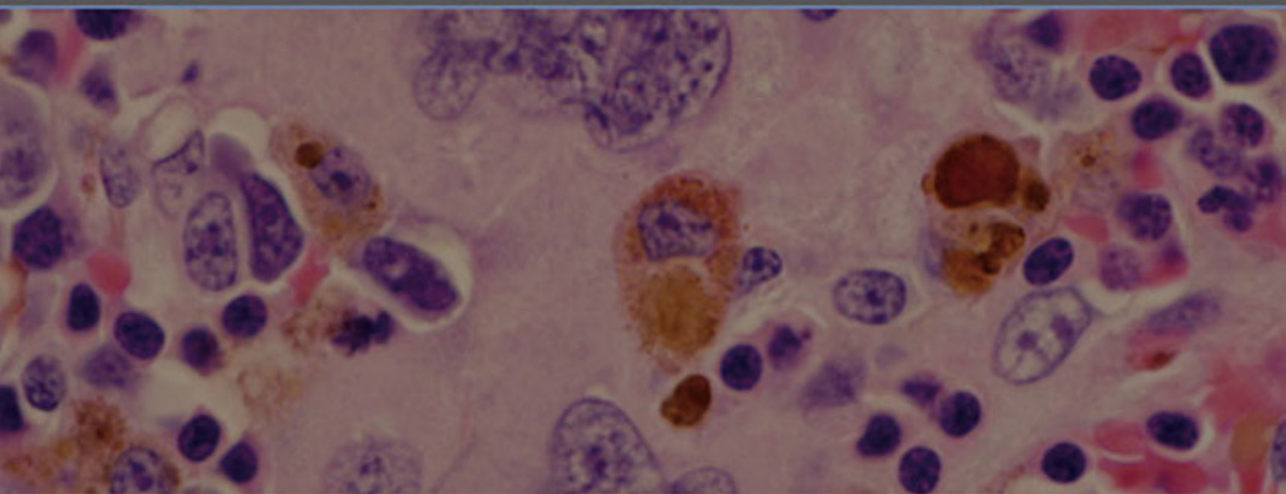
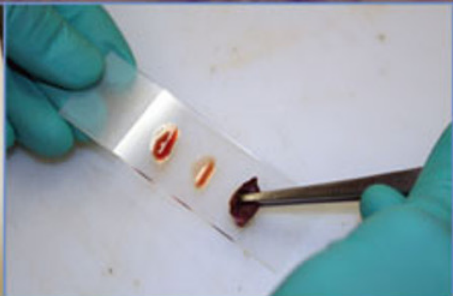


Second Edition

HEMATOLOGY TECHNIQUES & CONCEPTS for Veterinary Technicians



Gregg L. Voigt & Shannon L. Swist

 **WILEY-BLACKWELL**



Hematology Techniques and Concepts for Veterinary Technicians

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 **WILEY-BLACKWELL**

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This book has a companion website providing review questions, images from the book, and additional images only available online at www.wiley.com/go/voigt.

Preface

The major reason for the preparation of this second edition is to introduce the veterinary technology student to new technology, equipment, and test techniques that are becoming more available in the field of veterinary hematology. The intent of the new edition remains the same as in the previous edition. It is an introductory overview of veterinary hematology and a guide for the student to learn the fundamental concepts of collecting, handling, preparing, and testing blood and other samples in the hematology laboratory. It is not intended as a diagnostic aid, differential disease discussion, or complete atlas of blood cells.

Although changes have been made throughout the text, the basic format of the first edition has been retained for the benefit of those currently using the book in the classroom. The major change is the addition of a new chapter, Automated Laboratory Methods and Instruments, by Dr. S. L. Swist. Since publication of the first edition, automated analyzers specifically designed for the veterinary profession have become more available, affordable, accurate, and easier to operate and are more commonly seen in veterinary clinics. This new chapter describes the various types of automatic analyzers, the basics behind their operating systems, and compares the benefits and drawbacks of their use. Dr. Swist has also added her expertise to other areas of the text.

Other changes made to the new edition include a “Key Concept” area at the beginning of each chapter to inform the reader of the major concepts being presented. Many color photographs and photomicrographs have been added to help new students recognize blood cells, techniques, and equipment of the hematology laboratory. Whenever a new term is used in the text, it is accompanied by a definition or recognizable synonym. In addition, a glossary of terms has been added at the end of the chapters with a brief description or definition of terms.

Although the metric system is the standard throughout the medical field, many students accustomed to the American system have difficulty picturing metric units. In many places in the text where metric units are used that relate to animal size and weight, or blood volumes, the approximate US customary units are also given.

Since the original publication of this book, student use of the Internet has increased markedly and an accompanying website has been prepared by the publishers with further information, photographs, and study questions from the authors.

About the Review Questions

Review questions are provided at the end of each chapter and on the website. These questions are provided to stimulate the student to review the material in the chapter, and in any lecture or laboratory notes, in order to better understand the topic being presented by personal study or discussion groups with fellow students. Since the chapters themselves (with associated subheadings, figures, and diagrams) are the answers, a formalized list of answers is not provided. As the title of the book states, the concepts and techniques of hematology and how blood samples are tested are more important than the individual's ability to pick a word off a list of possible answers.

Acknowledgments

The authors would like to thank the personnel from the following veterinary clinics for their help in the preparation of this text:

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Hematology Techniques and Concepts for Veterinary Technicians

Second Edition

Chapter 1

Introduction to the Hematology Laboratory

Key Concepts

In the hematology laboratory, manual and automated test techniques are used in the study of blood and other body cells and fluids.

Blood tests obtained in the laboratory are used by the clinician for screening the health status of an animal or in making a diagnosis of a disease condition.

Quality control of laboratory procedures is essential to ensure that test results reflect the true status of the patient.

Hematology is the study of blood and the tissues that form, store, or circulate blood cells. Examination of the blood is a very common and useful procedure for several reasons. Blood bathes all the other cells of the body carrying nutrients, oxygen, and waste products, and is exposed to almost all metabolic processes of these cells, often reflecting any alteration from normal function. Blood is essential in water and electrolyte balance, temperature control, and the functioning of the immune system, which is the defense mechanism of the body. Obtaining, studying, and testing a blood sample is a relatively easy way of gathering information on many parts of the body.

Even though the term “hematology” literally means “the study of blood,” many of the techniques of collection, sample preparation, and cell identifications learned by the technician can be applied to other regions of the body, such as joint fluid, cerebrospinal fluid, thoracic and abdominal fluids, aspirants from abnormal growths or swellings, and cells collected from mucous membranes (e.g., the oral cavity, trachea, or vagina).

Veterinary technicians should expect to encounter a wide range of laboratory facilities and instrumentation in various veterinary clinics. This can vary from a separate room fully equipped with the latest automated analyzers to a small area on one counter with a microscope, slides, and stains in smaller clinics (Figs. 1.1, 1.2, and 1.3). Even if the clinic sends out all of its hematology tests to a commercial lab, or to the local human hospital, the technician is still usually responsible for correctly collecting, preparing, and mailing or delivering the sample as well as reporting and recording the results.

Hematology is one of several specialties in the field of clinical pathology, a field that encompasses any manual or automated laboratory procedure used on the animal to aid in diagnosing a clinical condition. Clinical chemistry, parasitology, and urinalysis



Figure 1.1 Some clinics may have limited laboratory equipment and may routinely send samples to hospital or commercial laboratories.



Figure 1.2 Technicians will find clinics with an area dedicated to laboratory work with some of the latest automated equipment.



Figure 1.3 Larger veterinary hospitals often have a complete laboratory room that may be staffed by one or more full-time laboratory technicians.

are additional classical aspects of clinical pathology. Other diagnostic tools used by the veterinarian that qualify as clinical pathology under this definition are microbiology, diagnostic imaging (radiology and ultrasound), and the microscopic interpretation of biopsies (tissue samples).

Uses and Benefits of Hematology Results

The most common use of the hematology laboratory is to screen the general health of an animal and to assess its overall ability to transport oxygen and defend against infectious agents. Hematology results provided by the technician are also used by the veterinarian as tools that, when combined with the history, physical examination, and other laboratory findings, help to form a diagnosis. Although occasionally a blood test will yield a definitive diagnosis (e.g., blood parasites), most laboratory results should be viewed as large or small pieces of a diagnostic puzzle that must be assembled.

Hematology results may also indicate a course of treatment for the animal. An example of this is the use of hematology to differentiate between anemia caused by internal hemorrhage and that caused by bone marrow depression. Another example is finding specific infectious organisms within blood cells. Laboratory findings may also suggest other beneficial tests, such as a bone marrow biopsy.

Serial sampling, which is the collection and testing of a series of blood samples over a period of time (hours, days, or even weeks), can demonstrate the severity of a disease process and the ability of the animal to respond. By combining these results with clinical evaluation, the veterinarian will be better able to understand the disease process and make a prognosis for the patient.

It has been said that a clinician who relies entirely on laboratory results to make a diagnosis is probably inexperienced and a clinician who claims not to need a laboratory is uninformed.

Limitations of Laboratory Findings

The validity and usefulness of both manual and automated laboratory results can be influenced by many factors that should be understood by the technician, so they may be eliminated or minimized as much as possible. Technical errors associated with individual tests are discussed in later chapters, but those of general laboratory procedures are addressed in this section.

The first potential problems arise in the collection and handling of the sample. The blood sample, or any other tissue sample, should not be subjected to traumatic physical forces, such as being forced rapidly through a small needle or violently shaken, or to extreme temperatures of heating or freezing. Care must be taken to avoid contaminating the sample with foreign material such as dirt, infectious agents, chemicals, or even water. Test results are always more meaningful with fresh samples, but when this is not possible samples should be refrigerated since blood, like other organic material, begins to degenerate after removal from the body. A more extensive list of sample handling errors is found in Chapter 4.

Quality control, or more accurately “quality assurance,” should be considered a laboratory commitment, rather than a laboratory problem. Whether the test procedures are performed manually, carried out by automated equipment, or even sent to a local hospital or a commercial or state laboratory, it is essential to know that the results reflect the patient’s status and not a difference in machines, technicians, or techniques.

Test results often vary with different test conditions, and the “normal” animal may routinely test higher or lower than published “normal” ranges. Commercial and state laboratories usually provide their established reference values for each test being performed on each species with the laboratory report. Clinical laboratories should establish reference values for any manual or automated test procedure performed in the clinic. For reference values to be statistically valid, a large number of tests must be performed, which may be difficult in a small clinical setting. By keeping a record of all tests run (and data on the patients), plus checking medical records for other similar test results, clinics can establish a database to compare with published values. At a minimum, tests on several known normal or control samples should be run for each procedure by each technician who will be performing the tests. These control samples can also be sent to the outside laboratory being routinely used and the results compared with the in-house results. Some slight variation in results should be expected, but they should fall within the established range for that procedure. Paired samples, half sent to an outside source and half run in-house for comparison, can also be quite instructive. Running such tests will ensure that current test procedures and results are reliable, or the tests can be used to establish reference readings for a new technique, technician, or a piece of equipment. The frequency of checking in-house tests will vary with the type and a number of tests performed and often varies from daily to weekly to monthly but should never be overlooked.

Another area of quality control that must not be overlooked is the variation in skill levels, accuracy, and care of the technician, especially in manual procedures. While the decision about which laboratory test to perform in the clinic or hospital and which to send out depends a great deal on the cost-effectiveness and timely availability of outside services, the number of tests being performed should also be considered. Routinely, if a test is not being done at least a few times per week, it does not provide the laboratory personnel with adequate opportunities to become skilled at, and comfortable with, the procedure or the equipment.

The veterinary technician is often responsible for collecting, preparing, and examining the sample and reporting the results. All counts or measurements should be done on a “blind” basis, that is, with no comparisons to normals or expectations of changes that could lead to bias in reported results. Since the technician may be the only person to actually see the sample, it is imperative that all observations, whether normal or abnormal, be recorded. Often the “comments” section of a lab report is as informative as the recorded results.

Laboratory Safety

Although most diseases of domestic animals routinely handled by the technician are not communicable to humans (zoonotic), the ones that tend to be quite severe, possibly fatal. All biological samples, whether blood or other body tissues and fluids, should be handled as if potentially infectious. Routine handwashing and disinfection of glassware and



Figure 1.4 A laboratory smock should be worn at all times, and protective gloves, eyewear, and confining of long hair are important when working around potentially infectious or hazardous conditions.

working areas is essential. There should be no drinking, eating, use of tobacco products, or other hand-to-mouth activities, nor any storage of food or beverages in the laboratory area. A laboratory coat should be worn at all times, long hair confined (especially around Bunsen burners and centrifuges!), and sandals, open-toed shoes, or canvas shoes should be avoided. Laboratory gloves should be worn whenever potentially infectious samples are being handled (Fig. 1.4). All disposable pointed or cutting instruments (e.g., needles and scalpel blades) should be placed in an appropriate “sharps” container, such as an empty gallon jug or commercial container, prior to disposal. Laboratory safety policies should not only be posted in the laboratory area but also be read and followed.

Review Questions

1. What is the major function of the hematology laboratory?
2. Compare the benefits and limitations of the hematology laboratory.
3. What is meant by the term “quality control” in relation to reporting test results?
4. Why is laboratory safety important to the hematology technician?

Chapter 2

Composition of Blood

Key Concepts

Blood is a tissue composed of a fluid called plasma and seven cellular elements called erythrocytes, neutrophils, eosinophils, basophils, lymphocytes, monocytes, and platelets.

The cellular elements of the blood can be identified by their individual characteristics and the staining qualities of their cellular contents on a prepared glass slide.

Blood is a type of connective tissue, and collecting a blood sample is essentially taking a tissue biopsy. Blood is composed of cells surrounded by a noncellular substance, just like other connective tissues, such as fibrous tissue, bone, or cartilage. The major difference is, of course, that the extracellular substance in blood is liquid, called plasma. This characteristic, plus the fact that much of this “tissue” is located near the surface of the animal, makes collecting a sample of blood comparatively easier than sampling deeper, more solid organs and tissues.

PCV, Buffy Coat, and Plasma

If drawn blood is kept from clotting by the addition of an anticoagulant (see Chapter 4), we can separate the blood into its various components. If allowed to set undisturbed (or placed in a centrifuge to speed up the rate of separation of the components), three distinct layers will appear: the heavy red cells on the bottom, the lighter white cells in the middle, and the plasma on the top (Fig. 2.1).

The bottom one-third to one-half of the tube will be dark red and will contain the heaviest (most dense) structures, the erythrocytes (red blood cells). The measurement of the percentage of these cells in the blood is called the packed cell volume (PCV) or hematocrit (Hct) and is commonly one of the first blood evaluations performed (see Chapter 6).

Directly on the top of the red blood cells will be a relatively thin, white to tan layer called the buffy coat. This layer contains the leukocytes (white blood cells) and the platelets. The thickness of this layer can be a basis for a rough estimate of the relative numbers of these cells in the sample. The occasional appearance of a light pink to red color in the buffy coat indicates the entrapment of some lighter density erythrocytes in this layer.

At the top of the tube will be the fluid portion of the blood sample, called plasma since the blood has been separated without clotting. If the blood had been allowed to

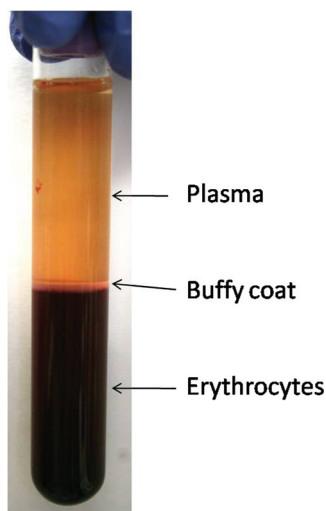


Figure 2.1 The components of unclotted blood.

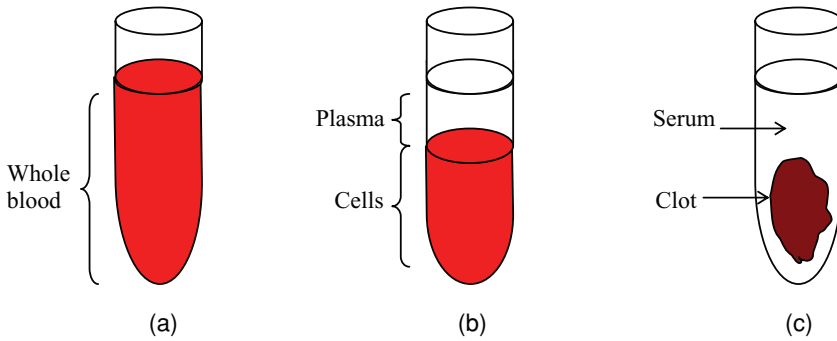


Figure 2.2 Physical difference between plasma and serum. (a) Mixed fluid and cells in whole blood. (b) With anticoagulant added, cells separate from plasma. (c) Without an anticoagulant, cells and clotting factors separate from serum.

clot, several proteins in the fluid would have been used to form the clot and the fluid would then be called serum (Fig. 2.2). The plasma color will vary from clear to straw to yellow-orange (due to species variation, diet, and physiologic or pathologic conditions) but should be transparent. The technician should always observe the visual characteristics of the plasma, especially any cloudiness, abnormal coloration, or layering, and write descriptive comments on the laboratory report. (Remember, the technician may be the only one to see the sample and should pass on the information!)

Blood Cells

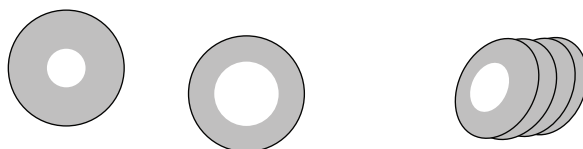
In order to see and evaluate individual blood cells, a drop of blood must be spread on a slide and stained. This is one of the most common and important hematology procedures. Techniques for making and staining the blood smear, as well as suggested methods of viewing the slide under the microscope to ensure optimum examination of the cells, are given in Chapters 5 and 6.

There are seven “formed elements” (cells or cell fragments) found in the blood:

- | | |
|--|-----|
| Erythrocytes (red blood cells or RBCs) | (1) |
| Leukocytes (white blood cells or WBCs) | |
| Granulocytes | |
| Neutrophils | (2) |
| Eosinophils (acidophils) | (3) |
| Basophils | (4) |
| Agranulocytes | |
| Lymphocytes | (5) |
| Monocytes | (6) |
| Thrombocytes (platelets) | (7) |

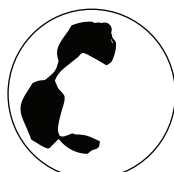
A diagram of the basic differences in appearance of each of these cells is shown in Fig. 2.3. A brief description of the appearance and function of each is then presented to

Red blood cells (erythrocytes)

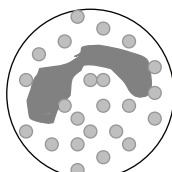


White blood cells (leukocytes)

Granulocytes



Neutrophil

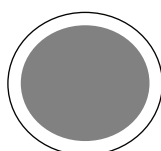


Eosinophil

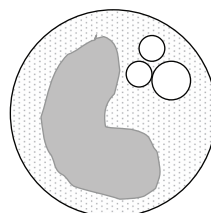


Basophil

Agranulocytes



Lymphocyte



Monocyte

Platelets (thrombocytes)

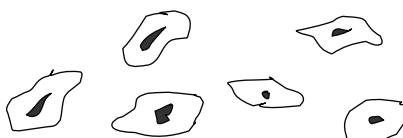


Figure 2.3 The seven “formed elements” (cells and cell fragments) of the blood.

allow the reader to become familiar with and begin to identify these cells. More detailed descriptions are given in later chapters.

Erythrocytes

The most common cell encountered in the blood is the **erythrocyte**; overall, there are approximately 1,000 erythrocytes for every leukocyte. On a slide, the erythrocyte of most mammals is a round, homogeneous, nonnucleated cell that stains pink to salmon to red (Fig. 2.4). In the dog, the center of the cell is thinner than the edges (biconcave) and more lightly stained, but this shape is less pronounced in the other common species. The

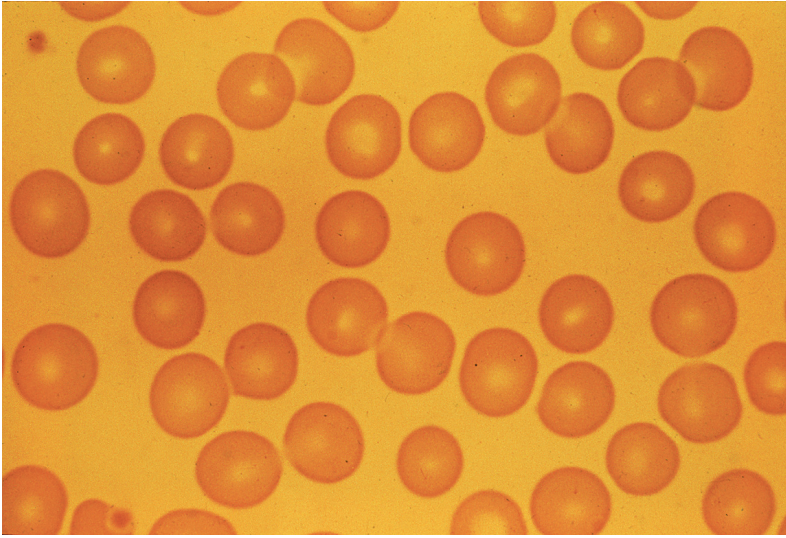


Figure 2.4 Normal canine erythrocytes. The central pale areas are due to the biconcave nature of the cells. In most other species of domestic animals, erythrocytes are flatter.

erythrocyte in the members of the camel family (camels, llamas, etc.) is routinely oval, and in birds, reptiles, amphibians, and fish, it is oval and nucleated (see Chapter 10).

The major function of the erythrocyte is to transport oxygen from the lungs to be released in the cells and tissues throughout the body. The carbon dioxide that is generated by the cells is then carried back to the lungs and exchanged for oxygen.

Leukocytes

Scattered among the erythrocytes on the slide preparation are nucleated cells of varying sizes, some of which contain granules that stain various colors. These are the **leukocytes**, of which there are five types. The neutrophil, eosinophil, and basophil routinely have granules present in the cytoplasm (cellular fluid) and are categorized as “granulocytes,” while the lymphocyte and monocyte are “agranulocytes.” Although the leukocytes have somewhat different individual functions, which are discussed in Chapter 8; in general, their activities are related to recognizing and responding to any substance foreign to the body, especially potential disease-causing agents, such as bacteria, viruses, and fungi.

The most common of the leukocytes in most animals, and the second most common in the rest, is the **neutrophil**. Even with slight differences in appearance between species, it is usually the most easily recognized leukocyte. The neutrophil is somewhat larger than an erythrocyte and is characterized by its densely stained, clumped-appearing nucleus, which is elongate and usually deeply indented or constricted (Fig. 2.5a). This clumping often makes the nucleus appear to have two to five separate lobes or segments, which leads to the common names of “segmenter” or “polymorphonuclear” (PMN), meaning many shapes to the nucleus. When stained, the cytoplasm is clear to pale blue or gray and contains scattered to numerous neutrophilic (gray to light pink) granules. In some

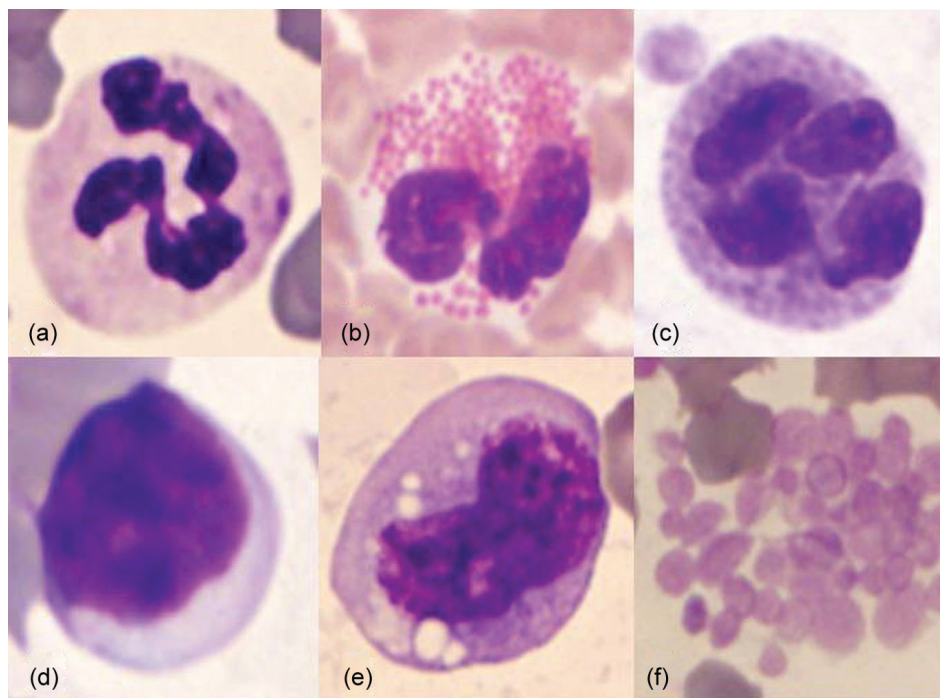


Figure 2.5 (a) Horse neutrophil, (b) cow eosinophil, (c) cat basophil, (d) cat lymphocyte, (e) sheep monocyte, and (f) horse platelets. The indistinct cell borders and cytoplasmic contents are typical of cells viewed on most microscopes under very high magnification. (Amount of magnification and final printed cell sizes do not reflect normal relative sizes.)

species, the granules are quite obvious, but in many species, they appear as a fine dust or are too small to see clearly with the light microscope, and so the cytoplasm will appear clear. If the nucleus is more ribbon-shaped, with parallel sides, it is called a band or stab neutrophil, and if more than five segments are seen, it is referred to as hypersegmented. Differentiating between these developmental stages is an important diagnostic aid and is discussed in Chapter 8.

The **eosinophil** (or acidophil) is the same size or slightly larger than the neutrophil and has a nucleus that may be band-shaped or constricted into a bilobed or trilobed appearance (Fig. 2.5b). The cytoplasm is usually a light blue but may be difficult to see due to the presence of distinctive granules. The size, shape, number, and staining qualities of these granules vary greatly between species and are so characteristic that the eosinophil can often be used to determine the species from which the sample was obtained. These cytoplasmic granules are called eosinophilic because they chemically attract eosin, the red dye used in staining. In general, they appear pink to orange to salmon colored, and occasionally bright red, in routine laboratory stains. They often refract (bend and scatter) light as it passes through the granules, which makes focusing directly on them difficult, and they may look like bright dots. Since overall staining qualities will vary with different techniques and species, a useful comparison is that the color of the granules should be similar to, or slightly lighter staining than, the surrounding erythrocytes.

The **basophil** is rarely encountered in normal blood smear preparations. Intermediate in size between the neutrophil and eosinophil, it has an elongate to slightly indented nucleus that has less densely staining chromatin. The nuclear outline is often obscured by basophilic granules in the cytoplasm that stain from light or dark purple to almost black (Fig. 2.5c). The number of granules may vary from few to densely packed and may be very small and light staining (as in the cat) or larger and very dark (as in the ruminant).

The **lymphocyte** is the most common leukocyte seen in many ruminants and rodents. It is unique among the leukocytes in that when it is stimulated it has the ability to change its size and shape into large, medium, and small forms, and it can form more oval cells called plasma cells. The mature lymphocyte is the smallest of the leukocytes, often barely larger than the erythrocyte, and has a round nucleus surrounded by scanty, blue-staining cytoplasm, which is often barely detectable (Fig. 2.5d). The nuclear chromatin is coarse and clumped, and a small indentation may be present at the nuclear periphery. Much larger lymphocytes are also seen, especially in cattle, with more abundant cytoplasm and a larger nucleus that may become more oblong or rectangular. These cells are easily distorted by surrounding cells. When the cytoplasm stains a more intense blue, the term “immunocyte” is often applied. Some lymphocytes will form plasma cells (plasmacytes), which are oval and have a round, eccentrically placed nucleus in which the clumped chromatin often has a cartwheel appearance. These cells are discussed in more detail in Chapters 8 and 9.

The **monocyte** is the largest of the leukocytes and is similar in appearance in all common species. It is characterized by a pleomorphic, or ameboid, nucleus that can assume various shapes from elongate to rounded and is often in kidney bean, horseshoe, butterfly, or H-shaped (Fig. 2.5e). The nuclear chromatin is fine, lacy, and smooth, with very little clumping, and therefore stains less densely than the other leukocytes (a characteristic often useful in identification). There is abundant, bluish-gray cytoplasm, which often has a foamy or ground glass appearance and often contains small to large vacuoles (holes or bubbles). Since the major activity of the monocyte is phagocytosis (the engulfing and digesting of particles), variable granules or particles, and sometimes other cells, may be found in the cytoplasm.

Thrombocytes (Platelet)

The final “formed element” seen in the blood smear is the platelet, or **thrombocyte**. Thrombocytes are not even complete cells but are simply pieces of the cytoplasm of a large cell found in the bone marrow (the megakaryocyte—see Chapter 14) and vary widely in size and shape in most mammals. Occasionally, they may be nearly as large as an erythrocyte (especially in the cat) but more commonly are small and lightly stained and may contain granules (Fig. 2.5f). They are usually scattered randomly throughout the smear but will occasionally be seen in small to large clumps that may also include other leukocytes, especially when slow or otherwise improper collection techniques were used in obtaining the sample. This clumping is due to the platelet’s essential function in blood coagulation.

Review Questions

1. Why is blood considered a connective tissue?

2. Differentiate between plasma and serum.

3. Name the seven cells and cell fragments of the blood and give a brief description and one major function of each.

Chapter 3

Blood Volume and Effects of Blood Loss

Key Concepts

Although difficult to accurately measure, approximately 7 percent of a typical adult animal's body weight is blood.

Up to 0.5 mL/kg (0.2 mL/lb) of blood can be safely drawn from a healthy animal.

Removal of more than 30 percent of an animal's blood volume can lead to dehydration, shock, and potential death.

Although it appears to be a simple concept, the total blood volume in any given animal is a difficult quantity to measure or even define. Blood is not a single substance but a mixture of components, most of which (water, proteins, electrolytes, even cells) routinely move in and out of the vascular channels. The water and dissolved substances even move into and out of other cells. Even if we limit our definition of “blood volume” to the amount present in the circulation at any given moment, this amount has proven difficult to measure or estimate with accuracy. Several methods have been devised in attempts to estimate the total active blood volume, mainly based on injection of a known amount of dye or radiolabeled molecules, and then measuring the amount of blood dilution.

As might be expected, total blood volumes vary significantly between the different species of animals and even different breeds or types within a species (e.g., “hot-blooded” horses, such as Arabians and Arabian crossbreeds, who have higher body temperatures and other physiological differences, versus “cold-blooded” horses, such as quarter horses; Table 3.1).

Also, fluid volumes in general are routinely higher in young animals and in lean animals compared with those with a higher fat percentage.

Approximate Total Blood Volumes

With slight variation, the amount of blood present in an animal is within the range of 5–11 percent body weight (40–110 mL/kg or 20–50 mL/lb). An average figure of 7 percent of body weight may be used to illustrate this volume of blood. For example, a 45 kg (100 lb) animal (or small adult human) would have 3,150 mL (6–7 pints, which is slightly less than a gallon) of blood. A 450 kg (1,000 lb) horse or cow would have about ten times this amount, 31,500 mL, or about 8 gallons of blood.

Effects of Blood Collection and Blood Loss

As a general rule, 0.5 mL blood/kg body weight can be safely removed from healthy animals with no detrimental effects (Fig. 3.1). This is about 1 mL from a 5 lb animal and

Table 3.1 Approximate blood volumes of animals.

Species	mL/kg	mL/lb	Body weight (%)
Dog	80–110	35–50	8–10
Cat	65–75	30–35	7–8
Horse			
Hot-blooded	90–110	40–50	9–11
Cold-blooded	60–80	28–35	6–8
Cow	60–70	25–30	6–7
Sheep	60–70	25–30	6–7
Goat	70–75	30–35	7–8
Pig	40–60	20–30	5–6



Figure 3.1 Three milliliters of blood is being safely removed from the jugular vein of this 15 lb (6 kg) dog.

20 mL from a 100 lb animal. Larger amounts up to 10 mL/kg (5 mL/lb) body weight can be removed when necessary, as in healthy blood donor animals, as long as the animal is given adequate time, nutrition, and care to rebuild its blood supply (Figs. 3.2, 3.3, and 3.4). By comparison, the average healthy human of 50 kg (110 lb) or more can donate 500 mL (1 pt) of blood every 8 weeks with minimal clinical effects. At this minimum acceptable weight, the amount of blood lost is 10 mL/kg, which represents between 10 and 15 percent of total blood volume.

Very small animals such as puppies, kittens, laboratory animals, small exotic pets, and caged bird may weigh only a few ounces and, therefore, have small total blood volumes. Extreme care should be taken when drawing blood from these animals as excessive blood loss can occur in the smallest of these with as little as a few drops. The same caution should be used with animals that are in shock, are anemic, or are otherwise debilitated.

The clinical effects of more extreme blood loss are related not only to volume of loss but the rate at which it is lost. With relatively rapid loss, most animals will begin to show signs of shock with the loss of 30 percent of their blood volume. With 40 percent or more loss, death rates increase rapidly. This is due to inadequate tissue perfusion even though other tissue fluids move into the vascular system over a short period of time. In a 45 kg (100 lb) animal, this means that about a liter (2 pt) may be lost (or collected from a blood donor) before signs of shock would be seen.



Figure 3.2 Supplies needed for blood collection from a donor dog.



Figure 3.3 Blood collection from the jugular vein of a donor dog.



Figure 3.4 Filled donor blood collection container.

With slower or more chronic blood loss, more time is available for fluid to shift from other parts of the body to the vasculature. Affected animals more typically demonstrate signs of anemia (low red blood cell values) rather than shock since the functional loss is in oxygen-carrying capacity, not fluid volume.

Review Questions

1. How much blood can be safely drawn from a healthy, adult animal weighing?

- 1 lb
- 10 lb
- 100 lb
- 1000 lb

2. What are two clinical conditions that can result from excessive loss of blood volume or blood cells?

Chapter 4

Blood Collection and Handling

Key Concepts

The proper collection and handling of a blood sample is important to prevent or minimize alterations of blood counts and measurements.

The equipment and techniques used for blood collection will vary with the size of the patient, selection of venipuncture site, and amount of blood to be collected.

Numerous types of anticoagulants are available for use in preventing blood clotting.

All blood tests begin with proper collection of the blood sample—improper collection and handling of the sample may alter blood measurements or cell counts and could lead to unreliable or, much worse, misleading results. A list of potential collection errors is presented at the end of this chapter.

Any events or circumstances occurring at the time of collection that may potentially influence the result, especially stressors such as patient excitement, exercise, apparent disease signs, and extreme weather conditions, should be noted (see “Physiologic Leukocytosis” in Chapter 8). Restraint of the animal should be minimal to reduce the effects of stress.

Venipuncture Site

The selection of the venipuncture site depends on the species of animal, the experience and preference of the technician, and the absence of pathology in the region (Figs. 4.1 and 4.2). Commonly used sites are listed in Table 4.1. In very small animals, such as laboratory animals or exotic pets, small amounts of blood can be obtained from the lateral marginal ear vein, the tail vein, or a toenail clip.

Any hair should be clipped from the venipuncture site and a suitable disinfectant applied. Skin is difficult to sterilize, but the site must be clean, and any possible contamination minimized. A surgical scrub and local anesthesia are recommended if placement of an intravenous (IV) catheter or use of a large bore needle is contemplated.



Figure 4.1 Placement of an intravenous catheter in the cephalic vein of a dog.



Figure 4.2 Blood collection from the saphenous vein of a dog.

Blood Collection Equipment

New needles should routinely be used, and any needle used should be sterile, dry, sharp, and free from any burrs. The size (gauge) and length of the needle used will vary but should not be smaller than 22 gauge. If a smaller needle is necessary due to the small size of the patient and/or vessel, very slow, deliberate aspiration must be used to prevent hemolysis (rupture of red blood cells) and leukocyte damage. Needles with a short bevel may be easier to place within small vessels. Some commonly preferred collection sites and needle sizes are presented in Table 4.1.

Table 4.1 Blood collection sites and needle sizes.

Species	Site	Needle size	
		Gauge	Length (in.)
Dog	Cephalic, jugular, or saphenous vein	20–22	1.0–1.5
Cat	Cephalic, jugular, or saphenous vein	22–23	1.0
Horse	Jugular vein	16–20	1.5
Cow	Jugular, caudal (tail), or mammary vein	14–18	1.5–2.0
Sheep	Jugular vein	18–20	1.5
Goat	Jugular vein	18–20	1.5
Pig	Anterior vena cava	18–20	2.0–4.0
	Ear vein	18–22	1.0–1.5

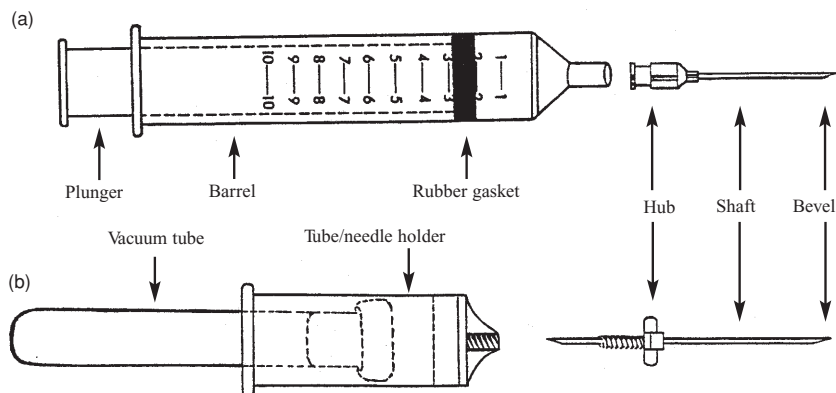


Figure 4.3 Blood collection equipment: (a) syringe and needle and (b) commercial vacuum tube and needle.

Blood may be collected directly from the needle hub to a glass slide or a capillary tube. More commonly, it will be drawn into a syringe (Fig. 4.3a) or directly into a commercial vacuum tube (Fig. 4.3b). Direct collection of the blood into a vacuum tube has the advantages of premeasured amounts of anticoagulant already present (if desired), the vacuum will draw in the correct amount of blood at a safe rate of aspiration, and multiple tubes may be filled from a single venipuncture site. Plastic equipment has the advantages of being relatively unbreakable, is less likely to stimulate clotting mechanism, and is less expensive. New plastic syringes should be used because autoclaving may cause the rubber plunger gasket to become too loose or too tight in the barrel and to either leak or bind. Anticoagulant (discussed later in this chapter) may be added to the syringe prior to collection, or the blood must be transferred to a suitable tube containing anticoagulant prior to the onset of coagulation.

Venipuncture Techniques

Techniques of venipuncture will vary with individual preferences and collection site, but with any technique the vessel should be occluded by an assistant or a tourniquet between the collection site and the heart. The technician should see the vein beneath the skin, or distinctly feel it and stabilize it prior to needle entry (Fig. 4.4). With experience, the technician will be able to pass the needle through the skin and fascia (subcutaneous connective tissue) and directly into the vein in a single motion. If blood is not present on the first attempt, the needle should be left motionless in the subcutaneous fascia, the location of the vessel redefined, and the needle advanced directly into it. The technician should not use the site as a pincushion in a blind attempt to “hit something.”

When only a small amount of blood is needed, the lateral marginal ear vein may be used in the dog, cat, and most laboratory animals in a manner similar to fingertip puncture



Figure 4.4 Obtaining a blood sample from the jugular vein of a horse. Note the dilation and delineation of the vessel from the pressure applied between the venipuncture site and the heart.

in humans. Long or matted hair must be clipped, and the area cleaned and disinfected. A thin layer of petrolatum spread on the skin will keep tissue fluid and debris out of the sample. A small puncture wound is made through the petrolatum and into the skin and vessel with a lancet or the point of a needle. Blood will well up at the site and may then be collected directly onto a slide, into a microhematocrit tube, or onto a specialized test strip or tube for use in an automated analyzer. The technician should avoid excessive pressure to the immediate area because this may damage cells or cause excessive amounts of tissue fluid in the sample.

Anticoagulants

When a blood sample is collected (or whenever blood encounters a substance other than the normal endothelial lining of the blood vessels), it will begin to coagulate, or form a clot, usually within 2 to 5 minutes. This mechanism is discussed in Chapter 13. Since we are concerned here with studying the total and relative numbers of cells, and the appearance or changes in these cells, we need to prevent the formation of a clot in order to leave the cells in suspension. This is done by adding an anticoagulant, a substance that interferes with the clotting mechanism. EDTA (ethylenediamine tetraacetic acid) is by far the most common anticoagulant used in routine hematology in mammals, but several types of anticoagulants

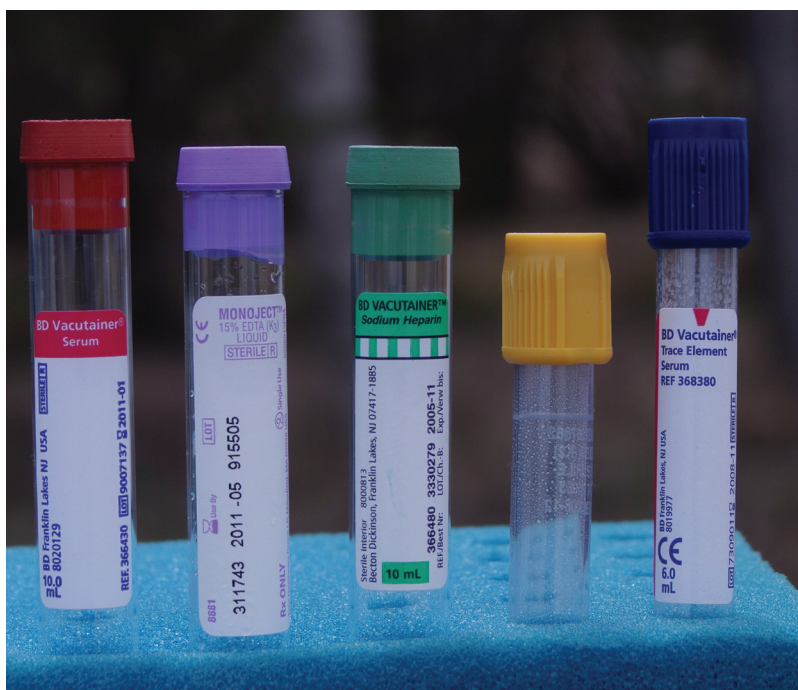


Figure 4.5 Examples of commercial blood collection vials. From the left, the red stopper indicates no anticoagulant (“clot tube”), the lavender one (often called “purple top”) has EDTA, the green has heparin, and the yellow and blue plastic capped tubes are specialized tubes for specific tests.

are available, each with its own advantages, disadvantages, and established use. (Table 4.2) The mechanism of action of almost all anticoagulants is binding of the calcium ion, which is critical in most steps of the clotting cascade. An exception to this is heparin, which inhibits formation of the key clotting protein, thrombin.

Commercial vacuum tubes are available containing appropriate amounts of anticoagulant for the size of the tubes. The anticoagulants are distinguished by the color of the rubber stopper in the tube: EDTA—lavender (often called “purple-top”); heparin—green; oxalates—black; citrates—blue. Tubes with a red stopper contain no anticoagulant and are routinely called “clot tubes” (Fig. 4.5). If anticoagulants are purchased in powder or liquid form, the concentration may vary between manufacturers, and the directions for measuring and mixing must be followed carefully to avoid cellular changes.

Potential Collection Errors

Careless or improper collection and handling of the blood sample can damage cell membranes, causing red blood cell hemolysis and white blood cells that are distorted or

Table 4.2 Anticoagulants.

Product	Mode of action	Amount required for 10 mL blood	Advantages	Disadvantages
EDTA (K or Na salts of ethylenediamine tetraacetic acid)	Forms insoluble Ca salts	10–20 mg (1 mL of 1% solution or 2 drops of 10% solution)	Excellent preserving power for 6 hours; recommended for routine hematology	Excess over 2 mg/mL shrinks cells; Na salt is less soluble than K EDTA
Heparin	Antithrombin and antithromboplastin	1–2 mg (0.2 mL of 1% solution); can moisten syringe and needle with concentrated (10 mg/mL) solution	Least effect on size and hemolysis of RBC; used for blood gas analysis	May cause clumping of WBC; unsuitable for smears-interferes with stainability of WBC; expensive; will not prevent clotting longer than 8 hours; not suitable for agglutination or prothrombin time tests
Sodium citrate	Combines with Ca to form insoluble salt of calcium citrate	10–20 mg; for some studies, one part 3.8% solution to 9 parts blood	Can be used for blood transfusion	Interferes with many chemical tests; prevents clotting for only a few hours; shrinks cells
Potassium oxalate	Unites with Ca to form insoluble calcium oxalate	20 mg or 2 drops of 20% solution	Very soluble	Causes 6–8% shrink in cell volume; poor for PCV and differential; excess interferes with precipitation of protein; low glucose levels; alters electrolytes; poisonous

(Continued)

Table 4.2 (Continued)

Product	Mode of action	Amount required for 10 mL blood	Advantages	Disadvantages
Sodium oxalate	Unites with Ca to form insoluble calcium oxalate	20 mg; for prothrombin time used 0.5 mL of 0.1 M sodium oxalate in 4.5 mL blood	Used mainly for prothrombin time	Same as potassium oxalate; shrinks cells
Ammonium and potassium oxalate (Heller and Paul's double oxalate)	Unites with Ca to form insoluble calcium oxalate	1 mL or 20 mg dried powder	Can be used for most hematologic procedures; produces less distortion and hemolysis than other oxalates	Ammonium oxalate off-sets RBC shrinkage caused by potassium oxalate: cannot be used for BUN study
Sodium fluoride and thymol (10:1)	Forms a weakly dissociated Ca complex	100 mg Na fluoride and 10 mg thymol	Both anticoagulant and preservative; excellent preservative for blood glucose	Interferes with enzyme methods for glucose and BUN (urease)
ACD solution B (acid citrate dextrose)		For transfusions use 25 mL ACD solution for 100 mL blood	Recommended for blood transfusions	

disrupted and therefore unreadable. Some of the more common errors in collection and handling that the technician should avoid are the following:

- Rapid or forceful aspiration of the blood, especially through a needle less than 22 gauge.
- Spraying the blood through the needle into a second vial. The needle and tube stopper should be removed and the blood runs gently down the inside of the vial.
- Water present in the syringe, needle, or tube, causing osmotic damage to cells.
- Collecting too little blood for the amount of anticoagulant present, resulting in dilution error or direct cellular damage from concentrated anticoagulant.
- Slow collection or delayed transfer to anticoagulant allowing clumping of platelets and clot formation.
- Improper or incomplete mixing of anticoagulant. The tube should be gently but thoroughly rotated by hand or on an automatic tube rotator, or it may be rolled across a flat surface.
- Excessive physical force, such as shaking, jarring, or dropping.
- Allowing the sample to overheat or freeze.
- Excessive time at room temperature between collection and utilization, allowing degeneration of cells (autolysis). If a sample cannot be prepared within an hour of collection, the sample should be refrigerated for optimum results.

