents, and then eluted in water or dilution buffer. Depending on the nature of the column, the extractions can be optimized for the concentration of RNA or DNA. Other critical steps of the extraction process, particularly of tissue samples, include lysis and digestion; these are usually achieved by some combination of mechanical homogenization and enzymatic digestion. For certain tissues very rich in nucleic acids (such as a liver and spleen), care should be taken to limit the size of the tissue sample extracted so as to not overwhelm the column with nucleic acid. The addition of additional enzymes, such as lyticase, can also facilitate the extraction of infectious agents with a cell wall, such as fungi.[10] Following nucleic acid extraction, the quantity and quality of extracted nucleic acids should be quantified spectroscopically or fluoroscopically. Extracts from samples containing non-host organic material (such as feces or environmental samples) may have carryover of PCR inhibitors that can interfere with downstream PCR, and in certain samples dilution of eluted nucleic acids is important to decrease this potential inhibition.[21] Lastly, it is always recommended to perform an extraction negative control concurrent to sample extractions. Extraction negative controls should be tested alongside extracted samples to rule out contamination during the extraction process.

TENETS OF PCR

While numerous variations of PCR exist, the central tenets of the technique are the same. PCR is cyclical and exponential amplification of nucleic acids by the action of thermostable DNA polymerases. These polymerases engage short oligonucleotides (primers) bound to complementary sequence in single-stranded DNA. With this cyclical amplification, target sequences present at incredibly low levels can be amplified to a point where they can be detected visually by gel electrophoresis and can be sequenced for analysis.

The required components of a typical PCR include the following: (1) DNA-dependent DNA polymerase; (2) a forward (sense) primer; (3) a reverse (antisense) primer; (4) deoxyribonucleotide triphosphate (dNTPs); (5) buffer for optimal polymerase activity; (6) template nucleic acids. The DNA-dependent DNA polymerases utilized in PCR were originally discovered in bacteria evolved to live at very high temperatures (geothermal vents and springs). Taq polymerase was originally isolated from the bacterium *Thermus aquaticus*.[5] Pfu DNA polymerase, a high-fidelity polymerase with proofreading activity also commonly used in PCR, was isolated from *Pyrococcus furiosus*.[15] The thermostable nature of these polymerases is essential to resist denaturation at the high temperatures necessary to promote the separation of double-stranded DNA into single strands. While the components necessary for PCR can be purchased separately, commercial master mixes containing all necessary elements other than primers and template DNA are commonly available.

Primers are the most important component of PCR in conferring assay sensitivity and specificity. Primer design, while greatly assisted by the availability of commercial software, is a process that combines science, art, and luck. There are many factors to consider in primer design, including but not limited to length, guanine and cytosine (GC) content, and secondary structure potential.[6] Degenerate primers can be designed

to decrease the specificity of a PCR. This can be incredibly useful if designing an assay to detect multiple strain or species variants of a nucleic acid target. In degenerate primers, at select positions multiple, mixed bases (such as an adenine [A] or a guanine [G]) are present in the primer mixture. While the decrease in specificity resultant of such degeneracies can increase the diversity of preferred targets for PCR, it can also increase the amplification of unwanted, nontarget sequences.[13]

Appropriate controls are necessary for accurate interpretation of PCR results. Assay positive controls as well as both assay and extraction negative controls are all worthwhile running for each batch of samples tested. Sources for potential positive controls include previously confirmed positive extractions, gel-extracted amplified nucleic acids from a prior assay, or commercially synthesized artificial controls (oligomers, gBlocks®, or plasmids). There are multiple benefits of using artificial controls, including the introduction of unique novel sequence that can be screened for to rule out laboratory sample contamination and accurate assessment of copy number for control curves.

End-Point PCR

End-point (also referred to as conventional, consensus, or qualitative) PCR can amplify short fragments of nucleic acids from a DNA template (Figure 145.1a,b). The components of an end-point PCR reaction are exactly as described in the earlier text, and include polymerase, primers, dNTPs, and template DNA. A typical end-point PCR cycle consists of the following stages: (1) Initial denaturation (94–96 °C); (2) 30–45 sequential cycles of (a) denaturation (94–96 °C), (b) annealing (variable), and (c) elongation (72 °C); (3) Terminal elongation (72 °C); (4) Holding (4–12 °C). The denaturation stages promote the dissociation of double-stranded DNA into single strands. The annealing stage is the period when the primers bind to the template DNA. A variety of temperatures can be used for this step. A general rule of thumb when establishing a new assay is to use an annealing temperature 5 °C lower than the lowest melting temperature (T_m) of the two primers. However, there is a lot of flexibility with this temperature, and it is an important variable to alter in assay optimization. Unique techniques varying annealing temperatures with cycle, such as touchdown PCR which utilizes annealing temperatures higher than calculated primer melting temperatures, can sometimes be useful in increasing assay specificity and sensitivity.[11] The elongation phase allows for template transcription from the bound primer, and occurs at the optimal temperature for polymerase activity. Amplicons are visualized by gel electrophoresis, and additional testing, such as sequencing, is required to confirm specific amplification.

Nested and Heminested PCR

A variation on end-point PCR that can increase assay sensitivity and/or specificity is the addition of a second round of PCR amplification using unique primers complementary to sequence internal to the primers used in the initial round of PCR (Figure 145.1b). In a fully nested PCR assay, both the forward and reverse primer sequences are unique and complementary to sequence internal to the round one primers. In a heminested assay, one unique primer with complementary sequence internal to either the round one forward or reverse primer is coupled with the converse round one primer. One or more primers may be degenerate to decrease specificity. In both instances, the input template DNA for the round two reaction is a small aliquot of the round one reaction. The products require visualization by gel electrophoresis for confirmation of appropriate amplification.

Reverse Transcription (RT)-PCR

For RNA targets, such as the genomes of RNA viruses or mRNA, RNA must first be transcribed to DNA before it can be amplified by PCR. This initial reverse transcription is achieved by another enzyme, reverse transcriptase. The reverse transcriptase of Moloney murine leukemia virus (MMLV) is commonly utilized for this, and there are many commercially available kits that include MMLV RT as part of an RT-PCR master mix.[8] Reverse transcription of the input RNA can be achieved utilizing either target-specific reverse primers, random oligonucleotide hexamers, or, for the case of mRNA, by an oligomer of poly-deoxythymidine that binds to the polyadenylated tail.[2] The reverse transcription step is performed prior to the initial denaturation step of PCR, as the high temperature step will denature the thermolabile RT. The sample can then proceed on with either end-point PCR or real-time PCR.

Real-Time (Quantitative) PCR

In contrast to end-point PCR, which requires gel electrophoresis following the PCR assay to assess for the presence of amplified nucleic acids, real-time PCR monitors for PCR amplification at the end of each PCR cycle via fluorescence.[1] Though not uniformly the case, real-time PCR assays are often more sensitive than end-point assays. Amplicon target sizes are also generally much shorter and often in the range of 100–200 nucleotides. The components of a real-time PCR are similar to an end-point assay in that there is still a requirement for a forward and reverse primer, DNA polymerase, and input template DNA (Figure 145.1c,d). The fundamental difference is the addition of a fluorescent signal as well as a passive reference dye. The type of fluorescent signal depends on the type of real-time PCR assay, which will be discussed in more detail in the following text. The PCR cycle stages are altered slightly and consist of (1) initial denaturation (94–96 °C); and (2) 30–45 sequential cycles of (a) denaturation (94–96 °C) followed by (b) annealing/elongation (generally 55–60 °C).

