In: 55th Annual Meeting of the American College of Veterinary Pathologists (ACVP) and 39th Annual Meeting of the American Society of Clinical Pathology (ASVCP), 2004 - Orlando, FL, USA, ACVP and ASVCP (Eds.). Publisher: American College of Veterinary Pathologists & American Society for Veterinary Clinical Pathology, Middleton WI, USA. Internet Publisher: International Veterinary Information Service, Ithaca NY (www.ivis.org), 13-Nov-2004; P1214.1104

Hematology of Lower Vertebrates

T. W. Campbell

Department of Clinical Sciences, College of Veterinary Medicine & Biomedical Sciences, Colorado State University, Fort Collins, CO, USA.

Introduction

The proper evaluation of the hemogram of any animal patient involves the determination of a total erythrocyte count [TRBC], packed cell volume [PCV], hemoglobin concentration [Hb], total white blood cell count [TWBC], white blood cell differential, and the evaluation of a stained peripheral blood film. The basic techniques used in mammalian hematology also apply to that of lower vertebrates, such as birds and reptiles. However, because lower vertebrates have nucleated erythrocytes and thrombocytes, there are a few modifications to the techniques. Also, the morphology of the hemic cells of lower vertebrates often varies from mammalians cells. The evaluation of the peripheral blood film involves the examination of the cellular components of the blood sample. Each cell group [erythrocytes, leukocytes, and thrombocytes] should be examined for any abnormal morphologic changes.

Evaluation of Erythrocytes

The total erythrocyte count can be obtained using either a standard manual method or an automated method, such as those used for mammalian blood. The packed cell volume is determined using the standard microhematocrit method with centrifugation at 12,000 G for 5 minutes. The hemoglobin concentration is best determined using the cyanmethemoglobin method following proper removal of free red cell nuclei by centrifugation. The mean corpuscular values [MCV, MCHC, and MCH] can be calculated once the TRBC, PCV, and Hb have been obtained using the standard formulas.

The normal mature avian erythrocyte is an oval cell with an oval centrally positioned nucleus. The cytoplasm stains orange-pink with Wright's stain and should have a homogenous texture. The erythrocyte nucleus should have uniformly appearing chromatin which becomes more condensed with age. The mature erythrocytes of reptiles are generally larger than erythrocytes of birds and mammals. They are ellipsoidal cells with centrally positioned nuclei. The nuclei often have irregular margins. The mature erythrocytes of fish and amphibians are also nucleated, elliptic discs. The erythrocytes of amphibians are large compared to other lower vertebrates.

Polychromatic erythrocytes are often seen in the peripheral blood films of normal birds. Usually these cells represent five percent or less of the erythrocyte population. Polychromatic erythrocytes as well as immature erythrocytes are occasionally present in the

peripheral blood films of reptiles, amphibians, and fish. Immature erythrocytes increase during periods of ecdysis and are more prevalent in young reptiles. [1] The degree of polychromasia or reticulocytosis in normal reptiles is generally low and represents less than one percent of the erythrocyte population. The reason may be that reptiles have a slower erythrocyte turn over rate compared to birds and mammals owing to their long red cell half-life which can be as long as 600 to 800 days in some species [2,3].

The degree of polychromasia is a good indicator of the erythrocytic regenerative response. For example, anemic birds showing ten percent or greater polychromasia [3+ or 4+ polychromasia] are demonstrating a good regenerative response to their anemia. Polychromatic erythrocytes have nuclei that are less pyknotic than mature erythrocytes and have cytoplasmic basophilia. In birds, the degree of polychromasia can be evaluated based upon the average number of polychromatic erythrocytes per 1000X monolayer field. A slight degree of polychromasia [1+] is represented by 2 - 10 polychromatic cells per 1000X monolayer field. A mild polychromasia [2+] is indicated by 11 - 14 polychromatic cells per 1000X monolayer field. A moderate [3+] and marked [4+] polychromasia are represented by 15 - 30 and greater than 30 polychromatic erythrocytes per 1000X monolayer field, respectively.

The avian reticulocyte has a distinct band of aggregated reticulum that encircles the nucleus when stained with vital stains, such as New methylene blue [4,5].Reticulocyte counts can also be used to evaluate the regenerative response of the erythrocytes.

Birds and other lower vertebrates with hypochromatic anemias usually have pale, hypochromatic appearing erythrocytes in the peripheral blood film. Chronic inflammatory diseases and iron deficiency anemia often result in hypochromasia in birds. The degree of hypochromasia can be rated based upon the average number of hypochromatic erythrocytes per 1000X monolayer field where a 1+, 2+, 3+, and 4+ hypochromasia is represented by 1 - 2, 3 - 5, 6 - 10, and greater than 10 hypochromatic cells, respectively.