Review Questions

1. Why are proper blood collection techniques important to the hematology technician?

2. List the equipment necessary for collection and handling of a blood sample.

3. Why is an anticoagulant added to most blood samples and how does an anticoagulant work?

Chapter 5

Blood Smears and Staining

Key Concepts

A properly prepared blood smear can provide valuable information in the diagnosis of many disease conditions.

Several approaches may be used to prepare a blood smear, but each requires correct technique and repetition.

Staining of a blood smear not only makes the cells more visible in the microscope but differentiates cellular components by including acidic and basic dyes.

The examination of peripheral blood smears can yield more information than any other single laboratory test, but only if the samples have been properly prepared and well stained. Blood smears will be used to determine the types and relative numbers of cells be determined, and to assess the size, shape, stage of development, depth of stain or changes in staining qualities, and cellular inclusions. Poor smearing or staining techniques may make these criteria difficult or impossible to evaluate.

Blood Smear Preparation

To make a good-quality blood smear, glass slides, and coverslips must be clean and dry, have no surface film or foreign material, and be free of any chips or etching. The use of new slides will routinely produce better results. Slides with a beveled edge are available, which are smoother and make for better smear preparation. Dipping slides in alcohol and wiping with a lint-free cloth or paper prior to use is recommended. Slides must be handled only by the edges or with forceps since even minimal amounts of grease deposited by fingertips will prevent adherence of cells to the slide, making slides unacceptable for use.

The blood sample should be as fresh as possible. The best smears, both in ease of preparation and in cellular morphology, are made from fresh blood with no anticoagulant. Blood collected in EDTA will show only slight changes if the smear is prepared within an hour, after which changes in staining characteristics and cellular detail will increase in relation to time and environmental conditions. Any sample that is retained more than an hour should be refrigerated. If smears are made from samples collected with anticoagulants other than EDTA, cells will often be distorted and difficult to interpret.

Slide Technique

To prepare a slide smear, the technician should place a clean slide on a flat surface or hold in his or her left (or nondominant) hand by the edges only. (This latter technique is more difficult to learn but is often useful where a clean, flat surface is not readily available—such as in field calls with large animals.) This first slide is often called the “smear” or “film” slide. A small drop of blood is transferred to this slide directly from the patient or by means of a needle, applicator stick, or capillary tube. The drop should be near one end of the slide and, if frosted end slides are used, should be near the upward-facing frosted end. The size of the drop is important in final smear thickness and must be learned by experience.

Immediately after the drop of blood is placed on the slide, a second slide (called the “spreader” or “pusher,” which must have clean, smooth edges) is picked up by the edges or frosted end and placed on the first slide in front of the drop of blood at an angle of about 30° back toward the drop. The spreader slide may be placed either vertically (Fig. 5.1a) or horizontally (Fig. 5.1b), depending upon the preferences and experience of the technician. The spreader slide is then drawn back to just contact the drop of blood, at which point the blood will spread between the edge of the spreader slide and the smear slide surface (Fig. 5.2). When the blood has spread approximately three-quarters of the way across the slide face, the spreader slide is quickly pushed in the opposite direction toward the open end of the smear slide, pulling the spreading drop of blood in the angle behind it. The edge of the

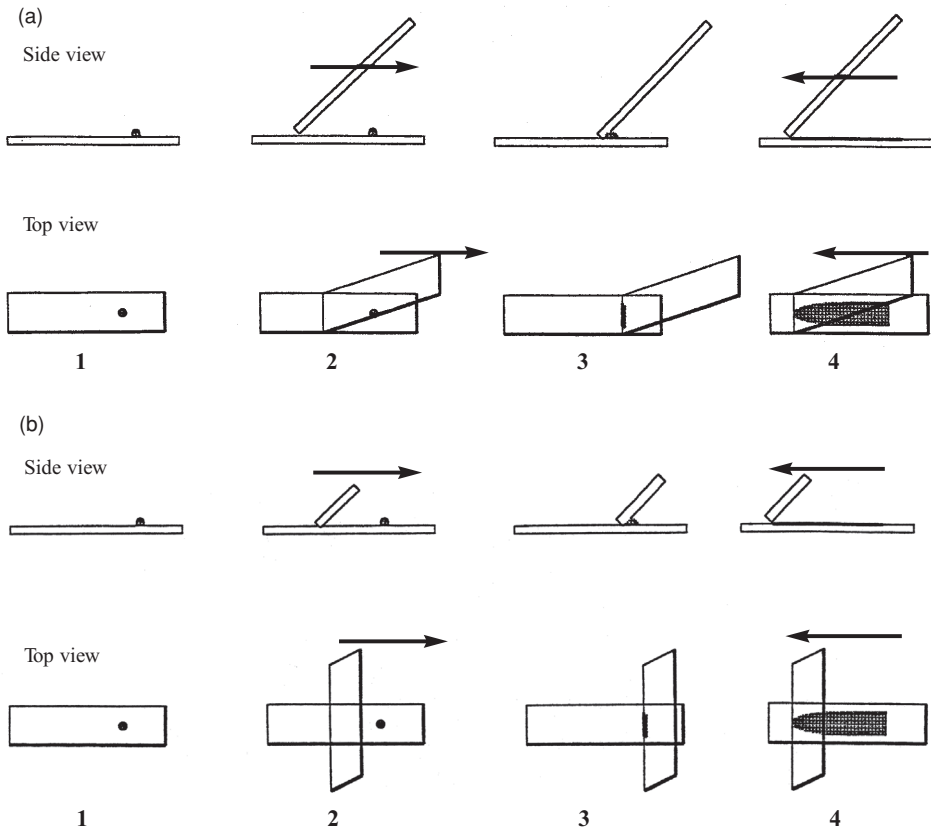


Figure 5.1 Techniques for making a blood smear with a monolayer suitable for cell evaluation.

spreader slide must remain flat on the surface of the slide and has only enough downward pressure on it to ensure continued surface contact; the weight of the slide and the surface tension of the blood are enough to form the blood smear. Too little pressure will allow the spreader slide to lift from the surface forming gaps in the smear; excess pressure will cause a wave appearance to the smear, with alternating thick and thin areas. Ideally, the blood will have spread laterally to within 2–3 mm of the edges and will “follow” the spreader slide nearly to the end of the film slide, where it will appear thin and feathered. This will provide a large area for optimum examination of cells that do not overlap but are abundant and demonstrate normal morphology and distribution (the monolayer).

The shape, thickness, and length of the smear (and, therefore, the size of the readable area) are influenced by (1) the size of the drop of blood, (2) the angle at which the spreader slide is held, and (3) the speed at which the drop is spread (Fig. 5.3). Using too large of a drop results in a smear that is too thick and dries slowly, which causes cellular distortion and leaves a very small area useful in interpretation. A large drop may even carry behind the spreader slide on past the end of the slide, making the slide unusable. A smear that is too thin will have the cells spread so widely it is difficult to survey adequate numbers. Similarly, varying the angle of the spreader slide will carry more or less blood along with it, and moving the spreader slide too rapidly or too slowly will alter the amount of blood

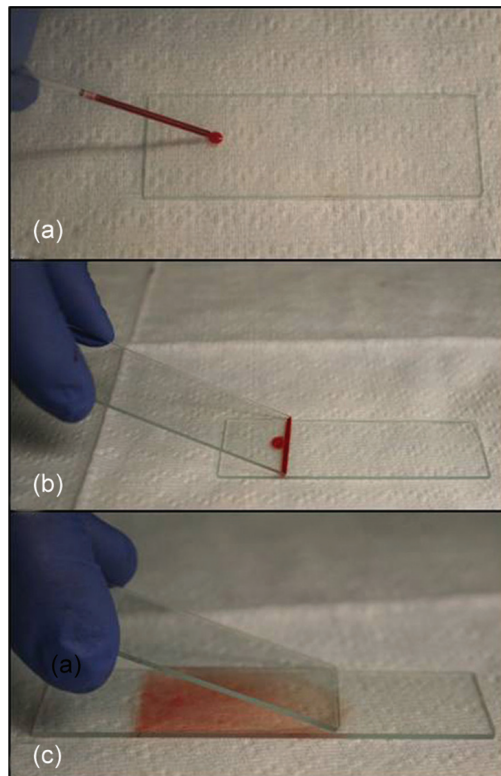


Figure 5.2 After the drop of blood is applied to the slide (a), the spreader slide is drawn back into the drop and the blood allowed to spread toward the edges of the slides (b), and the spreader slide is then slid forward to the other end of the smear slide (c).

that can be held behind the slide. The technician experiencing consistently poor results (too thick, too short, etc.) should intentionally alter one or more of these three variables to correct the problem. Slightly different approaches often work better in different hands, and these techniques can be learned only through experience.

The slide should be dried rapidly to avoid cellular distortion caused by gradual osmotic changes. Drying may be hastened by gently waving the slide in the air. The technician should avoid blowing on the smear or placing it near a radiator or other source of steam, which may condense on the surface.

Coverslip Technique

Blood smears may also be made on coverslips by placing a small (2–3 mm) drop of blood on one coverslip and then placing a second coverslip diagonally on top of the drop, forming an octagon (Fig. 5.4). After the blood has spread between the coverslips, they are gently slid apart laterally by grasping the corners, without lifting, forming two separate smears. As with slide preparations, the smears should be air-dried. Coverslip preparations result in uniform leukocyte distribution and excellent cell definition but, due to difficulties in handling, staining, and labeling, are not commonly used in veterinary medicine.

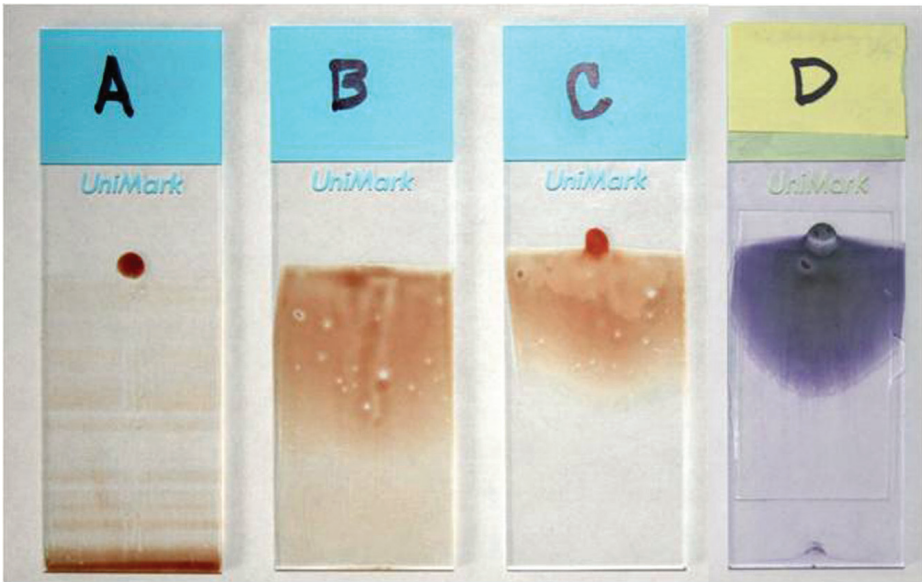


Figure 5.3 Examples of common errors in slide preparation: (a) Excess blood has been carried with the spreader slide and over the end of the smear slide, and the alternating thick and thin areas are caused by too much pressure on the top slide; (b) the slide is too thick for reading and the clear areas usually represent an improperly cleaned slide; (c) the smear is short, leaving too small of an area for cell identification (monolayer); (d) although a longer smear would be preferred, this stained smear shows an adequate monolayer area for cell identification.

Automated Smear Preparation

Methods of automatic blood smear preparation are available that spin the blood on a slide or automatically maneuver the spreader slide over the film slide. Automated staining equipment is also available. Use of this equipment is found primarily in larger veterinary hospitals, human medical centers, and laboratories.

Staining the Blood Smear

If the slide or coverslip smear is observed under the light microscope at this point, the cells will be colorless to lightly straw-colored, and differentiation of the cell type and morphology nearly impossible. Staining of the blood smears serves two basic purposes:



Figure 5.4 Blood smear preparation, coverslip technique.

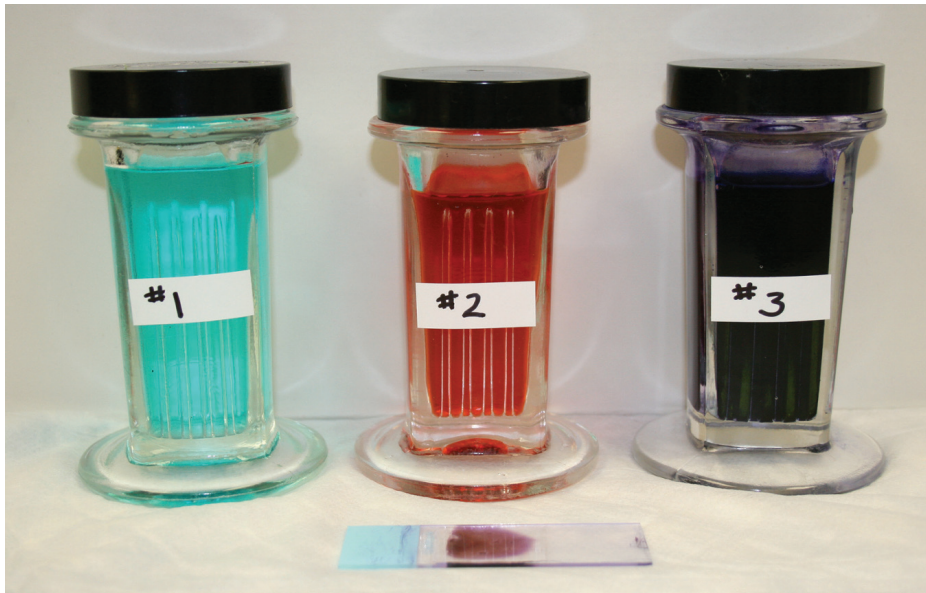


Figure 5.5 Typical appearance of containers used with commercial dip stains. Jar 1 contains the fixative, jar 2 has the acid stain, and jar 3 has the basic stain.

first, adding color to the cells makes them more visible, and second, by using stains with contrasting chemical properties, different cells or parts of cells will appear in different colors, allowing identification of cell types.

Most of the current stains for routine blood work are of the Romanowsky type, which incorporates basic (blue/purple) and acidic (red) dyes. This will stain some structures red and others blue, dependent upon the chemical nature of the cellular structure. Wright and Giemsa stains are examples of this type of staining technique and are the basis for the almost universal current use of commercial dip technique stains. These staining systems use (1) a fixative, such as methyl alcohol, (2) a red-colored, acidic dye (usually eosin), which is attracted to the basic components of cells, such as hemoglobin and the cytoplasmic granules in eosinophils, and (3) a dark blue to purple, alkaline, or basic dye (usually methylene blue), which will stain the more acid components, such as nucleic acids and the cytoplasmic granules in basophils. It is worth noting here that this is the basis for the naming of the granulocytic leukocytes: *eosinophils* (also called acidophils, acid staining), *basophils* (basic staining), and *neutrophils* (neutral). These dyes may be present as three separate solutions or incorporated into one or two solutions by various manufacturers (Fig. 5.5). Use of the stains involves dipping a slide into each solution for a length of time, or a certain number of times, specified by the manufacturers. The only maintenance needed for these stains is routine filtering to remove debris and replenishment or replacement from stock solutions. To prevent evaporation, the stains, and especially the fixative, must be tightly capped when not in use.

One other staining technique that is commonly used to demonstrate erythrocyte alterations or inclusions is the reticulocyte stain. This is called a supravital stain and must

be used on fresh blood that has not been placed in a fixative. New methylene blue (0.5 percent in distilled water) is most often used (a different dye from the methylene blue used in Romanowsky stains) and can be applied by two different, but extremely simple, methods. A drop of new methylene blue can be placed on a coverslip, which is then placed directly on an unfixed, unstained blood slide, or small, equal amounts of the stain and the blood sample are mixed prior to making the smear. The stain will outline the cells and incorporate into any DNA, RNA, or reticular fibers present. It will also demonstrate some types of erythrocyte parasites and inclusions and is discussed in Chapter 11.

Review Questions

- 1. Describe the method of preparing a blood smear on a glass slide.**
- 2. What are the functions of the different colored acidic and basic components in routine blood stains?**
- 3. What area of a properly prepared blood smear should be used for both counting and evaluation of cells?**

Chapter 6

Routine Hematology Laboratory Tests

Key Concepts

Even with automated testing equipment becoming more common and affordable, learning manual techniques of blood testing, and the mechanism and rationale behind the testing, is beneficial to the beginning student.

Numerous common laboratory tests can be performed in the veterinary clinic using manual and semiautomated techniques.

The hematology technician must be familiar with mathematical calculations to account for small sample sizes or dilution techniques and to express test results in commonly used units.

The numbers and types of cellular, serologic, and chemical tests that may potentially be run on a blood sample are continually expanding and are limited only by the designs and needs of the clinical pathologist, immunologist, toxicologist, and other clinicians and researchers. Most of these are beyond the scope of this text, and the technician will find that only a few of these tests are routinely used. For example, a routine CBC (complete blood count) should include a minimum of a hematocrit (PCV), total plasma protein, total leukocyte count, and differential leukocyte count. Other commonly requested tests are total erythrocyte count, erythrocyte sedimentation rate, hemoglobin concentration, and reticulocyte count. The technician should first understand how and why these tests are run. Techniques for these tests are presented in this chapter, with other more specific procedures (e.g., erythrocyte indices and bone marrow exam) discussed in later chapters. For the most part, manual or semiautomated methods are described here.

In many clinics and laboratories, these methods have been replaced by automated equipment (discussed in Chapter 7) or by reference laboratories that are located nearby or available through a courier service.

Both automation and reference laboratories can provide excellent results. Many clinics, however, still depend on their technicians for “blood work” using the techniques presented here. Many veterinary technician training programs believe that by having the students perform these manual techniques they will understand basic concepts, such as dilutions and calculations, become more familiar with the blood cells and fluids, and will see that there really *are* more leukocytes in the blood of an animal with a “high white count.” With experience the technician will begin to notice if the cells don’t “look quite right”: a large number of immature cells may be present, the cells may appear toxic, or other cellular changes may be important to the clinician that automated equipment has not as yet been able to evaluate. Once the technician understands the rationale for the test and the mechanics behind it, that knowledge can be applied to the operation of the various types of automated equipment available and can help him or her better understand and appreciate the displayed results.

Packed Cell Volume (PCV)/Hematocrit (Hct)

The purpose of measuring the packed cell volume, or hematocrit, is to determine the percentage of erythrocytes circulating in the peripheral blood at the time of collection (Table 6.1). It is one of the most common blood tests performed because it is simple to run, rapid, inexpensive, and highly accurate, and it also provides valuable information on the other components of the blood. The terms “packed cell volume” and “hematocrit” are generally used interchangeably, even though there is a slight difference in meaning. Hematocrit translates as “to split or separate blood,” so we are actually using hematocrit techniques to determine the packed cell volume. The real mechanism behind this test is the law of gravity: the more dense an object is, the more rapidly it will fall. The mature erythrocyte has a greater density than the other blood components, and when blood is collected and allowed to set undisturbed, erythrocytes will become “packed” at the bottom of a column of blood (Fig. 2.1). Because the rate of this separation is quite

Table 6.1 Common reference PCV percentages.

Species	Average	Range
Dog	45	37–55
Cat	37	24–45
Horse		
Hot-blooded	43	32–55
Cold-blooded	35	24–44
Cow	35	24–46
Sheep	35	27–45
Goat	34	24–48
Human female	42	—
Human male	47	—

slow (except in the horse) a centrifuge is used to speed the action (Fig. 6.1). Centrifuges specifically designed to produce the speeds and gravitational forces necessary to separate blood components should be used.

The microhematocrit method of determining the PCV is the most common manual method, because of its speed and accuracy. Capillary tubes are available either with an internal coating of anticoagulant (usually heparin) if blood is taken directly from the animal or with plain (no anticoagulant) if the blood has already been collected with an anticoagulant (Fig. 6.2). One end of the capillary tube should be placed into the surface of the blood source (the patient or sample vial), and the tube allowed to fill two-thirds



Figure 6.1 Many types and brands of centrifuges are available. Some are specialized for a single function but others (such as this one) have variable speeds and functions.



Figure 6.2 Technician filling a microhematocrit tube with fresh blood from a syringe. Note the plastic tray with clay used to seal the tube after it is filled.

to three-quarters full by capillary action. Holding the tube as horizontally as possible will allow more rapid and complete filling. Small air bubbles present in the column of blood denote poor technique but do not affect the test results. With the tube still held horizontally, the end of the tube should be sealed with clay, a plastic sealing cap, or a hot flame. It is recommended that two tubes be filled and tested simultaneously to provide sufficient plasma for later tests and as a safeguard against breakage or leaking.

The sample tubes are now placed into the radial grooves or slots of the microhematocrit centrifuge (Fig. 6.3), with the stoppered ends toward the periphery (outward). Samples centrifuged for 5 minutes at 10,000–12,000 rpm or 3 minutes at 15,000–16,000 rpm will usually show complete separation and packing of cells. The time for ruminants should be doubled, especially the sheep and goat, since the small size of their erythrocytes slows the rate of sedimentation. Running the centrifuge longer than the recommended time does not harm the sample or affect the results, but lessening the time can lead to erroneously high readings.

Some centrifuges have a percentage index, or reader, built into their heads, and the PCV can be determined prior to removal of the tubes if they have been filled exactly to the marks indicated by the manufacturers. More commonly, the capillary tubes are placed on a separate linear (Fig. 6.4) or circular (Fig. 6.5) scale for reading. The bottom of the red cell column (not including the clay or plastic plug) is placed on the lower, or 0, line. The tube is then moved across the reader until the top of the plasma falls on the line marked 100. The tube must remain perpendicular to the scale. The line that crosses the tube at the top of the packed red cells (not the buffy coat) is the percentage of red blood cells in the blood sample.

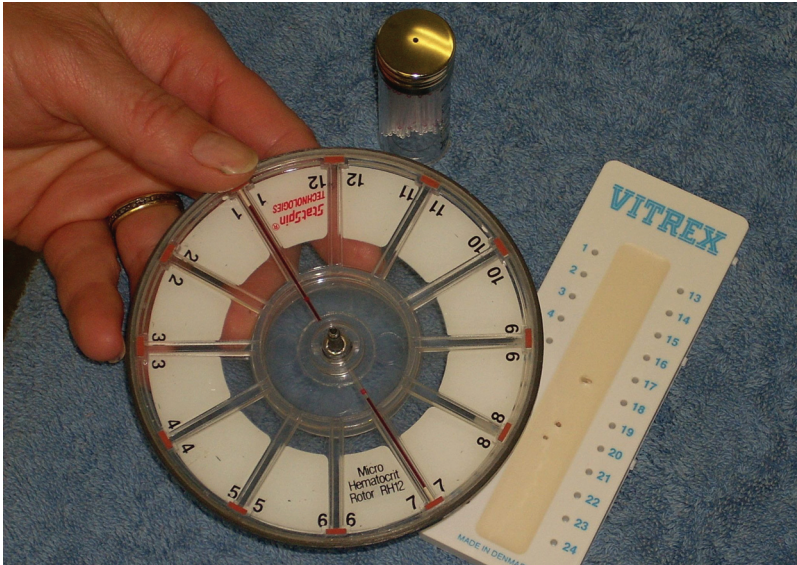


Figure 6.3 Place the microhematocrit tubes on opposite sides of the centrifuge head with the plugged ends outward.

The technicians may occasionally use a larger tube, such as a Wintrobe tube (Fig. 6.6), to calculate the PCV. If the tube is filled to the 10 mark on the right-hand scale with blood containing a suitable anticoagulant, the PCV can be read directly from the scale. Although rarely used, this method does have the advantage of a larger volume of plasma



Figure 6.4 Use of a rectangular microhematocrit tube reader for determination of PCV. Be sure to place the line between the blood and sealant clay on the zero line.



Figure 6.5 Measuring the PCV on a circular type of reader. Careful placement of the bottom of the blood column at “0” and the top at “100” is necessary for accurate measurement. This sample has a PCV reading of 46%.

available for testing, and changes in the size and character of the buffy coat are more easily evaluated. The disadvantages are the greater length of time required for the test (10–60 minutes), the volume of blood needed may not be available from small or debilitated patients, and cleaning nondisposable tubes is time-consuming.

Many types of electronic blood analyzers will determine the PCV value (see Chapter 7). The PCV obtained by electronic cell counters is computed by combining the electronically measured average red cell volume and the total red cell count. Since the average red cell size varies widely between species, the instrument must be carefully calibrated for the individual species.

Uses of PCV/Hct

The hematocrit provides the fastest, most accurate estimation of the oxygen-carrying capacity of the blood and, along with visual appraisal of a blood smear, is often the only red blood cell screening test performed. A rough approximation of the total red cell count in the dog and cat, assuming normal cell size, can be made by dividing the PCV by 6



Figure 6.6 Wintrobe tube.

and expressing the results as 10^6 red cells/cu mm. For example, a blood sample with a PCV of 39 percent would contain approximately 6.5×10^6 (6,500,000) RBC/cu mm in these species. The hemoglobin content of normal blood (in gm/dL) can be approximated by dividing the PCV by 3. The PCV of 39 percent in the example would represent approximately 13 g/dL hemoglobin. If anemia or other red cell problem is suspected or becomes apparent, actual total red cell counts and hemoglobin determinations should be made, and stains used to identify reticulocytes. Chapters 10, 11, and 12 discuss erythrocyte determinations and disorders.

In addition to measuring PCV, the technician should always examine and record the characteristics of the other two portions of the hematocrit, the plasma and the buffy coat. The plasma will vary from clear to yellow-orange depending on species and diet. Any reddish discoloration of the plasma indicates hemolysis of red cells, which is most often due to improper collection techniques. Any turbidity, cloudiness, or layering of the plasma may indicate fats or other substances in the blood. A brief description of the plasma, even if it appears normal, should be noted on the lab report. A change in the appearance of the plasma may aid in a diagnosis or may warn of interference with other test. For example,

cloudy plasma in a dog may be normal following a fatty meal but will make the total protein determination unreliable. A dark orange plasma of a Jersey cow may indicate red blood cell breakdown products in the blood, or it may be simply the plant pigment carotene from lush green feeds. A colorless, transparent plasma may appear normal but would be significant in an animal with acute hemolytic anemia. Descriptive, rather than interpretive, terminology should be used (“clear and transparent” rather than “normal,” “white, cloudy” rather than “fatty,” etc.) because interpretations may lead to misdiagnoses.

The buffy coat is the white to tan layer between the plasma and the PCV. It contains the leukocytes and platelets can also provide helpful information. It is normally about 1 mm thick in a Wintrobe tube and less than 1 mm in a microhematocrit tube. With experience, the technician will become familiar with the normal size and appearance of the buffy coat. Any increase or decrease in this width usually signifies a change in the number of white cells. The color of the buffy coat should also be noted. A red or pink tinge may indicate the presence of large, immature, or deformed erythrocytes (see Chapter 11).

Potential Errors in PCV/Hct Determination

Although the hematocrit is one of the most accurate hematologic tests, occasional errors in results may occur. Drawing blood from a tube that has been allowed to settle without remixing will give a nonrepresentative sample. Reading the scale above the buffy coat, or simply misreading the scale, is also a common error. A high concentration of anticoagulant will shrink erythrocytes and cause a false low reading. This can occur when the anticoagulant is premeasured but the amount of blood collected is less than anticipated.

The other major source of error may be the centrifuge itself. The controls on some machines, especially older models, may show only relative settings and not actual rpm. The rpm indicated on the controls may not be reached by the centrifuge because of its age or use. A simple method for testing the centrifuge is to run test samples of individual species for gradually increasing lengths of time and to record the PCV for each time period until there is no further decrease in PCV readings. The least amount of time necessary to reach the lowest PCV will be the optimum time for that species on that centrifuge.

Erythrocyte Sedimentation Rate (ESR)

As the name implies, this test is a measurement of the rate at which erythrocytes will separate from their own plasma in a column of blood containing an anticoagulant. Many different disease processes, especially those involving inflammation and/or tissue necrosis, increase the rate of sedimentation. This is apparently due to changes in the density of the plasma and increased red cell agglutination because of the alteration of the surface properties of the cell.

The ESR, although common in human medicine, is infrequently used in veterinary medicine due to extreme species variation and nonspecific results. Normal horse blood

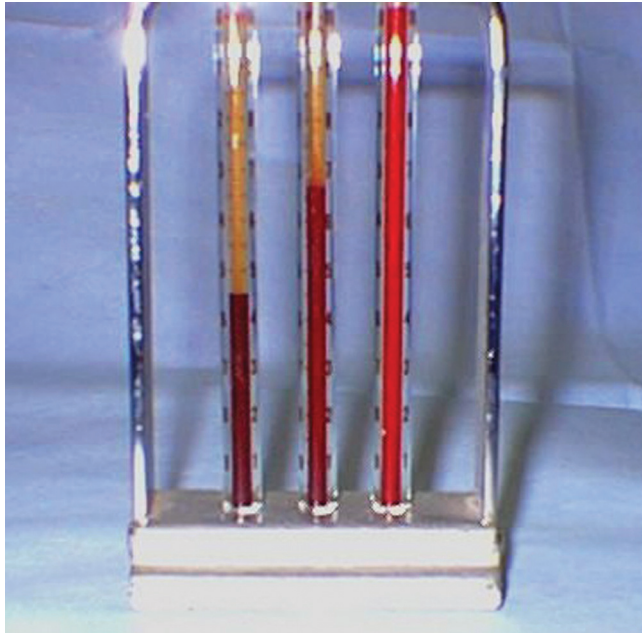


Figure 6.7 Blood samples in Wintrobe tubes placed in a stable rack to determine the erythrocyte sedimentation rate. Note the different amount of separation in the three samples.

will often separate in less than 20 minutes, the dog and cat in about an hour, and ruminants may take 24 hours or more.

The technique for the test involves carefully filling a glass tube, such as a Wintrobe, Westergren, or Cutler tube, to the 0 mark of the left-hand scale with blood to which EDTA or heparin has been added. Standardized charts of test results have been formulated for most of these tubes; however, if microhematocrit or other types of tubes are used, rates of change would be expected to be proportional but rates of fall cannot be compared directly due to variation in tube bore and length. The filled tube must be placed in a vertical position, usually in a special rack, because even slight deviation from vertical will increase the sedimentation rate (Fig. 6.7). After a specified length of time the upper level of the sedimenting erythrocytes is read on the left-hand scale, or the depth is measured in millimeters. The commonly used time intervals are 20 minutes for horses, 1 hour for dogs and cats, and up to 24 hours for ruminants, with results so variable in ruminants the test is rarely of significance (Table 6.2).

Because the normal rate of fall is influenced by the number of red cells present, the results obtained must be compared with tables of expected ESR levels for various PCV values (Table 6.3). For comparison, subtract the anticipated value from the observed value. Positive results indicate increased rates of sedimentation; negative results indicate decreased rates. For example, if a blood sample from a dog with a PCV of 25 percent had separated 40 mm after 60 minutes, this would represent an increased rate of 4 mm over the anticipated 36 mm.

Table 6.2 Typical reference values for erythrocyte sedimentation rate (Wintrobe method).

Species	mm of fall				
	10 min	20 min	30 min	1 h	24 hr
Dog			1–6	5–25	
Cat				7–23	
Horse	2–12	15–38			
Cow					2.25–4.0
Sheep					3.0–8.5
Goat					2.0–2.5
Pig			0–6	1–14	

Occasionally, the red cell column and plasma will lack a distinct line of separation, or a reddish color is present in the plasma that clears on centrifugation (i.e., it is not present in the hematocrit). This is called diphasic sedimentation and indicates the presence of abnormal erythrocytes (usually reticulocytes or poikilocytes, see Chapter 10), which do not form rouleaux and sediment more slowly.

Potential Errors in ESR Determination

Some common sources of error that may alter the ESR include:

- Tube not being vertical.
- Vibration or movement of tube.
- Presence of debris or chemicals in tube.
- Temperature variations.
- Improper anticoagulant concentration.
- Hemolysis.
- Air bubbles in blood column.
- Blood being stored too long at room temperature.

Table 6.3 Anticipated ESR values.

PCV (%)	Dog, Cat (60 min)	Horse (20 min)
10	79	86 ± 1
15	64	80 ± 1
20	49	70 ± 2
25	36	60 ± 3
30	26	47 ± 5
35	16	28 ± 15
40	10	11 ± 8
45	5	2.5 ± 1
50	0	0 ± 1

Hemoglobin Determination

Hemoglobin is the protein molecule in red blood cells responsible for transporting oxygen from the capillary beds of the lungs to the body tissues. Measurement of hemoglobin levels is indicated whenever erythrocyte disorders (e.g., anemia) are suspected. When coupled with the hematocrit and total red cell count, sizes, and hemoglobin contents of each cell can be calculated (see Chapter 10), which can be helpful in evaluating erythrocyte function.

A common manual method of hemoglobin measurement uses the Spencer hemoglobinometer (Fig. 6.8), which passes light through a hemolyzed blood sample to measure light absorption. Two small glass plates are used (Fig. 6.9), one with raised platforms and the other flat. Also used are applicator sticks with a hemolytic agent, usually saponin, dried onto one end. A large drop of blood is placed on one of the platforms and stirred thoroughly (at least 1 minute) with the applicator stick to hemolyze (burst) the cells, a process called “laking” the blood. The hemoglobin in the red cells is converted into oxyhemoglobin and should now be wine-colored and transparent. The flat cover glass is placed over the sample, and the blood must fill the entire space and be free of bubbles. Blood spilling into the moat will not affect the results. The two slides are placed into a clip and inserted into the instrument. Pressing the light switch button on the bottom of the case will illuminate two adjacent green fields. The tab on the side of the instrument should be slid back and forth several times to match the shades of green. The hemoglobin level is read from the top scale at the final position of the tab and expressed as grams per deciliter (Table 6.4).

Matching shades of green is a subjective evaluation and may lead to different results by different observers, although these have been shown to be minimal since gradations of green appear to be easier to match than those of other colors. Other potential errors



Figure 6.8 Proper use of the Spencer hemoglobinometer.

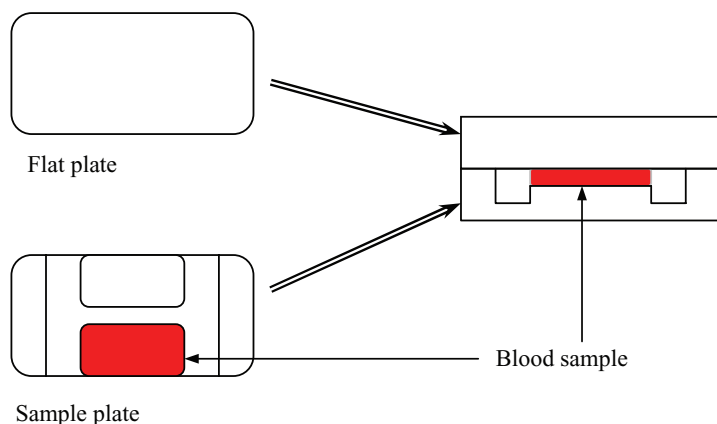


Figure 6.9 Placement of hemolyzed blood sample on plates used with Spencer hemoglobinometer.

may occur if the sample is not properly mixed, the sample is incompletely hemolyzed, the chamber is underfilled or has bubbles present, or the instrument has dirty lenses or weak illumination.

Another method of measuring hemoglobin is the cyanmethemoglobin method. This method involves mixing the blood with chemical reagents and measuring the light transmitted through the sample in a spectrophotometer. This method may be used in commercial or university laboratories, but rarely used in clinical practice.

Hemoglobin content can also be measured on various automated analyzers (see Chapter 7). Many of these are designed to measure hemoglobin (along with many other substances) by light refraction. Instructions may vary with different units, but commonly a drop of blood is placed at one end of a plastic strip and allowed to spread into a chemically treated area. The strip is then inserted into the analyzer, and after a time automatically determined by the coding of the strip, the amount of light refracted from the treated blood

Table 6.4 Common reference hemoglobin values.

Species	Average (g/dL)	Range (g/dL)
Dog	15.0	12–18
Cat	12.0	8–15
Horse		
Hot-blooded	14.4	11–19
Cold-blooded	11.5	8–14
Cow	11.0	8–15
Sheep	11.5	9–15
Goat	10.0	8–15
Pig	13.0	10–16
Human		
Adult female		12–16
Adult male		14–18
Children		10–14

surface is measured, converted to hemoglobin concentration, and displayed as grams of hemoglobin per deciliter of blood.

Total Plasma Proteins

Between 5 percent and 10 percent of the plasma is made up of a mixture of many protein molecules, the majority of which are classified as albumin, globulin, and fibrinogen. Among the other proteins present in trace amounts are hormones, enzymes, complement, and clotting factors other than fibrinogen. Most of these proteins are manufactured in the liver, with the notable exception of the gamma globulins (also called immunoglobulins), which are the antibodies produced by cells of the immune system. Alterations in plasma protein levels are not diagnostic of specific conditions but are indicators of metabolic or pathologic changes, especially those involving protein metabolism and fluid balance. Serum proteins can also be measured and are discussed under “Plasma Fibrinogen” (below).

In the clinical hematology laboratory, plasma protein can be measured manually with the Goldberg refractometer, or TS (total solids) meter (Fig. 6.10). The design of this instrument is based on the principle that light waves passing through a liquid will be bent from their original direction (refracted) by any solids present in the solution and the degree of this refraction is a function of the amount and type of solid present. The actual measurement being made is of total solids (the scale in the refractometer is usually labeled TS), but since proteins constitute the vast majority of the solids in normal plasma, the measurement adequately reflects plasma protein levels. In this test, a drop of plasma is placed on the face of the prism of the refractometer, either through an access slot in the cover plate or by opening the cover. The cover plate is held closed while holding the



Figure 6.10 Clinical refractometer (TS meter).

refractometer to the eye and orienting it toward a strong light source. A horizontal line between bright and dark areas should appear that crosses over several scales (number and type of scales are dependent on the manufacturer). Focusing of the eyepiece may be necessary. The protein level is read in grams per deciliter from the scale containing numbers from approximately 5 to 15. The scale is often labeled TS (some are labeled TP, SP, or PP, and some are not labeled). Normal plasma protein levels should range between 6 and 8 g/dL for most adult mammals and 4 to 6 g/dL for younger ones.

Most types of automated blood analyzers discussed in Chapter 7 will determine plasma protein levels and, in many cases, will separate the types of protein, such as albumin and globulin. Other methods of measuring the plasma or serum proteins are available that use chemical reactions, spectrophotometry, and electrophoresis to identify the individual types of proteins. These are especially useful in studies of liver metabolism and are part of the clinical chemistry laboratory.

Potential Errors in Manual Plasma Protein Determination

A common practice for obtaining the plasma sample is to break the capillary tube that has been used to determine PCV just above the buffy coat and to use the plasma in the top portion of the tube. When using this method, the technician should place the plasma on the prism through the unbroken end of the tube because any pieces of glass will interfere with test results. The plasma must be clear; any cloudiness, hemolysis, lipemia, or other abnormal solids will cause unreliable results. Another common problem encountered is the absence of a distinct line on the scale. This may be due to improper focusing, an inadequate or improperly oriented light source, interfering substances in the sample or on the prism, or an insufficient or improperly located sample. Many models automatically compensate for slight temperature changes, but extremes of temperature will alter readings. The prism must be kept clean and protected from damage; scratches, pits, etching, and foreign material will alter the results. The zero setting should be checked routinely by using distilled water as a blank sample; it can be adjusted with a set of screws.

Plasma Fibrinogen

Fibrinogen is a plasma protein manufactured by the liver that is important in blood coagulation. It also has been shown to move outside the blood vessels in response to inflammatory and infectious conditions to assist in localizing the disease process. Although a fibrinogen assay is not a routine procedure, and is more accurately measured with automated equipment, an estimate of fibrinogen levels is often useful in evaluating general inflammatory response.

A simple manual technique for estimating fibrinogen levels is to collect a pair of blood samples in EDTA and to centrifuge both as if for hematocrit determination. The plasma protein level is determined on the first sample while the second sample is placed in a water bath at 56–58°C for 3 minutes. Fibrinogen will precipitate at this temperature, while the

other plasma proteins remain in solution. The second tube is recentrifuged, causing the precipitated fibrinogen to collect just above the buffy coat. The protein level is determined on the fluid above the fibrinogen, and the reading subtracted from the amount found on the unheated tube. The results are reported as milligrams per deciliter or may be used to calculate a plasma protein-to-fibrinogen ratio (e.g., 6.0 g/dL plasma protein:500 mg/dL fibrinogen = 12:1 ratio).

Another estimate of fibrinogen levels can be made by doing total protein measurements of both plasma (anticoagulant present) and serum (no anticoagulant, allowed to clot) samples. The fibrinogen will be used in formation of the clot, along with very small levels of other clotting factors, and the difference between the two readings will closely approximate fibrinogen levels.

Microscopic Examination of the Blood Smear

Even if an automated blood analyzer is used for most laboratory work, a well-prepared blood smear that is properly examined by a knowledgeable technician can yield valuable information about cellular changes (e.g., erythrocyte inclusions, lymphoblastic changes) that are not shown in the instrument's printouts or screen. Some analyzers will even flag test results for visual examination if abnormal cells are detected. The information available on a blood smear can be easily overlooked unless a systematic approach is used to read the slide.

There will often be marked variation between slides in both the appearance of the smears and the quality of the stains. This can be best appreciated by observing the slide against a white piece of paper prior to placing it on the microscope. This will demonstrate general characteristics of the smear, such as the size and location of the monolayer and areas where the smear has gone off the edge of the glass or is otherwise unsuitable for examination, along with the overall depth and intensity of staining. The slide should then be scanned under the low-power (100 $\times$) objective, where the same characteristics should be noted and the optimum areas of study located. The ideal areas are where cells are abundant but do not overlap or appear crowded or distorted (Fig. 6.11). Although some individuals prefer to examine the blood under high, dry magnification (400 $\times$), more accurate cell identification of normal and abnormal cells is possible with the oil immersion objective (1,000 $\times$).

A common error in the microscopic examination of a blood smear is to immediately begin identifying and counting leukocytes and failing to observe the characteristics of the erythrocytes and the number and nature of the platelets. The hematologist should develop a routine to be used on each slide, which should include the following as a minimum:

1. Erythrocytes—size, shape, color, rouleaux formation, abnormalities, and inclusions
2. Platelets—distribution and estimated number
3. Leukocytes—distribution, estimated number, abnormalities, and inclusions
4. Other findings—overall depth and color of stains, unusual cells or parasites

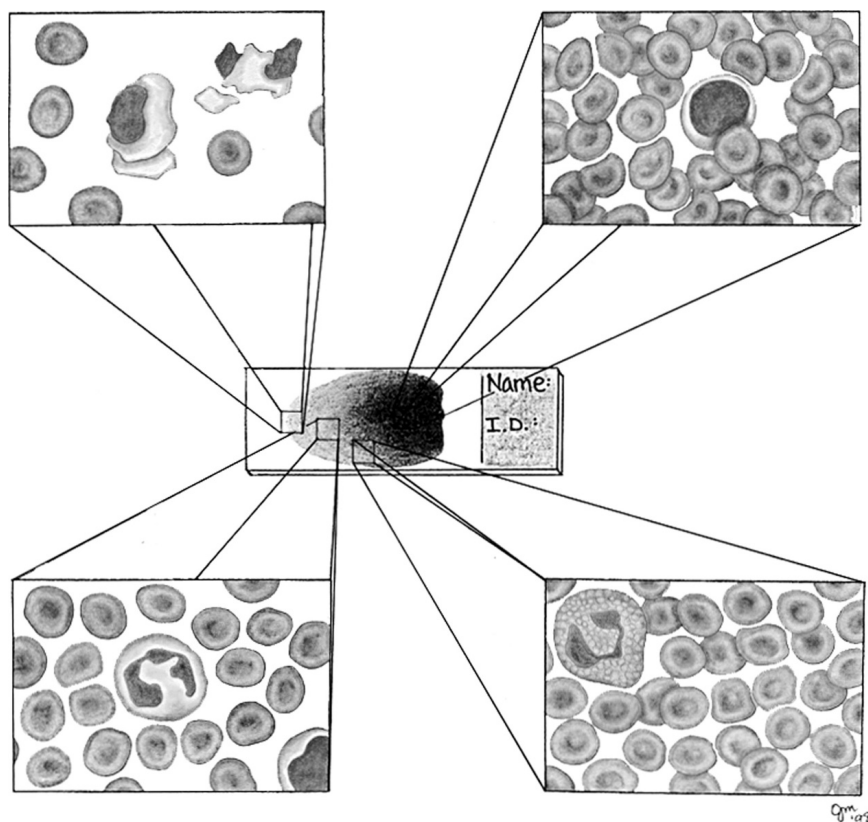


Figure 6.11 Diagram of the appearance of cells in various regions of a blood film. Leukocytes are drawn in large numbers to the feathered end of the smear (*upper left*) but are usually stretched, distorted, and broken. In the thick portion of the film (*upper right*), cells are closely packed, often overlapping and difficult or impossible to identify. Cells along the outer margins of the smear (*lower right*) are numerous and, although minor distortion may occur during preparation or from crowding by other cells, are usually identifiable. While total cell numbers may be smaller, optimum cellular morphology is seen in the monolayer between the thick portion of the film and the feathered edge (*lower left*).

Differential Leukocyte Count

After the initial examination of the smear is complete, a differential (relative) count is performed by counting and classifying at least 100 leukocytes. Since correct identification and enumeration of cell types is the essential function of this examination, the technician should avoid areas where the cells are overlapping and more than single layered or are distorted by surrounding cells or location on the smear (tip of the feathered edge). The leukocytes tend to move to the outer edges and end of the blood smear as it is made, mainly due to the size differential with erythrocytes.

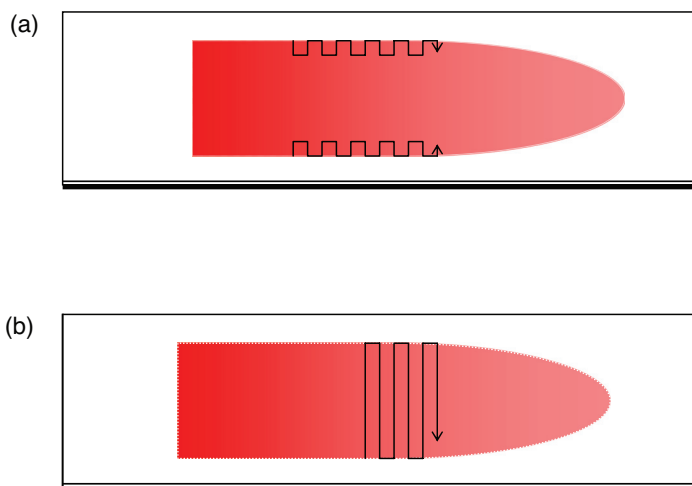


Figure 6.12 Techniques for performing a differential leukocyte count on a blood smear: (a) “battlement” technique and (b) crossing, or “wandering,” technique.

To ensure a random sampling of cells and to avoid recounting of the same field, one of two patterns of examining the slide should be used (Fig. 6.12). In the battlement method, the technician should examine the edge of the smear for 3–5 fields, move inward a short distance and parallel the edge for 3–5 fields, and then move back to the edge, repeating this procedure as often as necessary to identify the required number of cells. A second pattern (sometimes referred to as “wandering”) is similar, but the cells are counted across the entire monolayer (not in the thick area). Fewer leukocytes are present in the center of the film, but those present will usually be the most morphologically distinct.