Dye-based real-time PCR relies on the properties of a group of dyes, such as SYBR green, that emit a fluorescent signal only when incorporated into double-stranded

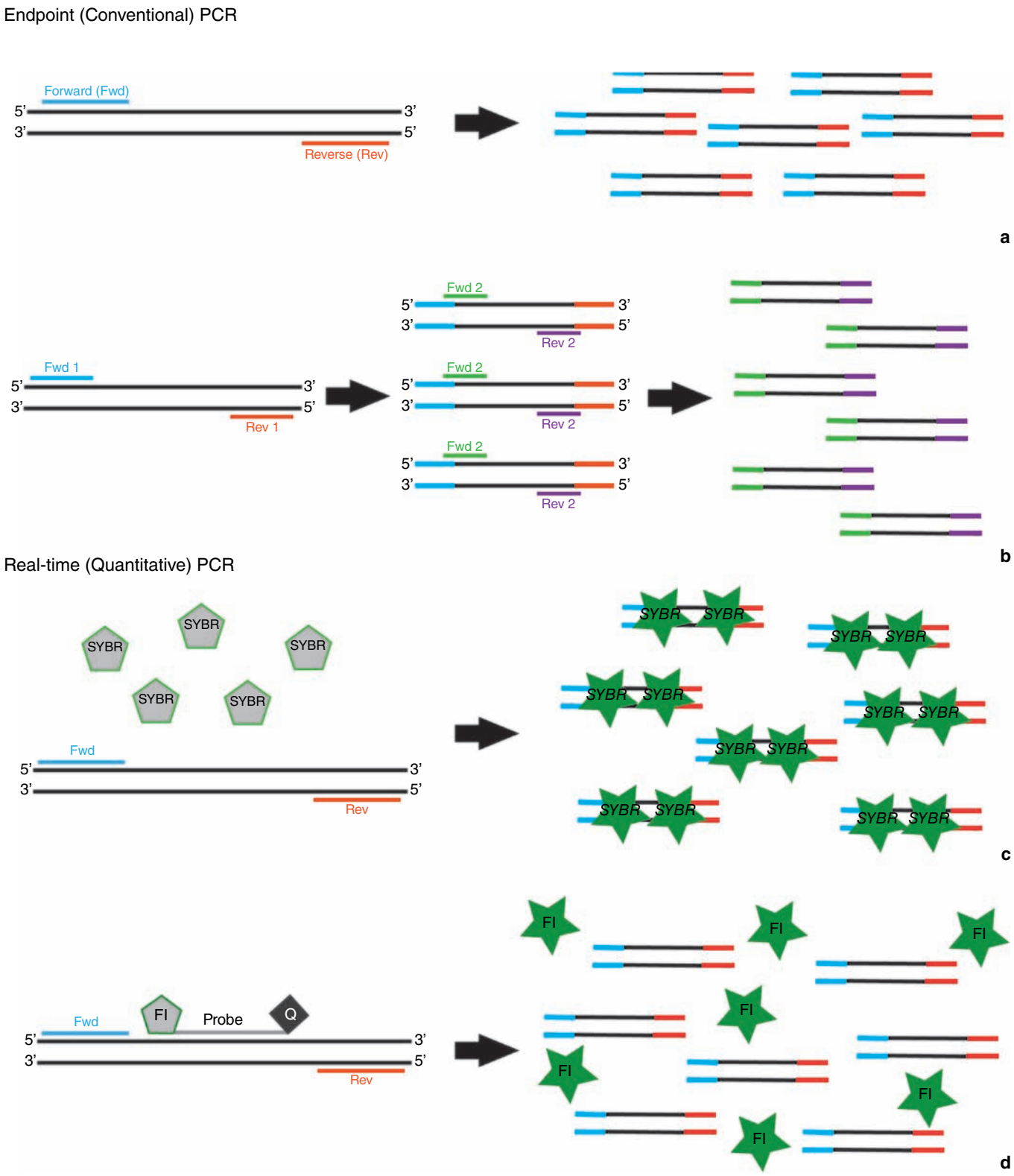


FIGURE 145.1 Schematic comparison of commonly used types of PCR. (a) and (b) End-point (conventional) PCR assays result in the exponential amplification of nucleic acid targets that must be visualized by gel electrophoresis. (a) In standard end-point PCR, a single pair of complementary oligonucleotides (primers) in a forward (sense) and reverse (antisense) orientation is utilized to amplify a known target; (b) In nested end-point PCR, a first round reaction is run using one pair of primers, and additional primers located internal to the round 1 primers are then utilized in an additional round of PCR to further amplify a known product. This technique can be used to both increase assay sensitivity and specificity. (c) and (d) Real-time (quantitative) PCR assays also result in exponential amplification of target nucleic acids, but the amplification is measured by changes in fluorescence over time. (c) In dye-based real-time PCR, the intercalation of dyes (SYBR) into double-stranded DNA results in the production of a fluorescent signal; (d) In probe-based real-time PCR, the liberation of a fluorophore (Fl) from a complementary probe and associated quencher molecule (Q) as a result of polymerase exonuclease activity results in the production of a fluorescent signal that increases with each round of PCR amplification in positive samples.

DNA.[29] At the end of each PCR cycle in a dye-based real-time PCR, the amount of incorporated dye is measured as a fluorescent signal. In a positive sample, the fluorescent signal will increase exponentially with each cycle due to an increase in the number of double-stranded DNA resultant from the PCR (Figure 145.1c). One major drawback of this style of real-time PCR is that the dye incorporation is nonspecific; if there is nontarget amplification, it will still be read as a positive signal. While techniques such as melt curve analysis can help differentiate true positives from false positives, it adds an additional step and can still complicate result interpretation.[25] The technique also has limited utility for the simultaneous detection of multiple target sequences.[9]

The advent of probe-based (also referred to as TaqMan or hydrolysis probe) PCR revolutionized real-time PCR.[12] In contrast to SYBR green real-time PCR, the fluorophore is attached to an oligonucleotide sequence complementary to the target DNA (Figure 145.1d). This probe also contains a quencher molecule that absorbs the energy emitted from the fluorophore. During transcription of the target DNA the bound probe is fragmented, liberating the fluorophore from the activity of the quencher molecule which allows for emission of the fluorescent signal. As in dye-based techniques, the fluorescent signal is measured at the end of each round of amplification. Not only does the addition of specific complementary probes increase the specificity of the assays, the availability of multiple fluorophore/quencher combinations with unique absorption/emission spectra allows for the detection of multiple target sequences in a single assay (multiplex real-time PCR).[27]

In both dye- and probe-based real-time PCR assays, positive samples exhibit a characteristic amplification curve, which will cross a baseline cycle threshold (C_t) value. The C_t value is inversely proportional to the amount of target nucleic acid in the initial sample. In other words, the more target nucleic acid, the earlier the amount of detected fluorescence will be higher than the threshold, and therefore the lower the C_t value. Positive samples may just be reported as positive, or may include a C_t value or count number. Count numbers are inferred from standard curves of known concentrations. Samples that do not cross the baseline threshold, cross the threshold beyond a certain cutoff (usually around 39–40 cycles), or exhibit atypical amplification curves are reported as negative or undetermined. Samples are often run in duplicate or triplicate to increase detection of weak positives.

Rolling Circle Amplification

Rolling circle amplification (RCA) is a technique useful for circular nucleic acid targets, including some viruses and plasmid DNA.[16,21] Unlike the previously described PCR techniques, the process is isothermal and does not require cyclical stages of heating and cooling. The polymerases utilized for rolling circle amplification do not have to be heat stable, and the most commonly used polymerase is phi29 from a bacteriophage. RCA does not require any predetermined sequence knowledge, and can be a great tool for viral discovery. However, the technique can be laborious and requires a substantial amount of processing and sequencing.