The degree of anisocytosis in birds can also be rated on a similar scale. A 1+ and 2+ anisocytosis are indicated by an average of 5 - 10 and 11 - 20 erythrocytes that vary in size per 1000X monolayer field, respectively. A 3+ and 4+ anisocytosis is base upon an average of 21 - 30 and greater than 30 erythrocytes that vary in size per 1000X monolayer field. The degree of poikilocytosis is also based upon the average number of abnormal cells in a 1000X monolayer field. A 1+, 2+, 3+, and 4+ poikilocytosis is indicated by 5 - 10, 11 - 20, 21 - 50, and greater than 50 abnormal erythrocytes, respectively per 1000X monolayer field. A sight anisocytosis and poikilocytosis is considered normal for most reptiles; however moderate to marked numbers of these cells are associated with regenerative responses or less commonly, erythrocyte disorders. Binucleated erythrocytes in birds are rare, but when present in large numbers suggest neoplastic, viral, or genetic disease [6]. Binucleated erythrocytes, anisokaryosis, or mitotic activity can be associated with marked erythrocytic regenerative responses, reptiles awakening from hibernation, severe inflammation, malnutrition, and starvation [7]. Punctate basophilic inclusions are often seen in the cytoplasm of erythrocytes of reptiles and may represent artifacts associated with degenerate organelles [8].

Erythroplastids are anucleated erythrocytes found in the peripheral blood of birds and other lower vertebrates [9]. In the bird, the degree of their occurrence is also based upon the average number found in a 1000X monolayer field. A 1+, 2+, 3+, and 4+ degree of erythroplastids seen is based upon 1 - 2, 3 - 5, 6 - 10, and greater than 10 erythroplastids found in an average 1000X monolayer field.

The number of immature erythrocytes should also be noted in blood films from lower vertebrate patients. These animals that are responding to anemia may exhibit an increase in the number of immature erythrocytes in the peripheral blood film. Immature erythrocytes, especially the rubricyte stages often appear smaller than mature erythrocytes and are round to slightly oval. The cytoplasm of immature erythrocytes stains more basophilic than mature erythrocytes. Rubriblasts and prorubricytes are rarely seen in peripheral blood films of birds and other lower vertebrates. These early immature erythrocytes may indicate a marked erythropoietic response or erythrocyte dyscrasia such as erythroblastosis. Birds suffering from heavy metal toxicosis, especially lead poisoning, often reveal an inappropriate release of immature erythrocytes in a nonanemic patient. This is reflected in the peripheral blood film by two distinct populations of erythrocytes, immature cells [i.e. metarubricytes and polychromatic erythrocytes] and old mature cells with pyknotic nuclei.

Evaluation of Leukocytes

The presence of nucleated erythrocytes and thrombocytes in the blood of lower vertebrates precludes the use of the automated methods used for counting white blood cells in the blood of mammals. Therefore, total white blood cell counts in lower vertebrates are determined either by the indirect method using phloxine B stain or the direct method using Natt and Herrick's solution with a hemacytometer [10,11]. The evaluation of reptilian leukogram is the same as those described for birds. In species that normally have low numbers of circulating acidophils, the Natt and Herrick's method is the preferred method over the phloxine B method because the later relies on large numbers of acidophils.

The granulocytes of birds and other lower vertebrates include the heterophil, eosinophil, and basophil. Normal mature avian heterophils have a lobed nucleus [usually two to three lobes] with densely clumped chromatin, colorless cytoplasm, and distinct rod-shaped eosinophilic cytoplasmic granules [heterophil granules of some species are spherical]. The heterophil granule often contains a central refractile body. The mature eosinophil also has a lobed nucleus, which often stains darker than the nuclei of heterophils in the same blood smear. Eosinophils have a blue cytoplasm that contains round eosinophilic granules [these granules are rod-shaped in some species] that have a staining quality that differs from heterophil granules on the same blood film. Avian basophils have a nonlobed nucleus which is often hidden by the basophilic cytoplasmic granules. The granules of basophils often stain poorly in alcohol-solubilized stains, such as Wright's stain.