As each white cell is encountered, it is identified and tallied, either on a multichambered counter or by hand (Fig. 6.13). Usually a total of 100 cells are counted, but this is mainly for convenience since results will automatically be expressed as percentages (Table 6.5). Counting 200 or more cells, which are then converted into percentages, will improve accuracy, especially in samples with elevated white cell counts.

In many blood smears, there will be intact but distorted or atypical cells that cannot be accurately identified. These should be recorded under a separate “unclassified” column and included in the total count with a brief description attached. With experience, the technician will rarely use this category, although occasional cells that defy classification will be found. Any cells that are disrupted or degenerated are termed “smudge” cells and are not counted. If the numbers of these cells are excessive, it should be noted on the report.

While the differential is a reflection of the quantity of the various leukocytes, the quality of the cells (changes from normal) should always be noted. This includes abnormal color or depth of staining, changes in characteristics of the cytoplasm, maturation-related changes, any cytoplasmic or nuclear inclusions, and other alterations. These may indicate a physiologic or pathologic process involving the leukocytes and are discussed in Chapter 8. An occasional nucleated red cell may be found in normal blood and should be noted,

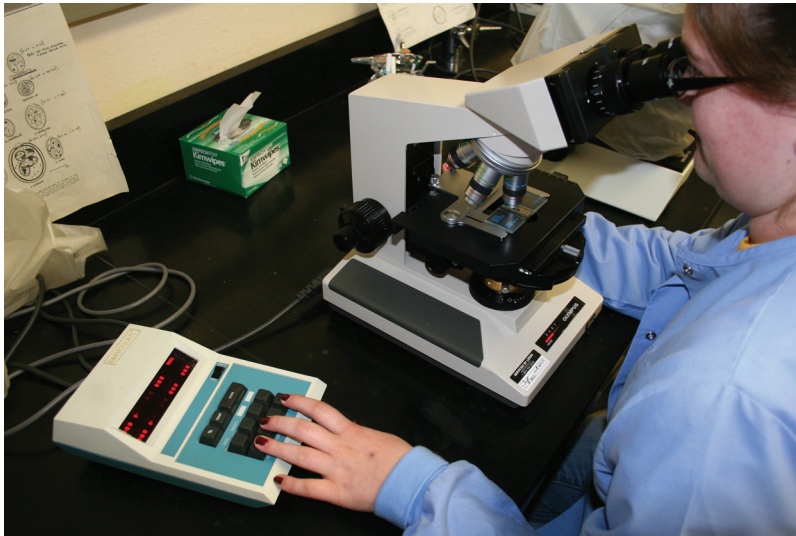


Figure 6.13 Veterinary technology student using a multichambered counter in performing a differential cell count.

but if more than four to five are seen during the differential count, a separate tally must be kept (not part of the total white cells). Since these red cells' nuclei will be counted on the hemocytometer, the total cell counting chamber, this number will be used to correct the total white cell count (see "Leukocyte Count" below).

Table 6.5 Reference values for differential leukocyte counts in various species—averages and (range).

Species	Band neutrophil	Segmented neutrophil	Lymphocyte	Monocyte	Eosinophil	Basophil
Dog	1 (0–3)	70 (60–77)	20 (12–30)	5 (3–10)	4 (0–10)	±0 (0–1)
Cat	0.5 (0–3)	59 (35–75)	32 (20–55)	3 (1–4)	5.5 (2–12)	±0 (0–1)
Horse						
Hot-blooded	0.5 (0–2)	49 (30–65)	44 (25–70)	2 (0–7)	4 (0–11)	0.5 (0–3)
Cold-blooded	0.5 (0–2)	54 (35–75)	35 (15–50)	5 (2–10)	5 (2–12)	0.5 (0–3)
Cow	0.5 (0–2)	28 (15–45)	58 (45–75)	4 (2–7)	9 (2–20)	0.5 (0–2)
Sheep	0.5 (0–2)	30 (10–50)	62 (40–75)	2.5 (1–6)	4.5 (1–10)	0.5 (0–3)
Goat	0.5 (0–2)	36.5 (30–48)	55 (50–70)	2.5 (1–4)	5 (3–8)	0.5 (0–2)
Pig	1 (0–4)	37 (28–47)	53 (39–52)	5 (2–10)	3.5 (0–11)	0.5 (0–2)

Note: All values are in percentages.

Automated differential leukocyte counts, based on differences in cell size and cytochemical reactions, are becoming quite common in the human medical field. They can be performed more rapidly, and in most cases, the results compare favorably with manual methods. In the veterinary field, the considerable species variation in both cell size and cytochemical content has limited their use, although more accurate counters are becoming more available. Even with the use of automated counting methods, examination of the stained smear for subjective cellular changes will remain a valuable and necessary technique.

Potential Errors in Differential Leukocyte Count

Errors in the differential count are mainly due to interpretation difficulties, which are rapidly minimized with careful practice. Since only a small sample of the total cells is being counted, however, the random distribution of the cells will always create a statistical variation. That is, counts done in different areas of the same smear, by the one or more observers, will usually not result in the same totals. The amount of difference between counts will become smaller as larger numbers of cells are counted.

Absolute Leukocyte Count

Although the percentages of cells reported in the differential leukocyte count reflect the relative populations of cell types, these percentages must be related to the total white cell count to calculate of an absolute value for each cell type. To determine the absolute count, the percentage of each cell type in the differential is multiplied by the total white cell count (techniques for calculating total counts are presented in the following section), which will show the number of each cell type per cubic millimeter of blood. For example, if the differential count showed 60 percent neutrophils and 30 percent lymphocytes, and the total white blood cell count was 12,000 WBC/cu mm, there would be 7,200 neutrophils/cu mm and 3,600 lymphocytes/cu mm.

To demonstrate the value of the absolute count, the following example can be used: blood samples from two different dogs had identical differential counts of 85 percent neutrophils, 10 percent lymphocytes, 3 percent eosinophils, and 2 percent monocytes. It would appear that both dogs had an elevated neutrophil level (neutrophilia). If the total leukocyte count for dog 1 is 10,000 WBC/cu mm and for dog 2 is 20,000 WBC/cu mm and absolute values are calculated for just the neutrophils and lymphocytes for comparison:

	Dog 1	Dog 2
Total count		
(WBC/cu mm)	10,000	20,000
Neutrophils (%)	85	85
(NP/cu mm)	8,500	17,000
Lymphocytes (%)	10	10
(LC/cu mm)	1,000	2,000

The calculations demonstrate that dog 1 has an absolute neutrophil count within the reference range (Table 6.6), even though it appeared quite high in the differential. However, the absolute lymphocyte count is quite low. With the same differential, dog 2 shows a marked increase in absolute numbers of neutrophils, but the lymphocyte numbers are in the normal range. The same type of effect can be shown for animals with similar total leukocyte counts, but different differential counts.

Potential Errors in Absolute Leukocyte Counts

Any errors in the total or differential leukocyte counts will be automatically compounded when the absolute count is calculated. As a check on mathematical or transcription errors, all the differential percentages should be added together, which will equal 100, and all the absolute counts, which will then equal the total count. If there is a discrepancy, all figures must be rechecked.

Total Cell Counts

The numbers of leukocytes and erythrocytes in the circulation can increase or decrease in response to physiologic needs or pathologic changes. The causes and mechanisms of these responses are discussed in Chapters 8 and 9, but in general, rapid increases in numbers will come from release of stored or normally noncirculating cells, with increased production of new cells usually taking a few days. Decreases in number are due to losses from rapid use or destruction of the cells or from decreased ability to produce new cells. Total cell counts can be a valuable diagnostic aid.

Automated Counters

Electronic cell counters are being used in veterinary hematology laboratories and some clinics for both red and white cell total counts and are described in greater detail in Chapter 7. Cell counts obtained by the use of automated methods are highly accurate compared with manual methods if appropriate adjustments are made in the calibration of the equipment for the cell size of the various species. Many of these cell counters operate by passing a dilute solution of blood in electrolytes through an aperture (small opening) with a weak electric field. As each particle passes through the aperture, it creates a voltage pulse that is counted electronically. If enough blood counts are being run to justify daily standardization and test samples, plus the cost of purchasing or leasing the equipment, electronic cell counters are faster and, if calibrated correctly, more accurate than manual counting.

Some veterinary clinics rely on manual blood counts, and many veterinary technology instructors feel that teaching manual cell counting techniques provides the student experience with laboratory equipment and procedures, handling of blood samples, dilution techniques, and mathematical calculations. Although operation of individual automated machines will vary, the technician who is adept at manual counting techniques should be able to readily understand and operate (and appreciate) automatic cell counters.

Table 6.6 Reference values for absolute leukocyte counts in various species—averages and (range).

Species	Band neutrophil	Segmented neutrophil	Lymphocyte	Monocyte	Eosinophil	Basophil
Dog	110 (0–330)	7,700 (6,600–8,500)	2,200 (1,300–3,300)	550 (330–1,100)	440 (0–1,100)	rare —
Cat	60 (0–360)	7,000 (4,200–9,000)	3,800 (2,400–6,600)	360 (120–480)	660 (240–1,400)	rare —
Horse						
Hot-blooded	50 (0–200)	4,900 (3,000–6,500)	4,400 (2,500–7,000)	200 (0–700)	400 (0–1,100)	50 (0–300)
Cold-blooded	40 (0–150)	4,600 (3,000–6,400)	3,000 (1,200–4,200)	425 (170–850)	425 (170–1,000)	40 (0–250)
Cow	40 (0–160)	2,200 (1,200–3,600)	4,600 (3,600–6,000)	320 (160–560)	720 (160–1,600)	40 (0–160)
Sheep	50 (0–180)	2,700 (900–4,500)	5,600 (3,600–8,800)	200 (90–550)	400 (90–900)	50 (0–270)
Goat	60 (0–240)	4,400 (3,600–5,800)	6,600 (6,000–8,400)	300 (120–480)	600 (360–960)	60 (0–240)
Pig	160 (0–640)	6,000 (4,500–7,500)	8,500 (6,200–9,900)	800 (320–1,600)	560 (0–1,700)	80 (0–320)

Note: All values are in cells/cu mm.

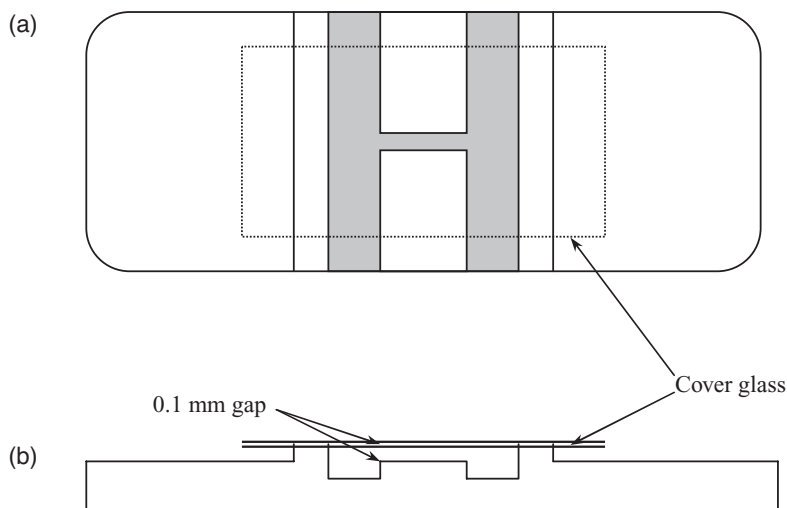


Figure 6.14 Hemocytometer slide and coverslip, top (a) and side (b) views.

Manual Counting Techniques

Because thousands of leukocytes and millions of erythrocytes are in even a cubic millimeter (cu mm = 0.001 mL, not quite the size of a pinhead!) of blood, manual counting requires the use of very small samples. Also used are precise dilution techniques and a calibrated counting instrument, the hemocytometer (Fig. 6.14). The hemocytometer is a thick glass plate with two raised, central platforms surrounded by a moat. A ridge on either side of the moat is designed to support an accompanying coverglass 0.1 mm above the level of the platforms.

On the surface of each platform is a 3 mm × 3 mm ruled area or grid, most commonly a Neubauer type, on which the cells will be counted (Fig. 6.15). The technician should become familiar with the appearance and dimensions of the rulings because various areas are used in counting cells and the size of the area counted determines the calculations needed to correctly report the concentration of cells.

Dilution Techniques

The blood sample may be collected directly from the patient or from a fresh sample collected with anticoagulant. If the sample has been refrigerated, it should be warmed to room temperature and thoroughly mixed prior to use. The sample is then diluted by one of several methods.

Manual Pipette Dilution

The Thoma pipette (Fig. 6.16) was the standard for cell counting for many years but is rarely used today. It is presented here as example of dilution techniques. The red cell pipette has markings of 0.5, 1, and 101 on the stem, which represent dilution ratios, not

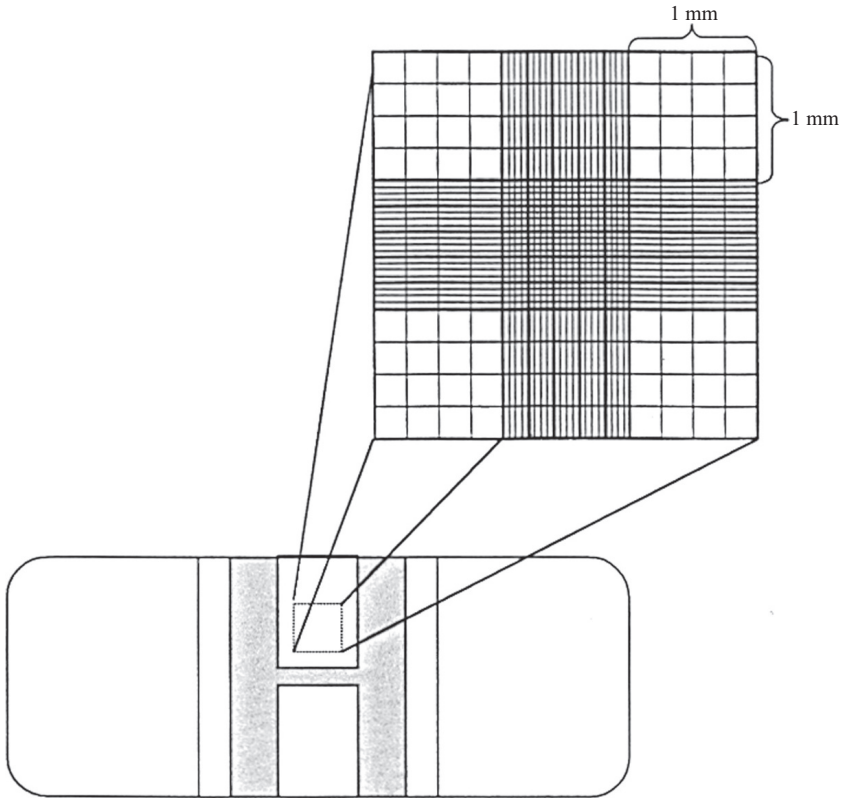


Figure 6.15 Location and dimensions of the Neubauer counting grid on the hemocytometer.

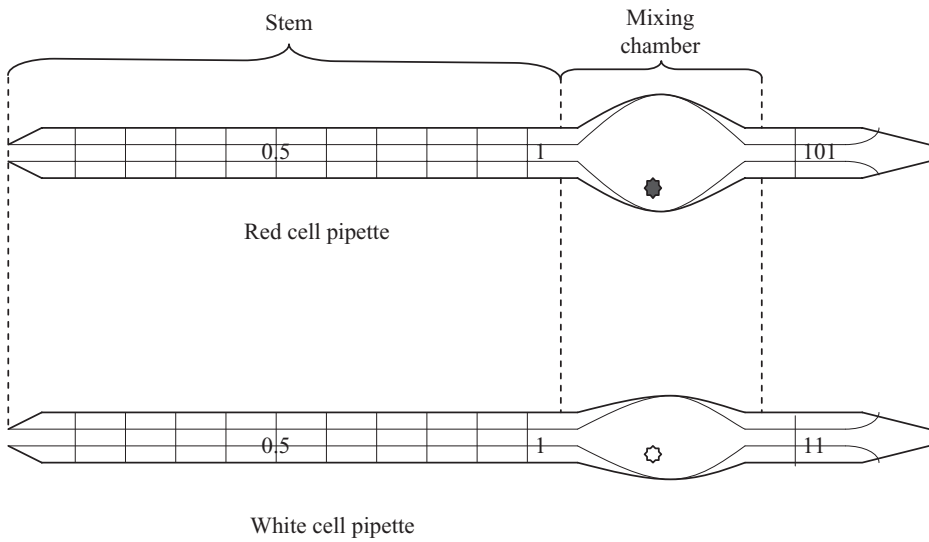


Figure 6.16 Thoma pipettes for red and white cell counts.

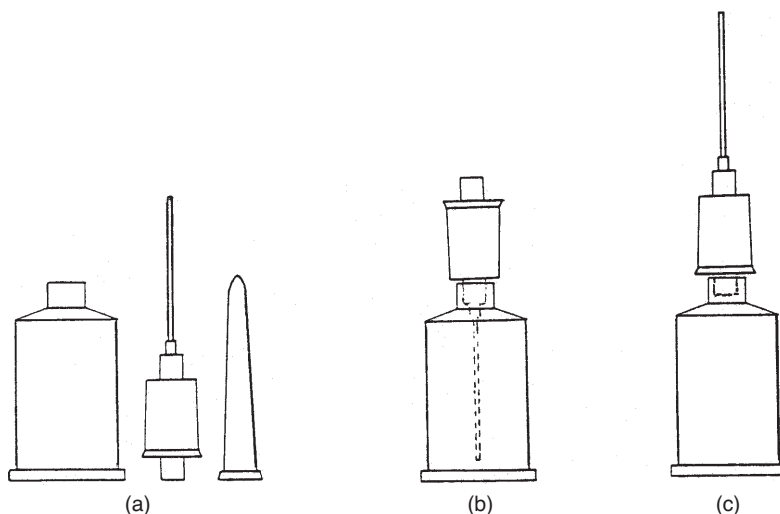


Figure 6.17 Commercial blood dilution system: (a) components; (b) filling the reservoir; and (c) dropper assembly.

specific volumes. Blood is drawn to the 0.5 mark by gentle suction and a sterile saline diluting solution (0.85 percent) is then drawn to the 101 mark. The sample is now a 1:200 (0.5:100) dilution.

The white cell pipette has a smaller mixing chamber and stem markings of 0.5, 1, and 11. When blood is drawn to the 0.5 mark and fluid added to the 11 mark, the dilution is 1:20. The diluent for white blood cell counting is a dilute acid that lyses (ruptures) cell membranes, making the nuclei of the white cells more visible.

Commercial Dilution Systems

Blood for either red or white cell counts may also be diluted with commercial plastic vial systems. These consist of a premeasured amount of diluent in a sealed plastic reservoir, a calibrated capillary tube with a hub at one end, and a pointed cap (Fig. 6.17a). The reservoir for red cell counting contains 1.99 mL of 0.85 percent saline and uses a 10 μ L (0.01 mL) capillary tube. This results in a 1:200 dilution ratio, which is the same as that for the Thoma pipette, so counting and computations are the same for both techniques. The white cell reservoir has 1.98 mL acetic acid and a 20 μ L (0.02 mL) capillary tube, resulting in a 1:100 dilution ratio. Other white cell counting reservoirs are available that yield a 1:20 dilution, as does the Thoma white cell pipette. Different areas of the hemocytometer grid will be counted and different computations will be necessary based on which technique has been used (see below).

The seal on the reservoir is punctured with the cap. The capillary tube is held horizontally with the tip in the blood source, from either the patient or a fresh sample in anticoagulant. The blood is allowed to fill the tube until it stops on its own at the end of the capillary tube, just past the tip of the hub. Any blood should be removed from the outside of the tube (when this is done, no blood should be drawn from the inside). The

sides of the reservoir are squeezed slightly and the tube is inserted (Fig. 6.17b). Releasing the reservoir will draw the blood into the solution. The reservoir is carefully squeezed 2–3 times to rinse the tube; fluid should not be forced out through the hub. The contents are thoroughly mixed by rotation or inversion. The capillary tube and hub are removed from the reservoir and reversed into a dropper assembly (Fig. 6.17c).

Charging the Hemocytometer

The special coverglass is placed on the supporting ridges of the hemocytometer. With either the pipette or plastic reservoir, the first 3–4 drops are discarded from the tip because these will be mostly diluent. To fill (charge) a chamber, the tip of the tube is placed between the edge of the coverglass and the chamber and the fluid allowed to draw into the space slowly and evenly. The chamber is filled completely but not allowed to overflow into the moat. If it does, the chamber must be cleaned, dried, and refilled. Both chambers are filled and the sample is allowed to set undisturbed on the microscope stage for 2–3 minutes to permit the cells to settle into a single plane of focus on the grid. In very dry conditions, it may be necessary to protect it from evaporation.

Erythrocyte Count

After the cells have been allowed to settle, the ruled area is located under low power ($100\times$) and reduced illumination. The entire chamber is scanned for incomplete filling, bubbles, or uneven distribution of cells; these will distort the count, and the chamber must then be cleaned, dried, and recharged. The center square of the nine large squares (Fig. 6.18) is moved into the center of the field. On low power ($100\times$), the technician will see that this center square contains 25 smaller squares, each of which is divided into 16 boxes. The red cell count will be done in five nonconsecutive sets of 16 boxes, most often the four corners and the center.

The first area to be counted (usually the upper left) is centered, and the magnification carefully switched to high, dry ($400\times$). A set of sixteen boxes should fill the field. With the large number of cells present, the technician must count in an orderly fashion to avoid

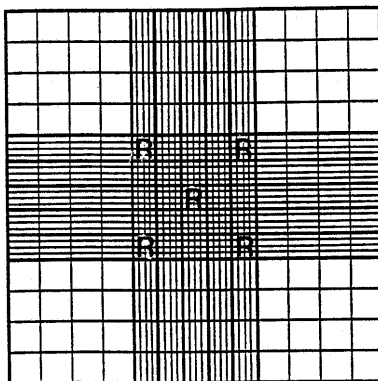


Figure 6.18 Areas of Neubauer grid counted for red blood cell total counts (R).

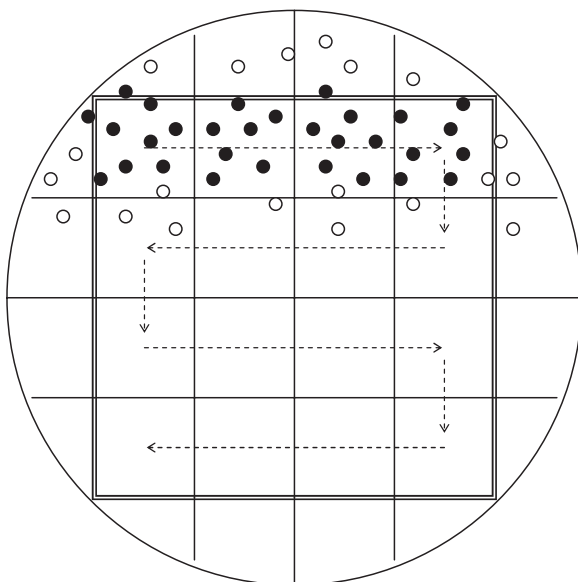


Figure 6.19 Technique of making total cell counts. The arrows indicate the direction of microscope movement. Filled circles represent cells counted in the first four squares; open circles are cells not counted with the first row.

missing cells or counting a cell twice. Starting in the upper left corner, the technician should count all cells in the first box, proceed along the top, counting the second, third, and fourth boxes, and then drop to the second row and count to the left, as shown in Figure 6.19. Within each box, any cell that touches the top or left-hand lines (whether it is mostly inside or outside the box) is counted, while any touching the bottom or right-hand line is not counted. When there is a triple line, the center line is used as the boundary. Double lines are less common, but if present, cells touching the outer top or left lines are counted, and those on the inner bottom or right lines are not. After all 16 boxes in the field are counted, the other four designated areas should be counted in a similar fashion.

Separate tallies should be kept for each of the five areas, either on paper or in a multichambered counter. If this is done, the entire count will not have to be repeated if an error is made in one area. Any large discrepancy in cell count between areas should be noted (if there is a >25 -cell difference between areas, the chamber should be cleaned and recharged).

Erythrocyte Calculations

In order to report the number of erythrocyte cells present in the original blood sample in RBC/cu mm, the dilution factors and chamber sizes must be considered along with the actual count of cells. The center square is 1 sq mm, but only 5 of the 25 squares, or one-fifth, were counted ($\times 5^a$); the space between the coverglass and chamber is 0.1 mm ($\times 10^b$); and the dilution ratio is 1:200 ($\times 200^c$).

Table 6.7 Total erythrocyte count reference values.

Species	Average ($\times 10^6$)	Range ($\times 10^6$)
Dog	6.8	6–9
Cat	7.5	5–10
Horse		
Hot-blooded	9.8	7–13
Cold-blooded	7.5	5.5–9.5
Cow	7.0	5–8
Sheep	12.0	8–15
Goat	13.0	8–17
Pig	6.5	5–8

Note: All values are in cells/cu mm.

Taking these three factors into consideration, if the hemocytometer count was 760 cells, the calculations are the following:

$$\begin{aligned}
 &\text{No. of cells} \times \text{no. of squares} \times \text{chamber depth} \times \text{dilution} \\
 &760 \times (5^a \times 10^b \times 200^c) = \\
 &760 \times (10,000) = \\
 &7,600,000 \text{ RBC/cu mm} \quad \text{or} \quad 7.6 \times 10^6 \text{ RBC/cu mm}
 \end{aligned}$$

Simply adding four zeros to the chamber count will give the correct answer, but in cases of severe anemia or hemoconcentration, different dilutions or counting areas may be used, which will alter the multiplier. Reference values for total erythrocyte counts are presented in Table 6.7.

Leukocyte Count

Allow the cells to settle on the hemocytometer for 2–3 minutes and then scan the field for filling defects as was done with the erythrocyte count. The count will be made on low power ($100\times$), and the areas to be counted will depend on the dilution technique used. If the Thoma pipette or reservoir with a 1:20 dilution was used, the four large, 1 mm $\times$ 1 mm corner squares will be counted (Fig. 6.20a). If the reservoir system was used that gave a 1:100 dilution, all nine large squares will be counted (Fig. 6.20b). Counting is done in the same fashion and using the same rules as with erythrocytes.

Because the blood has been in a lysing solution, cell membranes are disrupted, and the erythrocytes will not be seen or may appear as faint outlines (“ghosts”). The leukocyte cell membranes will also be removed, and the nuclei that remain will appear as black, irregular dots. Care must be taken to differentiate the nuclei from debris or other artifacts in the field of view. Reducing the illumination and closing the substage diaphragm on the microscope will help make the nuclei more distinct.

Leukocyte Calculations

Computation of reported results will vary with the dilution and counting technique used. With the 1:20 dilution, 4 sq mm were counted ($\times 1/4^a$), the coverglass-chamber distance

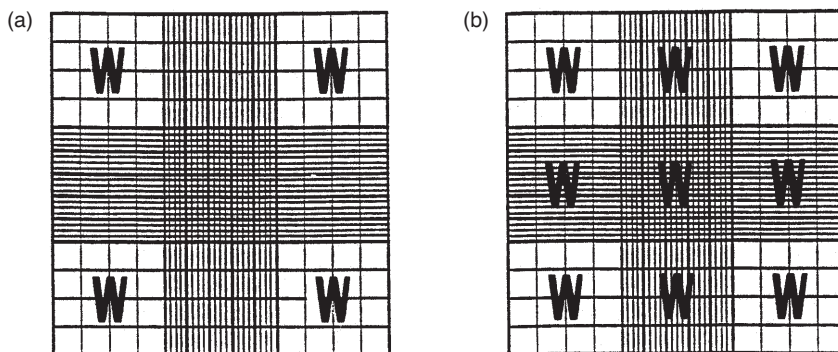


Figure 6.20 Areas of Neubauer grid counted for total leukocyte count. (a) Four corner squares counted for 1:20 dilution (Thoma pipette and some commercial systems). (b) All nine squares counted for 1:100 dilution (most common commercial system).

is 0.1 mm ($\times 10^b$) and the dilution is 1:20 ($\times 20^c$). If 146 nuclei were counted on the chamber, the calculations are the following:

No. of nuclei $\times$ no. of squares $\times$ chamber depth $\times$ dilution

$$146 \times (1/4^a \times 10^b \times 20^c) =$$

$$146 \times \frac{(10 \times 20)}{4} =$$

$$146 \times 50 = 7,300 \text{ WBC/cu mm}$$

With the 1:100 dilution, 9 sq mm were counted ($\times 1/9^a$), the coverglass-chamber gap remains 0.1 mm ($\times 10^b$), and the dilution is 1:100 ($\times 100^c$). For a count of 72 cells, calculations would be the following:

No. of nuclei $\times$ no. of squares $\times$ chamber depth $\times$ dilution

$$72 \times (1/9^a \times 10^b \times 100^c) =$$

$$72 \times \left(\frac{10}{9} \times 100 \right)$$

Instead of multiplying by 10 and then dividing by 9, the preferred, simpler method is to add 10 percent to the original count and then multiply by 100 (move the decimal two places to the right):

$$(72 + 10\%) \times 100 =$$

$$(72 + 7.2) \times 100 =$$

$$(79.2) \times 100 =$$

$$7,920 \text{ WBC/cu mm}$$

Although this will not give exactly the same answer, it is much easier to calculate and has been found to be statistically accurate.

Reference values for total leukocyte counts in various species are presented in Table 6.8.

Table 6.8 Total leukocyte count reference values.

Species	Average	Range
Dog	11,000	6–18,000
Cat	12,000	5–20,000
Horse		
Hot-blooded	10,000	7–14,000
Cold-blooded	8,500	6–12,000
Cow	8,000	4–12,000
Sheep	9,000	4–12,000
Goat	12,000	6–16,000
Pig	16,000	11–22,000

Note: All values are in cells/cu mm.

Correction for Nucleated Erythrocytes

Nucleated erythrocytes (nRBC) will occasionally be present in peripheral blood, especially in some anemias (see Chapter 11), and their nuclei will be counted along with the total white cell nuclei. If more than five nucleated red cells per 100 white cells were seen on the differential, the total leukocyte count must be corrected by using the following formula:

$$\text{corrected count} = \frac{\text{observed count} \times 100}{100 + \text{nRBC count}}$$

For example, if 72 total nuclei were counted on the hemocytometer, the observed WBC count is 7,920 WBC/cu mm. If 12 nRBC were tallied along with the differential count on the slide, the corrected leukocyte count is

$$\frac{7920 \times 100}{100 + 12} = 7,071 \text{ WBC/cu mm}$$

Eosinophil and Platelet Counts

Separate cell counts for eosinophils or platelets are occasionally useful for diagnosis. These can be most accurately determined by automated counters (see Chapter 7). Plastic reservoir systems are available for manual dilution and counting of either eosinophils or platelets, but more commonly, estimates of these cells are made that closely approximate total counts. For eosinophils, the concentration of cells calculated in the absolute count (total WBC $\times$ %eosinophils) will be a usable estimate. Platelets are estimated by counting the platelets in 10–20 microscopic fields, either at 100 $\times$ (“high dry”) or on oil immersion. If the average of these counts is between 5 and 25 on 100 $\times$, or between 2 and 5 on oil immersion, platelets are termed “adequate.” Counting the number of platelets present as the differential is counted can make closer estimates of platelets. This will give the number of platelets per 100 leukocytes, which will then be proportional to the total leukocyte count.

Potential Errors in Total Cell Counts

Manual cell counts have an inherent sampling and statistical error of 10–20 percent. Errors in technique that may occur include incorrect dilutions, inadequate mixing, blood on the outside of the pipette or capillary tube, improper chamber filling, lighting not adjusted, and failure to focus on cells that have not settled to the plane of focus. Distinguishing between cell nuclei and microscopic debris can be difficult but is minimized with experience. Errors in calculations should not occur, but occasionally do. All results should be double-checked.

Review Questions

1. Why is it important for the hematology student to perform manual blood smearing, staining, cell counting, etc. when these can be done by autoanalyzers?

2. Differentiate between relative, absolute, and total leukocyte counts.

3. Name or describe a manual or semiautomated laboratory test that could be used to test for each of the following blood contents:

- a. Percentage volume of erythrocytes
- b. Hemoglobin content
- c. Plasma protein levels
- d. Abnormal blood cells or blood parasites

Chapter 7

Automated Laboratory Methods and Instruments

Key Concepts

Automated chemistry analyzers have many advantages over manual techniques such as processing and analysis of a large number of samples quickly with minimal human assistance; however, final evaluation or interpretation of the results should still be performed or reviewed by a trained laboratory technician or medical professional.

Chemistry analyzers can be divided into four basic types: hematology, biochemistry, immuno-based, and miscellaneous analyzers.

Automated hematology and biochemistry analyzers use a variety of scientific techniques and calculations to process samples. The most common chemistry methods used can be broadly separated into electrical and optical methods of analysis.

Automated medical laboratory equipment is designed to process a number of samples quickly with minimal human assistance. These instruments are intended for *in vitro* (a test performed in a controlled environment and not in a living organism) diagnostic use in the quantitative measurement and analysis of biological samples. The results of these tests are useful in the diagnosis and prevention of disease. Automated laboratory testing is replacing many manual methods; however, the technician should never underestimate the need for human expertise. Technicians should understand the basic principles of both manual and automated techniques used in a clinical laboratory. This chapter will focus specifically on automated chemistry analyzers.

The specific tests that a technician will use in a clinical laboratory can often be performed both manually and by an automated analyzer. The manual form of the test requires that samples be prepared and then taken through each process of analysis manually. Contrarily, automatic analyzers work with minimal human assistance after loading the sample to report the details of analysis of the various samples rapidly and efficiently. Automated analyzers reduce the time for analysis for samples and decrease the occurrence of human error in analysis. Nonetheless, final evaluation of the results by the analyzer is still performed by a trained laboratory technician or medical professional.

Chemistry analyzers can be divided into four basic types: hematology, biochemistry, immuno-based, and miscellaneous analyzers. Hematology (blood) analyzers (i.e., cell counters and coagulometers) are used to conduct a complete blood count (CBC), erythrocyte sedimentation rates (ESRs), and coagulation tests. Biochemistry analyzers perform tests that measure enzyme levels (e.g., liver and kidney function tests), ion levels (e.g., sodium and chloride), and other chemical levels (e.g., glucose, lipase, or albumin). Immuno-based analyzers use antibodies to detect a specific substance or entity in various biologic samples. Miscellaneous analyzers are used less commonly and are unique in their function often requiring that a single analyzer be designed for a single or very few tests (e.g., DNA labeling and detection).

Hematology and Biochemical Analyzer System Overview

Over the last two decades the cost of automated machines has decreased allowing many veterinary practices to afford and use in-house, bench-top equipment. The major difference between automated equipment used in-house by the general veterinary practice (Fig. 7.1) versus larger commercial laboratories and/or veterinary colleges (Fig. 7.2) is the number and speed of samples that can be processed (rate), automated tube handling, and complexity of differential cell counting. There are a variety of manufacturers and machine types available for use depending on the needs of the laboratory (Table 7.1). Regardless of which automated equipment is used, always read and refer to the manufacturer's product reference and operating manual for detailed information regarding the system.

Hematology and biochemical analyzers have an internal computer to direct all system functions including test requisitioning, analyzer operation, data acquisition, data processing, report generation, and fault monitoring (systematic checking for errors and malfunctions in software). Samples are introduced into the analyzer either by placing tubes (e.g., test tube or manufacturer provided container/sample cup) into racks that are



Figure 7.1 Bench-top automated hematology, electrolyte, and biochemistry analyzers used at small animal veterinary hospital.

moved along a track, or by inserting tubes into circular carousels that rotate to make the sample available (Fig. 7.3). In smaller, bench-top machines the sample is placed into a tube or directly into a pipette that remains stationary and then the sample is dispensed (Fig. 7.4). To protect the health of laboratory staff, many analyzers feature closed tube sampling to prevent direct exposure to the samples. Both hematology and biochemical analyzers require specialized reagents for operation and analysis of biologic samples and consist of diluents, enzymatic cleaning agent, and multispecies lytic agents. These reagents are often specially developed for use in a particular manufacturer's instrument and are not compatible with other systems.



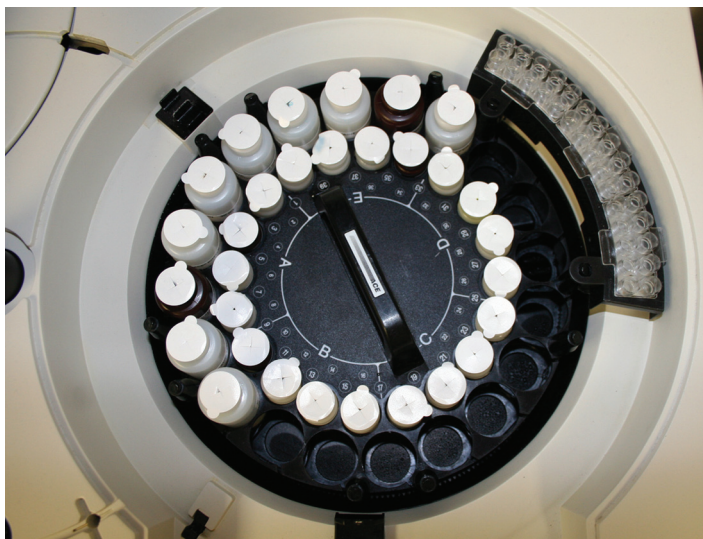
Figure 7.2 Bench-top automated hematology (a), electrolyte, and biochemistry (b) analyzers used at state veterinary diagnostic laboratory.

Table 7.1 Types of automated veterinary clinical hematology and biochemistry analyzers.

Company	Product
<i>Hematology analyzers</i>	
Abbott Laboratories	Aeroset, ARCHITECT, CELL-DYN
Bayer	ADVIA series
Beckman-Coulter	Coulter LH series, Coulter HmX, LH 1500
Drew Scientific	HemaVet 950
IDEXX	LaserCyte
<i>Biochemistry analyzers</i>	
Abbott Laboratories	Aeroset, AxSym
Alfa Wassermann	ACE Alera, VetACE, Starlyte III ISE
Beckman-Coulter	UniCel, Synchron series
IDEXX	VeTest
Olympus	AU series
<i>Immuno-based analyzers</i>	
Abbott Laboratories	IMx, AxSYM
Bayer	ADVIA Centaur
Beckman-Coulter	IMMAGE 800, Array 360

Hematology Analyzers

Hematology analyzers can be further subdivided into *cell counters* and *coagulometers*. Cell counters quantify, classify, and describe cell populations in blood and coagulometers measure the ability of blood to clot.

**Figure 7.3** Rotating circular carousel in biochemistry analyzer.

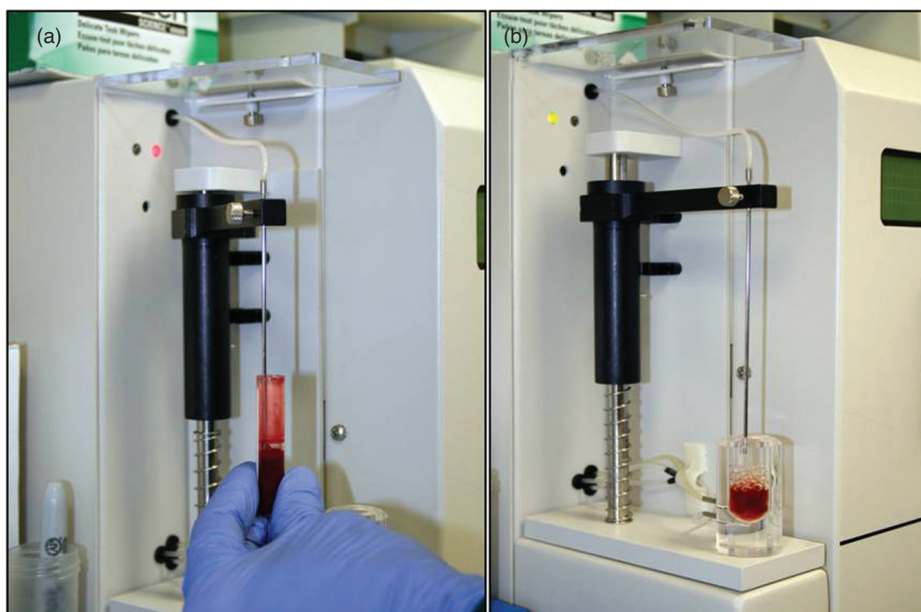


Figure 7.4 Hematology analyzer with stationary pipette for acquisition of desired sample. (a) Blood sample acquisition. (b) Blood sample dilution and mixing.

The complete blood count (CBC) includes a collection of tests used to quantify the number of red blood cells (RBCs), white blood cells (WBCs), and platelets, provide details about their size and shape, measure the hemoglobin (Hb) content of RBCs, determine the percentage and absolute number of the five types of WBCs, and identify immature and abnormal blood cells. Cell counters perform a CBC with unclotted whole blood (whole blood added to EDTA or heparin to prevent clotting), using electrical and optical methods to perform these tests simultaneously.

Automated cell counters calculate the hematocrit (Hct) or packed cell volume (PCV) by multiplying the red blood cell count by the mean red cell volume. The three main red blood cell indices (mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration; refer to Chapter 10) are used to determine the average size and hemoglobin content of the RBCs. The Hct and indices are important for characterizing and determining causes of anemia. The cell counters also provide a white blood cell count and a differential. A differential determines the percentage of each of the five types of mature WBCs. If the WBC is abnormal, the cell counter will flag that cell population so that a manual differential can be performed and compared to the automated counts.

Reticulocyte counts can now be performed by many hematology analyzers, using methods similar those in manual counts. A dye such as new methylene blue is used to stain the red blood cells containing reticulin prior to counting. Some analyzers have a slidemaker, which is able to then produce and stain blood films of high consistent quality, which will be reviewed by a medical professional.

Automated coagulation machines or coagulometers measure the ability of blood to clot. These machines require blood samples that have been drawn into tubes containing sodium citrate that acts as an anticoagulant; the anticoagulant effects of sodium citrate are reversible. Depending on the test being performed (partial thromboplastin time, prothrombin time), different chemicals can be added to the blood to trigger a clotting reaction. The progress of clotting can be analyzed by optical methods (absorbance spectrophotometry).

Biochemical Analyzers

Biochemical analyzers come in three categories: wet type, dry type, and biosensor. The wet type method involves mixing a test sample with a reagent to promote a chemical reaction. Analysis is performed using a reading device, such as a spectrophotometer that reads the color change before and after the reaction. The dry type method entails dispensing the test sample onto a test strip or cartridge that contains a chemical reagent that will react directly with the test sample (Fig. 7.5). Cartridges can contain single or multiple reagents (i.e., tests). It is often the smaller automated machines that have a stationary sample tube with manually loaded or mechanically moving cartridges whereas the larger machines rotate sample tubes only or also cartridges. Biosensor devices convert a biological response into an electrical signal. The devices contain a biological molecule (e.g., an enzyme or antibody) that is in direct contact with a signal transducer. For example, a widely used biosensor is the blood glucose biosensor that uses the enzyme, glucose oxidase (the biologic molecule) to break down (oxidize) glucose in a patient sample. The oxidation of glucose (which is the biologic response) is converted into an electrical signal by way of the transducer that transforms this into a concentration measurement.

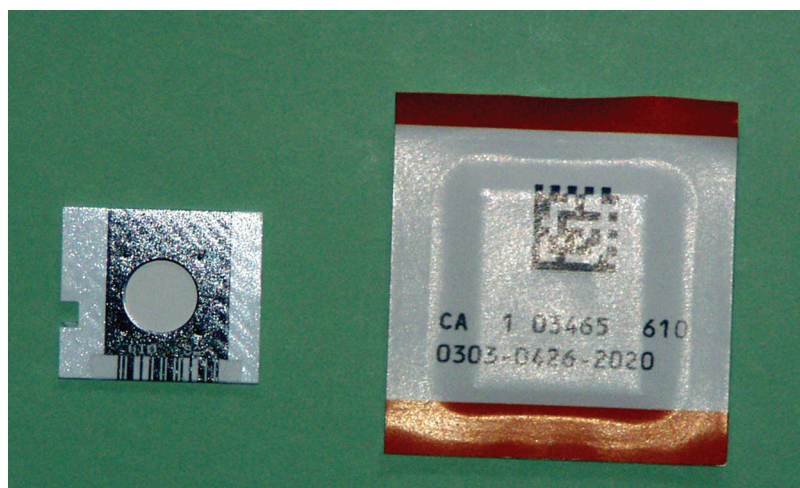


Figure 7.5 Cartridges used in dry type biochemistry analyzers. Cartridge contains a chemical reagent that will react directly with blood sample when a drop is placed on the cartridge.

Scientific Chemistry Methods

Automated equipment, whether used in a small or commercial laboratory, analyzes samples utilizing a variety of techniques and calculations. Due to the large scope of this topic, only the basic principles of the most common techniques will be discussed. It is not a necessity that the technician understands all the complexities of these techniques; however, an understanding of how these instruments work can help the technician comprehend the advantages and disadvantages of the types of equipment and trouble shoot if problems arise. The most common chemistry methods used can be broadly separated into electrical and optical methods of analysis. Hematology and biochemical analyzers use a single chemistry method or more advanced analyzers use a combination of electrical and optical methods.

Electrical Analysis

Electrochemical methods can be used to count cells in blood (e.g., white blood cell differential count and red blood cell count), measure blood pH, partial pressure of carbon dioxide or oxygen in blood, electrolytes (sodium, potassium, and chloride), and certain analytes (BUN—blood urea nitrogen). Electrochemical methods measure the potential (volts) and/or current (amps) in an electrochemical cell containing the analyte (substance desired to be measured). Figure 7.6 illustrates the basic scientific principles and components of an electrochemical cell with ion selective electrodes. These methods can be classified according to which aspects of the cell are controlled and measured. Three main classifications are potentiometry (the difference in electrode potentials is measured), coulometry (the cell's current is measured over time) and amperometry (the cell's current is measured while actively altering the cell's potential). Electrical analysis is based on the *principle of impedance* (i.e., resistance to alternating current flow).

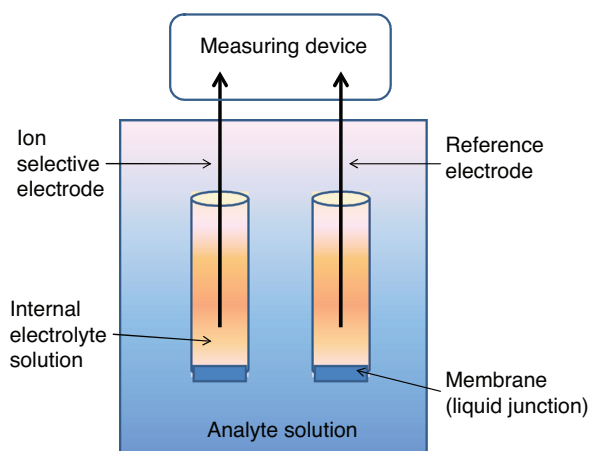


Figure 7.6 Electrochemical cell containing ion selective electrodes designed to measure sample analyte.