SEQUENCING MODALITIES

Sequence determination of PCR products (sequencing) is most commonly achieved by classical Sanger sequencing, a method developed by Sanger and Coulson in 1975.[20] At its core, Sanger sequencing is similar to PCR in that it requires template DNA, primers, and a DNA polymerase. Where it differs is that a subset of dNTPs are modified (dideoxynucleoside triphosphate; ddNTP) and fluorescently labeled. The amplification reaction is terminated when the polymerase incorporates a fluorescently labeled ddNTP into the strand. The fluorescent signal is detected and reported in a chromatogram.[7] Analysis of the chromatogram allows for determination of the input sequence. Sanger sequencing is widely commercially available, and single reactions can be run for just a few dollars. Sanger sequencing reactions are limited by length (500–700 bases) and the inability to differentiate mixed input sequences for which the initiation primer will bind.

Technological advances and decreased costs associated with next-generation nucleic acid sequencing have facilitated significant advances in science. The dramatic and rapid decrease in the cost of these techniques has made previously financially unfeasible testing modalities viable in the diagnostic and research setting. In next-generation sequencing modalities, including Illumina, PacBio, and pyrosequencing, no a priori knowledge of the sequence is required, and immense amounts of sequence data can be generated from mixed templates.[22] This is particularly powerful for metagenomic analyses. One of the challenges with these methods is the cost, which, while dramatically reduced in the past few years, can still be prohibitive for some. Another is the amount of bioinformatic analysis that can be required for interpretation of derived sequences. Despite the limitations, it is an exciting time for molecular diagnostics and pathogen discovery with the availability of these techniques.

IN SITU HYBRIDIZATION

In situ hybridization (ISH) is a powerful tool that can colocalize the presence or expression of nucleic acids to a certain cell type or within a lesion. Recent procedural and technologic advances have dramatically improved the sensitivity and specificity of the technique.[4,26] ISH works well on both cytologic and formalin-fixed paraffin-embedded preparations, and can be analyzed colorimetrically or fluoroscopically. Potential targets include both host mRNA and pathogen nucleic acids, with the technique being particularly powerful in placing novel pathogens within a putative lesion.[14]

Knowledge of target sequence and, ideally, the host genome are all that is required. Both a negative control probe and a positive host housekeeping gene control probe are important for accurate interpretation of results in most cases. The assay can also be complexed with immunohistochemistry to allow for concurrent detection of protein and nucleic acids.

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CHAPTER 146

Genetic Evaluation of Inherited Hematologic Diseases

NOA SAFRA and DANIKA BANNASCH

Definitions of Inherited Conditions
- Genotype Versus Phenotype
- Congenital Conditions, Phenocopies, and Epigenetics
- Penetrance and Variable Expressivity
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Acronyms and Abbreviations

For a glossary of relevant genetic terms, please see Table 146.1.

cDNA, complementary DNA;CNVs, copy number variations; DNA, deoxyribonucleic acid; GWAS, genome-wide association studies; IL-2RG, interleukin-2 receptor subunit gamma; Indels, insertion/deletion polymorphisms; NGS, next-generation sequencing; PCR, polymerase chain reaction; PRKDC, protein kinase, DNA-activated, catalytic subunit; RAG1, recombination activating gene 1; RNA, ribonucleic acid; RNAseq, RNA sequencing; SNPs, single-nucleotide polymorphisms; WES, whole-exome sequencing; WGS, whole-genome sequencing.

DEFINITIONS OF INHERITED CONDITIONS

Genetic information is inherited from parents to offspring. Typically, this information provides instructions to produce proteins that determine chemical and physical traits, including hematologic diseases. Genes that code genetic information are packed in paternal and maternal chromosome pairs in each cell's nucleus. Chromosomes are filament-like structures that contain tightly coiled DNA and histone proteins. The DNA sequence provides the blueprint for protein synthesis, and sequence alterations are called mutations. DNA mutations can be inherited from a parent, in which case they are found in all cells of an offspring. Other DNA mutations acquired during an individual's lifetime are called somatic mutations, and may affect only certain cells and underlie cancers. According to the central dogma of biology, the DNA template is transcribed into a RNA template and then the RNA template is translated into a protein product.

Genotype Versus Phenotype

The genetic basis for hematologic disease is determined by the inheritance of genes containing specific DNA mutations. The phenotypic expression of these genes, through synthesis of specific proteins, often includes interaction with environmental signals that trigger activation of typical molecular pathways. An individual's genotype is the complete set of genes, inherited from both parents. The individual's phenotype is all of its identifiable characteristics, which are influenced by its genotype, environmental factors, and interactions between the two.

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Congenital Conditions, Phenocopies, and Epigenetics

Disease phenotypes present at birth are termed congenital. These conditions can be inherited, or the result of environmental factors such as toxins, nutritional imbalances, or infections during gestation. Conditions that cause later onset of disease can also be inherited or acquired. Some inherited diseases are manifested under certain conditions. For example, inherited coagulation factor deficiencies (e.g., hemophilia A or B) lead to excessive bleeding post trauma or surgery. However, rodenticide poisoning can lead to acquired coagulation factor deficiencies with similar symptoms of excessive bleeding. Phenocopy describes conditions caused by environmental influences that mimic known inherited diseases.[29,93]. Alterations to gene activity in absence of changes in DNA sequence are termed epigenetic.[93] Any interference to the path from transcription to a physiologically functional protein is considered an epigenetic factor. Epigenetic modifications can be transmitted to offspring (inherited), or they can be reversed during reproduction.

Penetrance and Variable Expressivity

Penetrance is the proportion of individuals with a mutation that have phenotypic characteristics of a trait, or symptoms of an inherited disorder.[93] If <100% of individuals with a mutation exhibit the disorder, the penetrance is reduced or incomplete. When an inherited disorder is experienced in a wide range of severity, age of onset, or progression of disease, it is variably expressed.

Modes of Inheritance

The pattern in which a genetic trait or disorder is inherited is termed the mode of inheritance. Modes of inheritance for single-gene disorders are categorized based on a dominant or recessive pattern and chromosomal locations (i.e., autosomal, X-linked, or mitochondrial). Autosomal disorders typically cause equal numbers of affected males and females. When a mutation in a single allele is sufficient to cause a phenotype, it fits an autosomal dominant inheritance pattern. In autosomal recessive inheritance, mutations in both parental alleles are required to cause a phenotype. X-linked inheritance refers to disease causing mutations located on the X-chromosome. Larger proportions of affected males are observed because males have a single X chromosome. While females may be affected, they tend to be silent carriers that transmit the disorder.

Non-Mendelian disorders include mitochondrial inheritance, and complex or multifactorial inheritance. In mitochondrial inheritance, a mutation in the mitochondrial DNA, which is maternally transmitted, causes the disorder. Males and females are equally affected. Disorders with complex inheritance are caused by variations in multiple genes and their interactions with environmental factors. Susceptible individuals who exhibit the phenotype have sufficient numbers of certain genetic variations and are exposed to environmental factors that interact with their genetic variations, acting as triggers.

The mode of inheritance helps determine the risk of disease occurrence in related individuals and allows one to predict the disease state of progeny. Genetic selection against single-gene disorders using DNA screening tests in breeding animals is relatively straightforward. However, addressing complex conditions requires careful consideration of multiple genetic variants; sometimes with opposing effects on disease phenotype.[38] DNA mutations are found in every individual and inherited conditions affect both pure-bred and mixed-breed animals.[35] Typically, the frequency of animals affected by a recessive disorder is lower within mixed-breed population, but when considering an individual animal, inherited conditions should be listed as differential diagnoses regardless of breed status.[135]

ONLINE RESOURCES FOR INHERITED DISEASE INFORMATION

Online Mendelian Inheritance in Animals (OMIA): https://omia.org/home/
Inherited Diseases in Dogs: https://www.vet.cam.ac.uk/idid
Canine Inherited Disorder Prevalence Database: https://cidd.discoveryspace.ca/

GENETIC ANALYSIS

See Chapter 59 for cytogenetics review. DNA variations commonly used in genetic analyses include:

Single-nucleotide polymorphisms (SNPs) are substitutions of one nucleotide by another. SNPs are the most abundant form of genetic variation, found on average every 1000 bases in the dog genome.[81] SNPs may represent benign polymorphisms or mutations. Typically, polymorphic SNPs are found in noncoding sequence (i.e., introns) at a frequency of 1% or higher within a population. SNPs in coding DNA regions (i.e., exonic) may act as point mutations and alter protein structure or function; e.g., prekallikrein deficiency in Shih Tzu dogs caused by a single base substitution in exon 8 of the KLKB1 gene,[99] and factor VII deficiency in multiple breeds caused by a base substitution in exon 5 of factor VII.