The granulocytes of reptiles can be classified as acidophils [i.e. heterophils and eosinophils] and basophils. Reptilian heterophils are generally round cells with fusiform, bright orange cytoplasmic granules with Romanowsky stains. The cytoplasm of the normal mature reptilian heterophil is colorless and the nucleus is typically round to oval. Some species of lizards have heterophils with lobed nuclei. Reptilian eosinophils tend to be large round cells with spherical eosinophilic cytoplasmic granules. Some species such as green iguanas

[*Iguana iguana*] have eosinophils with cytoplasmic granules that stain blue with Romanowsky stains. The nucleus is variable in shape ranging from slightly elongated to lobed. Basophils are usually small round cells that contain basophilic metachromatic cytoplasmic granules that often obscure the nucleus. When visible, the nucleus is nonlobed. Like the acidophils, the size of basophils varies with species. Lizards tend to have small basophils and chelonians and crocodilians have large basophils. Basophil granules often partially dissolve in alcohol fixatives.

The percentage of heterophils in the leukogram of normal birds and reptiles varies with species. Heterophils may represent up to 40 percent of the differential count in some species [1,2,12,13]. Heterophil concentrations in reptiles and other ectotherms are also influenced by seasonal factors [highest numbers occur in the summer] [1]. Heterophils are primarily phagocytic and therefore are associated with inflammatory diseases, especially those associated with infectious diseases or tissue injury. Noninflammatory causes of heterophilias include stress and neoplasia.

The number of circulating eosinophils in birds varies with species. The number of circulating eosinophils in normal reptiles varies with species and seasonal changes. For example, in reptiles, eosinophil counts are usually highest in the winter during hibernation [1]. Basophils numbers also vary with species but are usually low. Some reptilian species, however, can range between 0 and 40 percent [some species of healthy tortoises have basophil numbers representing up to 40% of the leukocyte differential!] [1,2,12,13]. Reptilian basophils do not appear to vary with seasonal changes like the other granulocytes [14].

Abnormal appearing heterophils include immature cells and toxic cells. Immature heterophils [usually myelocytes and metamyelocytes] are found in the blood of birds suffering from conditions that result in excessive peripheral utilization of mature heterophils. These immature heterophils have cytoplasmic basophilia, nonlobed nuclei, fewer specific granules than mature cells, and occasionally immature granules [i.e. primary granules]. Typically, when immature cells are found in the peripheral blood, normal appearing mature heterophils can be found. Generally, when toxic heterophils are seen, all the heterophils in the film appear toxic and usually to the same degree unless the condition is caught in the peracute stage or is resolving. Toxic heterophils are classified on a scale of +1, +2, +3, and +4 depending upon the degree of toxicity. A +1 toxic heterophil shows increased cytoplasmic basophilia. A +2 toxic heterophil shows increased cytoplasmic basophilia, slightly abnormal granulation [i.e. partial degranulation, coalescing granules, or abnormal appearing granules], or vacuolation. A +3 toxic heterophil will show changes that are more severe than the +2 toxicity and the nucleus may show slight karyorrhexis or karyolysis. Finally, a +4 toxic heterophil will show marked changes in the cytoplasm and nucleus. Toxic heterophils are uncommon and usually seen in birds that are critically ill. Toxic heterophils can be associated with severe systemic diseases, such as septicemia, chlamydiosis, and bacterial toxemia. Reptilian heterophils exhibit toxic changes similar to those described for avian heterophils. They usually represent inflammatory diseases associated with infectious agents such as bacteria. Nuclear lobation in reptilian species that normally do not lobate their heterophil nucleus is also suggestive of severe inflammation.

The mononuclear leukocytes of the lower vertebrates include monocytes and lymphocytes. These cells resemble the same cell types found in the peripheral blood of mammals. Monocytes are the largest leukocytes found in peripheral blood. The monocyte nucleus is variable in shape ranging between round to oval to lobed. The cytoplasm of monocytes stains blue-gray, may appear slightly opaque, and may contain vacuoles or fine, dust-like eosinophilic or azurophilic granules. Highly vacuolated monocytes suggest increased phagocytic activity and may indicate a response to a systemic antigen. Reptilian monocytes that have numerous fine azurophilic cytoplasmic granules are often referred to as azurophils in the literature. Because these cells are morphologically and cytochemically identical to monocytes, they should be called monocytes rather than azurophils which implies that they are a separate cell type [2,5,15]. Snakes often have monocytes with distinct azurophilic granules. Monocytes generally occur in low numbers in the blood of most lower vertebrates and represent 0 to 10 percent of the differential. A monocytosis is suggestive of an inflammatory disease, especially a granulomatous inflammation.