For example, to perform a white blood cell differential count and red blood cell count on a hematology analyzer, a small sample of whole blood is diluted with an isotonic solution (e.g., saline) prior to analysis and is passed through a small aperture across which an electrical current is flowing. The electrical current is produced by two electrodes in the aperture and the isotonic solution conducts the current between the electrodes. The cells move through the aperture singly, displacing a volume of electrolyte equal to its size. This action causes an increase in electrical resistance and impedes the flow of current. Each time this occurs a voltage pulse is produced, the magnitude of which is proportional to the size of the cells. The cell counter is programmed to differentiate voltage pulses created by different cell types (e.g., red blood cells, neutrophils, or lymphocytes). A lytic reagent is then added to the diluted blood to specifically and rapidly lyse red blood cells freeing the hemoglobin and leaving white blood cells and platelets intact. The entire process is usually performed in triplicate for enhanced accuracy.

Additional electrochemical methods are *osmometry* and *electrophoresis*. Osmometers are used to measure the concentration of dissolved particles (e.g., sugars and salts) in fluid expressed as the osmolality or osmolarity. Electrophoresis, when used in veterinary medicine, is a method used to separate and measure proteins in serum (i.e., serum protein electrophoresis). Protein in serum is composed of albumin and globulin. Electrophoresis is based on the principle that charged molecules move at different rates when migrating through an electrical field. Serum is placed in or on a supporting substrate (e.g., agarose gel or cellulose acetate) and is exposed to an electric current. Proteins in the serum migrate or move along the substrate forming bands that show the type and amount of each protein fraction. The clinical significance derived from either of these methods is beyond the scope of this textbook; technicians in veterinary practices or small laboratories will likely not perform these techniques.

Optical Analysis

Optical methods of detection can be used to count cells in blood, measure the concentrations of hemoglobin, electrolytes (sodium and potassium), protein (albumin and globulin), total carbon dioxide (total CO₂), glucose, BUN, calcium, phosphorus, magnesium, and other organ enzymes/molecules (e.g., bile acids, cholesterol). Also, automated coagulation machines or coagulometers optically measure the ability of blood to clot. The most common types of optical techniques used in veterinary medicine are photometry and light-scatter techniques.

Photometry

In optics, photometry is the measurement of lights' brightness or intensity. Automated machines are equipped with different types of photometric techniques of which the most common and widely used include spectrophotometry (absorbance and reflectance) and fluorescence spectroscopy.

Spectrophotometry

A spectrophotometer measures light intensity (optical density) as a function of color (wavelength). The visible light spectrum is made up of different wavelengths each

Table 7.2 Visible light spectrum.

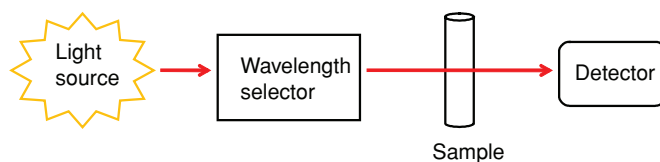
Color	Wavelength (nm)
None (infrared)	>740
Red	625–740
Orange	590–625
Yellow	565–590
Green	520–565
Blue	435–500
Violet	380–435
None (ultraviolet)	<380

representing a particular color (Table 7.2). The machine operates by passing a beam of light through a sample (which contains the desired analyte to be quantified) and measures the amount of light that is absorbed by the analyte or a product of the analyte (generated by an enzymatic reaction with a chemical reagent). A wavelength selector narrows the band of light to include only select wavelengths. A detector captures the absorbed light (absorbance spectrophotometry) or reflected light (reflectance spectrophotometry), converts it to an electrical signal and translates that signal to a corresponding analyte concentration (Fig. 7.7).

A good example of this technique is the measurement of hemoglobin. As previously mentioned, in order to count leukocytes and measure hemoglobin, a lysing agent is added to the sample of diluted blood. The lysing agent simultaneously lyses the red blood cells keeping leukocytes intact and releasing hemoglobin. The lysing agent contains potassium cyanide that reacts with and converts hemoglobin to a stable pigment (colored product) called cyanomethemoglobin. A spectrophotometer with wavelength set at the peak absorbance of cyanomethemoglobin measures the concentration of hemoglobin. The absorption of cyanomethemoglobin is directly proportional to the hemoglobin concentration of the sample. The cyanomethemoglobin method can also be performed manually to measure hemoglobin as discussed in Chapter 6. Pulse and CO (carbon monoxide) oximeters also use spectrophotometric technology to measure hemoglobin and blood gas levels.

Fluorescence spectroscopy (fluorometry)

Fluorescence spectroscopy is based on the principle that when an individual analyte or an analyte linked with a known fluorescing substance (dye) is exposed to a beam of light (at the correct wavelength), it will fluoresce. The light beam is absorbed by the analyte, which excites electrons in molecules of the analyte causing them to emit light (fluoresce)

**Figure 7.7** Basic scientific principle of spectrophotometry.

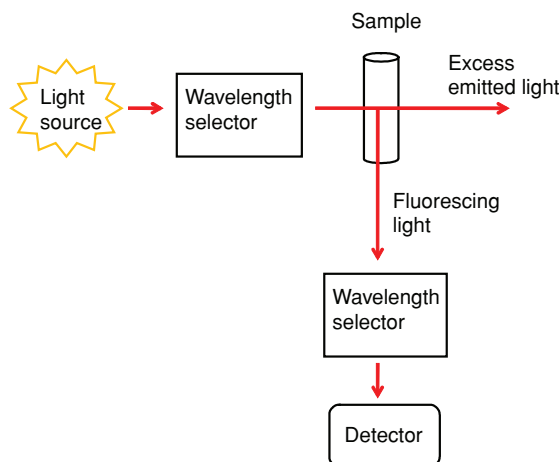


Figure 7.8 Basic scientific principle of fluorescent spectrometry.

of a lower energy than was absorbed. Then the wavelength and intensity of fluorescence are measured (Fig. 7.8). Fluorometry can also use fluorescent dyes that specifically bind nucleic acids for quantification of DNA or RNA in a sample.

Light Scatter

Light-scatter techniques are used to determine the concentration of particles (cells) in fluids (solution) and differentiate between different cell types. This optical method discriminates between cells on the basis of forward and side-scattered light (Fig. 7.9). Specifically, flow cytometers are used to measure the light-scatter and fluorescent properties of particles in a fluid. Flow cytometers can analyze the physical and/or chemical characteristics of up to thousands of particles per second. A sample in solution, for example, a blood sample is injected into a flow cytometer where the particles are randomly distributed in three-dimensional space. The blood sample must be forced into an ordered stream of single cells so that each individual cell's properties can be measured. The most common measuring systems used are electrical (measurement of impedance or conductivity) and optical systems using lamps (mercury, xenon) or lasers (argon, ultra violet). A stream of a single file of cells is passed through one or more beams of light (of a single wavelength) and an electronic detection apparatus. The detectors are aimed at the point

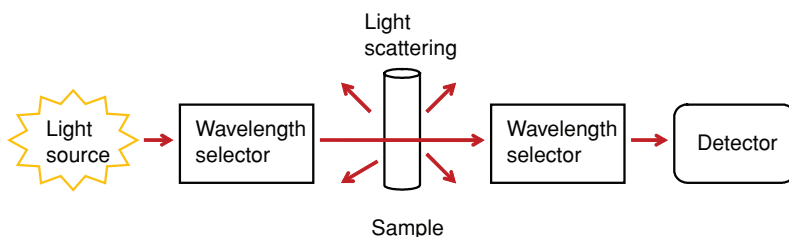


Figure 7.9 Basic scientific principle of light-scatter techniques.

where the stream passes through the light beam; one in line with the light beam (forward scatter) and several perpendicular to the light beam (side scatter) and/or one or more fluorescent detectors. Each suspended cell passing through the light beam scatters the light ray and/or fluorescent chemicals found in or attached to the cell are excited into emitting light at a longer wavelength than the light source. This scattered or fluorescent light is picked up by the detectors and analyzed on the basis of the fluctuations in brightness or fluorescence intensity at each detector. From this analysis, information about the physical and chemical properties of each cell can be derived. Forward light scatter correlates with the cell volume and side light scatter provides information about the internal structure of the cell (i.e., shape of the nucleus, amount and type of cytoplasmic granules). This is how a hematology analyzer is able to calculate the numbers of white blood cells and differentiate between the different types of white blood cells in a given blood sample.

Advantages and Disadvantages of Automated Equipment

Automated hematology and biochemistry systems provide numerous advantages to manual methods; however, these systems are not infallible and should never be used to replace human expertise. In general, the features and benefits that automated systems can provide involve ease of use, accuracy and precision, convenience, flexibility, and an economical alternative to manual methods. Depending on the clientele and needs of a particular veterinary practice, automated diagnostic equipment still requires a financial investment. The equipment may be purchased outright or the manufacturer might provide different payment plans. Also the technical life span of most equipment is on average 5–7 years and the economics of replacement considered. Many manufacturers provide lease plans that involve planned upgrades and replacement of equipment at predetermined intervals.

Automated equipment is able to replace many time-consuming and laborious manual methods by offering the ability to work with minimal human intervention to analyze various samples quickly and efficiently. High numbers of samples can be processed and multiple parameters analyzed simultaneously. For example, the ACE Clinical Chemistry System can process up to 165 tests per hour, use up to 40 different reagents and provide analysis on multiple parameters (e.g., concentration, cell size, and shape) of the sample at one time. Presentation of the analyzed data can be provided in different report styles. Each report can be viewed on screen, printed, stored, or transferred to a laboratory information system. Automated machines are also equipped to monitor all system functions and provide alerts for problematic analyses or system malfunctions.

Automated machines reduce the need to train veterinary technicians or other medical personnel in preparing and mixing samples for processing, calibration of control samples, and storage of reagents as this is often performed by and within the machine itself. These factors can reduce or even eliminate performance variation due to operator technique. Many automated machines offer closed tube sample processing to prevent direct exposure of personnel to biologic samples. The sample tubes, reagents, and other consumable

parts of the machine are often labeled with a bar code that contains information, such as expiration date, lot number, and other specifics pertaining to use.

Automated system design is becoming more affordable, compact (taking up a minimum amount of lab bench space), and flexible to accommodate most user preferences. Despite these factors, all automated equipment requires some knowledge of operation and maintenance. Just imagine that when the power fails and there is no electricity to operate the automated systems, what techniques will the veterinary technician or medical personnel be using to check the hematocrit on a critically ill patient?

Review Questions

1. Why should the analyzed results from an automated chemistry analyzer always be reviewed by a trained medical professional?
2. What are the four basic types of chemistry analyzers?
3. What is the primary function of a hematology analyzer and a biochemistry analyzer?
4. What are the two broad categories of scientific methods used by automated chemistry analyzers?
5. What is a spectrophotometer and how does it work?
6. Explain the principle of light-scatter technique.
7. What are the advantages and disadvantages of using automated chemistry analyzers versus manual techniques with regards to performing hematology and biochemical tests?

Chapter 8

Leukocyte Cell Types and Functions

Key Concepts

The hematology technician commonly encounters and measures the leukocytes in the blood stream, but they are also located in varying concentrations, called pools 1 throughout the body.

Neutrophils, eosinophils, basophils, monocytes, and lymphocytes all have numerous individual functions, and collectively are responsible for the body's reaction to foreign material (immune system) and damage (inflammation).

The leukocytes exhibit recognizable changes in appearance during normal maturation, when actively stimulated, and even between various species of animals.

Recognizing changes in the numbers and developmental stages of individual leukocytes in the blood sample, and the terminology associated with these changes, can be an important diagnostic aid.

The leukocytes, through many independent but interconnected functions, are responsible for the recognition, response, and removal from the body of foreign (“nonself”) material and damaged or dead cells or tissue. They are the major participants in both the inflammatory and immune response mechanisms. A brief description of each cell type is presented in Chapter 2. This chapter presents morphologic variations that may be encountered and discusses the functions of each cell type. Familiarity with the normal response mechanisms of the individual leukocytes will enable the technician to better understand the changes that occur in the blood picture under physiologic or pathologic conditions. The ability to identify changes in the appearance of the cells due to these responses (e.g., lymphoblast vs lymphocyte, band vs segmented neutrophil) are essential for accurate cell identification and formation of a leukogram, the overall picture of the status of the leukocytes in the body.

Leukocyte Distribution and Responses

The majority of leukocytes in the body are not freely circulating in the blood from which samples are drawn; rather, they are found in various collections throughout the body. These collections are called pools, and the cells can shift back and forth between these areas (Fig. 8.1). Two of these pools consist of cells not yet released into the circulation. These cells are called the **proliferation pool**, cells being formed in the bone marrow, which will be available upon maturation, and the **storage pool**, mature cells stored mainly in the spleen and bone marrow. Within the vasculature itself are two pools, the **circulating pool**, freely moving cells from which the sample was drawn, and the **marginal pool**, cells that are stuck to or rolling along the walls of small vessels and not freely circulating. In most animals, this marginal pool somewhat surprisingly contains 50–75 percent of the cells within the vasculature. Leukocytes that have left the blood vessels to enter various areas of the body make up the **tissue pool**, the site of functioning of most white blood cells.

The blood vessels function essentially as a means of transport between pools. Under disease conditions or hormonal influences, cells are shifted between pools, and it is

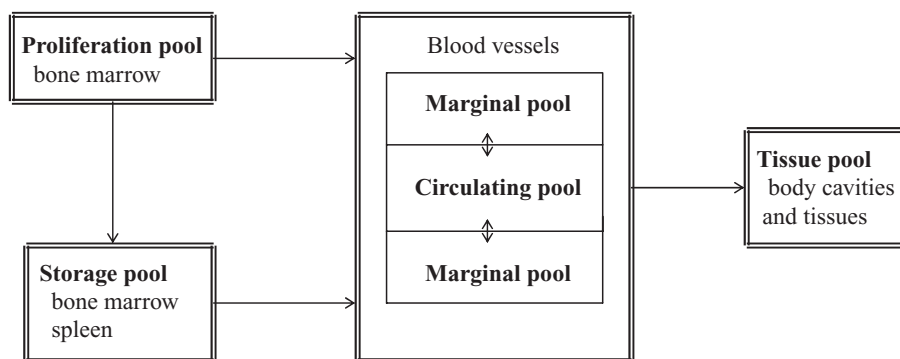


Figure 8.1 Distribution of leukocytes in “pools” in the body.

this shifting that causes the alterations seen in differential and total blood counts. The responses by the marginal and storage pools can be very rapid, within minutes to hours, in the presence of corticosteroids or epinephrine from stress, excitement, or external administration. Production of new cells by the proliferative (bone marrow) pool, however, is much slower. The number of leukocytes being produced from stem cells is increased, but the rates of mitosis and maturation are changed very little, so it may be several days before this response is seen in the circulating blood.

Any increase in the number of white blood cells above the normal range for a species is termed “leukocytosis.” An abnormally low total count is “leukopenia” (or leukocytopenia). Typical reference values for total counts of white blood cells in various species are presented in Table 6.8. The term “leukemia” may be applied when there are abnormal white cells present in the circulation, usually with neoplasia (cancer); the presence of abnormal cells without evidence of neoplasia is a leukemoid response. It is important to point out that not all changes in the leukogram result from pathologic or disease processes. Some increases in cell types and total numbers are due to normal metabolic processes and are called physiologic leukocytosis. Some of the conditions where physiologic leukocytosis may occur are the following:

1. Excitement, fear, apprehension, pain—most pronounced in the horse and cat, moderate in the dog.
2. Muscular exertion/exercise—mainly horse and cat but seen in all species.
3. Convulsive seizures—similar to muscular exertion.
4. Stress—may be considered physiologic or pathologic. Caused by production of adrenalin (epinephrine) and corticosteroids. Called a stress leukogram.
5. Digestion—carnivores and pig, peaks 3–4 hours after eating, not seen in herbivores.
6. Pregnancy—especially late term in cow and dog.
7. Estrus—common in cow around time of ovulation, not consistent in other species.
8. Age—newborn and very young, which animals routinely have a much higher white cell count than adults. This is not seen in the pig, which usually has a lower count at birth. Interestingly, much lower white cell counts are seen in animals delivered by Cesarean section than in those from natural birth.
9. Species—normal ranges vary considerably. For example, a white cell count of 20,000/cu mm would be quite high in most species but in the normal range for pigs.
10. Postimmunization—slight elevation possible for 1–2 weeks.

Neutrophils

The neutrophil (segmenter, or polymorphonuclear [PMN]) is produced in the bone marrow by mitosis and maturation of stem cells, a process that takes from 3 to 10 days. Neutrophils are present in the circulation for an average of 6–7 hours prior to migrating out of the blood vessels into the tissues and body cavities. They may live another 2–3 days out in the tissues or, in the presence of disease processes, only a few hours. Neutrophils are the

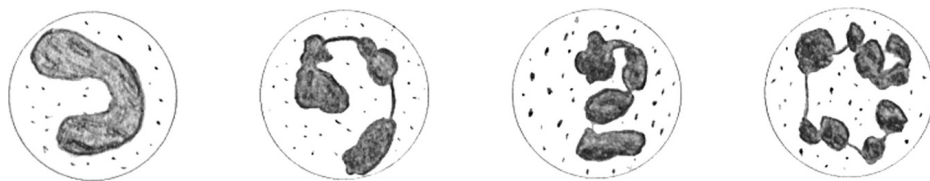


Figure 8.2 Examples of the typical appearance of neutrophils. The cell at far left is a less mature “band” cell, the two in the center are mature “segmenters,” and at far right is a hypersegmented neutrophil.

predominant leukocyte in the peripheral blood of dogs, cats, horses, and humans, but in ruminants and some laboratory animals, may be outnumbered by lymphocytes.

Neutrophil Morphology

The most easily recognized feature, the segmented nucleus with its 3–5 lobes, is present in all species, although the degree of segmentation may vary from simple constriction (dog, horse) to separate lobes connected by fine filaments (cow). The nuclear chromatin is coarse, clumped, and dark staining. The nucleus of a newly released or juvenile neutrophil in any species has a more ribbonlike or band appearance, with roughly parallel sides, and it stains less intensely. This stage of development is termed a “band” or “stab” cell (Fig. 8.2). Earlier stages of development will only occasionally be seen in the circulation (Fig. 8.3) and are discussed later in this chapter and in Chapter 13.

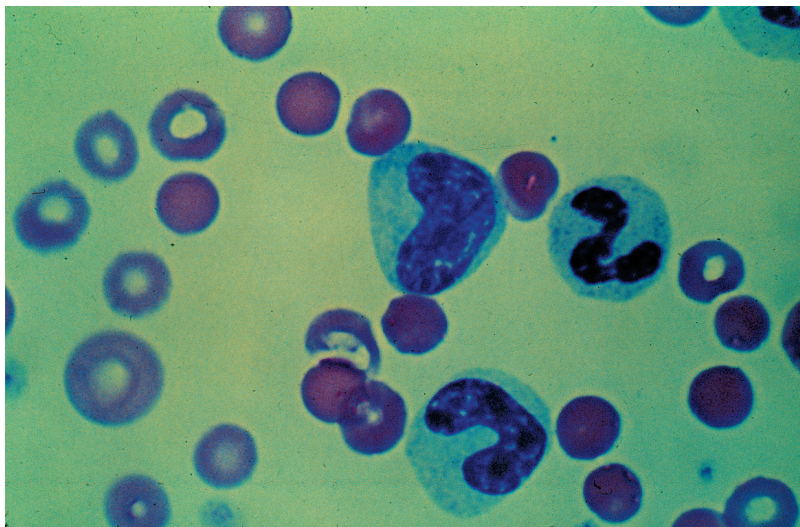


Figure 8.3 Segmented neutrophil, along with band, and metamyelocyte forms. The metamyelocyte stage is rarely encountered in peripheral blood. In the surrounding erythrocytes, note also the unequal cell sizes (anisocytosis), the smaller cells lacking central pallor (spherocytes), and the fragmented cell (schistocyte).

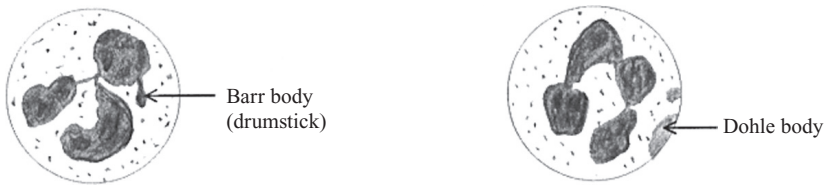


Figure 8.4 A Barr body attached to the nucleus of a neutrophil and a Dohle body in the cytoplasm of a neutrophil.

The color of the cytoplasm varies from light blue to pink or, more commonly, clear. Cytoplasmic granules in most species are fine and dust-like and stain light pink to gray but are usually too small to be visible on the light microscope. In some mammals (e.g., the rabbit and guinea pig) and most birds and reptiles, the granules will be conspicuous and more red staining; these cells are often referred to as heterophils.

A clublike appendage of the nucleus, called a Barr body or drumstick, may be seen in neutrophils collected from a female animal (Fig. 8.4). This appendage appears to be an inactivated X-chromosome and may occasionally be seen in other blood or body cells. Dohle bodies, bluish staining inclusions in the cytoplasm of the neutrophil, are occasionally seen, especially in the cat (Fig. 8.4). They are seen on the electron microscope as remnants of the rough endoplasmic reticulum.

Neutrophil Function and Pool Shifts

The major functions of neutrophils are associated with phagocytosis and inflammation. Toxins released by invading bacteria and chemical substances released by damaged tissue attract neutrophils to an area. Small particles and organisms are engulfed (phagocytized) and broken down by proteolytic enzymes in the neutrophilic granules. On large particles or surfaces that cannot be engulfed, the enzymatic granules are released outside the cell where they act on the invading organism and the local tissue. This increases the amount of inflammation and attracts more leukocytes to the area, with the hopeful outcome of controlling the infection.

Any increase in the number of circulating neutrophils is called neutrophilia. If this is associated with an increase in total leukocytes, “neutrophilic leukocytosis” is the correct term. Rapid increases in neutrophil numbers are due to shifting of cells from the marginal and storage pools into the circulating pool. Within days, cells from the proliferation pool are being more rapidly released into the circulation, sometimes prior to complete maturation (see description of left shift, below). Some of the common causes of neutrophilia are (1) infectious organisms, especially pyogenic (pus-forming) bacteria; (2) inflammation, especially with tissue necrosis (e.g., trauma); (3) neoplasia; (4) various intoxications; and (5) the presence of corticosteroids or epinephrine, whether administered or endogenous (produced internally) due to excitement or stress.

Neutropenia, or a decrease in neutrophils in circulation, is in general due to a rapid movement of the cells out into the tissue pool or a decrease in cells available from proliferation and storage pools. A temporary neutropenia in response to infection, when

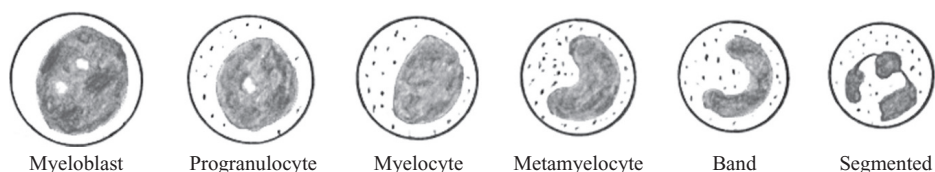


Figure 8.5 Schilling's maturation index of the neutrophil.

most of the available cells move rapidly out of the vessels, may be seen, but the total circulating count rebounds rapidly. This is most pronounced in cattle. Other causes of neutropenia include the following: (1) bone marrow suppression from viral or chemical agents; (2) overwhelming bacterial infections; and (3) sequestration of cells in capillary beds in endotoxic, septic, or anaphylactic shock.

Qualitative Changes

Changes in the stage of maturity on release from the bone marrow can be seen in any of the granulocytes but are most evident and best categorized in the neutrophil. The normal maturation sequence of the granulocytes in the bone marrow is discussed in Chapter 13 and is schematically depicted in Figure 8.5.

Normally, cells have reached the “adult” segmented stage prior to release into the circulation, although a small percentage of band cells is released. When there is a rapid loss and increased demand for neutrophils, as in infections and tissue damage, the increased release rate from the bone marrow will often contain a larger percentage of immature forms. This is termed a “shift to the left” or more simply a “left shift,” indicating the presence of cells to the “left” of the mature neutrophil in Shilling's maturation index of the neutrophil (Fig. 8.5), or in the graphs of neutrophil distribution in (Fig. 8.6). The degree of the left shift can be graded by the types of immature cells found: slight(1+)—band cells only; moderate(2+)—metamyelocytes; marked(3+)—myelocytes and promyelocytes; and extreme(4+)—myeloblasts.

When the total leukocyte count is elevated because of neutrophilia and the number of immature forms present remains equal to or less than the mature, segmented neutrophils, it is called a regenerative left shift (Fig. 8.6c). This indicates a proper bone marrow response where there is sufficient time and ability to respond to the increased demand.

In a degenerative left shift, the total leukocyte and neutrophil counts may be normal but usually begin falling, and the number of immature neutrophils exceeds the mature forms (Fig. 8.6d). Both the marginal and storage pools have been depleted, and the bone marrow has not had the time or cannot meet the demand for production. In most species, this indicates a poor prognosis. In cattle, however, a degenerative left shift is common and considered serious only if it exists for several days.

Any neutrophil with more than five nuclear segments is called hypersegmented and represents increased aging of the cell (Fig. 8.7). The presence of excessive numbers of these cells in the circulation is called a right shift (Fig. 8.6b). This may be seen when the neutrophil has remained in the circulation longer than normal due to the prolonged presence of corticosteroids from stress or a treatment regimen. Hypersegmentation may also be seen as an artifact in blood samples that have been stored too long.

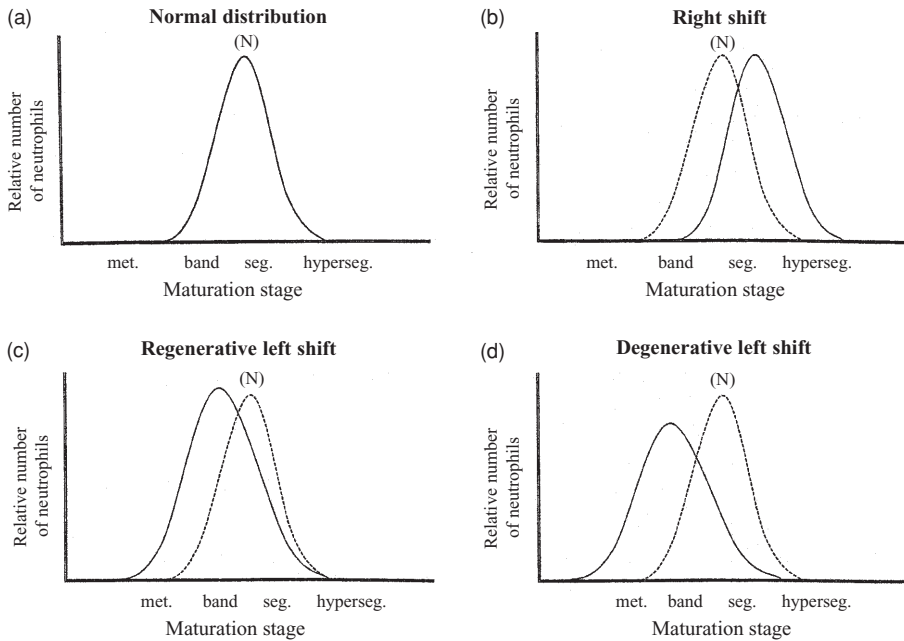


Figure 8.6 The normal distribution of neutrophils in the circulating pool and shifts in this relative distribution in physiologic and pathologic conditions. (met.; neutrophilic metamyelocyte, band; band neutrophil, seg.; segmented neutrophil, hyperseg.; hypersegmented neutrophil).

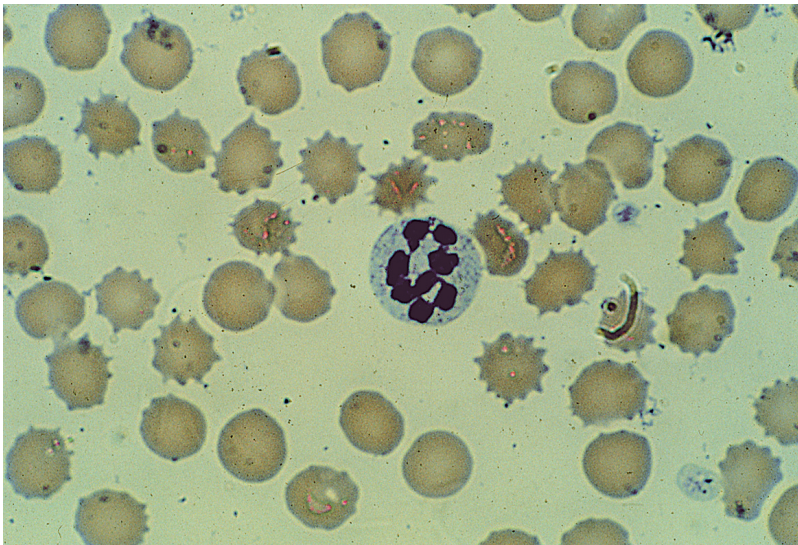


Figure 8.7 Hypersegmented neutrophil in peripheral blood. The basophilic crescent-shaped strand on the erythrocyte is an artifact.

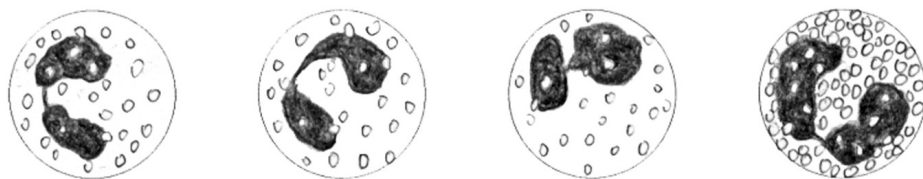


Figure 8.8 Examples of the typical appearance of eosinophils. The granules in the cytoplasm stain about the same color as the erythrocytes (red to orange) and vary in size, shape, total number, and staining intensity in different species.

With severe infections, toxicity or acidosis, neutrophils will often undergo characteristic changes known as toxic changes. The cytoplasm of toxic neutrophils will stain a darker blue (i.e., they will become basophilic) and will appear foamy or contain small vacuoles. Multiple dark-staining granules may also appear in the cytoplasm (toxic granulation) along with larger, blue-staining structures, called Dohle bodies. Toxic neutrophils often accompany a degenerative left shift and are a negative prognostic sign.

Eosinophils

Bone marrow production of eosinophils is similar to that of neutrophils and takes from 2 to 6 days. Many mature eosinophils remain in the bone marrow forming a large storage pool. Cells entering the blood stream circulate for 6–10 hours before migrating into body tissues or cavities, where they may persist for several days.

Eosinophil Morphology

The nucleus of the eosinophil varies from elongate (band) to bilobed or trilobed and is similar in appearance in the domestic species (Fig. 8.8). The cytoplasm stains a light blue and contains eosinophilic (red-staining) granules that are often refractile. The granules vary in size, shape, number, and staining intensity in the different species and are characteristic of most species (Fig. 8.9). In the dog, the granules are rounded and have the most variability in size and number. Vacuoles are often present, especially in the greyhound. Cat eosinophils have small rod-shaped or elongate granules that typically fill the cytoplasm. In the equine, the granules often stain a more salmon or orange color, are large and round, and completely fill the cytoplasm. They often crowd the nucleus and distort the cell membrane, giving the appearance of a thin plastic bag full of oranges. The granules of ruminant eosinophils are small, round, and numerous and often stain an intense red to lilac color, frequently described as appearing like jewels.

Eosinophil Function

The eosinophil has numerous, often unrelated functions, most of which are still poorly understood. It is attracted to histamine, antigen-antibody complexes (see Chapter 9), and foreign or degraded body protein associated with inflammation and allergic conditions. Eosinophils may play a role in controlling or regulating these responses. Most parasites,

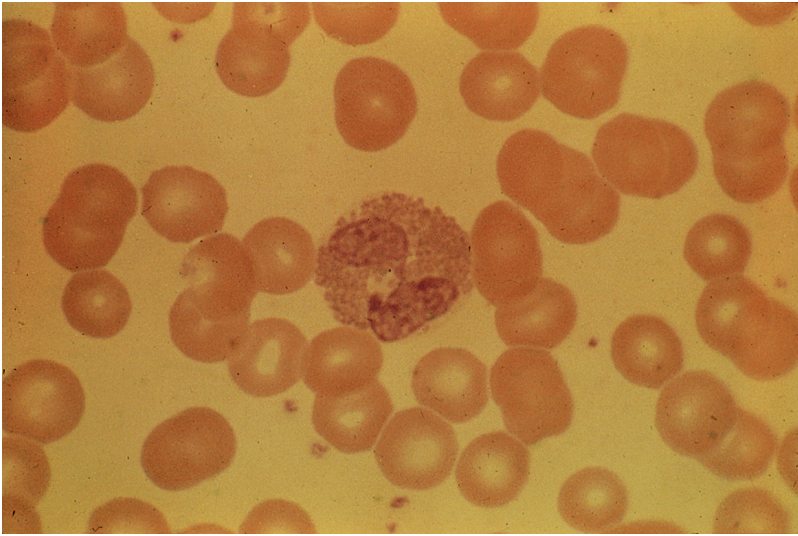


Figure 8.9 Mature eosinophil in peripheral blood.

especially migrating forms, activate eosinophils. By attaching to the parasite and degranulating, and with the presence of antibodies, eosinophils can kill the organism. Eosinophils also have some phagocytic ability and can detoxify some chemicals. They also appear to play a role in coagulation and fibrinolysis by activating steps in the clotting mechanism.

Peripheral eosinophilia is commonly associated with allergies and parasitism. A relative eosinophilia may occur in chronic infections, or in the recovery stages of acute infections, or whenever there is an increase in basophils or mast cells (see below). Certain conditions are characterized by eosinophilia, such as eosinophilic myositis or eosinophilic gastritis.

A decrease in circulating eosinophils (eosinopenia) is difficult to detect because differential counts for normal animals may reveal no eosinophils. Absolute eosinopenia occurs in stress conditions or with the administration of epinephrine or corticosteroids.

Basophils

Much less is known about the production, circulation, and function of basophils than the other leukocytes because of their rarity in blood and bone marrow. They are manufactured in the bone marrow, apparently in a manner similar to the other granulocytes, and have a total life span of 10–12 days.

Basophil Morphology

The basophil has an elongate nucleus that is often coiled and may be partially segmented (Fig. 8.10). The cytoplasm stains gray to blue and is partially (dog) or completely (ruminant, horse) filled with dark purple to nearly black basophilic granules (Fig. 8.11). The cat may have both light-staining (pink-gray) and dark-staining (purple) granules. When



Figure 8.10 Examples of the typical appearance of basophils. The cytoplasmic granules vary in number and depth of staining (lavender to dark purple to black) and often obscure the outline of the nucleus.

granules are numerous. they tend to obscure the nucleus. Basophils are slightly more common in the horse and ruminant than in other animals.

Basophil Function

The basophil is structurally and functionally similar to a cell found in the body tissues, called a mast cell. Other than a rounded nucleus and more numerous granules in the mast cell, the two cells appear to be chemically and functionally identical. The mast cell may represent a blastic transformation of the basophil after leaving the circulation, but this has not been proven. The function of basophils and mast cells is based on the sensitivity of membrane receptors to a variety of substances such as prostaglandins, immunoglobulins (antibodies), complement, endotoxins, and histamine. They often have, or may develop, receptors to allergens such as dust, mold, and other proteins, including some viruses. When these receptors are stimulated, the cells release their stored granules composed of histamine, dopamine, serotonin, heparin, peroxidase, and other enzymes that initiate an

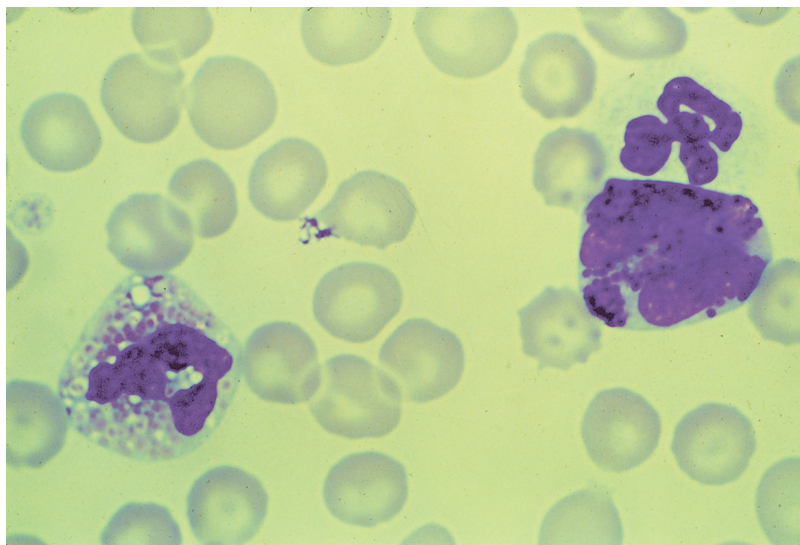


Figure 8.11 **Basophil** (with dark granules), **eosinophil** (with lighter granules), and **segmented neutrophil** from a dog. Canine basophils contain fewer granules than those in other species. Eosinophilic granules vary in size in the dog and often appear as clear or refractive areas.

acute inflammatory reaction. Normally, a protective mechanism, excessive degranulation, may result in local or generalized hypersensitive and allergic reactions. Both the basophil and mast cell can resynthesize their granules after degranulation.

A slight increase in basophil numbers may be seen in association with conditions causing eosinophilia, such as allergies and migrating parasites, and in some endocrine disorders. An apparent basophilia is often seen with mast cell sarcoma (mastocytoma/mastocytosis); differentiation between circulating mast cells and basophils is difficult. Since basophils are normally absent from the peripheral blood, basopenia has no clinical significance.

Lymphocytes

Although the sequence of development is the same, lymphocyte production is more widespread and complex than that of other leukocytes. Sites of production are the bone marrow; the lymphoid organs, which include the lymph nodes, spleen, and thymus (during fetal and early neonatal life); and the gut-associated lymphoid tissue, such as Peyer's patches, tonsils, and appendix. The major function of bone marrow and thymic production appears to be the seeding of the peripheral lymphoid tissue with precursor cells. Normal maturation time in the bone marrow is 2–5 days, but with antigenic stimulation in lymphoid tissues, it may be as short as 6–8 hours. Both short-lived and long-lived (memory) lymphocytes are present, with life spans varying from days to 20+ years. The lymphocytes are typically the most common leukocyte in the circulation of ruminants and are second to neutrophils in most of the other species. Lymphocytes are unique among the leukocytes in that after migrating out into the body tissues they routinely reenter the circulation through lymphatic channels.

Lymphocyte Morphology

The morphology of the lymphocyte often varies more within a sample than between species (Fig. 8.12). Most commonly seen is the mature lymphocyte, which is smaller than the other leukocytes and has a round or slightly indented nucleus with coarsely clumped chromatin. A thin rim of blue-staining cytoplasm surrounds the nucleus in small lymphocytes. Medium and large forms of lymphocytes also exist in all species but are more common in ruminants (Figs. 8.13 and 8.14). In these, the amount of cytoplasm is moderately to greatly increased and may contain variously colored (azurophilic) granules.



Figure 8.12 Examples of the typical appearance of lymphocytes. The cell at far right is a large lymphocyte that has been distorted by surrounding cells.

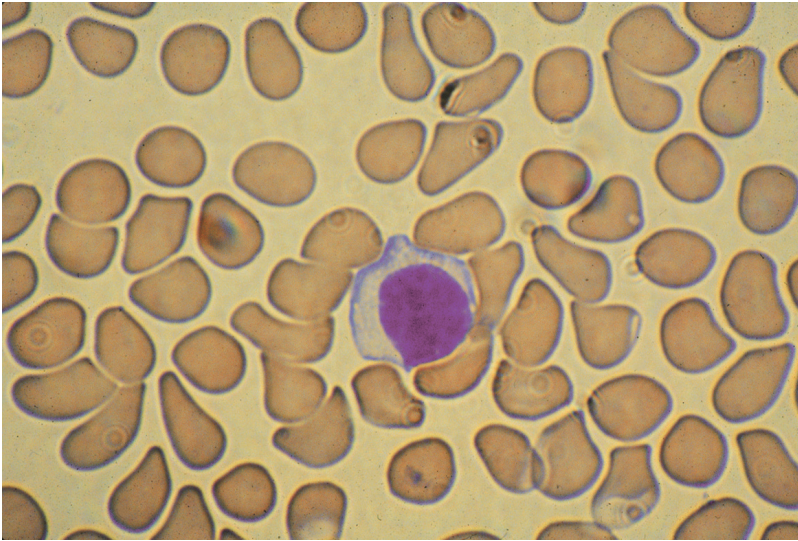


Figure 8.13 Mature lymphocyte. Note the coarse chromatin pattern in the nucleus. Many small lymphocytes will have smaller amounts of cytoplasm than is seen here. The distortion of erythrocytes is due to crowding of cells.

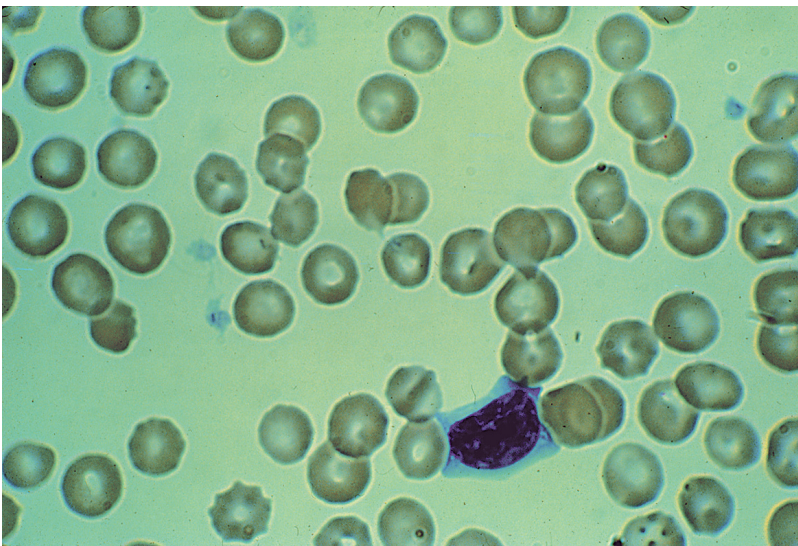


Figure 8.14 Large lymphocyte. It can be seen in any animal but is most common in ruminants. The cellular shape is easily distorted by surrounding cells.

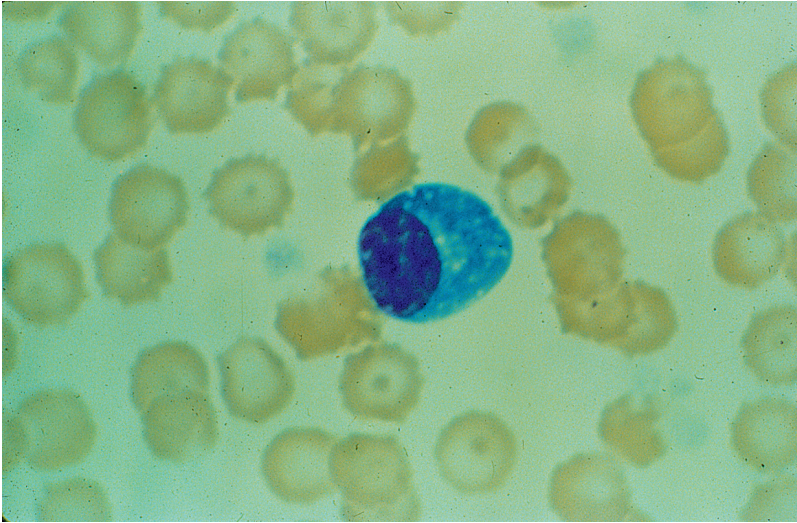


Figure 8.15 Circulating **plasma cell**. Uncommon in peripheral blood, the presence of more than one in a differential count may indicate lymphoid tissue activation.

The largest forms may be difficult to distinguish from monocytes. Lymphoblasts (immature lymphocytes) are large cells with a large, more open nucleus containing smoother-appearing chromatin and occasional nucleoli. The presence of circulating lymphoblasts should always be noted because they are often of important diagnostic significance.

In antigenically stimulated lymphocytes (immunocytes), the cytoplasm stains a more intense blue. The cytoplasm in a lymphocyte actively producing antibodies assumes an oval or egg shape with the nucleus at one end. The nuclear chromatin is often clumped to resemble the spokes of a wheel. This cell is often called a plasma cell (Fig. 8.15).

Lymphocyte Function

The lymphocyte is a major cell involved in the immune response. Three different types of lymphocytes are normally present, called B, T, and null lymphocytes, which appear identical on the light microscope (Fig. 8.16). The B lymphocytes are so named because in birds a structure called the Bursa of Fabricius must be present for proper functioning of these cells. Mammals do not have a Bursa of Fabricius, so the term “bursa-equivalent” is used, denoting most likely the bone marrow or Peyer’s patches as serving the same function as the bursa. The B lymphocytes are the antibody-producing cells of the humoral immune system (discussed in Chapter 9). The T lymphocytes are “thymus-associated.” Although the thymus atrophies by early adult life, it must be present during fetal and early postnatal life for proper development and functioning of the T lymphocytes in the cell-mediated portion of the immune response. It should be stressed that B and T lymphocytes are routinely manufactured throughout the lymphoid tissues of the body, not in the bursa or thymus, but these two structures must have been present for the subpopulations to exist. A small number of the lymphocytes in circulation are called the non-B, non-T, or null, lymphocytes since their surface markers differ from those of the B or T groups. The

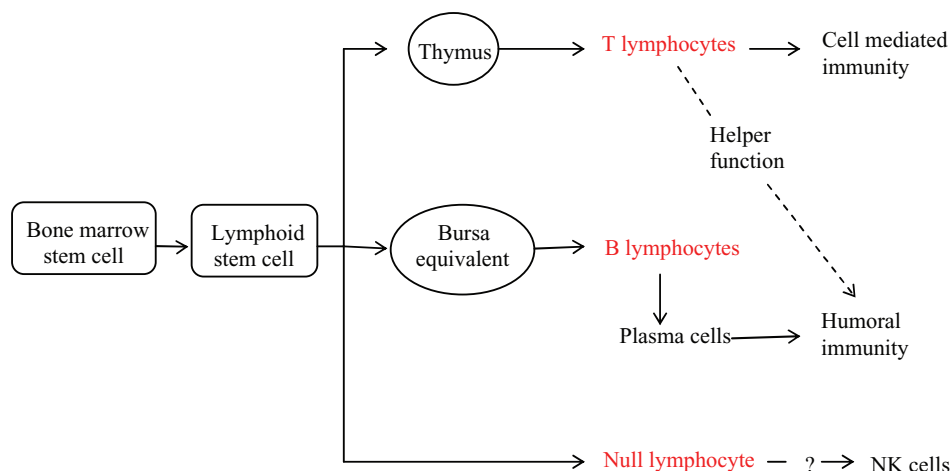


Figure 8.16 Kinetics of lymphocyte subpopulations in the body.

function of null lymphocytes has not been positively determined, but they are possibly the NK (natural killer) cells of the nonspecific immune defense mechanism. Discussion of the NK cells, as well as the B and T lymphocyte functions, is presented in greater detail in the next chapter.