Insertion/deletion polymorphisms (indels) are gains or losses of one or more nucleotides. Indels are less common than SNPs, found on average every 2400 bases; still they comprise a large proportion of DNA sequence variation. Exonic indels may cause frameshift mutations that can result in a premature stop codon; e.g., in dachshunds, a one-base deletion in exon 4 of the enzyme palmitoyl protein thioesterase 1 (PPT1). The frameshift

leads to a premature stop codon that causes neuronal ceroid lipofuscinosis-1.[7] An insertion in exon 7 of the factor XI gene causes factor XI deficiency in Kerry blue terriers.[114]

Copy number variations (CNVs) are genomic differences in copy number of DNA segments that range between hundreds to millions of bases. CNVs in mammalian genomes are common in gene families related to cell signaling and adhesion, olfactory receptors, sensory perception, immunity, and reproduction.[44] A duplication of 12.3 kilobases was recently discovered within the von Willebrand factor (VWF) gene in pigs affected by von Willebrand disease type III.[78]

Genetic analysis methods include:

Genome-wide association studies (GWAS) represent a powerful tool for investigating the genomic architecture of inherited traits. GWAS compare the frequencies of DNA variants between cohorts of cases (i.e., individuals affected by an inherited condition) and controls. GWAS typically utilize a hundred thousand to a million common DNA variants such as SNPs evenly spaced across the entire genome. Subsequent statistical analyses determine the likelihood of a variant to be associated with a disease. GWAS are hypothesis-free and do not require knowledge about mode of inheritance or pedigree information. With the complete sequencing of the entire genomes from most domestic species, high resolution SNP arrays were developed, which enable successful GWAS. In dogs, extensive linkage disequilibrium due to intense selection for breed-specific traits allows for remarkably effective mapping of loci for inherited traits by GWAS. Examples for successful GWAS include the discovery of a causative point mutation in ATP13A2 for neuronal ceroid lipofuscinosis in Tibetan terriers,[128] and mutations in CUBN that cause cobalamin malabsorption in beagles, border collies, and mixed-breed dogs.[36]

Whole-genome sequencing (WGS) is another method in which the order of DNA nucleotides in the individual's entire genome is determined. Next-generation sequencing (NGS), or high-throughput sequencing, describes several different modern sequencing technologies that offer much quicker and cheaper DNA and RNA sequencing compared to Sanger sequencing.[132] In general, the sequencing process involves the preparation of a library of short DNA fragments through either enzymatic or sonication techniques. These short DNA strands are then ligated to generic adapters in vitro. PCR amplification is performed using either emulsion PCR in oil–water emulsion micelles or bridge PCR on a solid surface coated with complementary primers. Subsequent amplicon sequencing is performed by pyrosequencing, sequencing by ligation, or sequencing by synthesis. The large number of short reads generated from this process must then be aligned against a reference sequence. A plethora of software have been developed not only to align the reads, but to also determine where deviations from a reference sequence exist.[91] These advanced techniques facilitate progress in the molecular study of genetic diseases, and allow breakthroughs in genomics; e.g., identification of a mutation in CLN8 that caused neuronal ceroid lipofuscinosis in a mixed-breed dog,[55] and a FAS-ligand variant associated with autoimmune lymphoproliferative syndrome in cats.[1]

In whole-exome sequencing (WES), all protein coding exons within an individual genome are sequenced. Next-generation sequencing can be used to determine this subset of DNA nucleotides called the exome, which is estimated to be only approximately 2% of an entire mammalian genome. Exome sequencing is proposed to be an efficient method to identify novel candidate mutations that cause inherited conditions, because most known disease-causing mutations occur in exons.[34]

Gene Expression

Over- and underexpressed genes in affected individuals compared to those in unaffected individuals can provide useful information to assist studies of simple as well as complex traits. Profiling of alternatively expressed transcripts can highlight a molecular pathway at play, identify biomarkers, or characterize response to therapy. Reliable evaluation of regulation of gene expressions is required for our understanding of the connection between genotype and phenotype. Technologies such as DNA microarray were developed in order to provide this type of expression analysis. Sample preparation begins with isolation of total RNA followed by reverse transcription to generate complementary DNA (cDNA). The sample cDNA sequences serve as nucleic acid probes that are covalently bound to glass slides. Known target sequences that are fluorescently labeled are hybridized to the probes and scanned.

Scanned images are converted to signal intensities and the data are processed using application-specific software. Software packages allow statistical analysis of the null hypothesis that a gene is not differentially expressed between two disease states. The sensitivity of microarrays enables a reliable detection of a twofold change in gene expression.

Microarrays can detect single-nucleotide polymorphisms, exon junctions, and gene rearrangements, but are limited to the transcripts bound to the array slides. Microarrays are only as good as the bioinformatic data available for the model organism's genome and transcriptome, and need to be updated to contain the most up to date sequence information.[112]

Gene expression profiling by microarray technologies has been successfully applied to study the transcriptional changes that occur in diseased versus normal tissues, such as heart, vessels, and blood cells in different cardiovascular disorders.[51]

Next-generation sequencing can also be used to sequence the complete expressed transcripts, or transcriptome, from cells or tissues; an approach called RNA sequencing or RNAseq.[112] RNAseq is independent of existing genome sequence data and has the capacity to sequence tissue-specific and novel transcripts. RNAseq can quantify transcript expression levels, predict exon–intron boundaries, and identify alternatively spliced

isoforms. RNAseq advantages over microarray gene expression platforms include higher resolution, detection of rare low-abundance transcripts, and polymorphism identification (i.e., SNPs) within transcripts.[112] Microarrays typically have a lower cost, have simpler computational analyses, and require less storage space for data.[112]

Genetic Testing

Screening for a genetic disorder is indicated when there is familial disease presentation, young age of onset, or when routine CBC, biochemistry, or urinalysis suggest known inherited disorders.[77] In dogs, genetic disorders should not be ruled out without further testing in pure and mixed-breed patients alike.[135] Screening of asymptomatic breeding animals is commonly requested prior to their use, as many breeders are aware of the availability of DNA tests, and strive to utilize them to apply selection against known inherited disorders.

ONLINE SOURCES OF AVAILABLE DNA TESTS

Orthopedic Foundation for Animals: https://www.ofa.org/diseases/dna-tested-diseases/all-dna-tests
University of Pennsylvania Inherited Disorder Test Search: https://www.vet.upenn.edu/research/academic-departments/clinical-sciences-advanced-medicine/research-labs-centers/penngen/penngen-tests

Phenotype Testing

Phenotype testing determines the individual's phenotype for a trait, and does not give information about the genotype; i.e., what an animal is capable of passing on to its offspring. The methods depend on the analyte tested, not the genetic or nongenetic origin, and frequently include antibody-based testing, though numerous modalities are possible. Serum cobalamin measurement by competitive protein-binding assay is used to diagnose cobalamin deficiency, regardless of the etiology.