Lymphocyte counts in lower vertebrates vary with species and, in the case of the ectotherms, environmental conditions. Lymphocyte counts can be as high as 80 percent of the leukocyte differential in some reptilian species [2]. Lymphocytes are lowest in the winter and highest during the summer in reptiles [2]. Lymphopenias occur with malnutrition and conditions of stress. Lymphocytosis occurs with wound healing, inflammatory disease, parasitic infections, and viral diseases. Reactive lymphocytes suggest the presence of systemic antigens. Reactive lymphocytes show increased cytoplasmic volume and basophilia. The nuclei of reactive lymphocytes may show smooth or delicate nuclear chromatin.

Evaluation of Thrombocytes

A total thrombocyte count or subjective estimate of the thrombocyte count from a blood film can be performed. Mature thrombocytes are small oval cells that appear more rounded than erythrocytes. The nuclear chromatin is densely clumped. The cytoplasm is colorless [an important feature that differentiates these cells from small mature lymphocytes], contains clear areas, and distinct red granules. Thrombocytes often clump together in blood smears making them easier to identify. An increased number of immature thrombocytes are seen in birds responding to thrombocytopenia. Immature thrombocytes are round cells with slight cytoplasmic basophilia [depending upon the stage of maturity] and round nuclei that appear less pyknotic than mature cells. Reptilian thrombocytes appear elliptical to fusiform and like those of birds are nucleated cells. Thrombocyte counts can be difficult to obtain; however, the procedure is the same as described for birds. Reptilian thrombocytes play a role in hemostasis. Immature thrombocytes of reptiles resemble those of birds. Thrombocytes with polymorphic nuclei are considered abnormal and may be associated with severe inflammatory disease.

Blood Parasites

The common blood parasites of birds are *Hemoproteus*, *Plasmodium*, Leukocytozoon, and *microfilaria*. *Hemoproteus* and *Plasmodium* have mature gametocytes within the cytoplasm of erythrocytes that have refractile pigment granules. Only the gametocyte stage occurs in the peripheral blood of birds with *Hemoproteus*; therefore, if schizogony is present then the parasite is a *Plasmodium*. The mature gametocyte of *Hemoproteus* occupies greater than 50

% of the host cell cytoplasmic volume and forms a characteristic halter-shape. In general, the mature gametocytes of *Plasmodium* are not as large and some species will dramatically alter the position of the host cell nucleus. *Plasmodium* gametocytes and schizonts may also be found in other cell types. *Hemoproteus* occurs only within the erythrocytes.

The gametocytes of Leukocytozoon lack refractile pigment granules and are the only form of the parasite found in the peripheral blood of birds. The mature gametocyte grossly deforms the host cell being parasitized.

Less common blood parasites of birds include *Atoxoplasma, Aegyptianella,* and *Trypanosoma*. *Atoxoplasma* is a coccidian parasite found primarily in passerine birds. It is identified by the intracytoplasmic inclusions [sporozoites] within lymphocytes. These large pale eosinophilic round to oval inclusions often indent the lymphocyte nucleus creating a "crescent-moon" shape to the nucleus.

Aegyptianella is a minute piroplasmid that lacks pigment granules. It appears in three forms, a small Anaplasma-like body less than one millimicron in diameter, a round to piriform inclusion that stains pale blue with a chromatin body at one pole, and a larger [two to four microns] round to elliptical inclusion.

Trypanosomes are recognized by their undulating membrane, slender tapering posterior end, and short anterior flagellum. They resemble those found in mammals are considered to be an incidental find.

Common hemoprotozoa of reptiles include hemogregarines, trypanosomes, microfilaria, piroplasmids, and Plasmodium. The hemogregarines consist of three genera, Hemogregarina which occur in aquatic turtles, Hepatozoon which occur in snakes, and *Karyolysus* which occur in old world lizards and tree snakes. The hemogregarines are identified by their sausage-shaped gametocytes that lack refractile pigment granules and distort the host cell by creating a bulge in the cytoplasm. Plasmodium gametocytes and schizonts in blood films of reptiles resemble those of birds. Piroplasmids such as *Sauroplasma* and *Serpentoplasma* are occasionally found in blood films of lizards and snakes respectively. They appear as small round to piriform, nonpigmented signet ring-like vacuoles in the cytoplasm of erythrocytes.

Summary

Hematology is frequently used to assess the health of fish, amphibians, reptiles, and birds. In general, interpretations of the hemogram of these lower vertebrates are considered to be the same as those for domestic mammals, with the consideration that external factors [e.g. environmental conditions] have a greater influence on the normal physiology and health of ectothermic vertebrates [fish, amphibians, reptiles] compared with endothermic vertebrates [birds and mammals]. Because of the challenges of obtaining cell counts in the lower vertebrates, where all hemic cells are nucleated, interpretation of the morphology of blood cells becomes an important part of the hematologic assessment.