A physiologic increase in circulating lymphocytes (lymphocytosis) similar to that seen with neutrophils will often be present in very young animals, animals expressing fear or excitement, or animals involved in muscular activity. Other causes of lymphocytosis are chronic antigenic stimulation—especially by viruses, blood parasites, and postimmunization. Lymphocytosis with the presence of lymphoblasts or other abnormal lymphocytes may be seen with lymphoid neoplasia (leukemia, lymphosarcoma).

Corticosteroid-induced lymphopenia (lymphocytopenia) may be seen with chronic stress. Viruses that suppress the immune system may produce a lymphopenia or pan-leukopenia (a decrease in all white blood cells). Radiation and chemotherapy associated with cancer treatment commonly lower lymphocyte numbers. Some cases of lymphocytic neoplasia will have a lowered lymphocyte count.

Monocytes

Monocytes are formed in the bone marrow with a production time of 2–4 days. Newly formed monocytes are released into the circulation as soon as they are produced and may circulate for up to 2 days. After moving into the tissues and body cavities, they become the fixed or free macrophages found in essentially all body tissue. Survival in tissues varies from days to possibly months.

Monocyte Morphology

Monocyte morphology is similar in most all domestic species (Fig. 8.17). The monocyte is usually the largest leukocyte, although very large lymphocytes may be similar. The

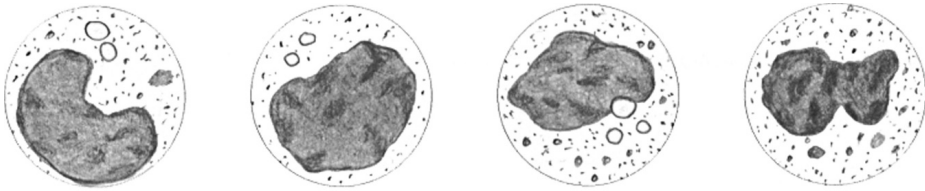


Figure 8.17 Examples of the typical appearance of monocytes.

nucleus can assume various shapes of round, oval, elongate, or kidney bean, or it may have multiple indentations (Fig. 8.18). The nuclear chromatin is lacy or reticular and appears smoother, with less clumping, than in other leukocytes. The cytoplasm is abundant, stains bluish-grey, and has a foamy or ground glass appearance. Variable numbers and sizes of vacuoles (empty spaces or bubbles) are common, and inclusions consisting of phagocytosed particles or even whole cells may be present.

Monocyte Function

The major functions of the monocyte/macrophage are due to their phagocytic ability. They engulf and destroy organisms that cannot be controlled by neutrophils, especially fungi, protozoa, intracellular organisms, and some bacteria. Macrophages remove tissue debris and foreign material from damaged areas and ingest dead or damaged cells and cell fragments. The macrophage plays an important role in the immune response by recognizing,

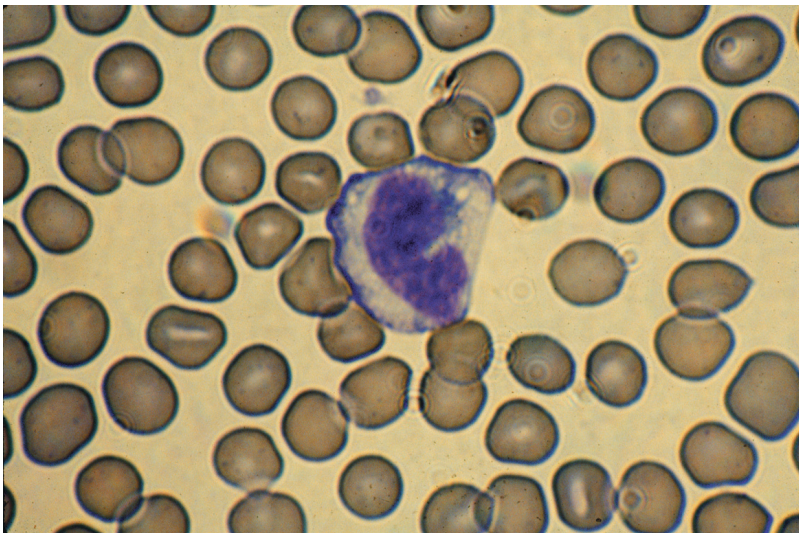


Figure 8.18 **Monocyte** in peripheral blood. The chromatin in the nucleus is less condensed and lighter staining than in other leukocytes. The cytoplasm is commonly grayish staining and granular, and often contains vacuoles. Note also the erythrocytes with elongate central pale areas (stomatocytes).

picking up, and processing foreign antigens throughout the body for presentation to the lymphocytes (see the following chapter).

Since monocytes are not mobilized as rapidly as other leukocytes, an increased monocyte count (monocytosis) usually indicates a chronic infection or inflammatory response, or it indicates the recovery phase of an acute process. Conditions with large amounts of necrosis and cellular debris, for example, pyothorax or retained placenta, will be highly chemotactic (chemically attractive) for monocytes. Monocytosis is also common in diseases that stimulate a granulomatous type of inflammatory response, such as tuberculosis, brucellosis, and most internal fungal diseases. An increase in monocyte numbers is seen in some species, predominantly the dog, in response to stress or corticosteroid administration.

A monocytopenia (decrease in circulating numbers) may occur during acute stages of infection or inflammation. It may also occur with corticosteroid administration in some species. Since the normal monocyte count is minimal, monocytopenia is of little clinical significance.

Review Questions

- 1. Discuss the various “pools” associated with the formation, distribution, and function of leukocytes.**
- 2. In relation to the neutrophil, what is the meaning and significance of the terms “left shift” and “right shift”?**
- 3. Name two conditions that will usually stimulate an increase in the number of eosinophils.**
- 4. The mast cell appears to be a tissue form of which leukocyte?**
- 5. Which adult leukocyte has the ability to transform into a more juvenile form when stimulated?**
- 6. What is the major function of the monocyte?**
- 7. List the terms used to signify above and below normal numbers of each of the leukocytes in the blood stream.**

Chapter 9

Introduction to the Immune System

Key Concepts

The body uses both nonspecific mechanisms and specific or acquired mechanisms to protect itself from infection or invasion.

In the humoral immune response, the B lymphocyte, after stimulation and assistance of other leukocytes, produces antibodies against specific antigens.

The cell-mediated immune response reacts to different types of antigens and is centered on the T lymphocyte and the monocyte.

Several manual and automated tests are available to test the blood for the presence of antigens or antibodies.

The immune system is by necessity a highly complex mechanism and is often difficult to understand on the first presentation. The study of immunology fills numerous large textbooks and is the subject of widespread research. Only a very condensed introduction to the cells, tissues, and processes of the immune system is presented here.

The immune system consists of all the organs, tissues, and cells concerned in the defense of the body from foreign material and infectious agents. Any substance (usually proteins) or organism that stimulates the immune system is called an antigen. Defense mechanisms against these antigens may be nonspecific, working against any foreign material, or specific (acquired), which are designed or “blueprinted” for individual antigens.

Nonspecific Immunity

The nonspecific defense mechanisms include physical or chemical barriers to prevent invasion by a wide range of antigens. Intact skin and mucous membranes provide physical protection, and the enzymes in tears and saliva, as well as the hydrochloric acid of the stomach, are chemical barriers against entry into the body. The body’s response to any cellular insult or damage is another nonspecific defense mechanism, called inflammation, which is characterized by warmth, redness, and swelling. This temperature elevation slows the rate of reproduction of some microorganisms. The increase in blood supply and capillary permeability seen with inflammation releases blood cells and fluid to dilute, dissolve, or destroy substances in the damaged area.

Among the cells present will usually be neutrophils, with the ability to phagocytize foreign material. Resident macrophages and circulating monocytes act as scavengers to remove cellular debris. Another nonspecific cell is called the natural killer (NK) cell. Exact cell lineage of this cell is not known, but it may represent the non-B, non-T (null) lymphocyte population introduced in the previous chapter. NK cells have the ability to destroy foreign cells, and some tumor cells, by direct cell-to-cell contact and lysis of cell membrane. In addition, in the presence of viral infection, body cells can release a substance called interferon, which helps the surrounding cells resist entry by the virus.

Specific (Acquired) Immunity

The mechanisms of specific (acquired) immunity are activated by the presence of antigens within the body. This response involves mainly the B and T lymphocytes and the monocyte or macrophage. These cells must first recognize all the normal cells, tissues, and chemicals within the body (“self”) and then recognize and respond to any substance that is not part of that particular animal (“nonself”). Two types of specific immune responses are seen. One is called the humoral (fluid) response, which is based on antibody production. The other type of response is called the cell-mediated response and involves cytotoxic (killer) lymphocytes and macrophages. These two systems are often interdependent and work in unison.

The humoral immune response mechanism is based on the B lymphocyte and its ability to manufacture antibodies. When a foreign antigen is present in the body, it will usually be first recognized by a tissue macrophage. The antigen is then processed in an

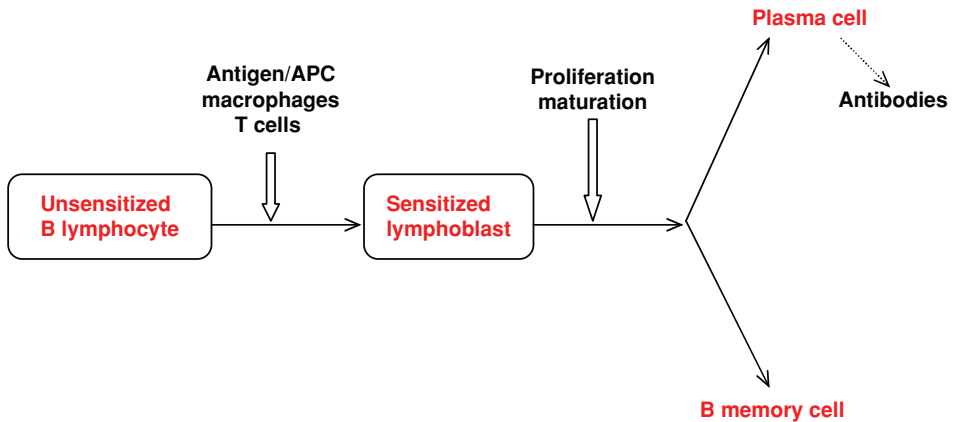


Figure 9.1 Response of B lymphocytes to antigenic stimulation (humoral immunity). APC; antigen-presenting cell.

incompletely understood manner by the macrophage with the assistance of a “helper” T lymphocyte (see below). The macrophage is then called an antigen-presenting cell (APC) and “presents” the processed antigen to a B lymphocyte. The stimulated B cell with the imprinted antigenic code transforms into a lymphoblast and begins to undergo repeated cell divisions (mitosis). Two types of cells result from these divisions (Fig. 9.1). One type is called a B memory cell that may or may not undergo further mitosis but retains the ability to respond to that antigen at a later date and can survive for months or years. The other cells produced are activated B cells (immunocytes), which transform into plasma cells programmed to produce antibodies specifically against that particular antigen. The antibodies are of various types and function in different ways (they are discussed later in this chapter) but in general act by attaching to the antigen and neutralizing or killing it.

The cell mediated immune response mechanism is centered on the T lymphocyte and its response mechanisms. Stimulation of the T cells is also by antigenic recognition by a macrophage (APC), which may stimulate both B and T lymphocytes. The T lymphocyte also undergoes blast transformation and mitosis. The resultant colony produces cells of several types (Fig. 9.2), one of which is a T memory cell, similar to the B memory cell, which is programmed for later response. The other types of T cells communicate with each other and with B cells by production of substances called lymphokines, but these T cells do not produce antibodies. T helper cells assist B lymphocytes in antigen recognition and antibody formation and also appear to assist other T cells. T killer cells (cytotoxic T cells) have the ability to attach directly to target cells and rupture cell membranes. Along with attacking invading organisms, these cells may also be seen in association with tissue or organ graft rejections, killing of cancer cells, and destruction of host cells infected with viruses. T suppressor cells control the overall immune response and limit the response of other T cells. A varied group of T cells from the colony, called T mediators, produces a wide range of lymphokines to assist in the immune response. Identified substances include lymphotoxin, interferon, migration inhibition factor, and chemotactic factor.

Initiation of either the humoral or cell-mediated mechanisms takes from 2 to 3 days, with peak response in 1 to 2 weeks, and begins to decline after that. Memory cells, however,

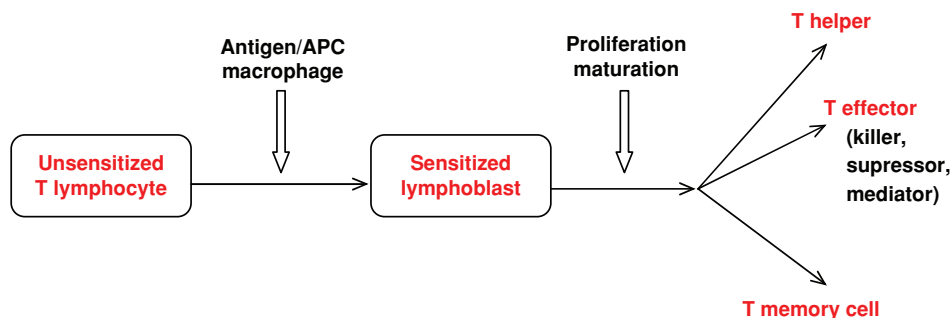


Figure 9.2 Response of T lymphocytes to antigenic stimulation (cell-mediated immunity). APC; antigen-presenting cell.

remain for much longer periods of time, and some may remain for the lifetime of the animal. When challenged by the same antigen, they can respond almost immediately and prevent further infection. Stimulation of this memory system is the basis behind vaccination (immunization) of animals and humans.

The integrated functions of the humoral and cell-mediated immune systems are shown in Figure 9.3.

Antibody Structure and Function

Antibodies produced by plasma cells are proteins that are present in solution in the bloodstream and are part of the plasma proteins measured in a routine complete blood count (CBC). Chemically, antibodies are globulins (globular proteins) and are categorized as gamma globulins, although they are more commonly referred to as immunoglobulins. The basic shape of an antibody is similar to the letter Y (Fig. 9.4). The “stem” is called the constant region, or constant fragment (Fc), and is similar in most antibodies. The “arms” are the variable regions, or antigen-binding fragment (Fab), which are specific for each particular antigen.

There are four types of antibodies normally found in animals, and with the term “immunoglobulin” abbreviated to Ig, they are labeled IgG, IgM, IgE, and IgA (Fig. 9.5). IgG is the most common antibody in blood vessels and tissues and makes up about 75 percent of the total. IgM is a large molecule confined to the vasculature and is about 5 percent of the total. Normally, less than 1 percent of the antibodies are IgE, but this can increase in the presence of allergies or parasites. IgA is found mainly on body surfaces and in secretions of the skin and the digestive and respiratory tract. It forms about 20 percent of the total antibodies. A fifth type, IgD (similar in structure to IgG), is present in humans but has not been identified in domestic animals.

The basic function of antibodies is to attach to specific antigens, forming an antigen-antibody complex, or “immune complex.” This antibody attachment and complex formation alters the antigen in one of several ways. It may cause lysis or rupture of the cell membrane of a microorganism by damaging the membrane structure. Other antibodies completely coat the entire surface of the antigen, preventing attachment, absorption of nutrients, or cell division, which renders the cell harmless. Some antibodies cause the

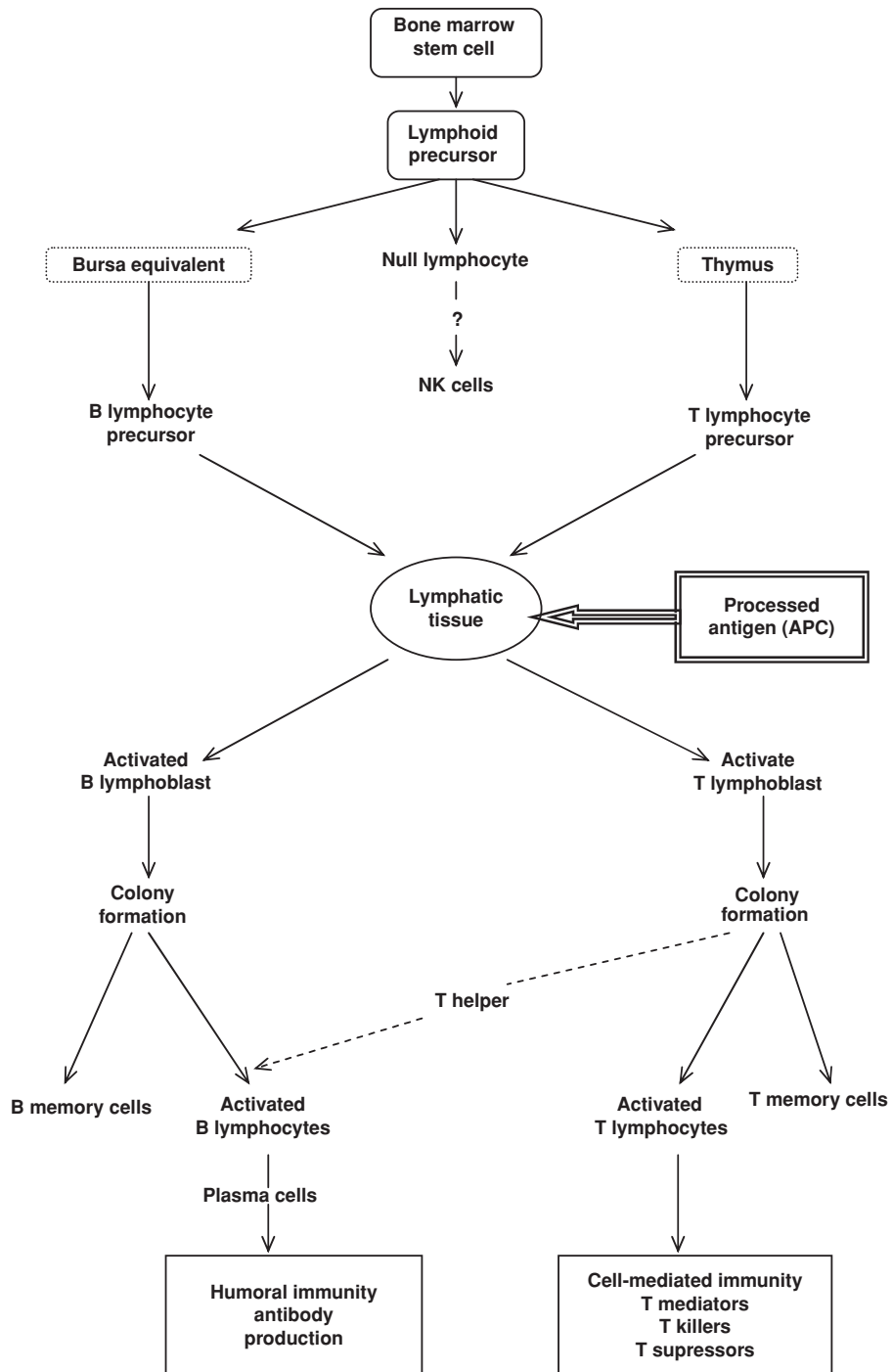


Figure 9.3 Diagram of the formation, functions, and interactions of the B, T, and null lymphocyte subpopulations in the immune response mechanisms.

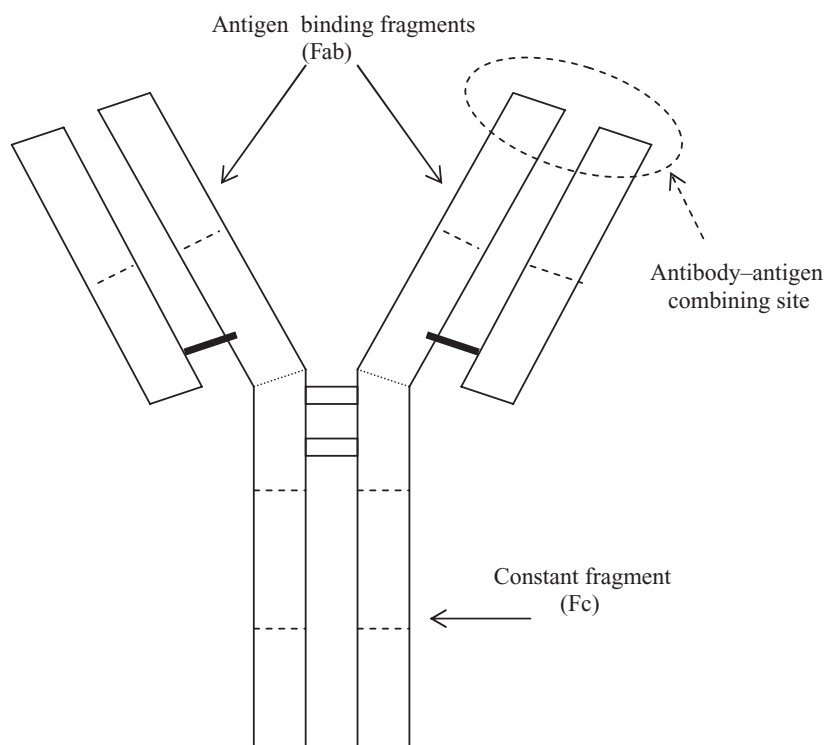


Figure 9.4 Diagram of an antibody molecule (IgG).

formation of insoluble immune complexes when they attach to antigens, which may then be excreted or phagocytized. Another mechanism is called opsonization, which makes the antigen easier to phagocytize and digest. The attachment of the antibody may also trigger a cascade of 25 soluble blood proteins called the complement system. The factors in this system have numerous effects, two of which are to attract leukocytes to the area and to coat the antigen to enhance phagocytosis by macrophages.

Testing Immune System Response

The total and differential leukocyte counts may be considered to be tests of the immune system by revealing generalized response (or lack of response) by the immune cells to antigenic stimulation. It is often necessary, however, to measure the overall humoral response capability of the immune system or the presence, absence, or level of response to a particular disease. Most of the tests are designed to detect the levels of antibody in the patient's serum or plasma and are termed "serologic tests." Many of the newer commercial test kits can also be designed to detect the presence of antigens in the patient. Some of the tests of immune system response are designed for use in the clinic, while others need specialized equipment or reagents found only in larger diagnostic laboratories, but it is

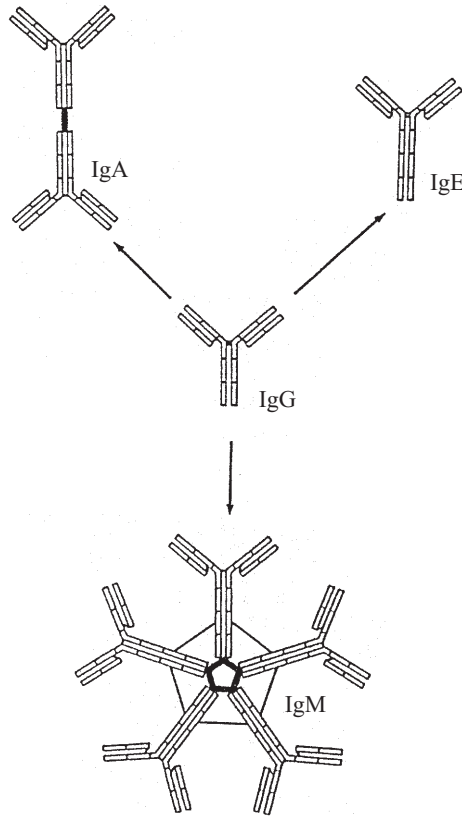


Figure 9.5 The basic structure of the major classes of immunoglobulins.

important for the technician to understand the basics behind these testing procedures. The various types of tests available usually fall into one of the categories discussed briefly below.

ELISA

Many commercial test kits available for use in the clinic utilize the ELISA mechanism. This term stands for “enzyme-linked immunosorbent assay” and new tests are routinely becoming available for detecting either antibodies or antigens in the patient’s serum for different diseases. ELISA test kits are commonly used to detect feline leukemia antibodies (or antigens). Individual procedures will vary between manufacturers, but the basis behind these tests is the inclusion of the test antigen in the structure of the plastic test wells (Fig. 9.6a, Indirect Test). If specific antibodies are present in the patient’s serum, they will form complexes with the antigen and remain after the well is rinsed. When a solution containing an anti-antibody chemically attached to an enzyme is added, it will selectively bind to the attached antibody. If the patient’s serum contained the specific antibody, the test will now have the built-in antigen, the patient’s antibody, and the enzyme-linked

Indirect Test for Antibody in Patient's Serum

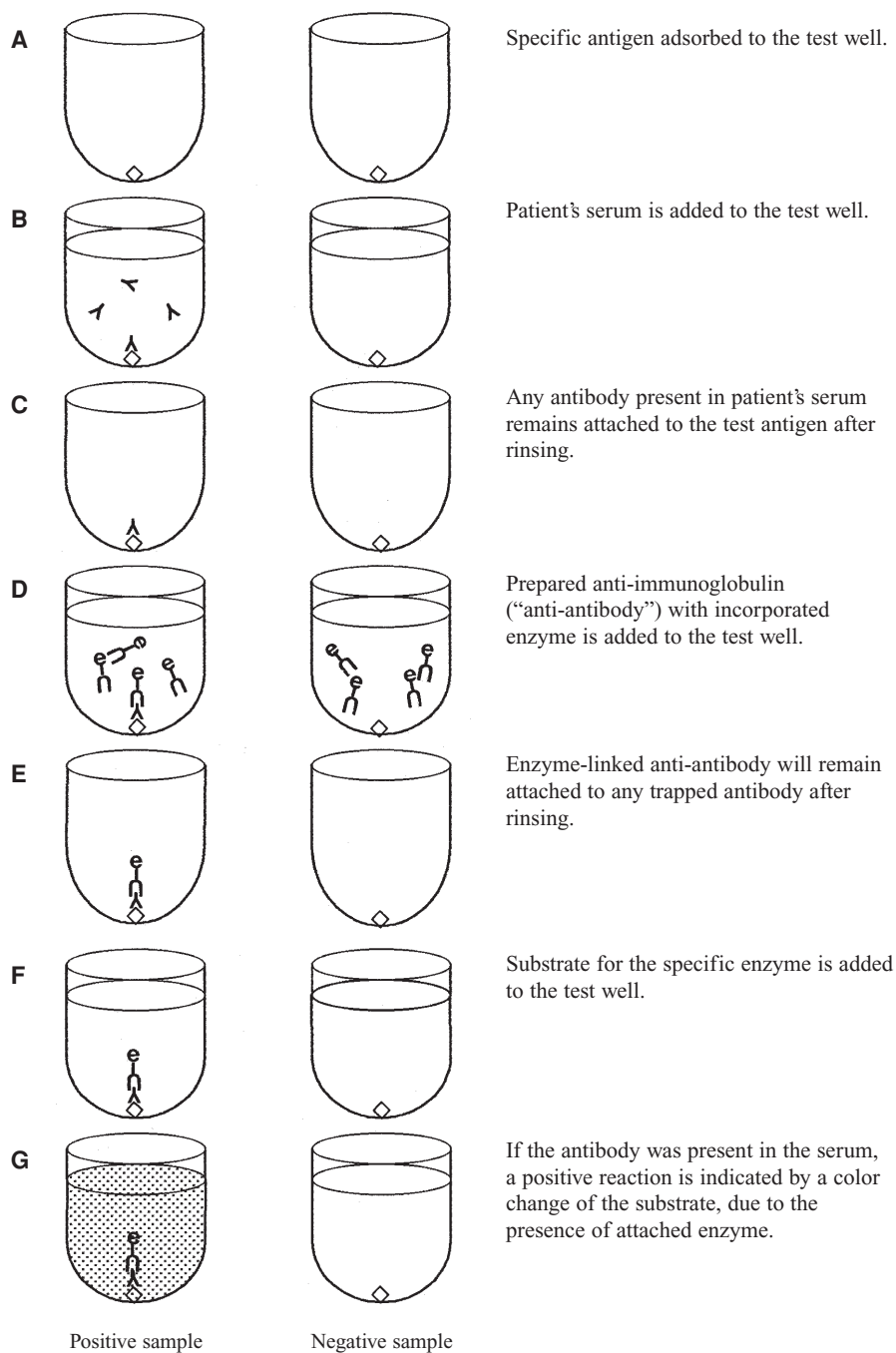


Figure 9.6 (a) Mechanisms of ELISA.

Direct Test for Antigen in Patient's Serum

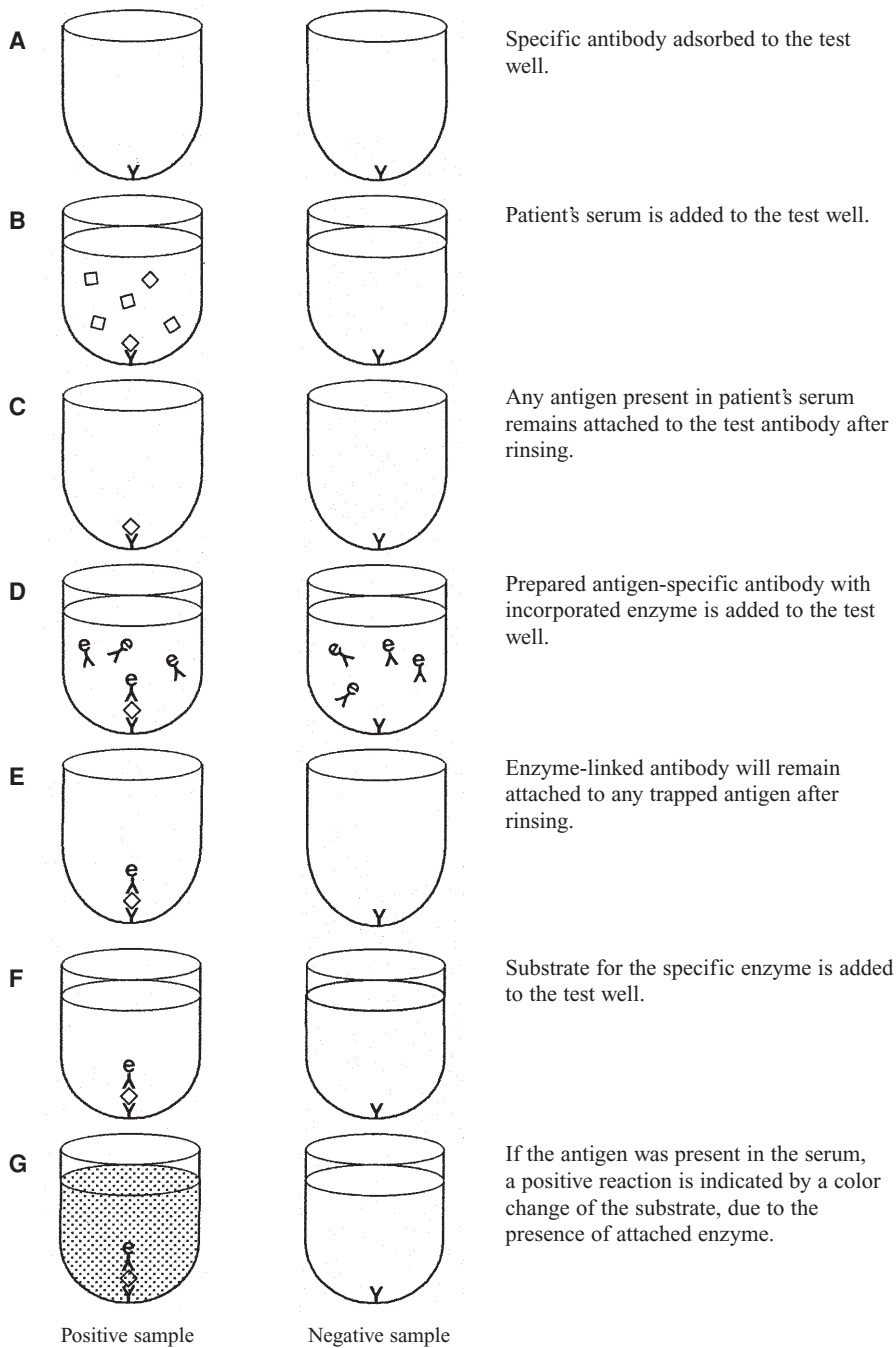


Figure 9.6 (b) Mechanisms of ELISA.

anti-antibody present. If the patient's serum had no antibody, the test well will be empty. The final step is to add a colored substance (substrate) the enzyme can alter if it is present, making the presence of the antibody visually apparent. If the test well had been designed to contain an antibody, the test would detect the presence of the antigen in the serum sample (Fig. 9.6b, Direct Test).

Precipitation

Several types of precipitation test are available for clinic and laboratory use and may be performed in liquid or semisolid media. The basic similarity in these tests is the formation of an insoluble immune complex that forms an observable layer or line of precipitation in the test medium. One of the more common variations of this technique is the Coggin's test, an agar gel immunodiffusion (AGID) test used to detect antibodies to equine infectious anemia. Several wells are cut into an agar plate around a central well, with tests performed on adjacent wells (Fig. 9.7). Commonly, antigen is placed in the center well, and serum samples placed in the surrounding wells. If patient serum is placed in the center well, several different test antigens may be placed in the peripheral wells. The samples and antigen solution will diffuse radially and eventually contact each other. If antibody is present in the serum, a line of precipitate will be evident in the agar between the two wells. Used in another way, if prepared antibody were placed in the center well, the test can detect the presence of antigen in serum samples.

Precipitation tests can also be performed in a liquid medium. By adding the patient's serum to a solution of test antigen (or antibody), the technician will see a positive reaction by the formation of a visible precipitate at the bottom of the tube or as a layer within the solution.

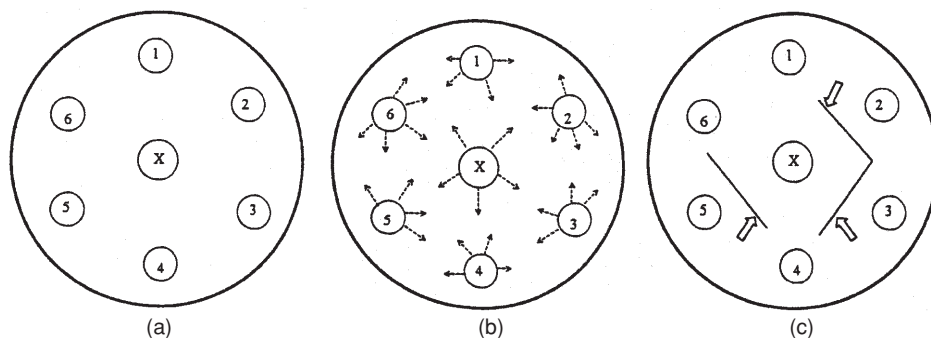


Figure 9.7 Technique of the agar gel immunodiffusion (AGID) precipitation test. (a) Agar gel in Petri dish with sample wells prepared. In this example, a known antigen is placed in the center well (X), and serum samples from different patients are placed in the peripheral wells (1–6). (b) The fluid samples diffuse outward from each well. (c) Lines of precipitation (open arrows) have formed between the known antigen and samples 2, 3, and 5, indicating the presence of specific antibody to the antigen used in the test. Samples 1, 4, and 6 show no evidence of precipitation.

Agglutination

With agglutination tests, the presence of antibody (or antigen) in the serum sample will cause visible agglutination, or clumping, when mixed with a prepared antigen (or antibody) solution. The prepared test solution often has the antigen or antibody bonded to a large particle, such as latex. When the antigen and antibody form a complex, the solution will contain small but readily visible lumps. The blood test in cattle commonly called a “Bang’s test” is an agglutination test for antibodies to the disease brucellosis.

A specialized type of agglutination test is Coombs’ test, designed to detect the presence of autoimmune hemolytic anemia (AIHA). This disease is caused by the formation of antibodies by the animal to its own erythrocytes and results in hemolytic anemia. The test is run by incubating the patient’s serum with Coomb’s reagent containing an anti-antibody. If the red cells are coated with antibody, Coombs’ reagent will cause a clumping of the cells. An indirect Coombs’ test incubates the patient’s serum with red cells from a normal animal, followed by the addition of Coombs’ reagent. If the indirect test is positive, the patient’s serum must have had antired blood cell antibodies present.

Fluorescent Antibody

Fluorescent antibody (FA) tests are routinely done only in larger diagnostic laboratories. Test solutions are available in which antibodies to specific diseases have been labeled with a fluorescent dye. In this test, the patient’s serum is spread on a slide then coated with the test antibody-dye solution that is allowed to react and then rinsed (Fig. 9.8a, Direct Test).

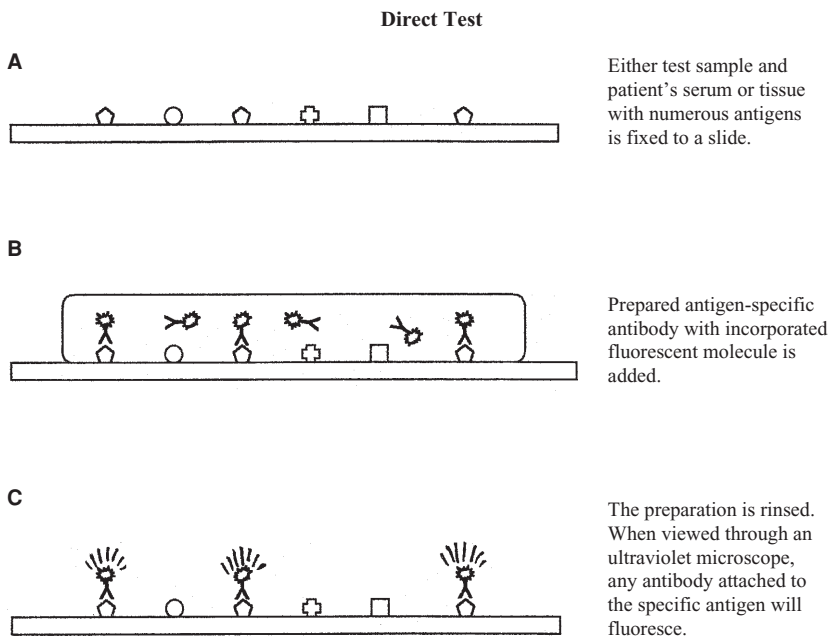


Figure 9.8 (a) Techniques of fluorescent antibody testing.

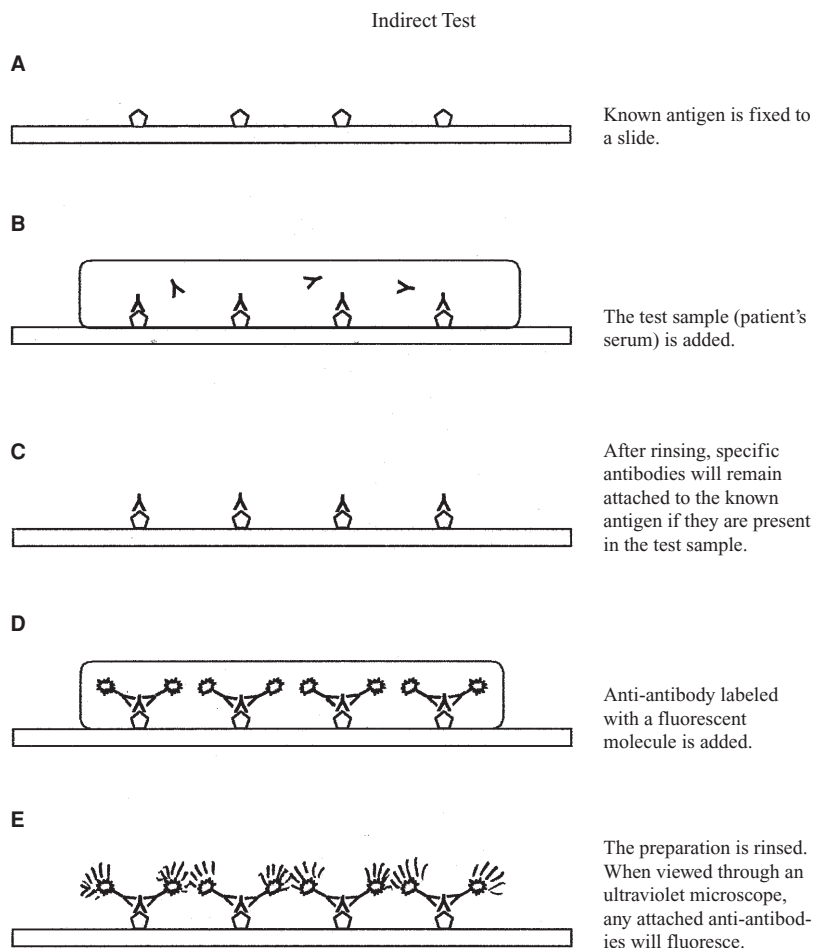


Figure 9.8 (b) (Continued) Techniques of fluorescent antibody testing.

If antigen is present in the test serum, it will attach to the labeled antibody and, through an ultraviolet microscope, appear as an apple green fluorescence. An indirect fluorescence is used to detect patient antibodies. The patient's serum is incubated with the antigen, and this sample is then placed on a slide with fluorescent anti-antibody (Fig. 9.8b, Indirect Test). If fluorescence occurs, the antibody must have been present in the serum.

Radioimmunoassay

The technique of radioimmunoassay is similar to FA and ELISA, except that the prepared test solutions are labeled with radioisotopes (mildly radioactive chemicals) and the results are determined by measuring the radioactivity of the sample. This technique is useful in

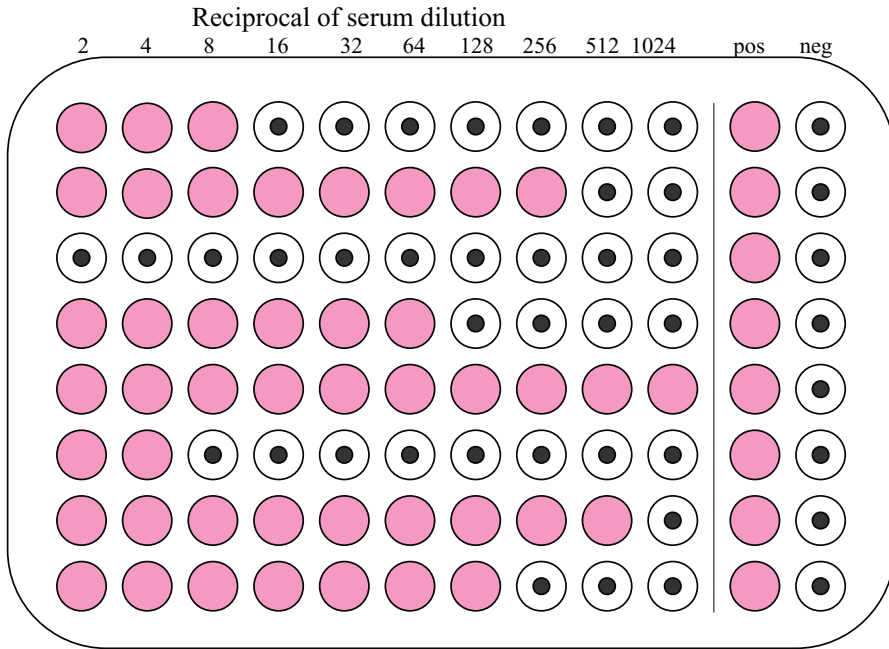


Figure 9.9 Example of antibody titer measurement of serum samples from eight different animals. The numbers at the top represent the amount of dilution of the serum sample (e.g., 16 = 1:16 dilution).

tests of thyroid function through the use of radioactive iodine, which incorporates rapidly into thyroid proteins.

Antibody Titers

The antibody titer is also routinely done in the diagnostic laboratory. It measures the relative amount of antibody present in the patient's serum and any changes in this level over time. These results are often important in differentiating between an active disease process and prior exposure to the antigen. The serum sample is gradually diluted and mixed with antigen in a series of steps until immune complexes are no longer formed (Fig. 9.9). The lowest dilution that still reacts with the test antigen is the patient's titer. The results are often reported as the reciprocal of the dilution; that is, a dilution of 1/8 would be reported as a titer of 8, 1/256 as 256. The higher the number reported, the greater the dilution that still showed a reaction, indicating a greater concentration of antibody in the patient's serum. Both the level of antibody titer and its change with time can be of diagnostic and prognostic importance.

Review Questions

1. Compare the functions of nonspecific immune mechanisms with the specific (acquired) immune mechanisms.
2. Which cells are involved in the humoral immune system and what are their major functions?
3. Which cells are involved in the cell-mediated immune system and what are their major functions?
4. Most tests of the immune system are designed to detect either antigens or antibodies. Discuss these two terms and how they can be measured in test procedures.

Chapter 10

Erythrocyte Form, Function, and Indices

Key Concepts

Although varying slightly in appearance among various species of animals, the major function of the erythrocyte in all species is the transport of oxygen and carbon dioxide between the lungs and body tissues.

By mathematically combining the measurements for packed cell volume, total erythrocyte count, and hemoglobin concentration the average cell size (MCV), hemoglobin concentration (MCHC), and weight of hemoglobin in the erythrocyte (MCH) can be calculated.

These erythrocyte indices are useful in the classification and diagnosis of anemia and polycythemia.

The erythrocyte, or red blood cell, is the most numerous normal body cell type. It has been estimated that there are about 1×10^{14} cells in the typical adult human body, of which 2.5×10^{13} , or about one-fourth, are erythrocytes. Production takes place in the bone marrow, with 6–8 days required to reach maturity. With increased demand, erythrocytes may be released into the vasculature in the “reticulocyte” stage in 3–5 days, prior to complete maturation. These cells are important in the measurement of response to anemia and are discussed in the next two chapters. Total circulating life span varies with individual species and ranges from 2 to 5 months (Table 10.1). Comparing the number of cells with the turnover rate indicates the average dog manufactures about 800,000 red blood cells every second.

Table 10.1 Average life span of erythrocytes in various species.

Species	Life span (days)
Dog	110–122
Cat	68
Horse	140–150
Cow	160
Sheep	70–153
Goat	125
Pig	63

Erythrocyte Morphology

The typical mature mammalian erythrocyte is a round, anucleate (contains no cell nucleus), homogenous cell that stains pink to salmon to red with common laboratory stains. The canine erythrocyte is distinctly biconcave (Fig. 10.1) with the thinner central area appearing pale on blood smears. There is less central pallor in the ruminant, and the cat and horse erythrocytes are nearly flat. Erythrocytes from the camel family (camels, llamas, alpacas, and vicunas) are more oval in appearance and in the deer family are often sickle-shaped. Birds, reptiles, amphibians, and fish have oval to elongate erythrocytes that contain a central, oval nucleus (Fig. 10.2).

The size of the red cell varies among the common domestic species; from the largest to the smallest are the dog, cat, horse/cow, sheep, and goat (Table 10.2). The goat erythrocyte is approximately half the diameter of the dog and often has unusual shapes (poikilocytosis).

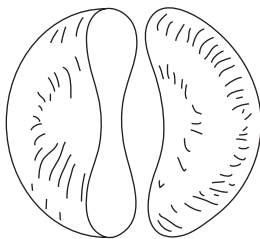


Figure 10.1 Erythrocyte that has been sectioned to demonstrate the biconcave appearance.