Genotype (DNA) Testing

Genetic or DNA testing identifies disease-causing mutations in genes. Genetic test results can confirm or rule out a suspected genetic condition and establish an individual's carrier status. A list of DNA tests available for dogs, cats, horses, livestock other than horses, and miscellaneous species at the time of writing this chapter is provided in Tables 146.2–146.7. It is noted that new DNA tests are constantly becoming available and readers are advised to remain updated by using online resources or by direct contact with testing laboratories. DNA tests have several advantages compared to phenotype tests. DNA can be collected from any cell other than RBCs in mammals. Noninvasive tools such as buccal or saliva swabs are used frequently and hair follicles or whole blood are sampling options. DNA can also be extracted from semen, urine, and feces. DNA samples from any aged patient can be tested; e.g., newly born animals, prior to clinical symptoms, and before an animal is sold. DNA from dead animals can also be tested, as long as an appropriate sample (e.g., frozen semen) that was collected while the animal was alive exists. DNA is very stable and there are no special shipping requirements, unlike other bioproducts such as RNA. RNA is extremely unstable; special conditions and stabilizing solutions are required in order to preserve RNA for diagnostic purposes.

Mutation Tests

DNA mutations have been identified for numerous monogenic disorders (Tables 146.2–146.7). DNA mutation tests for companion animals are being offered by a large number of commercial molecular genetic laboratories. DNA mutation tests are laboratory assays that need to be validated; however, validation is not commonly documented for DNA tests of veterinary species. Different laboratories use different assays that employ different primers sets, DNA concentrations, and number of PCR cycles, to name a few. Variations in assay false-positive and false-negative rates need to be taken into account when interpreting test results. Another important consideration for ordering and interpreting DNA mutation tests is that they are mutation-specific and breed-specific. Consequently, only the animals within breeds where the same exact mutation was identified are expected to have it. An absence of the tested mutation in all other breeds does not imply inherited disease-free status. For example, severe combined immunodeficiency is an autosomal recessive disorder in Parson Russell terriers, caused by a PRKDC gene mutation; however, in basset hounds and cardigan Welsh corgis, it is an X-linked disorder caused by several different mutations in interleukin-2 receptor subunit gamma (IL-2RG). Another dog breed with autosomal recessive severe combined immunodeficiency and yet another different gene mutation is Frisian water dog with a RAG1 mutation (Table 146.2). Unfortunately, some of the commercial DNA testing laboratories report DNA test results as "the animal is free of DNA mutations." This outcome merely reflects the limits of our current knowledge of DNA mutations. A more comprehensive statement should be "free of known mutations that are included in the assay." Results from 1000 human genomes revealed that people carry an average of 250–300 recessive mutations, and at least 50 mutations previously associated with inherited disorders.[37] It is reasonable to expect that the average dog will carry a similar burden

of disease-associated variants, many of which are not currently being interrogated by existing DNA tests.

When the exact mutation is unknown, DNA variations that segregate together with a presumed causative locus can be evaluated. These DNA variations, called associated markers, can be SNPs, indels, or CNVs. These marker tests may be inaccurate, since the mutant gene itself cannot be directly tested. It is important to consider this when prioritizing diagnostic procedures, and when reaching clinical decisions.

Odds Ratio Versus Genetic Risk Complex diseases are caused by a combination of DNA variations and environmental factors. For complex disease, a number of genetic mutations typically contribute to the risk for showing disease symptoms. The degree of contribution varies between different genes, gene combinations, and particular mutations. Some changes have large and some have small effects. The number and combination of changes in an animal's genome determine its overall risk of disease. Relative or genetic risk represents the proportion of individuals with DNA mutations that will suffer an associated inherited disorder in the future. An odds ratio represents the proportion of affected animals in which associated DNA mutations were detected. These terms are frequently used interchangeably; and odds ratios are often interpreted as if they were equal to relative risks. This ignores the actual meaning of a ratio of odds. It is common in the literature to describe an odds ratio of "2" as a "twofold risk" of developing disease. This inaccuracy is often misleading because the odds ratio tends to overestimate the relative risk.[122] Case-control studies are limited by design to odds ratio calculation because of their retrospective nature.

DNA Test Utility for Veterinarians

The clinical utility of a genetic test refers to the impact of the testing information on clinical care. Clinical utility depends on the availability of effective tools to treat or reduce clinical symptoms in the patient. The presence of a known disease-causing mutation in sick patients can assist in prognosis and anticipation of further symptoms or complications. Mutation identification also enables genetic counseling for animal breeders and farmers. An animal that carries a disease-causing mutation can be bred to an individual that does not carry the same mutation. Expecting breeding animals to be clear of all deleterious alleles is unrealistic, and it may negatively impact breed diversity.

The clinical validity of a genetic test (i.e., the ability of a genetic test to predict a disease phenotype) depends on the phenotype's penetrance and expressivity (Table 146.1). However, when no mutations are identified, it does not eliminate the possibility of an inherited condition. A negative result may mean that a genetic alteration still exists but cannot be identified with current available knowledge or technologies.

TABLE 146.1 Glossary of relevant genetic terms.

Term	Description
The central dogma of biology	Gene expression entails RNA transcription from a DNA template followed by messenger RNA (mRNA) translation into proteins
Genotype	The genetic makeup of an individual
Phenotype	The physical and biochemical manifestation of the genotype in an individual
Mutation	A change in the DNA sequence; a mutation may be beneficial, neutral, or harmful
Penetrance	The frequency of phenotypic characteristics caused by the genotype
Variable expressivity	The range of phenotypic manifestations (age of onset, severity, different signs and symptoms) caused by a specific genotype
Autosomal chromosomes	Species-specific number of paired sets other than the sex chromosomes
Sex chromosomes	The X and Y chromosomes that determine the individual's sex
Allele	One of two parental genes, arise by mutation, found at a certain location on a chromosome
Homozygous	The two alleles have the same DNA sequence (i.e., a mutation)
Heterozygous	The two alleles have different DNA sequence (i.e., a mutation is found only on one of the two alleles)
Congenital	A condition present at birth
Epigenetic	Altered protein activity without a change in the DNA sequence
Phenocopy	Conditions caused by environmental influences that mimic known inherited diseases

Source: Based on Morris-Rosendahl.[93]

Information needed to interpret and utilize DNA tests includes the following. The testing laboratory should provide clear and easy-to-follow information regarding the disease, mode of inheritance, gene mutation(s), and specific breeds that can benefit from testing. Peer-reviewed documentation is preferable. Information about the penetrance and expressivity of the tested mutation should also be available. For clinical cases, it is important to consider genetic variability (e.g., different genes or different mutations within a single gene) and phenocopies. For screening of breeding animals, knowledge of the current mutation's frequency within the breed is imperative.

TABLE 146.2 Inherited hematologic disorders in dogs.

Dog breed	Disorder	Inheritance	DNA test
greater Swiss mountain dog	Bleeding diathesis due to a P2RY12 mutation[19]	Heterozygous and homozygous dogs may bleed	Yes
Irish setter, red and white setter, mixed breed	Canine leukocyte adhesion deficiency due to an ITGB2 mutation (CLAD I) [121]	Autosomal recessive	Yes
German shepherd dog, mixed breed	Canine leukocyte adhesion deficiency due to a FERMT3 (kindlin-3) mutation (CLAD III) [61]	Autosomal recessive	Yes
Australian shepherd, giant schnauzer	°Cobalamin malabsorption due to AMN mutations[69]	Autosomal recessive	Yes
beagle, border collie, mixed breed	°Cobalamin malabsorption due to CUBN mutations[69]	Autosomal recessive	Yes
collie: rough, smooth; mixed breed,	Cyclic neutropenia due to an AP3B1 mutation[59]	Autosomal recessive	Yes
chow chow, golden retriever, Labrador retriever, mixed breed	Elliptocytosis due to a SPTB mutation[115]	Autosomal dominant	Yes
German hunting terrier	Exercise-induced metabolic myopathy due to a ACADVL mutation[80]	Autosomal dominant	Yes
Airedle terrier, Alaskan klee kai, beagle, Finnish hound, giant schnauzer, mixed breed, Scottish deerhound, Welsh springer spaniel	Factor VII deficiency due to a FVII mutation[67]	Autosomal recessive	Yes
Belgian Malinois, boxer, German shepherd dog, Havanese, mixed breed, Old English sheepdog	°Factor VIII deficiency due to FVIII mutations; hemophilia A[25]	X-linked recessive	Yes
Airedale terrier, German wirehaired pointer, Lhasa apso, Rhodesian ridgeback	°Factor IX deficiency due to FIX mutations; hemophilia B[54]	X-linked recessive	Yes
American cocker spaniel, Chihuahua, Jack Russell terrier	Factor X deficiency[96]	Presumed autosomal dominant	No
English springer spaniel, great Pyrenees, Kerry blue terrier	Factor XI Deficiency due to a FXI mutation[114]	Presumed autosomal dominant	Kerry Blue Terrier only
basenji, Irish wolfhound	Fanconi syndrome due to a FAN1 mutation[16,39]	Autosomal recessive	Basenji only
English springer spaniel	Fucosidosis due to a FUCA1 mutation[98]	Autosomal recessive	Yes
Alaskan husky, beagle, English springer spaniel, Portuguese water dog, Shiba Inu, Siberian husky	°Gangliosidosis (GM1) due to GLB1 mutations[74]	Autosomal recessive	Yes
Japanese chin, mixed breed	Gangliosidosis, (GM2, type I; B variant) due to a HEXA mutation[105]	Autosomal recessive	Yes
poodle (toy)	°Gangliosidosis, (GM2, type II; Sandhoff variant) due to HEXB mutations[105]	Autosomal recessive	Yes
bluetick hound, Cairn terrier, Dalmatian, Irish setter, kelpie, mixed breed, West Highland white terrier	°Globoid cell leukodystrophy (Krabbe disease) due to GALC mutations[53]	Autosomal recessive	Yes
Maltese	Glycogen storage disease Ia due to a G6PC mutation[71]	Autosomal recessive	Yes
Finnish Lapphund, Lapponian herder, Swedish Lapphund	Glycogen storage disease II due to a GAA mutation[105]	Autosomal recessive	Yes
curly-coated retriever	Glycogen storage disease IIIa due to an AGL mutation[130]	Autosomal recessive	Yes

TABLE 146.2 (Continued)

Dog breed	Disorder	Inheritance	DNA test
American foxhound, beagle, mixed breed	Hypocatalasia (or acatalasemia) due to a CAT mutation[94]	Presumed autosomal recessive	Yes
Cairn terrier, Cavalier King Charles spaniel, Norfolk terrier	Macrothrombocytopenia due to TUBB1 mutation[31]	Autosomal recessive	Yes
Akita	Macrothrombocytopenia[56]	Unknown	No
pug	May–Hegglin anomaly due to a MYH9 mutation[40]	Presumed autosomal dominant	Yes
Plott hound	Mucopolysaccharidosis I due to an IDUA mutation[89]	Autosomal recessive	Yes
Labrador retriever	Mucopolysaccharidosis II; unknown gene mutation[127]	X-linked	No
dachshund, New Zealand huntaway	°Mucopolysaccharidosis IIIA due to SGSH mutations[131]	Autosomal recessive	Yes
Chesapeake bay retriever, Great Dane, miniature pinscher, poodle (miniature, toy), schnauzer (miniature), Welsh corgi	°Mucopolysaccharidosis VI due to ARSB mutations[125]	Autosomal recessive	Yes
Brazilian terrier, German shepherd dog, mixed breed[¶]	°Mucopolysaccharidosis VII due to GUSB mutations[62]	Autosomal recessive	Yes
poodle	Niemann–Pick disease, type A; unknown gene mutation[21]	Unknown	No
boxer	Niemann–Pick disease, type C1; unknown gene mutation[76]	Unknown	No
cane corso, dachshund	°Neuronal ceroid lipofuscinosis-1 due to PPT1 mutations[72]	Autosomal recessive	Dachshund only
dachshund	Neuronal ceroid lipofuscinosis-2 due to a TPP1 mutation[7]	Autosomal recessive	Yes
American Staffordshire terrier, bull terrier, mixed breed	Neuronal ceroid lipofuscinosis-4A due to an ARSG mutation[2]	Autosomal recessive	Yes
Australian cattle dog, border collie, mixed breed	Neuronal ceroid lipofuscinosis-5 due to a mutation in CLN5[88]	Autosomal recessive	Yes
Chinese crested, chihuahua	Neuronal ceroid lipofuscinosis-7 due to a MFSD8 mutation[6]	Autosomal recessive	Yes
Alpine dachsbracke, Australian cattle dog, Australian shepherd, English setter, mixed breed[$], saluki	°Neuronal ceroid lipofuscinosis-8 due to mutations in CLN8[55]	Autosomal recessive	Yes
American bulldog, mixed breed	Neuronal ceroid lipofuscinosis-10 due to a CTSD mutation[8]	Autosomal recessive	Yes
Tibetan terrier	Neuronal ceroid lipofuscinosis-12 due to an ATP13A2 mutation[128]	Autosomal recessive	Yes
Australian shepherd, Australian cattle dog, basenji, border collie, Boston terrier, cocker spaniel, Coonhound, Danish/Swedish farmdog, foxhound, German shepherd dog, mixed breed, Samoyed	Pelger–Huët anomaly; unknown gene mutation[120]	Autosomal dominant	No
American cocker spaniel, English cocker spaniel, English springer spaniel, German spaniel (wachtelhund), mixed breed, whippet	°Phosphofructokinase (PFK) deficiency due to PFKM mutations; glycogen storage disease VII[64]	Autosomal recessive	Yes, excluding German Spaniel
Mixed breed, shih tzu	Prekallikrein deficiency due to a KLKB1 mutation[99]	Autosomal recessive	Yes

(*Continued*)

TABLE 146.2 (Continued)

Dog breed	Disorder	Inheritance	DNA test
Clumber spaniel, Sussex spaniel	Pyruvate dehydrogenase deficiency due to a PDP1 mutation[22]	Autosomal recessive	Yes
basenji, beagle, Cairn terrier, Labrador retriever, mixed breed[£], pug, West Highland white terrier	°Pyruvate kinase deficiency due to PKLR mutations[52]	Autosomal recessive	Yes
German shepherd dog	Scott Syndrome: Platelet dysfunction due to a TMEM16F (ano6) mutation[20]	Autosomal recessive	Yes
Parson Russell terrier	Severe combined immunodeficiency due to a PRKDC mutation[10]	Autosomal recessive	Yes
Frisian water dog	Severe combined immunodeficiency due to a RAG1 mutation[100]	Autosomal recessive	Yes
basset hound, Welsh corgi (Cardigan)	°Severe combined immunodeficiency due to IL2RG mutations[100]	X-linked	Yes
golden retriever	Spectrin deficiency; unknown gene mutation[111]	Autosomal dominant	No
great Pyrenees, mixed breed, otterhound, Scottish deerhound	°Thrombasthenia type I (Glanzmann) due to ITGA2B mutations[17]	Autosomal recessive	Yes
basset hound, Eskimo spitz, Landseer, mixed breed[€]	°Thrombopathia due to RASGRP1 (CalDAG-GEFI) mutations[18]	Autosomal recessive	Yes
border collie, mixed breed	Trapped neutrophil syndrome due to a VPS13B mutation[92]	Autosomal recessive	Yes
barbet, Bernese mountain dog, coton de Tulear,	von Willebrand disease I due to a VWF mutation	Presumed autosomal dominant	Yes
Dobermann pinscher, Drentsche patrijshond, Dutch partridge dog, Dutch shepherd, French water dog, Havanese, German pinscher, Irish red and white setter,			
Irish setter, Kerry blue terrier,			
kromfohrländer,			
Manchester terrier (regular, toy), mixed breed, papillon, phalène, poodle (standard, miniature, toy),			
Welsh corgi, stabyhoun, West Highland white terrier			
Chinese crested dog, German shorthaired pointer, German wirehaired pointer	°von Willebrand disease II due to VWF mutations[73]	Autosomal recessive	Yes
Dutch kooiker, mixed breed[¥], Shetland sheepdog, Scottish terrier	°von Willebrand disease III due to VWF mutations[104]	Autosomal recessive	Yes