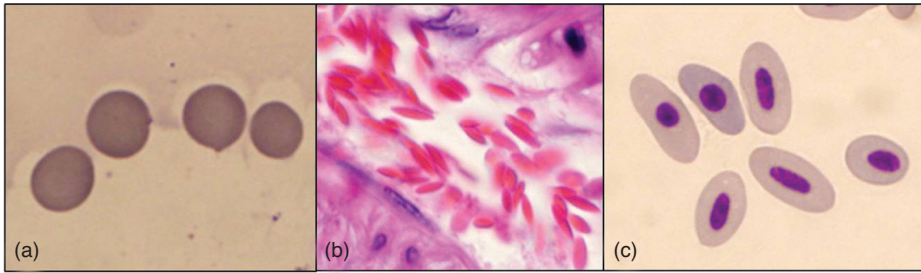


Figure 10.2 Normal erythrocytes from various species: (a) dog, (b) alpaca, (c) bird.

The erythrocyte cell membrane is flexible, allowing the cell to alter its shape when passing through small capillaries, but it is relatively inelastic, having little ability to stretch. Erythrocytes will lose internal fluids and contract (crenate) in hypertonic solutions but will swell only slightly before the cell membrane ruptures (hemolysis) in hypotonic solutions. A process called rouleaux formation (Figs. 10.3 and 10.4) in which the red cells stack on top of each other (appearing like a stack of coins that has tipped over on the blood smear) is most commonly seen in horse blood. Although less commonly seen in the dog and cat, rouleaux may increase with an inflammatory disease. Rouleaux are rarely seen in the ruminant.

Erythrocyte Function

The function of the erythrocyte is to transport the respiratory pigment hemoglobin (Hb) between the lungs and the body tissues. Because hemoglobin readily attracts and releases oxygen, the essential function of erythrocytes is to distribute oxygen throughout the body. The red blood cell is approximately 60–65 percent water and 30–35 percent hemoglobin (95 percent of the dry weight), with the remainder consisting of inorganic material and a limited number of metabolic enzymes. Hemoglobin is composed of four molecules of heme, an iron containing porphyrin pigment, attached to globulin, a protein with two peptide chains of about 150 amino acids each. It has been estimated that there are 400,000,000 hemoglobin molecules in a single canine erythrocyte. Each

Table 10.2 Erythrocyte diameter in various species.

Species	Average (μm)	Range (μm)
Dog	7.0	6.9–7.3
Pig	6.0	4.0–6.0
Cat	5.8	5.4–6.5
Horse	5.8	4.0–8.0
Cow	5.8	4.0–9.6
Sheep	4.5	3.5–6.0
Goat	3.2	3.0–4.2

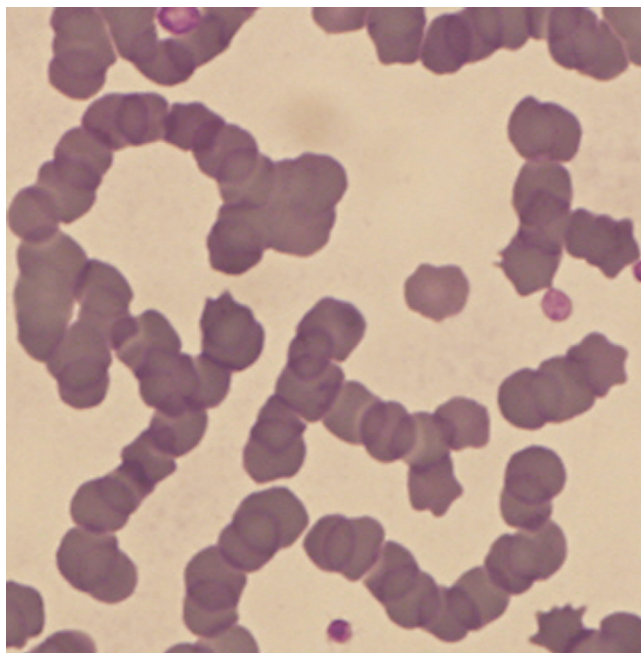


Figure 10.3 Rouleaux formation of erythrocytes in a smear.

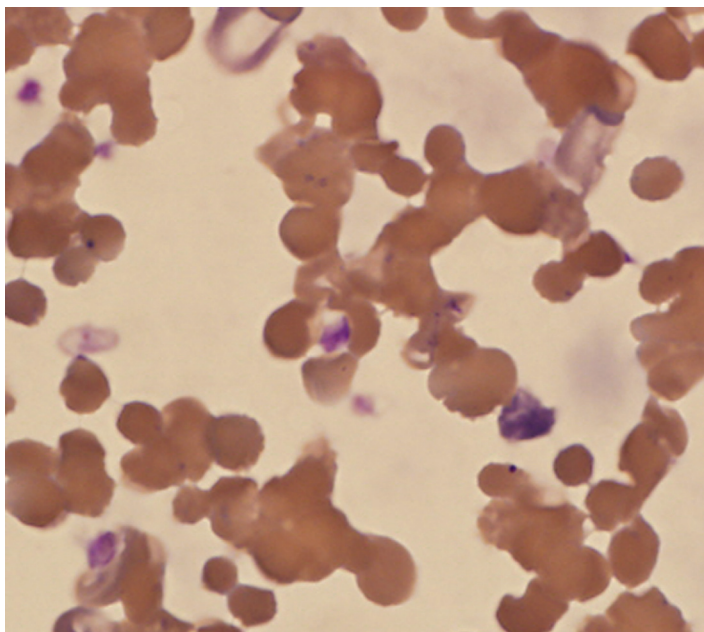


Figure 10.4 Clumping of erythrocytes. Compare to Rouleaux formation in the previous picture.

molecule of hemoglobin can attach to four oxygen molecules, and the structure of the hemoglobin molecule allows it to bind or release this oxygen depending on the oxygen concentration of the microenvironment. Therefore, in the lung alveoli, where oxygen concentration is high, the hemoglobin can become 95–98 percent saturated with oxygen. Hemoglobin can carry 60–70 times more oxygen than a similar quantity of water under the same conditions. In the tissue capillaries, the oxygen tension is lower, and the oxygen can be released. Hemoglobin has a somewhat lesser affinity for carbon dioxide but does transport the excess from the tissues (high concentration) to the lung (low concentration).

The population of red blood cells is constantly changing, with rapid production and, under normal conditions, an equal rate of removal. “Erythron” is a term often used for the mass of circulating erythrocytes plus the erythropoietic (red cell-forming) tissue of the bone marrow and the cell removal systems. For the erythron to remain stable or respond to increased or decreased demands, the oxygen level in the blood is monitored by cells in the kidney. In response to low oxygen levels in the blood (hypoxemia), these cells release erythropoietin, a hormone that increases the rate of red cell production in healthy bone marrow. Essential nutrients must be available for production, including adequate quantities of protein, minerals (especially iron, copper, and cobalt), and vitamins, particularly those of the B series. Removal of damaged or old erythrocytes occurs primarily in the spleen, although fixed and free macrophages in the vasculature, tissues, and liver also participate. The iron and structural components of hemoglobin are retained and reused by the bone marrow.

Red Blood Cell Indices

The common measurements of the red blood cells are the hematocrit (PCV), hemoglobin concentration, and total red blood cell count, all described in Chapter 6. Individually these results provide valuable information, but when combined to form the red cell indices (plural form of the word *index*). Together they define the size and hemoglobin content of individual cells and are an important aid in the diagnosis of the causes of anemia. The indices are used to classify the type of anemia and are referred to in the discussion of anemia and polycythemia in Chapter 11. The three indices are the mean corpuscular volume (MCV), the mean corpuscular hemoglobin concentration (MCHC), and the mean corpuscular hemoglobin (MCH). When combined with a careful examination of the erythrocytes on the blood smear, they present a description of red blood cell morphology useful to the veterinarian.

Mean Corpuscular Volume (MCV)

The MCV expresses the average volume of the individual erythrocyte and is calculated from the formula:

$$\text{MCV} = \frac{\text{PCV (whole numbers)} \times 10}{\text{Total count (millions/cu mm)}}$$

Table 10.3 Typical reference values for erythrocyte indices—average and (range).

Species	MCV (fL)	MCHC (gm/dL)	MCH (pg)
Dog	70 (60–77)	33 (31–34)	21 (19–23)
Cat	45 (40–45)	33 (31–35)	15 (13–17)
Horse			
Hot-blooded	42 (37–50)	33 (31–55)	13 (11–15)
Cold-blooded	44 (39–52)	33 (31–35)	17 (15–19)
Cow	50 (40–60)	30 (26–34)	16 (14–19)
Sheep	32 (23–48)	32 (29–35)	11 (9–13)
Goat	20 (18–24)	32 (30–35)	6 (5–8)
Pig	63 (50–68)	33 (31–34)	19 (16–22)
Human	87 (82–92)	34 (32–36)	29 (27–31)

The units of the resulting cell size are in femtoliters (fL). For example, if the PCV = 46 percent and the total red cell count = 8,000,000/cu mm:

$$\text{MCV} = \frac{46 \times 10}{8} = 57.5 \text{ fL.}$$

The calculated cell volume is then compared to the normal range for that species (Table 10.3).

Cells within the normal ranges are termed “normocytic” (normal sized). If they are larger than normal, they are “macrocytic”; smaller than normal, “microcytic.” These terms are routinely used in morphologic descriptions of anemia (Table 10.4).

Mean Corpuscular Hemoglobin Concentration (MCHC)

The MCHC determines the concentration of hemoglobin in the average erythrocyte and is calculated from the formula:

$$\text{MCHC} = \frac{\text{Hb (gm/dL)} \times 100}{\text{PCV (whole numbers)}}$$

Table 10.4 Use of erythrocyte indices in morphologic—classification of anemias.

Classification	MCHC normal	MCHC decreased
MCV normal	Normocytic Normochromic	Normocytic Hypochromic
MCV increased	Macrocytic Normochromic	Macrocytic Hypochromic
MCV decreased	Microcytic Normochromic	Microcytic Hypochromic

The resulting concentration is in grams/deciliter (g/dL). For example, if hemoglobin = 15 g/dL and the PCV = 45 percent:

$$\text{MCHC} = \frac{15 \times 100}{45} = 33.3 \text{ g/dL}.$$

The calculated MCHC is then compared with the normal range for that species (Table 10.3). If it is within the normal range, the cells are termed “normochromic” (normal color). If the value is below the range, the cells are “hypochromic.” The term “hyperchromic” would be used for values greater than normal, but since normal erythrocytes are completely saturated with hemoglobin, there are no described conditions where the cells can become supersaturated. The term is occasionally applied to cells that appear a more intense red than normal on blood films, but the cells are usually thicker than normal, and the concentration of hemoglobin per unit volume is not increased.

Mean Corpuscular Hemoglobin (MCH)

The MCH is used to demonstrate the amount of hemoglobin, by weight, in the erythrocyte and is calculated from the formula:

$$\text{MCH} = \frac{\text{Hb (g/dL)} \times 10}{\text{Total count (millions/cu mm)}}$$

The resulting weight of hemoglobin is in picograms (pg). For example, if hemoglobin = 12 g/dL and the total red cell count = 8,500,000/cu mm:

$$\text{MCH} = \frac{12 \times 10}{8.5} = 14.1 \text{ pg}$$

This weight calculation is then compared with the normals for that species (Table 10.3). The same terms are applied as with the MCHC (normochromic and hypochromic, Table 10.4). The MCH is much less commonly used than the MCHC in veterinary medicine due to the wide species variation in red blood cell size and expected hemoglobin content.

Since the accuracy of all three indices is no better than the original values used in calculations, any errors in deriving the PCV, hemoglobin concentration, or total red cell count will be compounded. Computations should always be rechecked for potential errors.

Review Questions

1. Discuss the similarities and differences in structure and function of erythrocytes among different species of animals.

2. The major component of the erythrocyte is hemoglobin. What is this molecule and why is it important?

3. The terms MCV, MCHC, and MCH are commonly seen on laboratory reports. What do these terms mean and how are they calculated?

Chapter 11

Erythrocyte Abnormalities

Key Concepts

Variations in erythrocyte cell size and shape are often seen in blood smears from both healthy animals and in disease conditions.

Alterations and abnormal inclusions are also seen within the cytoplasm of the erythrocyte.

The terminology associated with these changes is important in describing the changes and in identifying the possible cause of the condition.

Alterations in erythrocyte size, shape, or cytoplasm will be encountered even in healthy animals and should always be noted on laboratory reports. Often the precise basis of the abnormality is not known but may involve changes in the fundamental properties or components of the cell, such as membrane composition or hemoglobin structure. These changes may indicate a physiologic response, chemical or metabolic damage, mechanical fragmentation or an infectious organism. The technician should only evaluate structural changes in the monolayer area of the blood smear, where cells are not overlapping or distorted in smear preparation. Descriptive terminology has been developed for most of the common cell alterations, and the technician should use it whenever possible to more rapidly and precisely describe the condition seen.

Variations in Cell Size

Unequal size of erythrocytes in a blood film is termed **anisocytosis** (Figs. 8.3 and 11.1). This can indicate the presence of macrocytes (cells larger than normal) and/or microcytes (smaller than normal). It is usually difficult to evaluate which of the cells on the slide are of normal diameter or volume, but average cell size can be determined by calculating the mean corpuscular volume (MCV). A high MCV indicates macrocytes, and a low MCV microcytes. If the obvious anisocytosis is present but the MCV is normal, both macrocytes and microcytes may be present, a condition seen normally in cattle. Macrocytes are usually juvenile red cells and may indicate a regenerative response to anemia (see Chapter 12), although they may also be seen with cell maturation disorders. Red cells that are more than twice as large as normal cells are called gigantocytes. The presence of microcytes is most often associated with deficiencies of essential erythrocyte nutrients, especially iron.

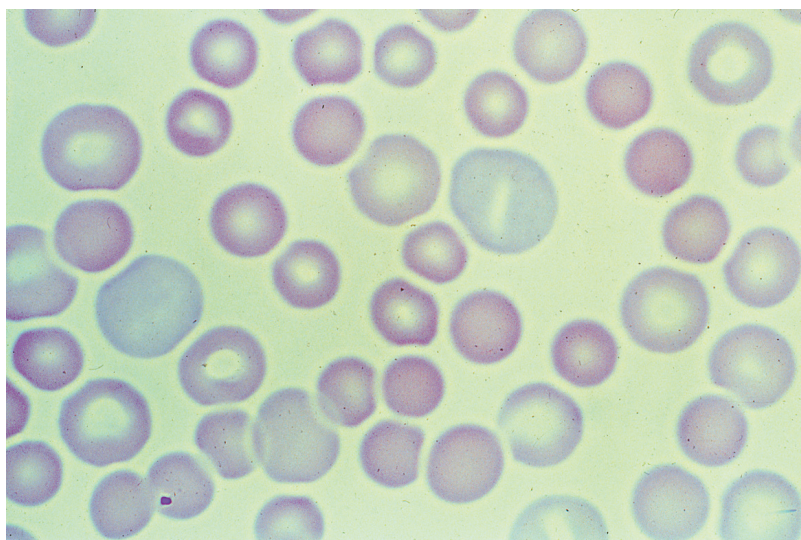


Figure 11.1 Anisocytosis (unequal cell size) in erythrocytes. The variation in colors in different cells is polychromasia, with the larger cells having a grayish-blue tinge. The smaller cells with no central pale areas are spherocytes.

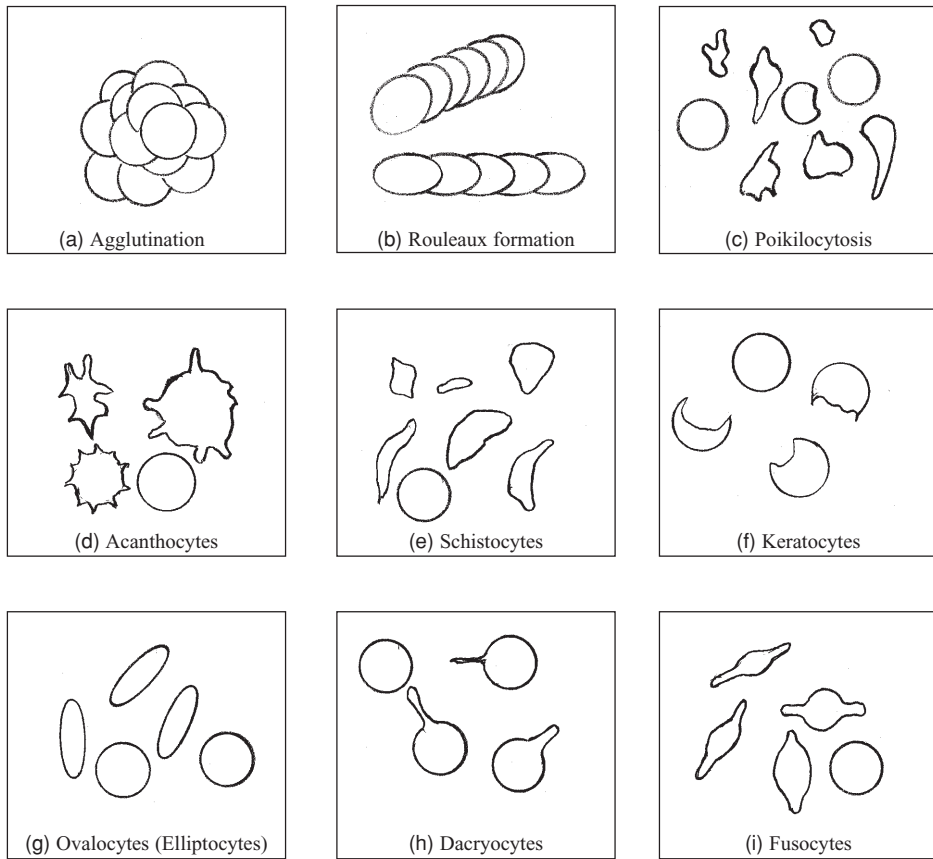


Figure 11.2 Examples of variations in the appearance of erythrocytes.

Although not truly a size variation, any **agglutination** of erythrocytes should be noted (Fig. 11.2a). Agglutination is defined as an unorganized three-dimensional clustering of erythrocytes and is often associated with immune system disorders. Agglutination must be distinguished from **rouleaux** formation (Figs. 11.2b and 10.3), where the cells are in a more linear arrangement that is associated with the charges on the cell membrane. Rouleaux are common in normal equine blood, are occasionally found in dog and cat blood, and can be increased in number in many inflammatory diseases.

Variations in Cell Shape

The normal erythrocyte is termed a **discocyte** (Fig. 10.1). The term for the presence of any abnormally shaped erythrocytes in the circulation is **poikilocytosis** (Fig. 11.2c). This is a general term and should be used when a more specific descriptive term is not available (see below). Imaginative modifiers are often added when describing poikilocytes, such as “apple stem,” “clover leaf,” or “budding.” Poikilocytes may represent formational defects or mechanical or toxic damage to the mature cell. Abnormally shaped cells are relatively

common in the blood of newborn animals, especially the ruminant. This section describes some of the more common types of poikilocytes.

Crenated red blood cells, sometimes called echinocytes, can be the most common altered form encountered but are artifacts of preparation and not due to cellular changes in the body. Crenated cells have blunt projections evenly distributed around the periphery of the cell. Since their shape is due to slow drying of the smear, with pH and osmotic changes causing shrinkage of cells, the majority of red blood cells in any given area of the smear will appear crenated.

Acanthocytes, also called burr cells or spur cells, resemble crenated cells and must be differentiated. The acanthocyte has multiple, irregularly spaced rounded or clublike projections over the entire surface of the cell (Figs. 11.2d and 8.7). As opposed to crenated cells, acanthocytes are commonly seen adjacent to relatively normal erythrocytes. They result from alterations in the cell membrane and are often seen with kidney or liver disease.

Leptocytes are thin, flattened red cells, with large surface areas compared with cell volumes, that tend to fold or wrinkle and can assume several forms. The **target cell (codocyte)** has a thicker, dark-staining center surrounded by a lightly stained area, and a darker periphery (Fig. 11.3). A **bar cell (knizocyte)** is similar to the target cell, but the center darker-staining area extends across the cell with a pale area on each side of it. The **stomatocyte** has an elongate, often-curved, central pale area resembling a mouth (Fig. 8.18). Leptocytes may also begin folding over into bowl shapes or with part of one edge folded completely over, similar to a folded leaf. Leptocytes are most common with chronic diseases, especially anemia.

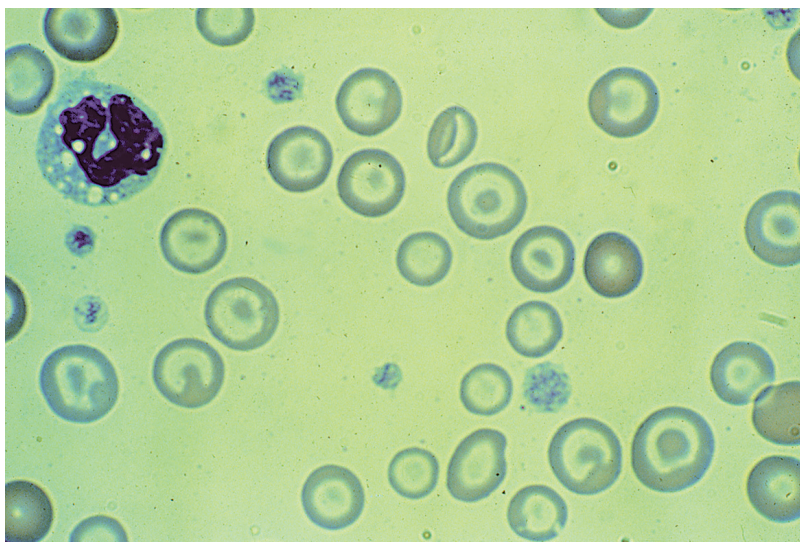


Figure 11.3 The cells' widened areas of central pallor and lightly stained edges (hypochromasia), along with the folded cell near the center, indicate thin erythrocytes (**leptocytes**). The cells with secondary central staining areas are target cells (**codocytes**). A monocyte and numerous platelets are also present.

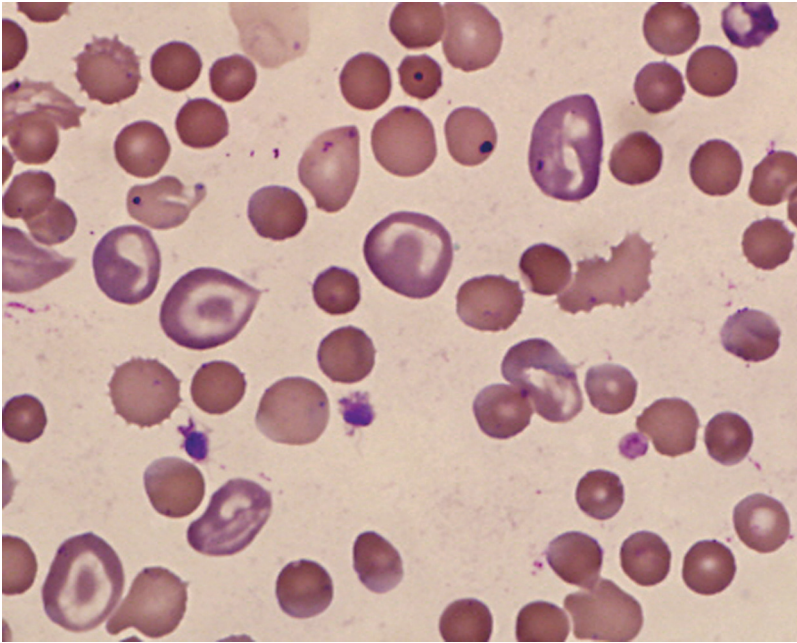


Figure 11.4 The smaller erythrocytes with no evidence of lighter staining in the center are **spherocytes**. **Leptocytes** and various other **poikilocytes** are also present.

Spherocytes are essentially the opposite of leptocytes in that they are thickened or rounded and have decreased surface areas compared with cell volumes. They appear smaller than normal red cells (microcytic) and have densely staining hemoglobin and loss of any evidence of central pallor (Figs. 11.1 and 11.4). Spherocytes are seen with autoimmune diseases and occasionally with splenic disorders.

A **schistocyte**, or **schizocyte**, is an irregular red blood cell fragment due to mechanical damage to the cell membrane (Fig. 11.2e). It may be a red cell with a portion removed, termed a **keratocyte**, “spiculated RBC,” or “helmet cell” (Fig. 11.2f), or as small, highly irregular cell fragments. This fragmentation may be the result of abnormal fibrin strands in the vessels damaging the cells or from phagocytosis of portions of the cell due to damage or aging changes.

An **ovalocyte**, or **elliptocyte**, is an oval-shaped red cell that may occasionally be elongated into the shape of a cigar or matchstick (Fig. 11.2g). Ovalocytes are sometimes seen with anemia. Ovalocytes are the normal form of erythrocytes in the camel family, and nucleated ovalocytes are characteristic of birds, fish, and reptiles (Fig. 10.2).

The **dacryocyte**, or teardrop-shaped red cell, results from the failure of the cell to resume its normal shape after passage through the capillaries (Fig. 11.2h). It can also be an artifact of smear preparation.

Two very similar alterations are the **fusocyte** (accuminocyte) and the **drepanocyte** (sickle cell). The fusocyte (Fig. 11.2i) is a spindle-shaped cell that tapers to a point at both ends. The drepanocyte is a more elongate, thread-like cell. Either of these may be seen in the sheep and goat (especially the Angora) but is rare in other species. The blood cells of

deer may form sickle shapes with oxygenation after collection. The cellular elongation is due to polymerization of hemoglobin into long chains.

Cytoplasmic Variations and Inclusions

A subtle but important cytoplasmic variation between cells is the depth of staining. Cells that are lighter staining or have larger pale central areas than accompanying cells are called hypochromic, a condition that should be verified with a low mean corpuscular hemoglobin concentration (MCHC) value (see Chapter 10). Cells that stain more intensely are often called hyperchromic but since a true increase in hemoglobin concentration does not occur, these cells are usually thicker than normal (spherocytes).

Polychromasia is the presence of erythrocytes with a faint to obvious blue coloration. These cells are usually macrocytic and represent immature red cells (Fig. 11.1). With a new methylene blue stain, many of these polychromatophils will be reticulocytes (discussed below), and, when numerous, indicate an increase in bone marrow production. The term **polychromatophilia** is used when patchy areas of variable colors are seen in the individual cell.

Another variation involves the presence of objects or organisms within the cytoplasm. **Nucleated red blood cells** are rarely encountered in healthy animals but may become numerous with increased production of erythrocytes by the bone marrow in response to erythropoietin (blood formation hormone) stimulation. The nucleus of the developing red blood cell, which is usually removed prior to release from the bone marrow, remains present in these circulating cells. The nucleated red cell may be the same size or slightly larger than the mature cell with a round, dark-staining nucleus surrounded by bluish-red cytoplasm (Fig. 11.5). Care must be taken to distinguish the nucleated red cell from small lymphocytes.

A **Howell-Jolly body** is a small portion of a nucleus that has been retained in the cell and has the same staining qualities as the original nucleus. It is usually single, round, and randomly located within the cell (Fig. 11.6).

A **Heinz body**, also called an erythrocyte refractile (ER) body, is a small, round to irregular, refractile inclusion that may be single or multiple within the cell or bulging from the surface (Fig. 11.7). Heinz bodies stain poorly in most routine lab stains and may appear as a bright, clear area that is slightly darkened at the edges. They are readily stained with new methylene blue (Howell-Jolly bodies are not). Heinz bodies are areas of denatured hemoglobin and are associated with toxic oxidative damage in most species. They are common in the cat and have been found in over 50 percent of the red cells in some healthy cats.

On routine Romanowsky-type stains, the **reticulocyte** is slightly larger than mature red cells (macrocytic) and stains bluish-red (polychromasia). With a new methylene blue stain an irregular network (known as a reticulum) or clumps of dark-staining material become evident (Fig. 11.8). This reticulum and clumping are remnants of ribosomal RNA and other organelles that were present in the developing cell but are normally absent or in very small amounts in the circulating mature cell. A reticulocyte count is made with the new methylene blue stain by counting 1,000 red cells and determining the number

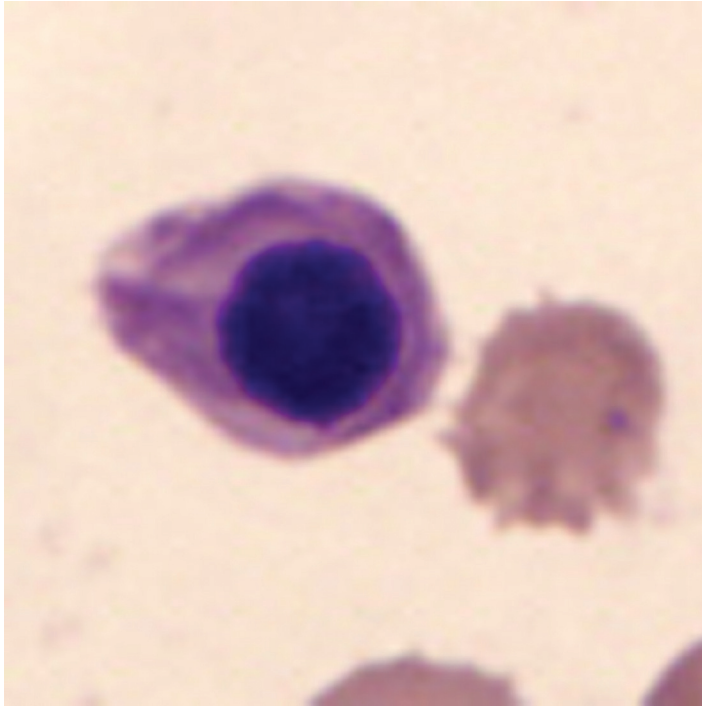


Figure 11.5 Nucleated erythrocyte on a blood smear. The indistinct appearance is due to high magnification.

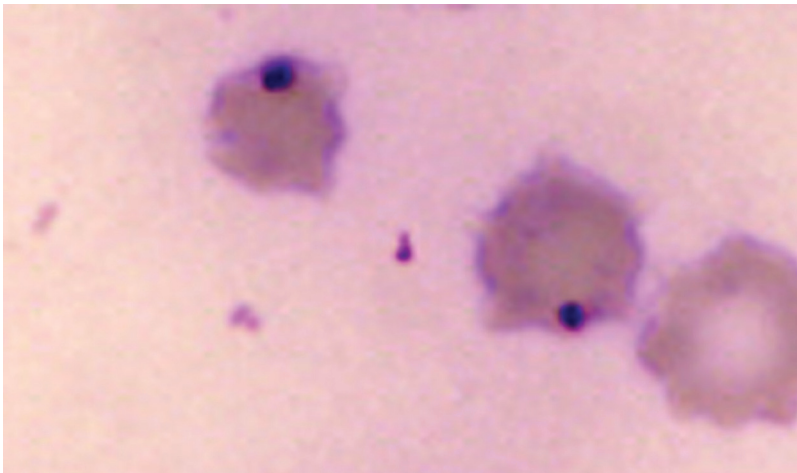


Figure 11.6 Howell-Jolly bodies in erythrocytes under oil immersion (1000 $\times$) magnification.

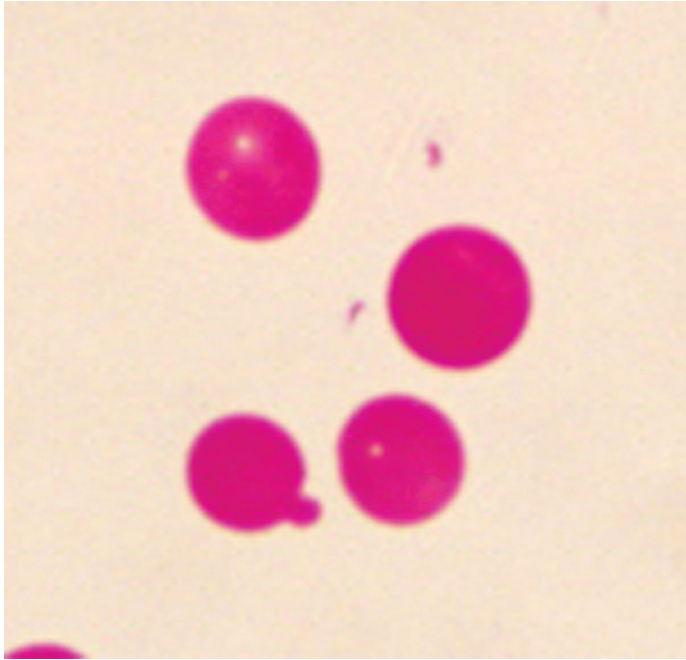


Figure 11.7 Heinz bodies in cat blood with routine laboratory stain.

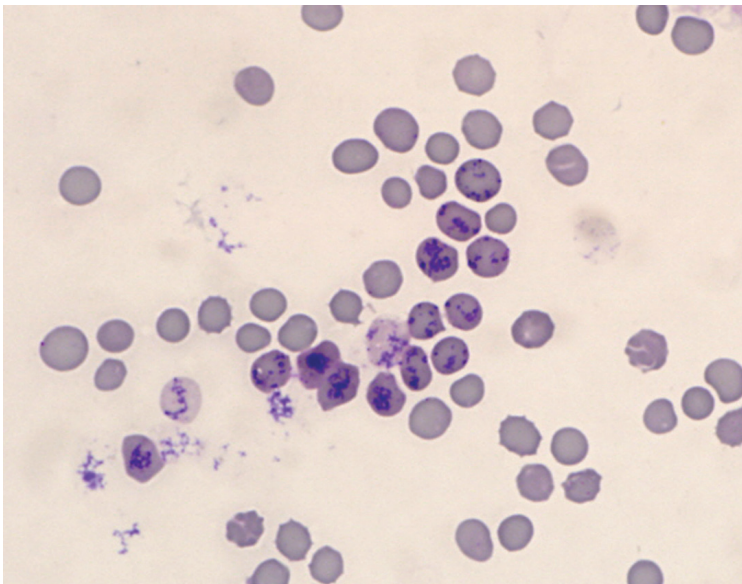


Figure 11.8 Reticulocytes stained with new methylene blue. The coarse network of cellular organelles is apparent with this stain. Note that the reticulocytes are large (macrocytic) compared with the accompanying mature erythrocytes.

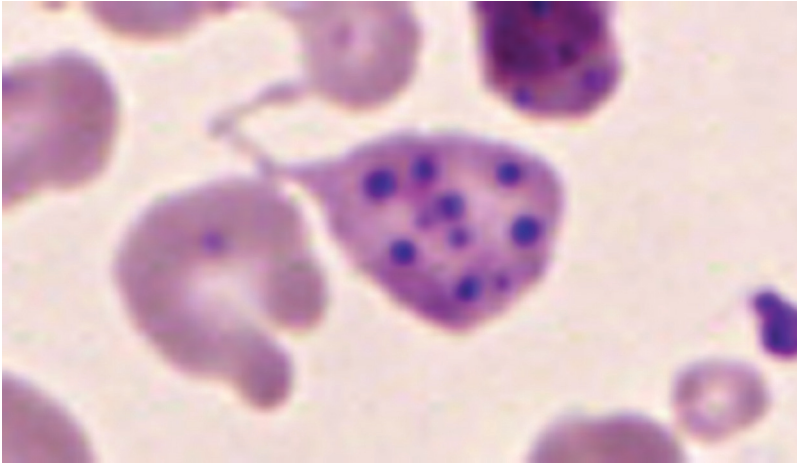


Figure 11.9 Basophilic stippling in the erythrocytes under oil immersion (1000 $\times$) magnification.

of these that are reticulocytes. This can be expressed as a percentage, or the percent can be multiplied by the total erythrocyte count to show the count in reticulocytes per microliter. The degree of polychromasia and number of reticulocytes are proportionally increased with more rapid bone marrow production. The presence of increased numbers of reticulocytes in circulating blood is usually considered a positive prognostic sign of red blood cell regeneration. Two forms of reticulocytes are seen in the cat: aggregate, with abundant reticulum, and punctate, with only isolated clusters that do not coalesce. Reticulocytes are rarely seen in the horse, and other criteria must be used to evaluate regenerative response.

Basophilic stippling of red blood cells appears somewhat similar to reticulocytes, with small- to medium-sized, blue-staining dots scattered in the cells (Fig. 11.9). In contrast to reticulocytes, though, the dots stain well with Romanowsky stains (reticulocytes do not) and less intensely with new methylene blue. The basophilic spots are retained, altered RNA and are occasionally seen in normal ruminant blood. In most species, erythrocytes can contain the spots during regenerative responses by the bone marrow. Basophilic stippling can also result from poisoning by heavy metals, especially lead.

Siderocytes also contain blue-staining granules in the cytoplasm, but these usually stain a lighter blue. These small bodies are aggregates of iron accumulation and are known as Pappenheimer bodies. A Prussian blue stain, which is specific for iron, is the only way to confirm the identification.

Some cellular inclusions in erythrocytes are organisms that infect or parasitize blood cells and usually cause an acute, chronic, or relapsing hemolytic anemia. These organisms may be seen as differentially stained areas within the cell but are often difficult to demonstrate in routine blood smears unless the animal is tested in the acute stages of a widespread, heavy infection. Routinely, only a small percentage of the cells contain parasites and are quite diluted in the blood stream. Some parasites are a bit more abundant in capillary blood and may be more evident in samples from peripheral capillary beds, such as the lateral ear margin. Once located, parasites may be difficult to differentiate

from various inclusions and artifacts. Scrupulously clean slides and stains are essential. Only the more common parasitic inclusions are described here.

A parasite that may be seen in both the dog and cat erythrocyte is *Hemobartonella* (*H. canis* and *H. felis*). In both animals, the parasite is very small (less than 1 μm), coccoid or rod-shaped, and may be seen individually or forming chains across the surface of the red cell. Ring forms are also seen in the cat. Attachment to the cell membrane is not very strong, and the organisms may be found in the background of the slide. *Hemobartonella* is difficult to distinguish from stain precipitate.

Babesia canis is occasionally seen in dogs in the southeastern part of the United States. It is a large, intracellular parasite that is oval to pear-shaped. Two or more organisms may be present in the cell. With careful staining and microscopy, the organism will have a light blue cytoplasm with a reddish nuclear region in Wright's-type stains.

Anaplasmosis, caused by *Anaplasma marginale*, is a relatively common disease of cattle. The organism is coccoid-shaped and stains dark purple or black, and one or more organisms are usually located at the periphery of the cell. Differentiation from Howell-Jolly bodies is based on location in the cell and uniform size of the organism.

Viral inclusions may occasionally be found in red blood cells (and white blood cells) in dogs with distemper. They appear as relatively large, homogeneous, red to light blue, round to oblong structures in the cytoplasm. Unless the stain is intensely red, they may be difficult to differentiate from other inclusions.

Although not intracellular, microfilaria of the vascular parasites *Dirofilaria immitis* (heartworm) and *Dipetalonema reconditum* may also be encountered in canine blood smears (and occasionally in feline). Both are elongate, wormlike, extracellular (outside and between blood cells) organisms approximately the diameter of erythrocytes (Fig. 11.10). The parasites are much more common in the southeastern part of the United

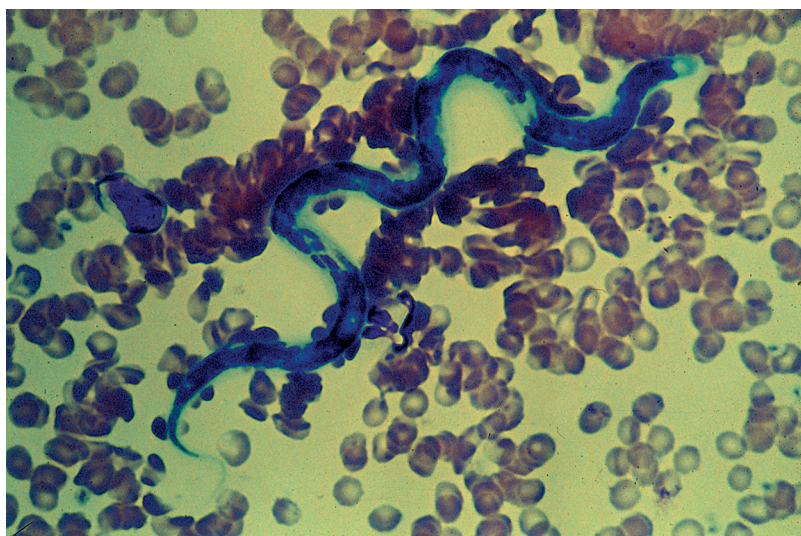


Figure 11.10 Microfilaria of the heartworm, *Dirofilaria immitis*, in a peripheral blood smear. If fresh blood is examined under a microscope prior to drying, the microfilaria will be evident by the movement of cells caused by their active swimming motions.

States but may now be found in virtually all parts of the country. Differentiation between the two species is based on slight morphological differences that can be properly evaluated only on wet mount or fixed samples. Other metazoan (multicellular) parasites may also occasionally be encountered, such as various *Trypanosoma* species. These are elongate, ribbonlike, and tapered at both ends, with a thin membrane often evident along one side. The finding of microfilaria or trypanosomes in the peripheral blood should always be reported, even though several nonpathogenic (nondisease-causing) strains exist, since differentiation between strains may be impossible in a blood smear.

Several artifacts (changes caused by improper slide preparation) can be confused with red cell inclusions or parasites. The most common artifact is stain precipitate that will be seen as variably sized, pink to purple granules on the red and white blood cells as well as in between the cells. The variability in size and the distribution help differentiate the precipitate from true inclusions or organisms. Routine filtering of the stains through laboratory filter paper will reduce the amount of precipitate. Another confusing artifact is the presence of one or more platelets on or under the erythrocyte, which may distort the cell membrane. Comparison with other platelets in the area should identify the platelet. The other common artifact that may appear in some areas or on an entire film is refraction of the erythrocytes themselves. Small or large areas of the erythrocytes will look like bubbles and remain out of focus, or refractile, when the microscope is focused up and down. The exact cause of these refractile areas is not known but is related to smear preparation.

Review Questions

1. What are the general terms used to denote unequal erythrocyte cell sizes and abnormal erythrocyte cell shapes seen on a blood smear?

2. Name and describe five abnormal erythrocyte cell shapes.

3. Name and describe five erythrocyte cytoplasmic abnormalities.

Chapter 12

Anemias and Polycythemias

Key Concepts

Anemia and polycythemia represent alterations in the number, size, and/or hemoglobin content of the circulating erythrocytes and can have numerous causes.

Classification of anemia as regenerative or nonregenerative by laboratory methods and clinical signs is important in diagnosis of the condition.

Polycythemia is less commonly seen and is classified as either absolute or relative.

Anemia and polycythemia are alterations of the erythron, the mass of circulating erythrocytes and the erythropoietic (red blood cell forming) tissue of the bone marrow. *Anemia* is defined as a decrease, and polycythemia as an increase, in the number, volume, and/or hemoglobin content of erythrocytes. Anemia and polycythemia are not disease entities by themselves but should be considered as lesions or clinical effects of an underlying disease process. There are a large number of conditions that can cause an animal to become anemic or polycythemic (which is much less common), so the diagnostic goal of the veterinarian is to determine the cause of the anemia so treatment may be oriented toward the original problem. Blood smear interpretation and other test results obtained by the laboratory technician are essential in narrowing the number of possible causes and establishing an etiology. As an example, Figure 12.1 demonstrates the variability in appearance of the hematocrit in several of these disease conditions.

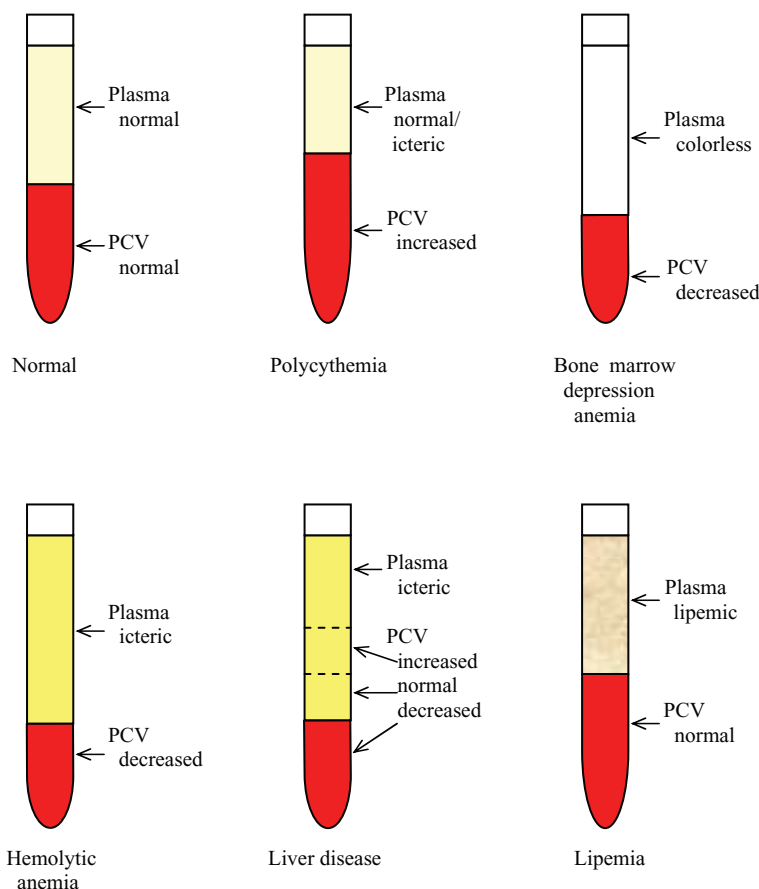


Figure 12.1 Hematocrit evaluation of various disease conditions. PCV, packed cell volume.

* Excess fat in the bloodstream.

Anemia

The term “anemia” literally means “no blood” but clinically means low total blood hemoglobin. Whether the defect is in total number, volume, or hemoglobin concentration of the red blood cells, the overall effect is a decrease in functional hemoglobin to transport oxygen throughout the body. A relative anemia may develop when the total red cell mass is normal but the amount of fluid in the plasma has been expanded. This hemodilution may be seen in newborn animals and in overhydration during fluid therapy. Absolute anemia, with a true decrease in the red cell mass, is much more common and clinically important. It is usually caused by a failure to produce adequate numbers of cells or by a loss of cells at a rate greater than can be produced (e.g., from bleeding or red cell destruction). Both careful physical examination and laboratory determinations are necessary to identify the cause of the anemia, and to even determine the presence of anemia in some cases. For example, an anemic animal that is also dehydrated may have relatively normal laboratory results since there is a decrease in both the cellular and fluid portions of the blood. Also, lab tests performed shortly after an animal loses a significant amount of blood may still be within normal limits, since the body has not had time to respond to the reduced volume and the remaining blood will be unchanged.

Clinical Signs of Anemia

The clinical signs seen with anemia are related to the reduced oxygen-carrying ability of the blood and the body’s attempt to compensate. The signs will vary somewhat depending upon the rate of onset, the degree of hemoglobin loss, and the cause of the anemia. Common signs presented in most cases of anemia are pale mucous membranes, reduced exercise tolerance, tachycardia, and an increased inappropriate respiratory rate with exertional dyspnea (Fig. 12.2). In hemolytic anemias the mucous membrane pallor may be masked by icterus, a yellow-orange color due to red cell breakdown products in the blood stream. With more chronic anemias a low-grade fever and rough, dry hair coat will often develop. Animals may become listless but exhibit hypoxic dementia when stressed.

Classification of Anemias

The purpose of classifying anemias into categories is to help reduce the large number of potential mechanisms behind a particular case to determine its probable cause. The two most common methods of classification are based on erythrocyte morphology and degree of bone marrow response. Both of these methods rely to a great degree on the measured values and slide interpretation obtained from the laboratory. Care must be taken in all phases of the lab tests to ensure reliable results.

Morphological Classification

Morphologic classification groups together those anemias that are similar in cellular size and hemoglobin content based on changes seen and measured in the erythrocytes.

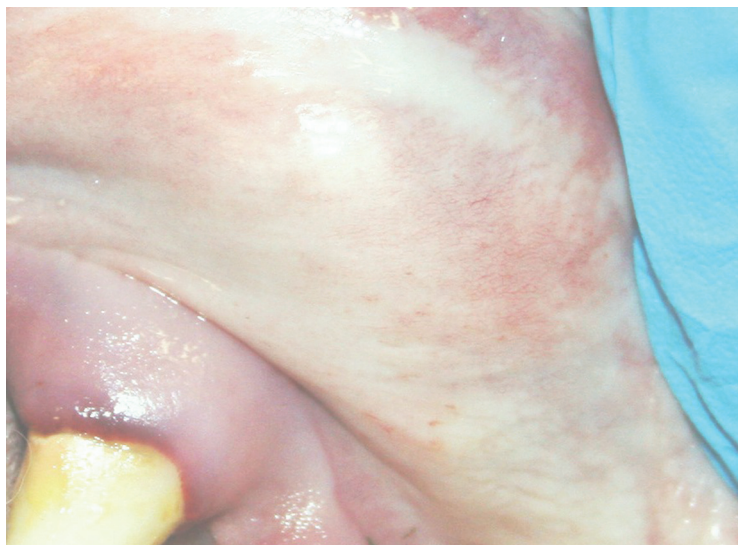


Figure 12.2 Pale mucous membranes are a common sign of anemia.

Maxine M. Benjamin established a flow chart using this method to separate the various causes of anemia into similar smaller groups (Fig. 12.3). Determining how to classify an anemia is based mainly on the mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) values (see Chapter 10) and other test results (see Chapter 6) determined by the technician. From the MCV, the cells are classified as within the normal size range (normocytic), larger than normal (macrocytic), or smaller than normal (microcytic). The MCHC demonstrates either normal hemoglobin levels (normochromic) or decreased hemoglobin levels (hypochromic). Combining these terms presents a description of erythrocyte morphology (Table 10.4). These major groups can then be separated into smaller groups with further laboratory tests, such as reticulocyte counts, plasma protein levels, leukocyte response, thrombocyte counts, and microscopic slide evaluation.

Bone Marrow Response Classification

Anemias can also be classified as regenerative or nonregenerative based on the presence, absence, or degree of bone marrow proliferation evident in the peripheral blood. A regenerative anemia implies that the bone marrow is capable of manufacturing new erythrocytes and has actually increased the production rate, but since the animal is anemic, the cells are apparently being lost at a rate greater than that of production (usually through hemorrhage or hemolysis). A nonregenerative anemia means that the bone marrow is not responding sufficiently to the need for erythrocyte production due to primary intrinsic bone marrow failure or other secondary factors such as a lack of nutrients or the lack of erythropoietin stimulation (Fig. 12.4).

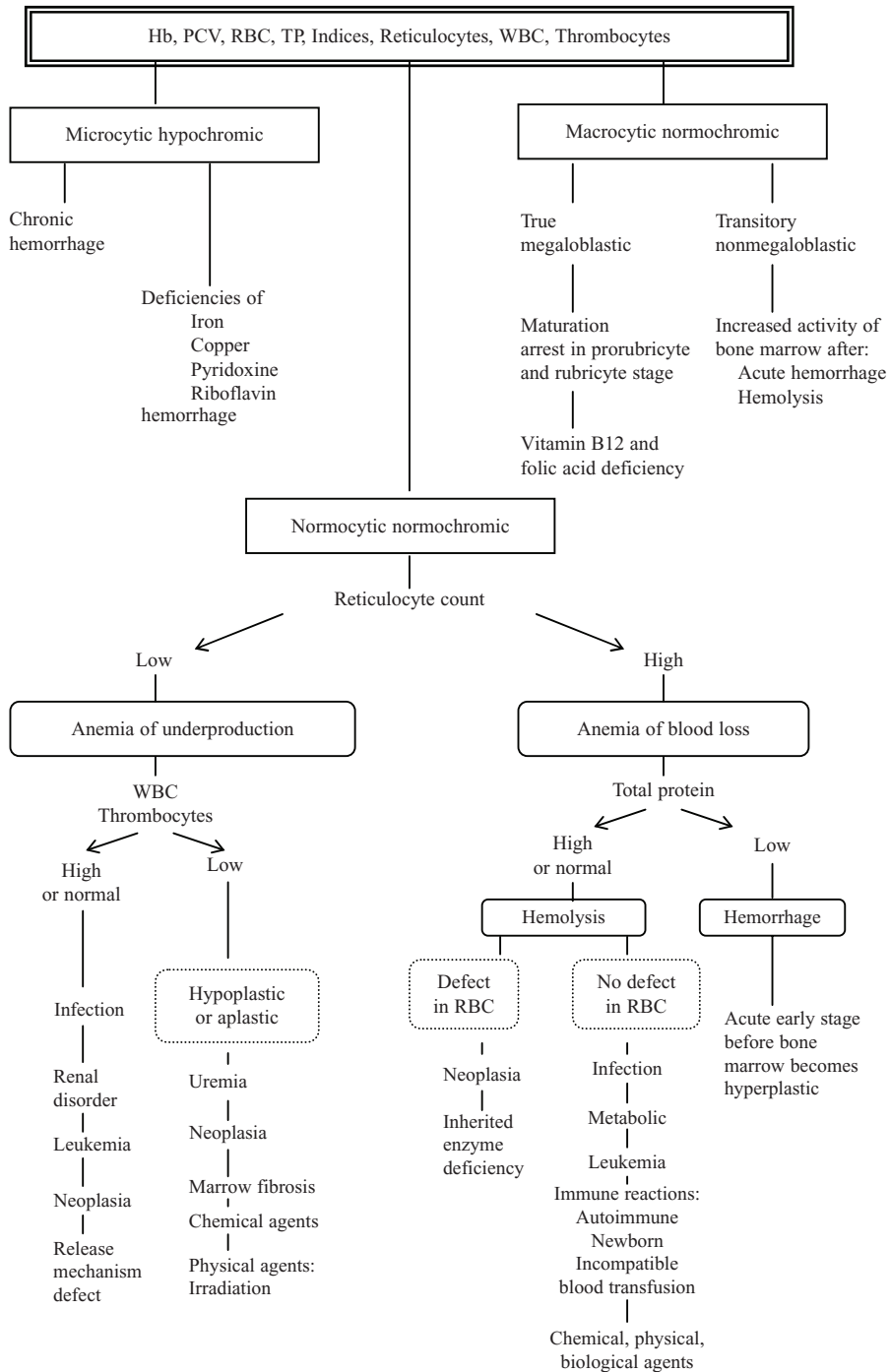


Figure 12.3 Classification of anemias based on morphologic characteristics of the erythrocytes. Hb, hemoglobin; PCV, packed cell volume; RBC, red blood cells; TP, total protein; WBC, white blood cells. (Adapted from Maxine M. Benjamin, 1989)

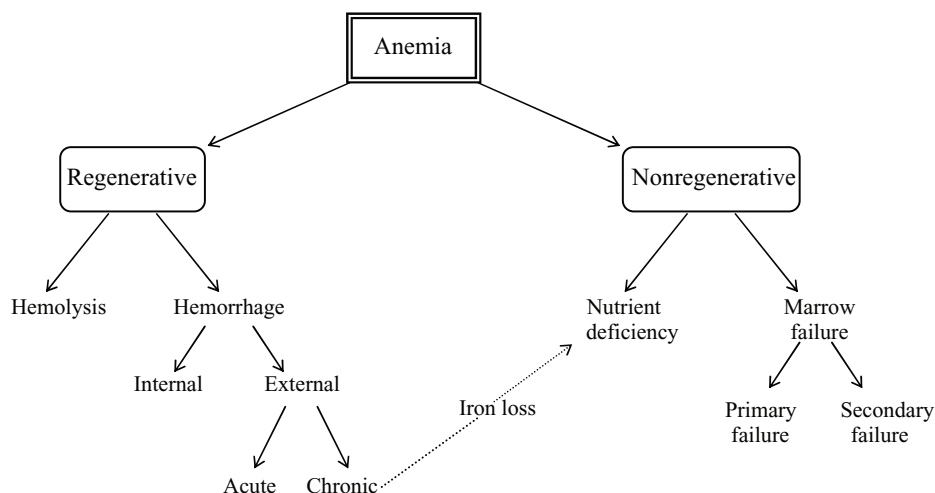


Figure 12.4 Classification of anemias based on bone marrow response.

Regenerative Anemias

Anemias are classified as regenerative when, even though the animal is anemic, the rate and amount of erythrocyte production and delivery to the circulation are increased, leading to the presence of larger numbers of juvenile red cells in the blood sample. The blood picture in regenerative anemias usually presents polychromasia, nucleated red blood cells, and anisocytosis with an increased MCV, indicating the presence of macrocytes. With new methylene blue staining, the polychromatic macrocytes can be identified as reticulocytes in all species except the horse, where reticulocytes are not seen. The reticulocyte count or percentage is proportional to the amount of bone marrow response.

The two major causes of regenerative anemia, hemorrhage and hemolysis, can often be differentiated clinically. In the laboratory, examination of the plasma from an animal with regenerative anemia may give a clue to the cause of the anemia. In hemolytic anemia, the plasma is commonly dark yellow to orange (icteric) due to the presence of red blood cell breakdown products. The plasma remains clear with hemorrhage. Plasma protein levels remain normal or increase slightly with hemolysis for the same reasons. In hemorrhagic anemia, plasma proteins are being lost along with the erythrocytes and will decrease in proportion to the packed cell volume (PCV). In addition to reticulocytes, polychromasia, and nucleated red blood cells, blood smears from animals with hemolytic anemia may contain schistocytes, spherocytes, and other specific changes related to the cause of the hemolysis, such as parasite inclusions or agglutination with autoimmune conditions.

The time frame of response for regenerative anemia must also be considered. Immediately following acute, nonfatal hemorrhage (up to about 1 hour) in an otherwise healthy animal, no changes are detectable in the PCV or hemoglobin of the patient since the blood volume decrease is proportional in fluid and cellular components. In other words, even though blood has been lost, the blood that remains is still relatively normal, just decreased

in total amount. (This is particularly significant when assessing a trauma or surgical patient immediately following hemorrhage.) Within the next few hours, fluid is shifted from other body compartments into the vasculature to restore blood volume. The diluting effect of this fluid will cause a drop in PCV, hemoglobin, total red cell count, and plasma protein. Any stored erythrocytes in the spleen or bone marrow will be released; with a small amount of hemorrhage, this may be enough to restore the PCV. While this is occurring, the kidney is stimulated by the low oxygen levels to release erythropoietin (erythrocyte production-stimulating hormone) to the bone marrow. Erythrocyte production takes 3–5 days before the earliest significant number of new cells is seen in the circulation. Peak response to a single bleeding episode is on the fifth to sixth day. Complete recovery may take 2–3 weeks.

Nonregenerative Anemias

In nonregenerative anemia, the bone marrow production of erythrocytes is decreased (or possibly completely stopped) and insufficient to meet the body's needs. The blood picture in nonregenerative anemia shows a lack of the bone marrow response cells seen with regeneration, or fewer than expected for the degree of anemia. Poikilocytes may be present, with anisocytosis and a normal or decreased MCV and MCHC. Bone marrow examination may be necessary to evaluate hematopoietic capabilities.

Nonregenerative anemias may be the result of nutrient deficiencies, especially iron, protein, and B vitamins. Iron deficiency is common in baby pigs raised on concrete since the sow's milk is deficient in iron. Nutrient deficiencies are also seen with improper diets, malabsorption syndromes, and chronic blood loss, all of which can deplete the body's stores of iron.

Nonregenerative anemia may also be due to bone marrow failure. Secondary bone marrow failure is a lack of response due to a disease process outside the bone marrow, usually involving the kidney and a lack of erythropoietin production. Primary bone marrow failure is called hypoplastic or aplastic anemia and may be due to bone marrow infection, toxins, neoplasia, or bone marrow exhaustion from overstimulation. True aplastic anemia, indicating a failure of any production, has a grave prognosis.

Polycythemia

Polycythemia, also called erythrocytosis, is an increase in the red blood cell count, hemoglobin content, and/or volume in the blood. At first it would appear that this would enable the blood to carry more oxygen but as the mass of red cells (PCV) rises above 50 percent, tissue oxygenation rapidly diminishes. The increased number of cells raises the viscosity (thickness) of the blood, which increases pulmonary and tissue vascular resistance. This results in sluggish blood flow, reduced cardiac output, and decreased tissue oxygenation.

Polycythemia may be absolute, indicating a real increase in total body erythrocytes, or relative, an apparent increase due to loss of plasma volume. Absolute polycythemia may also be primary or secondary.

Absolute Polycythemia

Primary polycythemia, known as polycythemia vera, is rare in animals but has been seen in the dog, cat, and cow. Unregulated overproduction of red cells occurs in the bone marrow with a mild increase in leukocytes and platelets. As blood volume increases, blood pressure becomes elevated, and vascular damage and blood clot formation are common. Laboratory examination of the blood reveals normocytic, normochromic erythrocytes plus an increased PCV, and total red blood cell count.

Secondary polycythemia can develop in response to hypoxemia and erythropoietin stimulation. This may be a physiologic response, as in the increase in PCV commonly seen in animals moved to a higher elevation. It also occurs as a pathologic response to any decrease in blood flow to the kidney, causing excessive erythropoietin production. Conditions that the kidney may register as hypoxemia include heart defects, congestive heart failure, and thrombosis or constriction of renal vessels. Excessive erythropoietin may also be seen with renal neoplasia.

Relative Polycythemia

Relative polycythemia is more common and clinically significant than the absolute form. In relative polycythemia, the total body mass of erythrocytes remains constant but appears to elevate on laboratory testing. The most common cause of relative polycythemia is hemoconcentration. This is seen when the red cell mass remains constant, but the fluid portion of the blood is decreased because of dehydration or fluid shifts. Any condition associated with excessive water loss, such as vomiting, diarrhea, polyuria, excessive sweating, or water deprivation, can lead to an apparent polycythemia. Hemoconcentration commonly occurs because of vascular pooling in shock and measurement of the PCV provides a valuable assessment of the degree of shock. As PCV values become greater than 60 percent, capillary circulation and tissue oxygenation diminish, decreasing the animal's chances for survival.

Another mechanism for this apparent increase is related to the large splenic and bone marrow storage reserves of red blood cells possessed by some animals, mainly the horse, cat, and to a lesser extent, ruminants and dogs. With stress, excitement, or muscular exercise, stored red cells are released into the blood stream, and the PCV can rise several percent, although the total number of red cells in the body has not changed.

Review Questions

1. Define the terms *anemia* and *polycythemia*.

2. What would you expect to see on a blood smear from an animal with regenerative anemia?

3. What would you expect to see on a blood smear from an animal with nonregenerative anemia?

4. Compare absolute and relative polycythemia.

Chapter 13

Hemostasis and Coagulation

Key Concepts

The processes of blood clot formation are critical both in daily life and during veterinary procedures to prevent excessive loss of blood.

The hemostatic mechanism involves an interconnected response by the blood vessels and surrounding tissues, the platelets, and the clotting factors of the coagulation cascade.

Laboratory tests can be used to evaluate the blood clotting ability of the animal.

The complex series of physiologic and biochemical events that are activated to stop the loss of blood whenever vessels are damaged is called the hemostatic mechanism. It prevents the minor bumps and bruises of daily life from being fatal. The veterinarian and technician (and the patient!) rely on this process every time a surgery is performed and a blood sample is collected. The technician should know the basic mechanisms behind hemostasis and routine laboratory tests; these mechanisms can be used to estimate the patients clotting ability.

Hemostatic Mechanisms

Hemostasis is a combination of three separate mechanisms, each with a different function, working in unison. These three factors are the response of the blood vessels to injury, the activation of platelets, and the stimulation of the clotting cascade.

Vascular Response

Arteries and arterioles contain varying amounts of smooth muscle that contracts almost immediately when the vessel is damaged. This contraction is due to local stimulation of the muscle fibers and a sympathetic nervous system reflex. The effect of the contraction is a narrowing of the lumen of the vessel, which decreases the rate of blood loss and creates a turbulence in which platelets may begin to attach. Most capillaries and veins lack smooth muscle and rely on contraction of the surrounding tissues. Hydrostatic pressure is much lower in these vessels, however, and peripheral hemorrhage is usually well controlled by the following two mechanisms.

Platelet Response

Mammalian blood platelets (thrombocytes) are small, irregular cytoplasmic fragments (often containing visible granules) that are formed and released in the bone marrow by giant cells called megakaryocytes (Figs. 13.1 and 13.2). The formation of platelets and other blood cells is discussed in Chapter 14.

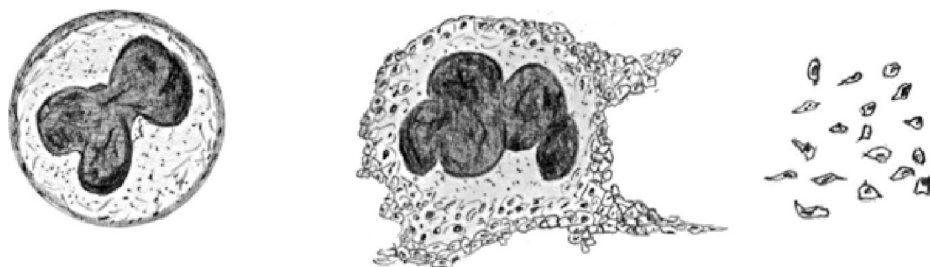


Figure 13.1 Megakaryocytes and platelets. The large megakaryocytes are confined to the bone marrow, where they mature and release cellular fragments, called thrombocytes or platelets, into the circulation.

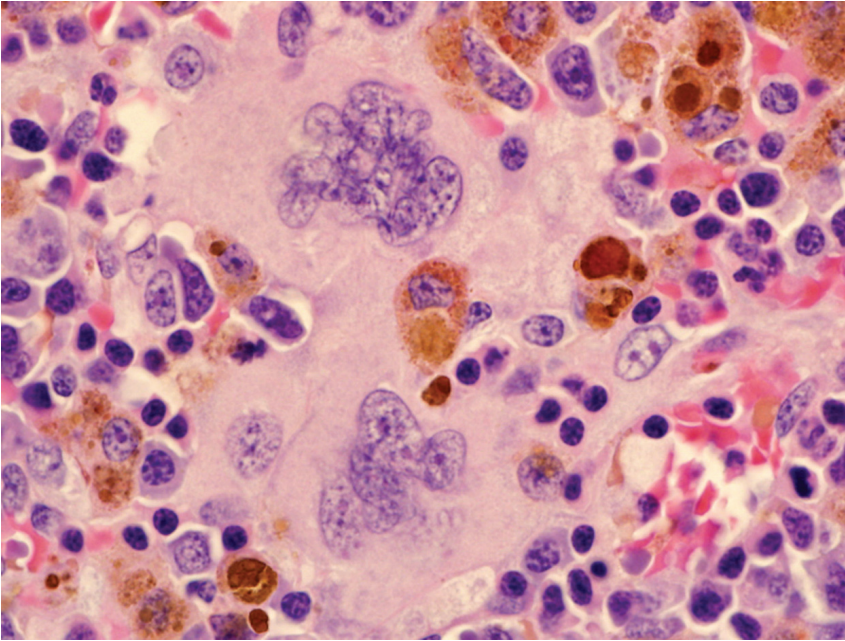


Figure 13.2 Megakaryocytes are the very large cells seen in the center of this bone marrow sample from a dog.

Platelets have a normal life span of 5–10 days in the vasculature, during which they are exposed only to the linings of the blood vessels (endothelium) and heart (endocardium) and the sinusoids of the liver. Despite their relatively simple appearance, the platelet has several functions of major importance in hemostasis. When a blood vessel is injured, platelets are exposed to the subendothelial basement membrane and connective tissues. This exposure activates the platelets to alter surface membrane properties (become “sticky”) and to adhere to the vessel wall and each other (Fig. 13.3). As the platelets aggregate, several products are released. One of the chemicals is ADP (adenosine diphosphate), which stimulates rapid adherence and aggregation of more platelets. Serotonin and histamine release maintains the vasoconstriction initiated by the trauma. In small vessels, the vasoconstriction and platelet plug may be enough to control the hemorrhage. A phospholipid, called platelet factor 3 (PF3), is also released, which is important in activating the coagulation cascade (see the following section).

Later, after the blood clot has been formed, platelets also play a major role in clot retraction. As can be readily observed in a sample tube, a blood clot will gradually shrink to less than half its original size over a period of time, expressing trapped serum (this process is called syneresis). The effect of this shrinkage in vessels is to decrease the size of the vascular defect. The platelet substance responsible for this contraction is called thrombosthenin.

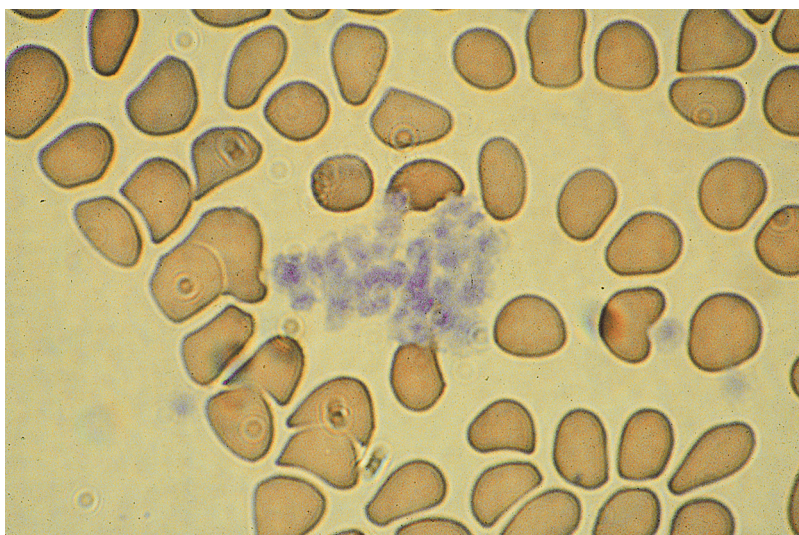


Figure 13.3 Platelet aggregation in blood sample. Clumping of platelets in a blood sample indicates the onset of the hemostatic mechanism, possibly due to slowed collection technique or inadequate mixing of the anticoagulant in the sample.

Coagulation Mechanism Response

Blood coagulation is quite similar in appearance to the changes seen when gelatin is mixed with water. The initial mixture is a colored liquid, but with proper activation (chilling) the protein fibers in the gelatin organize into a structural framework, converting the liquid into a semisolid substance. A very similar change occurs in the blood stream. Numerous soluble proteins and other substances are present in the blood stream in an inactive form, which, when activated, can change the physical form of the blood in the region from a liquid to a semisolid substance. This is called the coagulation mechanism or clotting cascade. The substances in the blood are called clotting factors (Table 13.1). The factors

Table 13.1 International nomenclature of blood clotting factors.

Factor	Synonym
I	Fibrinogen
II	Prothrombin
III	Tissue Thromboplastin
IV	Calcium
V	Labile factor, Ac-globulin, proaccelerin
VII	Proconvertin, stable factor
VIII	Antihemophilic factor(AHF), thromboplastogen
IX	Plasma thromboplastin component (PTC), Christmas factor
X	Stuart-Prower factor, Stuart factor
XI	Plasma thromboplastin antecedent (PTA)
XII	Hageman factor, glass activation factor
XIII	Fibrin stabilizing factor (FSF), Fibrinase, Laki-Lorand factor

Note: Factor VI is not included in this list because it is no longer considered a clotting factor.

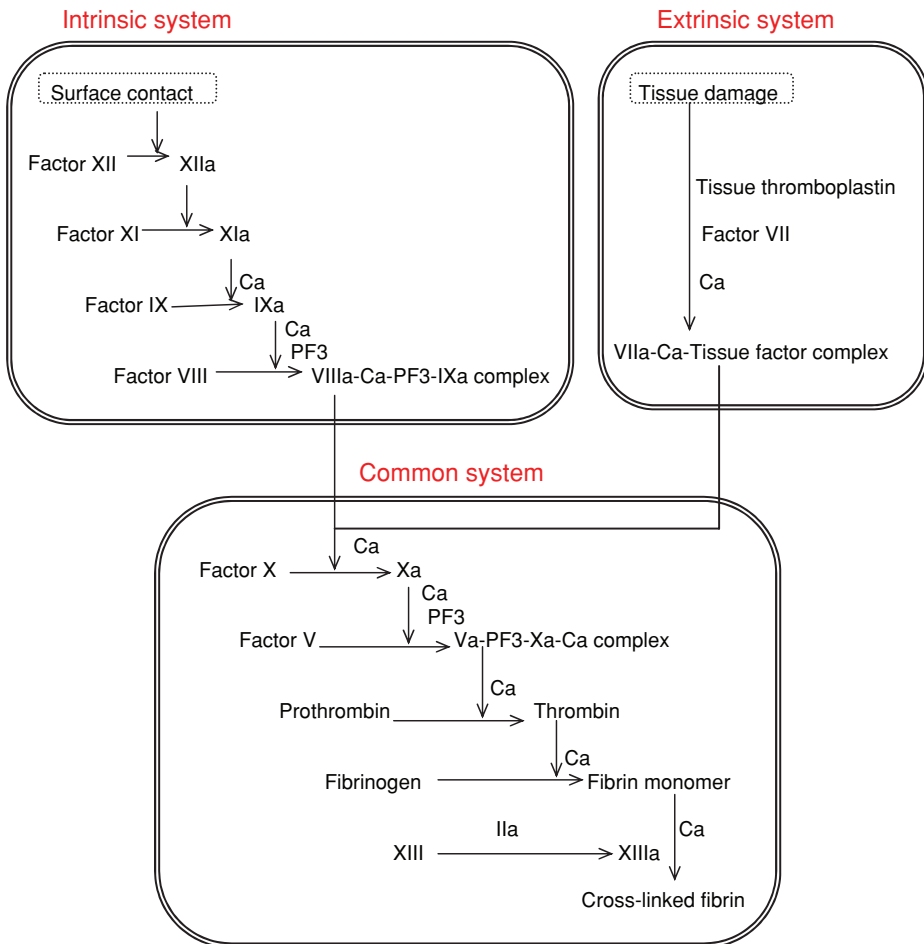


Figure 13.4 Coagulation cascade: the interaction of clotting factors in hemostasis.

are numbered in the order of discovery, not the order they appear in the coagulation sequence. One nonprotein element, calcium (factor IV), is necessary in almost all steps in the cascade.

Formation of a clot can be activated by two pathways (Fig. 13.4). The intrinsic pathway consists of factors present within the blood stream that can be activated without tissue contact. Examples of this would be clot formation around vascular deposits or an indwelling IV catheter without proper anticoagulant. The extrinsic pathway is activated by contact with cells and tissue fluid outside the vessel. Tissue thromboplastin (factor III) is a major factor in this activation. Either pathway then activates the common pathway, the overall effect of which is to convert the soluble fibrinogen molecule into an insoluble fibrin strand.

The fibrin strands formed by the coagulation mechanism crosslink into a meshwork or net that attaches to the platelet plug. Any cells still escaping through the damaged area will be trapped and added to the clot being formed. This process starts in as few as 15

seconds and, if not physically disrupted, is usually completed in approximately 5 minutes. A summary of the hemostatic mechanism is presented in Figure 13.5.

As each factor is activated to stimulate the next step in the cascade, it will be rapidly destroyed by local enzymes. This is to contain the coagulation to the damaged region and not allow it to spread throughout the vasculature. Similarly, an enzyme called fibrinolysin (also called plasmin) is present to begin degrading the clot as it is forming. Clot formation is actually a dynamic balance between stimulation for formation and removal. After the damaged vessel is repaired, the clot will be removed.

A blood clot formed within a vessel is called a thrombus if it remains at the site of formation. If it detaches into the blood stream, it becomes an embolus (thromboembolus), which may block blood flow when it reaches a smaller diameter blood vessel. Thrombi and emboli are common causes of heart attacks and strokes in humans and potentially in animals. A blood clot that forms on the skin surface contracts and dries and is called an eschar (scab).

Hemostatic Defects

Regardless of cause, defects in the hemostatic mechanisms lead to excessive loss of blood from even small injuries and are potentially fatal. These defects may be either hereditary or acquired. Hereditary defects in blood coagulation (coagulopathies) are occasionally seen in animals but appear to be much less common than in humans, possibly due to the decreased survival of newborn animals with impaired hemostasis. Inherited defects have been identified for essentially all of the clotting factors. The most common hemostatic defect, von Willebrand's disease, is not a clotting factor defect but a lack of a protein necessary for the platelets to adhere to the endothelium, markedly slowing the formation of a clot.

Acquired defects are somewhat more common in animals. Thrombocytopenia (a decrease in platelet number or function) is the most commonly identified defect. Numerous infectious agents, therapeutic or toxic chemicals, and immune system disorders are associated with decreased platelet activity. Another common acquired clotting defect is due to vitamin K deficiency or antagonism. Vitamin K, which is essential in the formation of several clotting factors, may become deficient in fat malabsorption syndromes, long-term antibiotic treatment, and liver disease or obstruction. Vitamin K antagonism is the basis behind rodenticides containing coumarin and related chemicals, and ingestion by non-target species (pets) occasionally occurs. Additionally, dicoumarol is a fungal metabolite found in sweet clover, and surgical procedures (e.g., dehorning and castration) on animals consuming sweet clover may lead to excessive hemorrhage.

Coagulation Tests

Numerous tests have been devised to determine specific coagulation defects that are beyond the scope of this text, and the tests are only occasionally practical or necessary in clinical practice. A few relatively rapid and inexpensive screening tests, however, can

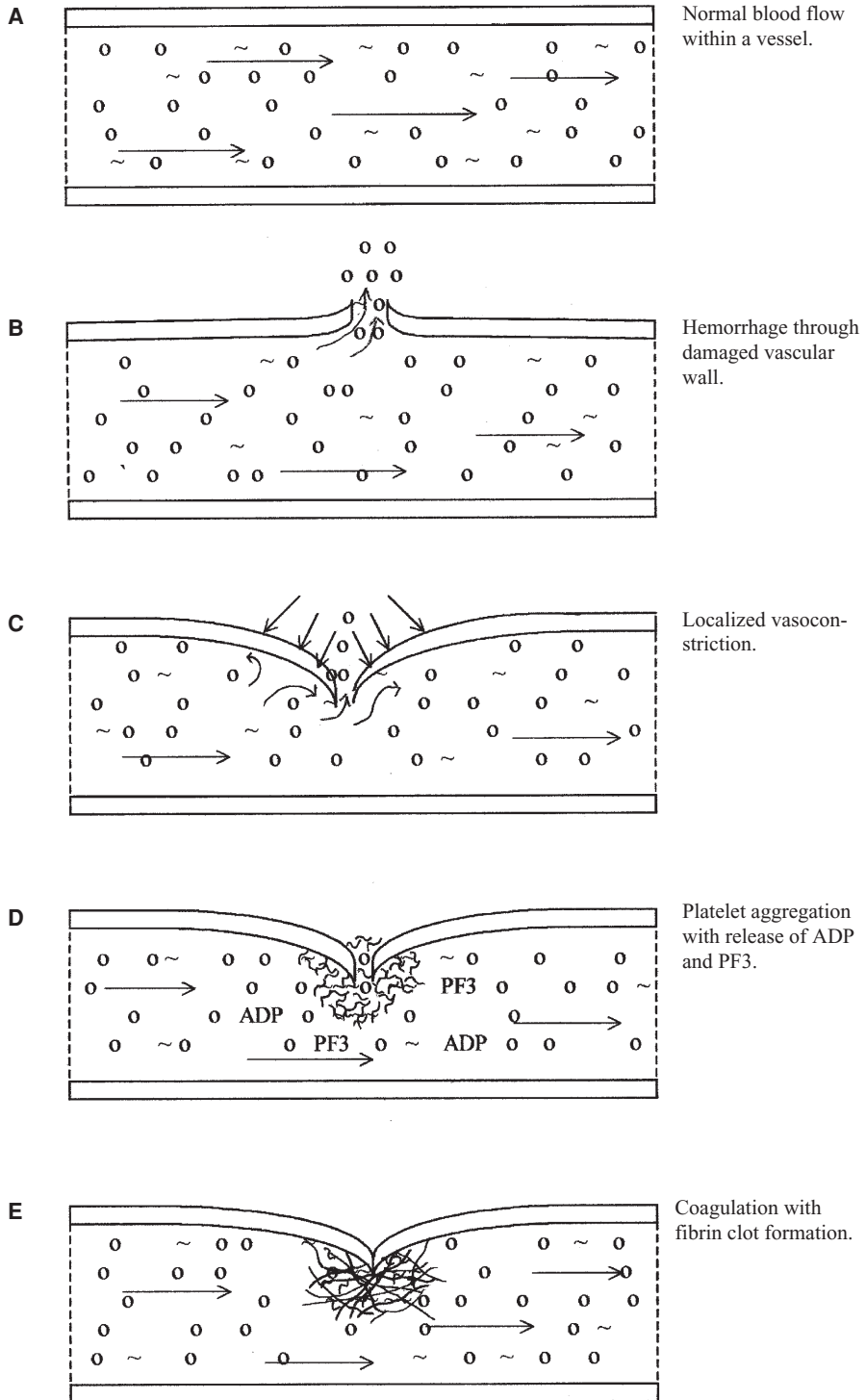


Figure 13.5 Diagram of the events in the hemostatic mechanism.

be performed in the clinic as part of a presurgical workup or if a hemostatic disorder is suspected. Tables of expected values are available for most tests, but since conditions and techniques often vary considerably in different clinics or laboratories, it is highly recommended that several known normal samples be run to verify similarity with the standards or to establish normal ranges for the species and type of animal (e.g., age group) in each laboratory setting.

Bleeding Time Test

A simple test to determine how long it takes for blood to stop flowing from a clean, fresh, controlled wound is called the bleeding time test. The test can be run on any clean, relatively hairless area of the skin or, preferably, on the mucous membrane of the upper lip in the region of the largest molar (carnassial) tooth. By comparison, the test is performed on humans on the ear lobe or forearm. A small, deep puncture wound is made with a lancet, needle, or 11 scalpel blade, and a timer is started with the appearance of blood. As blood accumulates at the site, it should be removed every 30 seconds by gently touching the surface of the blood with filter paper, being cautious not to disturb the wound or the surrounding tissue. The timer is stopped when blood no longer appears. Bleeding time in most animals with this technique is 2–5 minutes.

A similar technique in the anesthetized or unconscious small animal is cuticle-bleeding time. A nail is cleaned with alcohol, allowed to dry, and trimmed just short enough for blood to appear. The nail is allowed to bleed freely while a timer is run. The endpoint is the cessation of bleeding. Normal cuticular bleeding time is slightly longer than skin or mucous membrane bleeding time, 3–9 minutes, possibly due to the decreased vasoconstriction in the solid nail tissue.

Capillary Tube Method

Another relatively simple test for assessing coagulation time is the capillary tube method. Three to four small capillary tubes (similar to microhematocrit tube but *without* anticoagulant!) are filled with blood (or one extra long 15 cm capillary tube may be used instead). If blood is collected from capillary puncture, the first drop of blood is removed because it will contain large amounts of tissue fluid, which will increase the rate of clotting. Blood from venipuncture is preferred. The timer is started when blood first enters the tube. Every 30 seconds a small piece of the tube is carefully broken off, with the endpoint reached when a strand of fibrin is present between the fragments. Normal clotting times with this method are 3–5 minutes in the dog, cat, and pig, and wider ranges up to 12 minutes in the horse and ruminant (Table 13.2).

Lee-White Method

The Lee–White method of measuring coagulation time is a more precise technique. Three 13 × 75 mm glass or silicone tubes (blood coagulates much faster on a glass surface) are rinsed with sterile saline and maintained at 37°C (body temperature), in either a water bath or other device. A minimum of 3 mL of blood is collected from the patient with a

Table 13.2 Typical clotting times measured by the capillary tube method.

Species	Time (min)
Dog	2–4
Cat	2–5
Horse	3–15
Cow	3–15
Sheep	1–6
Goat	3–12
Pig	2–4

syringe previously rinsed with sterile saline. Care should be taken not to allow air to mix with the sample because this will increase the coagulation rate. A timer is started as soon as blood enters the syringe. With the needle removed from the syringe, one-third of the sample is carefully placed in each tube. Remaining at 37°C, the first tube is tilted every 30 seconds until the tube can be inverted without movement of the sample. When this occurs, the second tube is checked in the same way. The undisturbed third tube will usually clot shortly after the second and should be gently tilted every 10–20 seconds at this point. The endpoint is reached at the earliest time that the third tube is coagulated.

Typically, coagulation times by the Lee–White method are quite variable between species and between glass and silicone tubes (Table 13.3), and each laboratory must establish its own reference values in clinically normal animals. The only generality to note is that in most cases, the clotting time is two or more times as long as with the capillary tube method.

Platelet Estimates

Platelet estimates should be made on any blood film examination performed in the laboratory. There should be approximately 3–10 platelets per oil immersion field. Estimates should be made using freshly collected blood that has been well mixed with anticoagulant because any aggregation or clumping of platelets will interfere with even

Table 13.3 Typical clotting times measured by the Lee–White method.

Species	Tube	Time (min)
Dog	Glass	3–13
	Silicone	15–45
Cat	Glass	5–10
	Glass	15–30
Horse	Silicone	25–86
	Glass	4–15
Cow	Glass	5–15
Sheep	Plastic	5–27
Pig	Glass	6–7

distribution of platelets throughout the slide. With potential hemostatic defects, either manual or automated total platelet counts are recommended (see Chapters 6 and 7). Platelet counts in apparently normal animals can be quite variable and range from 200,000 to 500,000/cu mm.

Fibrinogen

Fibrinogen is an important molecule in coagulation, and its levels are occasionally altered in coagulation defects. See Chapters 6 and 7 for methods of measurement of fibrinogen.

Review Questions

1. Describe the vascular, platelet, and coagulation mechanism response to blood vessel injury.

2. Name and describe three laboratory tests useful in estimating blood clotting time.

Chapter 14

Hematopoiesis and Bone Marrow Examination

Key Concepts

Although blood cells can form in many areas of the developing fetus, most of the blood cells in the adult animal are formed in the bone marrow in a process called hematopoiesis.

Multipotent stem cells in the bone marrow can divide to produce immature cells of all blood cell types that normally pass through several maturation stages before being released into the blood stream.

A sample of bone marrow tissue can be collected to evaluate the relative rates of blood cell formation.

In the embryo, undifferentiated cells within the body are called pluripotent stem cells since they can differentiate into any of the body tissues. These cells begin to form tubes that eventually become the blood vessels and heart. The cells in the center of the tubes begin to form the cells of the blood. With further development of the embryo, these stem cells form centers of hematopoiesis (blood formation) in the liver, spleen, and thymus. At this point, they are termed multipotent stem cells since they can still form any of the blood cells but not other tissues. With the development of the skeletal system, the stem cells move to the bone marrow cavities, where production of blood cells remains throughout life. Erythrocytes formed early in embryonic and fetal life remain nucleated, but those formed in the bone marrow have their nuclei removed prior to release. In the young, growing animal, almost all the bone marrow is active and highly cellular (“red marrow”). As animals age, the amount of space in the cavities of the bones increases, and much of the marrow cavity fills with fat cells (“yellow marrow”) with decreased production of blood cells in these areas. Active, cellular marrow remains in the flat bones (ribs, sternum, pelvis, and skull), the vertebrae, and the ends of the humerus and femur.

Hematopoiesis

All stages of development of blood cells are present in normal bone marrow, including the multipotent stem cells, which can still be stimulated to develop into any of the blood cell lines. Most blood cell formation, however, is from mitosis and maturation of blast cells already committed to a single cell line. The marrow produces erythrocytes, neutrophils, eosinophils, basophils, and thrombocytes (platelets) and is a minor site for production of lymphocytes.

As leukocytes, erythrocytes, and megakaryocytes (platelet-producing cells) mature, recognizable stages of development are assigned names. Historically, several different naming schemes have been proposed and may be seen in older texts. The most common current terminology used with hematopoietic cells is presented in Figure 14.1.

It should be emphasized that the developmental alterations from one stage to the next are gradual processes, and assigning names is just a convenient way to express the levels of development. Familiarity with each stage of all cells is achieved only through repetition and in-depth study and is beyond the scope of this text. However, a few generalities hold true for most of the formative cells. Younger cells tend to be larger than the more mature forms with a larger nucleus to cytoplasmic ratio. The nuclei of juvenile cells are usually round or slightly oval. As cells of the granulocytic series mature, the nuclei begin to indent and elongate and eventually become segmented. Erythrocyte nuclei become smaller and markedly condensed and are removed from the cells prior to complete maturation. The chromatin of the immature forms is dispersed in a fine, delicate network and often stains red to reddish-purple. As cells mature, the chromatin becomes more coarse and irregularly clumped and stains dark purple to black. Nucleoli are commonly visible as light blue structureless masses within the nuclei of immature cells but disappear as the cells mature. The cytoplasm of juvenile cells contains active RNA and stains an intense blue color. In leukocytes, this blue color gradually lightens with maturation, and in erythrocytes it is mixed with, and gradually replaced by, a red tinge as hemoglobin is formed.

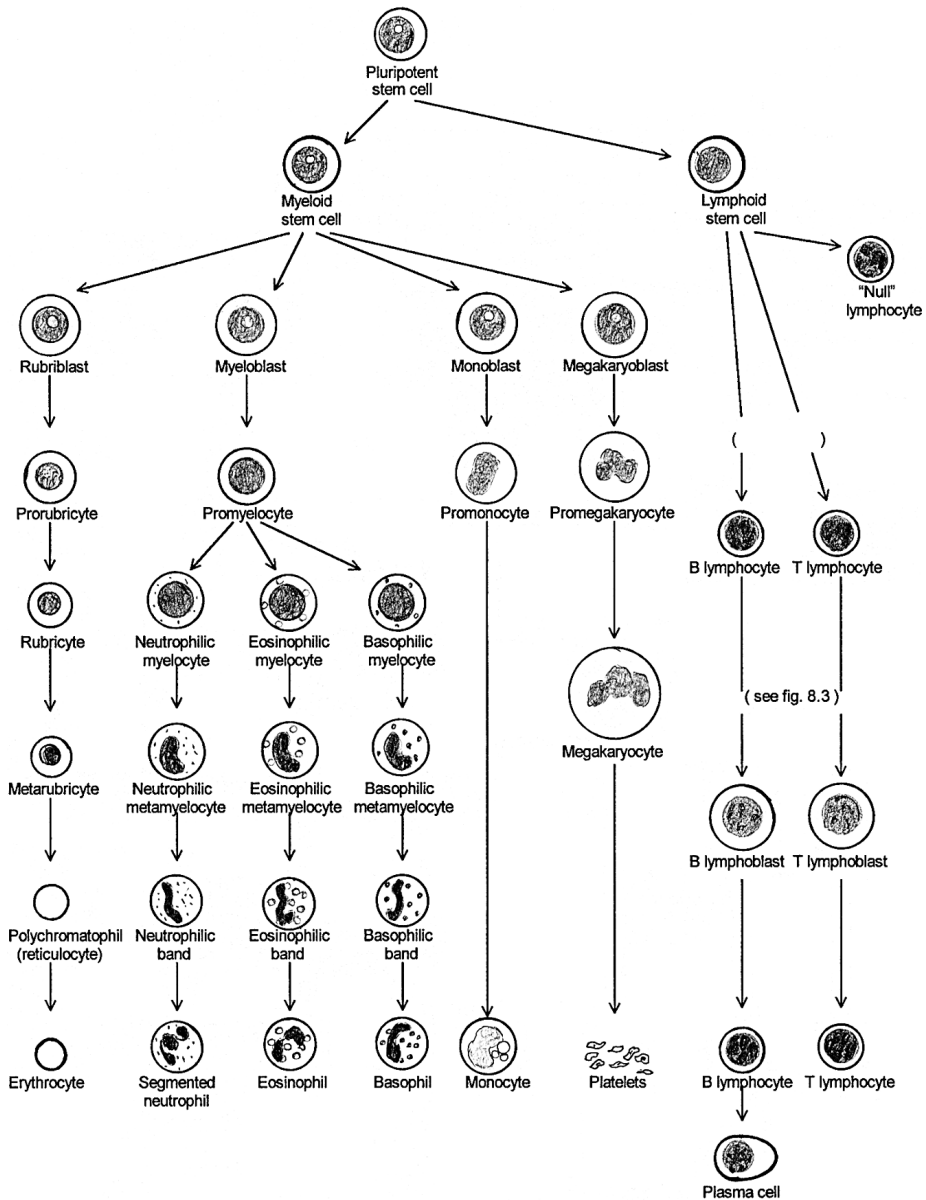


Figure 14.1 Developmental stages of cells in hematopoiesis.

The most recognizable characteristic of juvenile cells in the erythrocytic series is the dark purplish-blue color imparted to the cytoplasm by the mixture of hemoglobin and RNA. As the cell matures, the red color begins to predominate, and the nucleus becomes pyknotic (a dark, shrunken, central mass with no distinguishable chromatin pattern). In the reticulocyte stage, the nucleus has been removed, and clumps or fibrils of retained RNA can be stained with new methylene blue or other supravital stain.

Cells of the granulocytic series (neutrophils, eosinophils, and basophils) all go through similar developmental stages. The first identifiable differences between cell types is in the myelocyte stage, where cytoplasmic granules begin to assume their characteristic staining qualities for neutrophilia, eosinophilia, and basophilia. In the metamyelocyte stage, the nucleus indents and resembles a kidney bean or thick sausage. The band cell is characterized by an elongate, often coiled, nucleus with approximately parallel sides. The latter two stages (metamyelocyte and band) are occasionally present in peripheral blood and their recognition is important in assessing neutrophilic response mechanisms (see Chapter 8).

Fewer formational stages are evident in the agranulocyte series. Most lymphocytes are formed in lymphoid tissues throughout the body, including the spleen, lymph nodes, tonsils, and Peyer's patches. Lymphoblasts are similar in appearance to other blast forms and prolymphocytes appear intermediate in size, shape, and internal structure to the mature lymphocytes previously described (see Chapters 2 and 8). The monoblast is often slightly larger than, but otherwise similar to, other blast cells, and developing monocytes remain larger than other white cells of similar maturity. The nuclei of monocytes condense less than in other cells and retain an open, lacy-appearing chromatin pattern, with occasional clumping near the nuclear periphery. The nuclei stain a pale reddish-purple.