° Multiple breed-specific mutations identified.
Mutations identified within mixed breed dogs:
^ Cairn terrier and West Highland White terrier mutation.
ꟻ German Shepherd dog mutation.
$ Australian Shepherd mutation.
£ Beagle and West Highland White terrier mutations.
€ Basset Hound and Eskimo Spitz mutations.
¥ Shetland Sheepdog mutation.

TABLE 146.3 Inherited hematologic disorders in cats.

Cat breed	Disorder	Inheritance	DNA test
Domestic cat, Ragdoll	Blood group antigen AB due to CMAH mutations[15]	Autosomal	Yes
Persian cat	Chédiak–Higashi syndrome; unknown gene mutation[30]	Presumed autosomal recessive	No
Domestic cat	Dihydropyrimidinase deficiency due to a DPYS mutation[24]	Presumed autosomal recessive	Yes
British shorthair, domestic cat	Factor IX deficiency due to FIX mutations; hemophilia B[50]	X-linked	No
Domestic cat	Factors IX and XII, combined deficiency; unknown gene mutation[33]	Unknown	No
Domestic cat	Factor X deficiency; unknown gene mutation[49]	Unknown	No
Domestic cat	Factor XI deficiency; unknown gene mutation[118]	Unknown	No
Domestic cat	Factor XII deficiency due to FXII mutations[11,86]	Autosomal recessive	No
Domestic cat	Feline leukocyte adhesion deficiency due to ITGB2 mutation (FLAD I)[9]	Autosomal recessive	Yes
Domestic cat, Korat, Siamese	Gangliosidosis (GM1) due to a GLB1 mutation[84]	Autosomal recessive	Yes
Domestic cat, Korat, Siamese	Gangliosidosis (GM2) due to a GM2A mutation[85]	Autosomal recessive	Yes
Burmese, domestic cat, Japanese domestic cat, Korat	Gangliosidosis (GM2, type II; Sandhoff variant) due to HEXB mutations[68]	Autosomal recessive	Yes
Domestic cat	Globoid cell leukodystrophy (Krabbe disease); unknown gene mutation[110]	Unknown	No
Norwegian Forest cat	Glycogen storage disease IV due to a GBE1 mutation[43]	Autosomal Recessive	Yes
Domestic cat	Hyperlipoproteinaemia due to a LPL mutation[48]	Autosomal recessive	Yes
Domestic cat	Hypothyroidism due to a TPO mutation[45]	Autosomal recessive	Yes
British shorthair	Lymphoproliferative syndrome, autoimmune (ALPS) due to a FASLG mutation[1]	Autosomal recessive	Yes
Domestic cat, Persian	Mannosidosis-alpha, due to a MAN2B1 mutation[12]	Autosomal recessive	Yes
Domestic cat	Mucolipidosis II due to a GNPTAB mutation[46]	Autosomal recessive	No
Domestic cat	Mucopolysaccharidosis I due to an IDUA mutation[57]	Autosomal recessive	Yes
Birman, domestic cat, Ragdoll, Siamese	Mucopolysaccharidosis VI due to ARSB mutations[83]	Autosomal recessive	Yes
Domestic cat	Mucopolysaccharidosis VII due to GUSB mutations[124]	Autosomal recessive	Yes
Domestic cat	Niemann–Pick disease (C1) due to mutations in NPC1[87]	Autosomal recessive	No
Domestic cat	Niemann–Pick disease (C2) due to a mutation in NPC2[133]	Autosomal recessive	No
Domestic cat, Siamese	Porphyria, acute intermittent (AIP) due to mutations in HMBS[27]	Autosomal dominant and recessive	Yes
Domestic cat	Porphyria, congenital erythropoietic (CEP) due to UROS mutations[28]	Autosomal recessive	Yes
Abyssinian, Bengal, domestic cat, Egyptian Mau, LaPerm, Maine Coon, Norwegian Forest, Savannah, Siberian, Singapura, Somali	Pyruvate kinase deficiency due to a PKLR mutation[52]	Autosomal recessive	Yes
Himalayan	von Willebrand disease III; unknown mutation[42]	Autosomal	No

TABLE 146.4 Inherited hematologic disorders in horses.

Horse breed	Disorder	Inheritance	DNA test
American Quarter Horse, Arabian, Standardbred, Tennessee Walking Horse, Thoroughbred	Factor VIII deficiency; hemophilia A[97]	X-linked	No
Dales pony, Fell pony, Gypsy horse	Foal immunodeficiency syndrome due to a SLC5A3 mutation[41]	Autosomal recessive	Yes
American Saddlebred	Glucose-6-phosphate dehydrogenase deficiency[113]	Unknown	No
American Quarter Horse, American Paint Horse	Glycogen storage disease IV due to a GBE1 mutation[126]	Autosomal	Yes
Austrian, Icelandic, Peruvian Paso	Neuronal ceroid lipofuscinoses; unknown gene mutation[119]	Unknown	No
Arabian	Pelger–Huët anomaly; unknown gene mutation[47]	Unknown	No
Arabian, Caspian horse	Severe combined immunodeficiency due to a PRKDC mutation [108]	Autosomal recessive	Yes
American Quarter Horse, Oldenburg, Peruvian Paso, Thoroughbred, Warmblood	Thrombasthenia type I (Glanzmann) due to ITGA2B mutations [26]	Autosomal recessive	Yes
American Quarter Horse, Thoroughbred	von Willebrand disease; unknown gene mutation[103]	Unknown	No

TABLE 146.5 Inherited hematologic disorders in livestock.

Breed	Disorder	Inheritance	DNA test
Cattle			
Blonde d'Aquitaine, Limousin	Bovine erythropoietic protoporphyria due to a FECH mutation[65]	Autosomal recessive	Yes
Fleckvieh, Holstein Friesian, Jersey	Bovine leukocyte adhesion deficiency due to an ITGB2 mutation (BLAD I)[109]	Autosomal recessive	Yes
Japanese Black	Chédiak–Higashi syndrome due to a LYST mutation[75]	Autosomal recessive	Yes
Japanese Brown	Factor VIII deficiency due to a FVIII mutation; hemophilia A[70]	X-linked	Yes
Holstein, Japanese Black, Sahiwal	°Factor XI deficiency due to FXI mutations[90]	Autosomal recessive	Yes
German Fleckvieh, Swiss Braunvieh	Fanconi syndrome due to a SLC2A2 mutation[66]	Autosomal recessive	Yes
Brahman, Droughtmaster, Shorthorn	°Glycogen storage disease II due to GAA mutations[32]	Autosomal recessive	Yes
Angus, Brangus, Galloway, Murray Grey	Mannosidosis-alpha, due to MAN2B1 mutations[116]	Autosomal recessive	Yes
Devon	Neuronal ceroid lipofuscinosis-5 due to a CLN5 mutation[60]	Autosomal recessive	Yes
Hereford	Niemann–Pick disease type A; unknown gene mutation[106]	Unknown	No
Japanese Black	Spherocytosis (BAND3 deficiency) due to a SLC4A1 mutation[63]	Autosomal dominant	Yes
Fleckvieh-Simmental	Thrombopathia due to a RASGRP2 (CalDAG-GEFI) mutation[18]	Autosomal recessive	?

TABLE 146.5 (Continued)

Breed	Disorder	Inheritance	DNA test
Sheep			
Swiss Alpine	Factor VIII deficiency due to a FVIII mutation; hemophilia A[101]	X-linked	No
Romney Marsh	Gangliosidosis (GM1) due a GLB1 mutation[3]	Autosomal Recessive	No
British Jacob sheep	Gangliosidosis (GM2, type I; B variant) due to a HEXA mutation[117]	Autosomal Recessive	Yes
Southdown	Gaucher disease, type I due to a GBA mutation[134]	Autosomal Recessive	No
Polled Dorset	Globoid cell leukodystrophy (Krabbe disease); unknown gene mutation[102]	Presumed autosomal recessive	No
German Blackface	Porphyria cutanea tarda due to a UROD mutation[95]	Autosomal Recessive	No
Goats			
Nubian	Mannosidosis-alpha, due to MAN2B1 mutations[79]	Autosomal recessive	No
Nubian	Mucopolysaccharidosis IIID due to a GNS mutation[23]	Autosomal recessive	Yes
Pigs			
All pigs	Blood group ABO due to a GGTA1 mutation[129]	Autosomal Recessive	Yes
Norwegian Yorkshire	Porcine dense deposit disease; hereditary factor H deficiency[58]	Autosomal Recessive	No
Yorkshire	Severe combined immunodeficiency due to DCLRE1C mutations[123]	Autosomal Recessive	Yes
Mixed breed	von Willebrand disease III due to VWF mutations[78]	Autosomal Recessive	?

° Multiple breed-specific mutations identified.

TABLE 146.6 Inherited hematologic disorders in miscellaneous species.

Miscellaneous species	Disorder	Inheritance	DNA test
American mink	Chédiak–Higashi syndrome[4]	Autosomal recessive	No
Guinea pig	Mannosidosis-alpha due to a MAN2B1 mutation[13]	Autosomal recessive	No
Rhesus monkey	Globoid cell leukodystrophy (Krabbe disease) due to a GALC mutation[82]	Autosomal recessive	No
Emu	Gangliosidosis (GM1); unknown gene mutation[14]	Unknown	No
Emu	Mucopolysaccharidosis IIIB due to a NAGLU mutation[5]	Autosomal recessive	Yes

TABLE 146.7 Inherited hematologic disorders in birds.

Avian species	Disorder	Inheritance	DNA test
Emu	Gangliosidosis (GM1); unknown gene mutation[14]	Unknown	No
Emu	Mucopolysaccharidosis IIIB due to a NAGLU mutation[5]	Autosomal recessive	Yes

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SECTION 10 GLOSSARY

The following definitions and formulas are provided to ensure that the terms used subsequently are understood by all readers.

Bias: Systematic difference between a measured value and a value representing true analyte concentration or activity. Bias can be due to analytical error within the test system or reflect disagreement between a test system and a comparative method due to inherent methodologic and/or operational differences. Bias can be calculated from method comparison, quality control (QC), or proficiency testing data. See systematic error.

Biological variation: Variation in blood values between individuals and groups of the same species.

Control limits: Numerical limits calculated using a control rule and used during statistical QC to interpret control data and define unacceptable results (probable analytical error).

Control rule: Mathematical rule used in statistical QC to calculate control limits. Westgard defined a logical sequence of multiple control rules known as "Westgard rules" that can be applied for generic statistical QC. Individual control rule(s) for a given method can also be selected during a process of QC validation.

Decision limits: A predetermined test threshold that distinguishes between condition-present and condition-absent subgroups of a target population. Analyte concentration or activity at which medical interpretation and patient care will be impacted.

External quality assessment (EQA): An interlaboratory comparison program in which specimen aliquots are periodically measured by participants, who receive a report from the program provider comparing their results to those from a peer group using statistical analysis or comparing their results to a known target using statistical quality control tools. Often used synonymously with proficiency testing (PT).

Imprecision: Variability of data from a set of replicate measurements expressed as standard deviation (SD) or coefficient of variation (CV).

Peer group: Participants using the same method and/or instrument in an interlaboratory comparison.

Probability of error detection (P_{ed}): Probability that control data will be rejected (deemed unacceptable) when analytical error is correctly identified. Ideally, P_{ed} should be 100%; in clinical laboratory practice, $P_{ed} \geq 90\%$ is considered adequate.

Probability of false rejection (P_{fr}): Probability that control data will be rejected (deemed unacceptable) when no analytical error is present. Ideally, P_{fr} should be 0.00; in clinical laboratory practice, $P_{fr} < 0.05$ is considered adequate.

Proficiency testing (PT): An interlaboratory comparison program in which specimen aliquots are periodically measured by participants, who receive a report from the program provider comparing their results to those from a peer group using statistical analysis or comparing their results to a known target using statistical quality control tools. Often used synonymously with external quality assurance.

Quality control (QC): Laboratory procedures ensuring that accurate patient results are released; includes statistical and nonstatistical procedures.

QC material (QCM): Assayed or unassayed test material having known stability and used for statistical QC of laboratory testing. Data resulting from QCM measurement are known as control data.

QC validation: The process of tailoring statistical QC to a given analytical method, measurand, and quality goal.

Random error: Synonymous with imprecision.

Repeat patient testing (RPT): A statistical QC method using patient results obtained from repeated patient sample testing.

Reference interval: The interval between, and including, two reference limits. The reference interval is designated as the interval of values from the lower reference limit to the upper reference limit. In some cases, only one reference limit is important, usually the upper limit and the corresponding reference interval are zero.

Reference limit: A value derived from the reference distribution.

Reference value: The value (test result) obtained from a reference individual (e.g., calcium result from the blood sample of a study participant).

Statistical QC: Application of statistical principles, control rules, and control limits to control data in order to detect analytical error. Control data are most commonly derived from repeated measurement of stable QCM over time in internal or external quality assessment. The principle underlying statistical QC is that, in a stable test system, control data over time should approximate a Gaussian distribution.

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Systematic error: Dysfunction of a test system causing disagreement between a measured result and some representation of true measurand concentration/activity; mathematically, systematic error is represented by difference and occurs in one direction (positive or negative). Systematic error can be constant over a range of concentrations/activities or can change as concentration/activity changes proportionally. See bias.

Total error (TE): The sum of random and systematic error.

Total error allowable (TE_a): An analytical quality requirement specifying the amount of total error that is acceptable, as it will not change clinical decisions.

Total error observed (TE_{obs}): The sum of random and systematic error of an analyte as measured by a given method or instrument. TE_{obs} is unique to an individual instrument/method and may vary across analyte range, by species, and over time.

BASIC CALCULATIONS

$$\text{Mean} = \frac{x_1 + x_2 + x_3 + \ldots + x_n}{n}.$$

$$\text{Standard deviation}(\text{SD}) = \sqrt{\frac{\Sigma(x_i - \bar{x})^2}{(n-1)}}.$$

$$\text{Coefficient of variation}(\text{CV}) = \frac{\text{SD}}{\text{Mean}} \times 100.$$

$$\text{Bias in}\% = \frac{\text{Mean}_{\text{target}} - \text{Mean}_{\text{measured}}}{\text{Mean}_{\text{target}}} \times 100.$$

Total error observed (TE_{obs}) = 2 × CV + Bias (%) or
2 × SD + Bias (unit).

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