The megakaryocyte series will usually be the first cells noted on a marrow smear due to their large size. Unlike the other hematopoietic cells, the megakaryocyte become larger as it matures and can reach 10–15 times the diameter of adjacent cells. This is due to a process called endomitosis, in which the nucleus undergoes mitosis without cytoplasmic division. The clumped, multiple nuclei may appear irregular, multilobed, or ring-shaped. The cytoplasm contains light-staining (azurophilic) granules and becomes cloudy and has a roughened outline at maturity. The cytoplasm eventually fragments into numerous irregular pieces that are released into the circulation as platelets (thrombocytes).

Other cells may occasionally be seen in normal bone marrow smears. Fat cells, which appear as large, round, empty spaces, are quite common. The reticulum cell, which may be the stem cell, is a light-staining, round to oval cell with a round, open nucleus. Plasma cells, which are activated B lymphocytes (see Chapter 8), may be present. Osteoblasts (bone-forming cells) are oval cells with round, eccentric nuclei and closely resemble the plasma cell. Osteoclasts (bone-dissolving cells) are nearly as large as megakaryocytes and have multiple, separated nuclei. Fibroblasts and endothelial cells from marrow capillaries are occasionally seen. The presence of cells normally external to the bone, such as epithelium or muscle cells, indicates improper collection techniques.

Indications for Bone Marrow Examination

A bone marrow examination is not a routine procedure since representative samples are somewhat difficult to obtain and interpretation of results dependent upon the experience and ability of the person examining the smear. Bone marrow sampling is indicated in cases of hematopoietic disorders in which changes in the peripheral blood are not specific or signify general bone marrow dysfunction, as with pancytopenia, a decrease in all blood cells. Anemias of undetermined cause or those that are unresponsive to treatment may



Figure 14.2 Sites for collection of bone marrow samples in the dog: (a) proximal humerus, (b) iliac crest, and (c) proximal femur.

require bone marrow study. In the horse, since reticulocytes are not present in peripheral blood, a bone marrow examination may be the only way to determine regenerative response. A marrow examination is also indicated when abnormal cell forms are found in peripheral blood or there is clinical or radiologic evidence of primary or metastatic bone marrow neoplasia. With osteomyelitis (infection of the bone and bone marrow), bone marrow extraction may be necessary for both the examination of the cellular reaction and for microbiological culture and sensitivity testing.

Bone Marrow Collection Techniques

Adequate amounts of active hematopoietic tissue are found in only a few accessible sites in the adult animal (or human). Common sites for bone marrow collection in the dog and cat are the proximal femur (hip) and the proximal humerus (shoulder). The iliac crest (lower back) is also used in dogs but due to the smaller size is less common in the cat (Fig. 14.2). In large animals, the most common collection site is the proximal portion of the rib with the least amount of tissue covering, usually the 10 to 13 rib. In all animals, the site and surrounding area must be clipped and thoroughly scrubbed and disinfected.

An assortment of bone marrow aspiration and biopsy needles is available; the size and type of needle used depends on the patient (the species, size, and site selected) and personal

preference. Bone needles must be of strong steel, have a short bevel and a seated stylet, and attach readily to a syringe. The person reading the aspirant should perform the collection, or at least be present, to be aware of any difficulties or possible contaminations present. The procedure may be performed under local or general anesthesia. If local anesthesia is used, care must be taken to infiltrate the sensitive periosteum. A local anesthetic containing epinephrine is preferred since the vasoconstriction produced lessens the chance of dilution of the marrow sample with peripheral blood.

A stab incision is made in the sterile field over the selected site. Any minor hemorrhage encountered should be controlled with gauze and pressure to avoid its inclusion in the sample. The bone needle with stylet in place is inserted perpendicular to the center of the target site on the bone. When the desired location on the bone is found, the needle is passed through the cortical bone with constant pressure and a twisting motion. When the needle penetrates into the marrow cavity, a distinct “give” will be felt. In older or very large patients, it may be necessary to use a sterile bone pin or drill bit to penetrate the cortex. A 3/32-in. pin or bit produces an opening appropriate for a 12-gauge needle.

With the needle placed securely in the marrow cavity, the stylet is removed, and a 10–20 mL dry syringe attached. A short, rapid aspiration should produce a small amount of marrow tissue in the tip of the syringe. If unsuccessful, the needle should be rotated and/or moved slightly up or down and reaspirated. The stylet may be briefly reintroduced into the needle to remove possible obstruction. If marrow is still not collected, another site should be selected. A large amount of fluid flowing readily into the syringe indicates entry into a vascular channel with minimal marrow tissue, and the needle must be repositioned. If the technique has been successful, the sample is immediately transferred to one or more glass slides (cover slips may be used if preferred). Introduction of 1–2 mL of air into the syringe prior to aspiration will make expelling of the sample easier. If the sample is to be cultured for microorganisms, a portion should be placed in the appropriate media prior to any contact with nonsterile surfaces or other potential contamination.

Bone Marrow Slide Preparation

Marrow tissue will vary from semisolid tissue fragments to a more fluid consistency. Smears may be made by use of a spreader slide (see Chapter 5) or a “squash” or “pull” preparation by placing two slides (or coverslips) together with the sample between and sliding them apart laterally (Fig. 14.3).

Any small particles (spicules) of bone present will interfere with the smear preparation but may be used for making imprints or brushed across a slide. Excess bone marrow collected may be mixed with EDTA and saved for later examination, although cell morphology may be less distinct. Peripheral blood smears should always be prepared at the same time for comparison.

If a bone marrow biopsy needle has been used, a small core of tissue has been removed. This tissue is routinely placed in an appropriate fixative solution and delivered to a laboratory for histopathologic examination. Tentative examination may be done on imprint slides made from the tissue core, but extreme care is needed to prevent damaging the tissue.

After the slides are thoroughly dried, they may be stained by several methods. Use of the same stain routinely used with peripheral blood smears is suggested to minimize

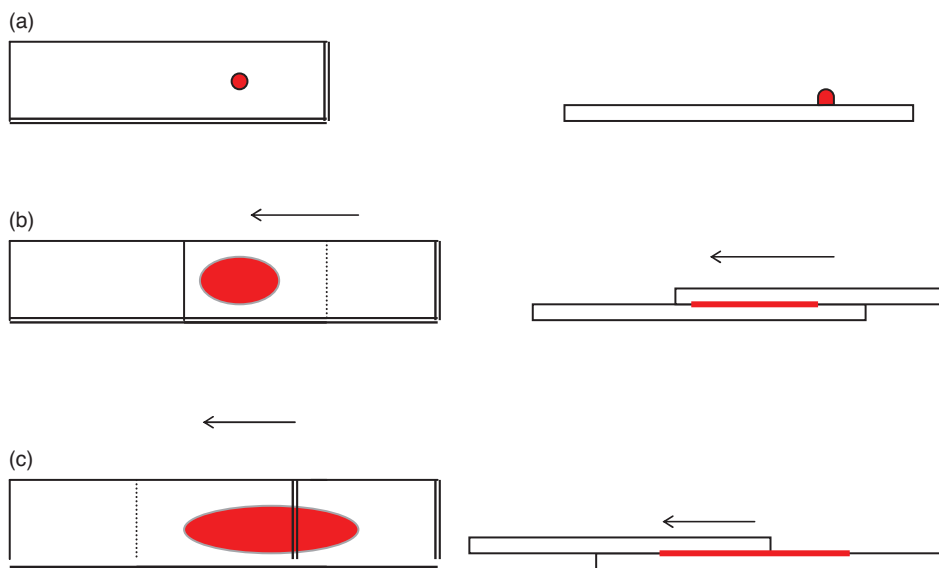


Figure 14.3 The pull ("squash") technique for making a bone marrow smear.

difficulties in cell recognition. Other stains may also be indicated, such as new methylene blue for reticulocytes or Gram's stain for microorganisms. Bone marrow smears should be stained slightly longer than peripheral blood smears because of the increased thickness.

Examining the Smear

The smear should be carefully scanned under low power (100 $\times$) and an assessment made of the number and distribution of cells (Fig. 14.4). With experience, the observer will be able to rate the sample as high, moderate, or low cellularity. This is a subjective judgment and evaluates the amount of material for study on that slide and not necessarily the quality of the bone marrow. By switching to high, dry power (400 $\times$), the observer can make a general evaluation of the types of cells. An estimate of relative percentages of mature and immature forms should be noted, and the slide scanned for the presence of obviously abnormal cells, especially if present in groups or sheets of similar cells.

Individual cell identification is done on oil immersion (1,000 $\times$). This is a challenging task for the beginner and even for those with more experience: some cells are present that are undifferentiated and difficult or impossible to classify. These are usually identified by "the company they keep"; that is, they are classified with nearby similar but more differentiated cells. Between 500 and 1,000 nucleated cells should be observed and must be determined to be either leukocyte (myeloid) or erythrocyte (erythroid) lineage cells. From these figures, a myeloid-to-erythroid (M:E) ratio can be calculated by dividing the number of erythroid cells into the number of myeloid cells. For example, if 1,000 cells were counted and 550 were myeloid, 450 erythroid, the M:E ratio is 550/450 or 1.22:1 (usually expressed simply as 1.22). Similarly, if 400 myeloid cells and 600 erythroid cells were seen, the M:E ratio is 0.67:1 (or 0.67). The M:E ratio varies somewhat between

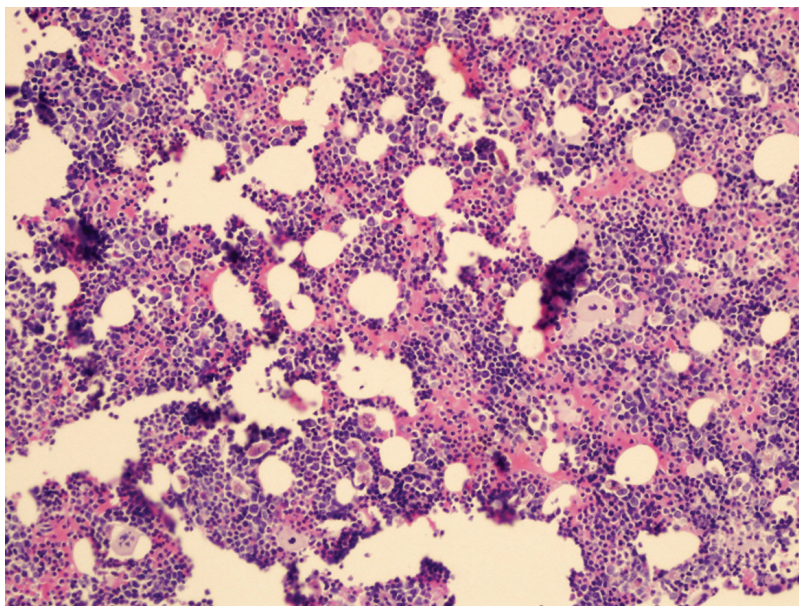


Figure 14.4 Example of cells seen in a bone marrow smear.

species but is close to 1:1 in many; the dog, cat, and pig are slightly higher than 1:1, and the ruminants slightly lower than 1:1. Any increase in this ratio above the normal range for that species indicates an increase in white cell production or a decrease in red cell production. A decreased M:E ratio has similar, but opposite, implications.

Bone marrow differential counts of all cell types and stages of development are laborious and should be performed only by laboratory personnel experienced in bone marrow examination. A reliable differential count can yield objective information with which to compare future changes. No normal range of values has been universally accepted since recognition and interpretation of cell types and stages varies between observers. Each laboratory, and each individual, must establish their own base line values.

Review Questions

1. The marrow cavities of which bones usually contain the most active blood cell formation in the adult animal?
2. Describe the general changes in size and appearance of blood cells as they mature.
3. Which blood cell is commonly formed outside the bone marrow in the adult animal?
4. What is meant by the term M:E ratio in relation to a bone marrow sample?

Chapter 15

Collection and Handling of Cytology Samples

Key Concepts

Cytology is the study of cells collected from various areas of the patient's body.

Using laboratory techniques and equipment similar to blood collection, the hematology technician can assist in the collection and handling of cytology samples.

With the knowledge of circulating blood cells, the hematology technician can help to make initial evaluations of cytology samples.

The term “cytology” simply means the study of cells. Clinically, cytology involves the collection and examination of cells and fluids from body surfaces, cavities, and tissues. The continuous growth of epithelial tissues on the body’s surface (skin and mucous membranes) and the linings of body cavities and organs produces a continual supply of exfoliating, or sloughing, cells. Collection and study of these cells is “exfoliative cytology.” Obtaining cytology samples from within the body by use of needles and syringes is “aspiration cytology.” Bone marrow sample collection in the previous chapter is an example of aspiration cytology. The basic purposes of any cytologic examination are to characterize the type of fluid or cells present and to determine the presence or absence of inflammation or neoplasia.

The field of cytology is usually not considered part of hematology since interpretation of samples usually falls under the realm of diagnostic pathology. The technician will find, however, that the techniques of collecting and handling samples (i.e., syringe aspiration and slide preparation) are essentially identical to those in hematology, especially bone marrow collection. Also, the initial cell identification of the sample is based on knowledge of the blood cells (e.g., are they inflammatory or not), although it must be stressed that final evaluation should be done at a pathology laboratory.

The major advantages of cytology are the rapidity of the procedure, often providing useful information in a matter of minutes, and the minimal amount of equipment and expense involved. A cytologic examination may provide a positive diagnosis when definitive cells are present, and so may preclude the use of more time-consuming or expensive testing. Sterile cytology samples may also be used for microbiological culture and sensitivity testing. The major drawback to cytology is the loss of normal tissue architecture, and the alterations in the cells themselves, whether pathological or artifactual, make slide interpretation quite difficult. Histopathological confirmation of results is so often necessary that the two collection techniques are often combined. Along with the cytology specimen, tissue samples may be obtained by removal of a core of tissue with a biopsy needle, or localized lesions may be removed by surgical excision. A small portion of the sample may be used to make an impression smear by gently pressing a cut edge of the mass to a glass slide one or more times (see Surgical Specimens below). The slide is allowed to dry and then stained for study under the microscope. The bulk of the tissue will be sent to a diagnostic pathology laboratory for interpretation.

Sample Collection

Equipment Needed

The supplies necessary for cytology sample collection are readily available in most veterinary clinics. For surface cell collection, a scalpel blade or swab will usually be used to transfer the sample to the glass slide (Figs. 15.1, 15.2, and 15.3). For aspiration cytology, an assortment of needles and syringes is needed since the size of animal and collection site will vary greatly. Needles of 22 gauge or less should be used for soft tissue sample collection (unless the sample is a viscid or thick fluid) to lessen the chance of taking a



Figure 15.1 Collecting material from a skin scraping using a scalpel blade.

core of overlying epithelium and connective tissue into the sample area. A spinal needle with stylet will be necessary for obtaining cerebrospinal fluid and may be useful in other collections. Sterile swabs, glass slides, coverslips, and a selection of stains of personal preference will also be necessary.

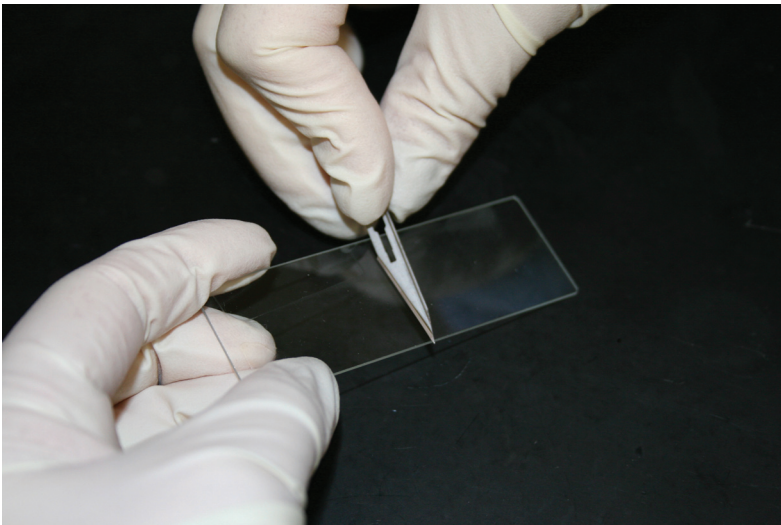


Figure 15.2 Transferring skin scraping to a glass slide.

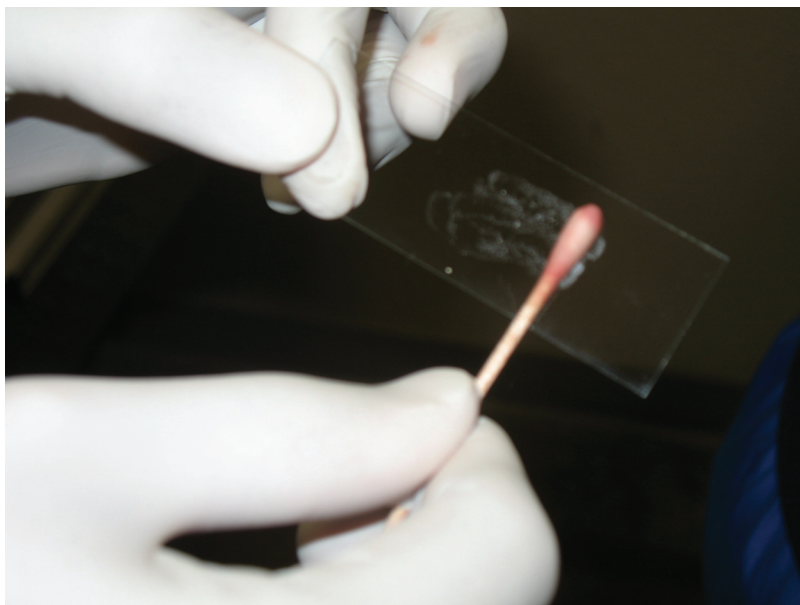


Figure 15.3 Material collected on a moistened swab may be directly transferred by rolling the swab on a glass slide.

Collection Sites

Cytology specimens from the skin may be collected by skin scrapings or by direct impressions. Cells from the mucous membranes of the digestive, respiratory, or reproductive systems are collected with a sterile swab or by “washing” the area with fluids that are reaspirated. Direct needle aspiration is used in joints, body cavities, the spinal column, abnormal subcutaneous swellings, and other fluid accumulations.

General Approach

All collection sites and the surrounding areas should be clipped and surgically scrubbed and disinfected. Local or general anesthesia is used as necessary, although in small superficial lesions the sample collection takes less time and is less stressful than anesthesia. If a needle larger than 22 gauge must be used, a small stab incision over the point of needle entry is recommended to prevent potential contamination or obstruction of the bore of the needle. If very small samples are anticipated, drawing 1–2 mL of air into the syringe prior to sample aspiration will aid in the removal of the sample from the syringe tip and needle hub. If large amounts of fluid are apparently present or encountered, half the sample should be placed in a tube containing EDTA and the other half in a plain tube for evidence of clotting. When few cells are present in a fluid sample, it may be centrifuged, and smears may be made from the sediment. When the sample is to be cultured for identification and/or sensitivity testing of microorganisms, this procedure should be performed as soon



Figure 15.4 Collecting fluid from a subcutaneous mass with a needle and syringe.

as the sample is collected and prior to any further handling or testing, or a sample of the fluid or tissue should be aseptically transferred to a sterile container.

Sample Collection Techniques

Palpable Masses

Abnormal cellular or fluid masses in the skin or palpable in underlying tissues may often be identified by needle aspiration. This is rather descriptively referred to as “lump and bump” cytology. Local anesthesia and/or sedation are often unnecessary unless indicated by the temperament of the animal or location of the lesion. After surgical preparation of the site, the mass is localized and stabilized with the nondominant hand. A 25–22-gauge needle is introduced into the center of the mass, and negative pressure applied to the attached syringe (Fig. 15.4). The needle may be moved to several different areas in the mass to ensure the sample is representative. Care should be taken not to penetrate the margins of the mass. Prior to withdrawal of the needle, the negative pressure on the syringe must be released. Slides are prepared in a manner similar to that for a blood smear, or, if the sample is highly cellular or thick, by the squash, or pull, technique used with bone marrow samples (Fig. 14.2).

Abdomen

Collection of needle aspirant samples from the abdominal cavity is called abdominal paracentesis, or simply a “belly tap.” This is often indicated in cases of excessive fluid

accumulation (ascites), acute abdominal pain with potential hemorrhage or rupture of internal organs, or the suspected presence of abnormal cells. For this procedure, the animal should be standing to allow fluids to collect ventrally. Dependent upon the patient, sedation or local anesthesia may be necessary. The puncture site should be in the lowest point of the abdominal profile, usually between the xiphoid cartilage and the umbilicus. In many horses, the lowest point may be somewhat further posterior. Extreme care must be taken to avoid the abdominal organs. Collection slightly to the right of the midline will help avoid the spleen and liver. A 22–18-gauge needle is advanced through the skin (or stab incision), muscle, and peritoneum at an angle with the beveled surface of the needle facing downward to help prevent blockage of the needle by omentum. Placing the needle through a sterile gauze sponge prior to sample collection will help prevent sample contamination by fluid or debris on the skin. Immediately upon the needle entering the abdominal cavity, fluid should be present at the needle hub. If no fluid appears, injection of 1–2 mL of air will help dislodge any tissue blocking the needle. Fluid may be collected by gentle aspiration, or the syringe may be disconnected, and the fluid allowed to flow into a sterile container. Slides may be made directly from the sample, or it may be centrifuged and the sediment used.

Thorax (Chest)

The presence of fluid in the thoracic cavity will decrease respiratory capacity, and care must be taken to avoid stressing the animal. The use of a 3-way stopcock between the syringe and needle will prevent air from being drawn into the chest. With the animal standing, a 20–16-gauge needle is inserted as far ventrally as possible between the eighth and ninth, or ninth and tenth, ribs (intercostal space) with the beveled surface toward the parietal pleura (chest lining). The puncture should be in the middle of the intercostal space to prevent damage to vessels or nerves adjacent to the ribs. Movement of the needle should be minimal once the cavity has been entered to avoid lacerating the lung. Handling of the sample is the same as with abdominal fluid.

Joints

Excessive fluid in a joint cavity may be due to inflammation or infection and is often quite thick, necessitating the use of a 16–18-gauge needle. Sterility is essential to prevent introduction of infectious agents into the joint. Specific techniques will vary with the animal and joint involved, but in most cases, flexion of the joint will allow more space for insertion of the needle into the opposite, extended cavity. Care must be taken not to damage the cartilage surfaces with the needle. Local anesthesia may be used but is often unnecessary. Joint fluid is commonly cultured if any evidence of infection exists, and smears are prepared for staining and examination.

Trachea

Transtracheal aspiration is a method used for obtaining cytology or culture samples from the lower respiratory tract. The beginning technician should be familiar with the technique,

but actual collection should be done only after considerable experience assisting with the procedure. A needle and long catheter are selected of appropriate dimensions so that the catheter will pass through the bore of the needle. On the ventral surface of the neck, a notch can be palpated at the base of the larynx (cricothyroid ligament). The area is surgically prepped, and a local anesthetic administered. The needle is inserted through the notch and directed down the trachea. The catheter is threaded through the needle until it reaches the point where the trachea divides into bronchi. The needle is then removed, and 5 mL of warmed, sterile saline solution is injected through the catheter. Negative pressure on the syringe will withdraw some fluid plus mucus and air. The syringe is then disconnected, the excess air expelled, and the aspiration technique repeated several times in order to collect about 0.5 mL of sample. The catheter is then withdrawn, and the sample handled as previously described. The fluid that remains after aspiration is rapidly absorbed.

Cerebrospinal Fluid

Collection of cerebrospinal fluid is not a common procedure and should be done only by experienced personnel with a thorough knowledge of spinal column anatomy. In small animals, the most common collection site is the space between the base of the skull and the first vertebra (atlando-occipital joint), although some technicians prefer collection between the vertebrae of the lower back (lumbar or lumbosacral). General anesthesia is usually necessary and the animal is placed in lateral recumbency. In large animals, fluid may be collected between the first two to three tail (caudal or coccygeal) vertebrae after the pelvis and may require local anesthesia. The collection site must be clipped and surgically scrubbed and disinfected. A 20–22-gauge spinal needle is used, which has a short bevel and a seated stylet. If the atlanto-occipital joint is used, the head is flexed ventrally 90°; any site used must be accurately located by palpation. The needle is carefully inserted between the vertebrae, or vertebrae and skull, and stopped as soon as it is felt to pass through the fibrous tissue surrounding the spinal canal (dura mater). Without moving the needle, the stylet is removed, and fluid should appear at the hub. Fluid is collected without aspiration, if possible by allowing it to drip into a sterile container. A maximum of 1–2 mL of fluid should be collected for bacteriology, cytology, and/or chemical analysis.

Vaginal Cavity

Collection of cytology samples from the vaginal cavity for evaluation of reproductive disorders may be indicated in any species, but the procedure is most common in the bitch to determine the stage of the estrous (heat) cycle. To obtain the sample, the animal is restrained in a standing position with the tail elevated. The lips of the vulva are cleaned, and a lubricated vaginal speculum is used to access the vaginal cavity. A sterile swab, which may be moistened with sterile saline if the vaginal cavity is dry, is inserted through the speculum above the urethral opening. The swab is rotated around the vaginal wall near the cervix, withdrawn, and rolled across the surface of clean glass slides. Cells recovered from the front of the vaginal cavity will be released from the swab and adhere to the glass slides, which can then be stained and examined.

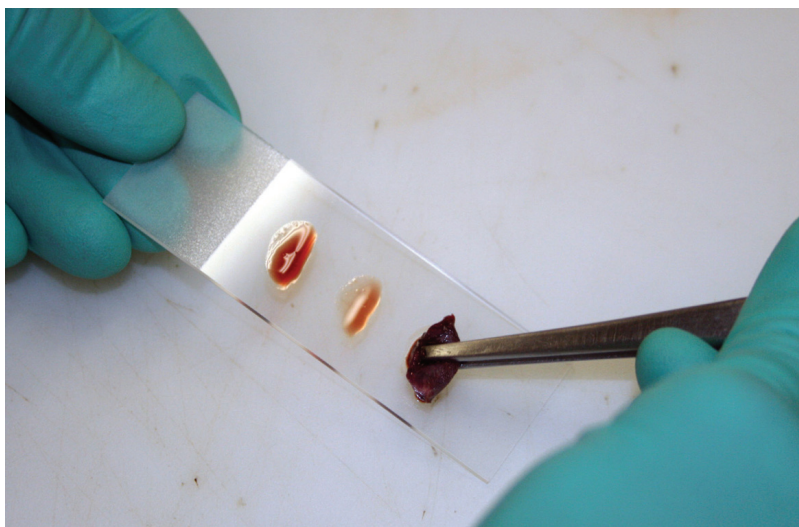


Figure 15.5 An impression smear being prepared by pressing the cut surface of a surgical specimen to a glass slide.

Surgical Specimens

Cytology preparations can be made directly from tissue removed surgically prior to fixation for shipment to a pathology laboratory by making an impression smear. The sample is sectioned with a scalpel, and the freshly cut surface is dried by briefly touching it to a paper towel or filter paper. Do not distort or damage the sample to be sent to the laboratory; if possible, make the impression from a portion of the sample not being sent. Touching the sample to a glass slide in one or more places will cause cells from the tissue to adhere to the glass slide (Fig. 15.5). Care must be taken not to move or slide the tissue on the glass because this may distort the cells. After air drying, the slides can be stained. At all times the sample must be handled gently to avoid damaging the cellular structure necessary for the histopathology examination.

Sample Evaluation

Fluids—Transudates versus Exudates

The major purpose of laboratory examination of fluid samples is to determine the type of fluid present (transudate or exudate, described below) and its potential source or cause. If culturing of the sample is indicated, a portion of the fluid is aseptically placed in culture media or appropriate transport media before further tests are performed. The physical characteristics of the sample should be noted at this point, including color, transparency, odor, sediment, and any additional findings. This step will occasionally be quite significant; for example, an abdominal paracentesis that is yellow and smells like ammonia or urine and another abdominal sample that contains small particles of ingested food

Table 15.1 Differential characteristics of transudates and exudates.

Characteristic	Transudate	Exudate
Appearance	Clear, thin and watery, colorless to light yellow	Clear to cloudy, thick to creamy, often colored
Odor	Usually odorless	Odor usually present
Coagulation	None	Usually clots
Specific gravity	<1.017	>1.017
Protein (g/dL)	<3.0	>3.0
Cells	Few to none, erythrocytes, lymphocytes, resident cells	Numerous, mixed leukocytes, erythrocytes, abnormal cells
Bacteria	Absent	Often present

are both rather definitive. Descriptive terms should be used such as “colorless,” “white,” “red,” “cloudy,” “hazy,” or “clear” rather than interpretive terms such as “bloody” or “fatty.”

Depending on the amount of fluid available, several routine tests should be performed next, including total protein, specific gravity (which measures the amount of any substance dissolved in a fluid compared with distilled water and is another scale on the refractometer), presence or absence of coagulation, and a slide evaluation of any cells present. This will help to identify the fluid as a transudate or an exudate (Table 15.1). A transudate is defined as a normal body fluid that is present in excess amounts or in an improper location in the body. An exudate is an abnormal fluid that should not normally be present in the body. Differentiation between these two classifications is often an essential early step in diagnosis. Transudates are noninflammatory in origin and are usually associated with a blockage or change in osmotic pressure in the circulatory system (blood or lymph) or in the viscera (e.g., twisting or impaction of the intestine). These fluids are usually sterile, do not clot, and have low protein and specific gravity levels, with very few cells present. Exudates are inflammatory reactions commonly due to infection, trauma, foreign bodies, and some tumors. Numerous inflammatory blood cells are present, bacteria are often seen, specific gravity and total protein are high, and the sample tends to clot. The initial treatment of an animal may be based on identifying the sample as a transudate or exudates.

Cells

Smears made from cellular aspirants will vary widely in shape, thickness, cellularity, and difficulty of interpretation. Numerous stains may be used, but it is recommended to begin with the same stain used for routine hematology to lessen the confusion created by color variations. Other stains, such as Gram’s stain for bacteria or new methylene blue for reticulocytes, may also be used.

It is easy to be overwhelmed by the large number of unusual, abnormal, and unfamiliar cells present, especially in exudates. Identification of these cells must be left to experts in cytology. The technician can make some basic evaluations of the sample, however, by

following a simple protocol in observing the slide. The initial goals of cytologic evaluation are to determine (1) if the sample appears inflammatory or not, (2) if there are any bacteria present (septic), and (3) if there is any evidence of neoplasia (cancer). Inflammation is characterized by the presence of cells the technician should recognize—red and white blood cells. Neutrophils are usually the most numerous inflammatory cell, with larger numbers of macrophages (monocytes) in more chronic conditions. Variable numbers of lymphocytes, plasma cells, eosinophils, and basophils may be present, depending on the cause and nature of the inflammation. Noninflammatory conditions (transudates) may have a few of these cells present (cell counts less than 1,000/cu mm) but total numbers will be markedly higher (cell counts often greater than 50,000/cu mm) with inflammation (exudates).

If the aspirant is inflammatory, the next step is to check for the presence of bacteria or other microorganisms. These will be much smaller than the cells and appear as round to elongate “specks” of uniform size between and occasionally within the cells. Specific identification of the microorganisms is usually not possible with the slide (this will be done by culture techniques), but a Gram’s stain will help with classification or the organism into important groups. The use and interpretation of this stain is discussed in standard microbiology texts.

The third step in examining the smear, studying for evidence of neoplasia, is somewhat more difficult, but with practice, accuracy and confidence can be increased. The major problem encountered is that the technician is observing cells with which he or she are not familiar mixed with varying proportions of blood cells. The fact that these are *not* familiar cells is the first indication that the condition may not be a strictly inflammatory response. The technician should be alert for the presence of obviously abnormal cells (e.g., multiple nuclei, extremely large cells, unusual shapes), any cells undergoing cell division (mitosis), or a large number of similar cells, especially if they are in sheets or clusters. These *may* be signs of neoplasia. Under no circumstances should the technician attempt to diagnose the condition, but these types of cells should be described in the report and brought to the attention of the veterinarian. The technician with a special interest in cytology should retain duplicate slides of those sent to the pathologist and restudy the slides while comparing them to the pathology report.

Vaginal Cytology

During the course of the female reproductive cycle, numerous anatomic, physiologic, and behavioral changes occur. These complex neuroendocrine interactions are beyond the scope of this book and are well described in several texts dealing with reproductive physiology. Four stages in the normal estrous (heat) cycle—diestrus, proestrus, estrus, and metestrus—are recognized by characteristic changes that occur during that time period. Only those changes that occur in and around the vaginal cavity and affect the cells seen in cytology preparations are discussed here.

Diestrus is the normal period of reproductive inactivity between heat cycles. The term “anestrus” is used when this time period becomes abnormally long. Since the normal

period of diestrus in the bitch is hard to predict with accuracy, varying from 5 to 12 months, the terms are often used interchangeably. During diestrus/anestrus, the epithelium lining the vaginal cavity is two to four cells thick and essentially nonsecretory, so the cavity will appear dry during sample collection. Cytology smears from diestrus/anestrus will contain plump, round to oval cells with centrally located, round nuclei (the cells are similar in appearance to fried eggs). The nuclei often contain coarsely clumped chromatin interspersed with clear areas (i.e., the nuclei are vesicular). The cytoplasm stains a light to dark blue depending upon the age of the cell. A few of the epithelial cells may have slightly shrunken, darker nuclei and a reddish-purple cytoplasm and appear flattened. Occasional neutrophils and cellular debris may be seen.

In proestrus, the first signs of reproductive activity are seen. The vulva begins to swell, and vaginal secretion begins, which may appear straw colored to red tinged. The bitch begins to attract males but will not allow mating. During proestrus the vaginal lining begins to thicken to several cells thick and a process called keratinization, or cornification, accelerates. This is a process in which a protein called keratin is deposited in the outer layer of epithelial cells. On the slide, these outer cells will appear markedly flattened, with angular, wrinkled margins (somewhat similar to corn flakes), and the nuclei become dark and shrunken (pyknotic) and are often completely absent from the cell. This keratinization (cornification) and thickening of the lining increase during proestrus, increasing the numbers of these cells seen on the slide. Red blood cells are commonly present, often in large numbers, but neutrophils and other white blood cells are usually absent. The length of this stage is variable, and the changes gradual, but signs of proestrus are usually apparent for an average of 7–10 days.

Estrus is the period of time during which the female will allow copulation, and the period is therefore commonly called standing heat. This stage may last from 4 to 14 days with ovulation usually occurring near the end of estrus. The epithelial thickening and cornification of cells is maximal during estrus, and flattened, dark-staining cells with pyknotic or absent nuclei will be the predominant cells seen on the prepared slides. The number of erythrocytes rapidly decreases, and in late estrus, usually within 24–36 hours after ovulation, leukocytes will often increase in number quite noticeably. The technician should also be alert for the presence of sperm cells at this and the next stage, indicating that mating has occurred.

Metestrus is the period of time following estrus when the bitch will no longer accept the male. Physiologic evidence of metestrus may last for 2–3 months but cytologic changes occur more rapidly. The epithelial lining of the vaginal cavity begins to regress in thickness dramatically over the first 2–3 days in the nonpregnant animal and there is a similar rapid drop in the number of cornified cells and total numbers of epithelial cells on the slide. A large number of neutrophils are present in early metestrus and may persist for 1–3 weeks.

The technician should be aware that all these changes occur gradually and represent typical cases. Individual animals will vary somewhat and may not exhibit every cellular change. Familiarity with the normal cells in the cycle will allow the observer to recognize abnormalities in vaginal cytology possibly indicating inflammatory, infectious, or neoplastic conditions. The technician with a special interest in vaginal cytology, or other cytological techniques, should consult reference texts dealing directly with cytology.

Review Questions

1. Name five areas of the body where cytology samples may be obtained.
2. What equipment or supplies are routinely needed for collection of a cytology sample from soft tissue?
3. In the microscopic evaluation of a cytology sample, which cells would the hematology technician be most likely to recognize and what is the significance of these cells?

Glossary

Acanthocyte: (burr cell, spur cell) abnormal erythrocyte with projections on the surface

Acidophil: cell attracting an acid dye, equivalent to eosinophil in hematology

Agglutination: irregular three-dimensional cluster of erythrocytes

Agranulocyte: classification of leukocytes that typically do not contain granules (lymphocytes and monocytes)

Albumin: protein present in plasma, distinct from gamma globulins (antibodies)

Anemia: decrease in oxygen-carrying capacity of the blood due to a decrease in number, volume, or hemoglobin content of erythrocytes

Anisocytosis: unequal cell sizes of erythrocytes

Antibody: specific protein formed by B lymphocytes to inactivate or destroy an antigen

Anticoagulant: substance added to the blood to inhibit coagulation

Antigen: any substance (usually foreign or “nonself”) that stimulates the immune system

Antigen-presenting cell (APC): macrophage that encounters an antigen and stimulates B and T lymphocytes of the immune system

Artifact: any alteration to a blood sample caused by techniques of handling and preparation

Autolysis: natural breakdown of cells and tissues due to internal enzymes

B lymphocyte (B cell): lymphocyte important in the production of antibodies in the humoral immune system (B = bursa equivalent)

Band cell: stage in the development of a neutrophil where the nucleus has parallel sides and the appearance of a band

Barr body (drumstick): club-like appendage on the nucleus of a neutrophil usually found in females

Basophil: granulocytic leukocyte with an elongate nucleus and dark granules

Basophilia: increase in the numbers of basophils in a sample

Basophilic stippling: scattered dark staining areas in an erythrocyte representing abnormally retained RNA

Buffy coat: the white to tan layer between the plasma and erythrocytes in a centrifuged blood sample that contains leukocytes and platelets

Bursa of Fabricius: structure found in birds necessary for functioning of B lymphocytes, mammals have “bursa-equivalent” tissue

Cell-mediated immunity: the immune response mechanism mediated by T lymphocytes and macrophages that inactivate or destroy antigens by cell-to-cell contact

Clotting factors: substances in the blood that interact in the coagulation mechanism to form a blood clot

Coagulation: the process of turning liquid blood into a semisolid gel by activation of clotting factors

Complement: substance in the blood that combines with antigen-antibody complexes to destroy cellular antigens

Crenation: notching and projections on the surface of erythrocytes that is an artifact of drying during preparation of a blood sample

Cytology: the study of cells from body surfaces (exfoliative cytology), drawn from deeper tissues with a needle (aspiration cytology), or surgically removed and pressed onto a slide (impression smears)

Dacryocyte: teardrop shaped erythrocyte

Discocyte: normal disc-shaped erythrocyte

Dohle body: bluish-staining inclusions occasionally seen in the cytoplasm of a neutrophil

Drepanocyte: elongate, thread-like erythrocyte

Echinocyte: crenated erythrocyte

EDTA (ethylenediaminetetraacetic acid): common anticoagulant

ELISA (enzyme-linked immunosorbent assay): type of testing the immune system response

Elliptocyte (ovalocyte): oval to elongate erythrocyte

Embolus: blood clot that moves in the blood stream from its point of formation

Eosinophil: granulocytic leukocyte, usually with a bilobed nucleus and acidophilic (red-staining) granules, also called acidophil

Erythrocyte (red blood cell): hemoglobin contain blood cell responsible for transporting oxygen and carbon dioxide between the lungs and body tissues

Erythrocyte indices: mathematical expressions of the average size and hemoglobin content of erythrocytes (indices = plural of index)

Erythrocytosis: less common term for polycythemia

Erythron: total of circulating erythrocytes, erythrocyte-forming tissue in the bone marrow, and storage areas (mainly in the spleen)

Erythropoietin: hormone that stimulated erythrocyte production

ESR (Erythrocyte Sedimentation Rate): rate at which erythrocytes will settle in a column of blood

Exudate: abnormal body fluid, usually due to inflammation

FA (Fluorescent antibody): test for the presence of an antibody by use of antigens with an attached fluorescent dye

Fibrinogen: protein present in the blood important in blood coagulation

Fibrinolysin (plasmin): enzyme present in the blood that breaks down blood clots

Fusocyte: spindle-shaped erythrocyte that taper to a point at both ends

Gigantocytes: erythrocytes that are more than twice the normal size

Globulin: proteins present in the blood mainly associated with antibody production, gamma globulins (immunoglobulins) are antibody proteins

- Granulocyte:** leukocytes that contain cytoplasmic granules (neutrophils, eosinophils, and basophils)
- Hct (hematocrit):** percentage of erythrocytes in the blood, or the techniques used to calculate this percentage
- Heinz body:** areas of denatured or abnormal hemoglobin in an erythrocyte, also called erythrocyte refractile body (ER body) due to its staining qualities
- Hematopoiesis:** formation of blood cells
- Hemocytometer:** glass instrument with a calibrated grid used for counting blood cells
- Hemoglobin:** protein in the erythrocyte that carries and releases oxygen and carbon dioxide
- Hemoglobinometer:** instrument used to measure the amount of hemoglobin in the blood
- Hemolysis:** release of hemoglobin into the blood due to rupture of cell membranes
- Hemostasis:** the stopping of blood loss
- Howell-Jolly body:** dark staining area in an erythrocyte consisting of a retained portion of a nucleus
- Humoral immunity:** the portion of the immune response mediated mainly by B lymphocytes involving antibody production
- Hyperchromic:** greater than normal coloration
- Hypersegmentation:** formation of greater than normal number of lobes, usually describing a neutrophil with more than five nuclear lobes
- Hypochromic:** less than normal coloration
- Immunocyte:** antigenically stimulated lymphocyte
- Immunoglobulin:** gamma globulin proteins (antibodies) in the blood
- Keratocyte:** erythrocyte with a portion removed, also called helmet cells
- Knizocyte (bar cell):** form of leptocyte with a wide thickened area at the center having the appearance of a bar
- Left shift:** increase in the percentage of immature neutrophil in the blood
- Leptocyte:** thin, flattened erythrocyte, edges may be folded over
- Leukocyte:** any of the five types of nucleated blood cells (white blood cells) usually associated with disease protection—neutrophil, eosinophil, basophil, monocyte, lymphocyte.
- Leukocytosis:** above normal numbers of leukocytes in the blood
- Leukocytopenia (leukopenia):** below normal numbers of leukocytes in the blood
- Lipemia:** presence of fat in the blood
- Lymphoblast:** formative, immature stage of a lymphocyte; term also used to describe regressional stage seen after antigenic stimulation
- Lymphocyte:** agranulocytic leukocyte with a round to indented nucleus filling most of the cell
- Lymphocytosis:** above normal numbers of lymphocytes in the blood
- Lymphocytopenia (lymphopenia):** below normal numbers of lymphocytes in the blood
- Macrocyte:** larger than normal cell size
- Macrophage:** cell that engulf (phagocytize) cells or cellular debris in the body
- Mast cell:** tissue cell important in inflammation, possibly the tissue form of a basophil

- MCH/Mean Corpuscular Hemoglobin:** calculation of the amount by weight of hemoglobin in an erythrocyte
- MCHC/Mean Corpuscular Hemoglobin Concentration:** calculation of the concentration of hemoglobin in an average erythrocyte
- MCV/Mean Corpuscular Volume:** calculation of the average cell volume of erythrocytes
- M:E ratio (myeloid:erythroid ratio):** comparison of the relative numbers of leukocyte and erythrocyte precursors in a bone marrow sample
- Megakaryocyte:** large cell in the bone marrow that produces platelets
- Metamyelocyte:** immature form of a leukocyte
- Microcyte:** smaller than normal cell
- Monoblast:** immature stage of a monocyte
- Monocyte:** agranulocytic leukocyte, often larger than surrounding cells with a lobed or H-shaped nucleus and bluish-staining cytoplasm
- Monocytopenia:** below normal number of monocytes
- Monocytosis:** above normal number of monocytes
- Myeloblast:** immature stage of a leukocyte
- Myelocyte:** immature stage of a leukocyte
- N K (natural killer) cells:** lymphocytes associated with nonspecific immunity, destroy antigens through cell-to-cell contact
- Neoplasia:** unrestricted growth of cells, cancer
- Neutopenia:** below normal number of neutrophils
- Neutophilia:** above normal number of neutrophils
- Neutrophil:** granulocytic leukocyte with a clumped or segmented nucleus and small to medium sized, lightly staining cytoplasmic granules; also called segmenter, PMN/polymorphonuclear
- Null lymphocyte:** lymphocyte antigenically different than a B or T cell
- Osteoblast:** bone-forming cell found in the bone marrow and elsewhere
- Osteoclast:** bone-digesting cell found in the bone marrow and elsewhere
- Ovalocyte (elliptocyte):** oval to elongate erythrocyte
- PCV (Packed Cell Volume):** percentage of erythrocytes in the blood measure after settling or centrifuging
- Pappenheimer body:** areas of iron deposits in an erythrocyte
- Plasma:** liquid portion of unclotted blood
- Plasma proteins:** soluble proteins in the blood, mainly albumin, globulin, and fibrinogen
- Plasmacyte (plasma cell):** activated, antibody producing B lymphocyte
- Platelet (thrombocyte):** cell fragment in the blood important in clotting
- P M N (polymorphonuclear cell):** neutrophil
- Poikilocyte:** abnormally shaped erythrocyte
- Polychromasia:** variation in coloration or staining between erythrocytes
- Polychromatophilia:** variation in coloration or staining within an individual erythrocyte
- Polycythemia:** above normal percentage of erythrocytes in the blood
- Progranulocyte:** immature stage of a granulocyte
- Prolymphocyte:** immature stage of a lymphocyte

- Refractometer:** instrument used for measuring dissolved solids in a liquid based on the refraction (bending) of light
- Reticulocyte:** erythrocyte with irregular network of material in the cytoplasm that is dark staining with vital stains
- Right shift:** increase in the percentage of mature or hypersegmented neutrophils in the blood
- Rouleaux formation:** side-by-side stacking or clusters of erythrocytes
- Schistocyte (schizocyte):** irregular erythrocyte fragment
- Serum:** liquid portion of blood after clotting has occurred
- Siderocyte:** erythrocyte containing light staining areas of iron deposits
- Spherocyte:** erythrocyte with a thickened center, rounded appearance, and often smaller diameter
- Stab cell:** less common name for band cell, immature neutrophil
- Stem cell:** earliest form of blood cells, capable of developing into any of the blood cell types
- Stomatocyte:** form of leptocyte with a curved, central pale area shaped like a mouth
- T lymphocyte (T cell):** lymphocyte that assists B cells in humoral immunity and functions with macrophages in cell-mediated immunity (T = thymus associated)
- Target cell (codocyte):** form of leptocyte with a thicker center, giving it the appearance of a target
- Thoma pipette:** calibrated glass pipette for diluting blood for cell counters
- Thrombin:** protein important in the blood clotting mechanism
- Thrombocyte:** platelet
- Thrombocytopenia:** below normal numbers of platelets
- Thrombocytosis:** above normal numbers of platelets
- Thromboplastin:** protein present in the blood and tissues important in blood clotting
- Thrombus:** blood clot that remains at its point of formation
- Titer:** the concentration of a substance (i.e., antibody or antigen) in a solution that produces a reaction with a known concentration of another test substance
- Transudate:** fluid accumulation in abnormal amounts or locations in the body
- TS meter:** instrument for measuring total solids in a liquid
- Vacuole:** small space or cavity (bubble) in the cytoplasm of cells, commonly seen in monocytes and macrophages
- Wintrobe tube:** calibrated glass tube for determining packed cell volume and sedimentation rate
- Wright stain:** staining method using separate acidic and basic dyes to differentially stain cells and cell contents

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