# 1 Identifying Blood and Bone Marrow Abnormalities in the Laboratory

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## Overview

Automated analysis of peripheral blood samples for complete blood count (CBC) and white blood cell (WBC) differential evaluation was introduced in the early 1950s and since that time has developed into the primary approach by which blood samples are analyzed for cellular constituents and the main avenue through which hematologic abnormalities first come to clinical attention. Prior to that time, the CBC, one of the most commonly ordered laboratory tests, was performed using entirely manual methods. For example, WBC, red blood cell (RBC), and platelet counts were performed using dilution of whole blood samples followed by manual counting under the microscope using a hemocytometer counting chamber; hematocrit was determined by high-speed column centrifugation; and WBC differential analysis always required examination, classification, and enumeration of cells under the microscope. Inarguably, these methods proved very time-consuming in a high-volume laboratory. Furthermore, manual WBC differential analysis is susceptible to errors related to consistency of cell classification between different observers, cell distribution artifact that results in larger cell types (e.g., neutrophils, monocytes, and eosinophils) being spread along the edges of a blood smear, and the inherent statistical limitation that stems from enumerating a relatively small number of cells, typically in the range of 100–200 per sample.

Modern-day hematology instrumentation comprises multichannel analyzers that employ a combination of methods, including electrical impedance, light scatter, radiofrequency conductivity, and/or cytochemistry, to perform cell counts [1]. The technology has evolved to the point that automated approaches to CBC and WBC differential analysis have proven to be efficient and cost-effective, as well as accurate and reliable in detecting clinically significant abnormalities. However, automated cell counters come with their own sources of error, confounding variables, and artifacts that may impede in the timely detection of peripheral blood abnormalities or result in falsely abnormal test results. In addition, it is generally accepted that these instruments serve a dual purpose as both diagnostic tools and screening devices, the latter implying that certain numerical abnormalities or analyzer-specific operator alerts (also known as "instrument flags") should trigger microscopic blood smear review by a skilled laboratory technologist or pathologist, particularly at the time of their initial occur-

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S.A. Wang, R.P. Hasserjian (eds.), *Diagnosis of Blood and Bone Marrow Disorders*,
https://doi.org/10.1007/978-3-319-20279-2_1

rence. For these reasons, a general understanding of the methods underlying automated CBC and WBC differential analysis is critical to a sound diagnostic approach to disorders involving the blood and, by extension, originating from the bone marrow.

This chapter provides an overview of automated analysis of whole blood constituents, including RBCs, platelets, and WBCs, with a focus on interferences, sources of error, and findings for which microscopic review or confirmation should be considered. In addition, situations in which peripheral blood flow cytometric analysis may be warranted will be addressed. Finally, appropriate preparation and microscopic evaluation of peripheral blood and bone marrow aspirate smears and trephine biopsies will be discussed.

## Analysis of Red Blood Cells and Associated Parameters

The standard CBC includes the following RBC parameters: RBC count, hemoglobin concentration (HGB) measured in grams/deciliter (g/dL), hematocrit (HCT), mean corpuscular volume (MCV) measured in femtoliters (fL), mean corpuscular hemoglobin (MCH) measured in picograms/cell, mean corpuscular hemoglobin concentration (MCHC) measured in g/dL, and red cell distribution width (RDW). Reference ranges vary by age and gender and are readily obtained by consulting reference hematology or laboratory medicine texts [1]. Typical reference ranges for adults are listed in Table 1.1. However, it is recommended that individual laboratories confirm standard reference ranges for the specific patient population(s) they serve, as reference ranges for certain parameters may be affected by ethnic and geographic variations.

Most automated cell counters measure HGB using a spectrophotometric method, following RBC lysis and conversion of the HGB molecule to a derivative that can be measured using light absorbance at a specific wavelength. Historically, this process has required the use of potassium cyanide to convert HGB to hemiglobincyanide; however, cyanide-free measurement methods now exist [1]. RBC count is determined in many instances via electrical impedance, whereby cells

**Table 1.1** Red blood cell indices: calculation, normal ranges, and interpretation in patients with anemia

| Red blood cell (RBC) parameter | Definition/calculation | Normal range[a] | Abnormal values and some common associations |
|---|---|---|---|
| **Mean corpuscular volume (MCV) (fL)** | Average size of RBCs =HCT/RBC × 10 | 80–100 | **Decreased MCV and decreased MCH:** iron deficiency, lead poisoning, thalassemia |
| **Mean corpuscular HGB (MCH) (pg)** | Amount of HGB per RBC =HGB/RBC × 10 | 26–33 | **Normal MCV and normal MCH:** sudden blood loss, chronic inflammation, kidney failure, pregnancy<br>**High MCV:** vitamin B12 or folate deficiency, certain drugs, MDS, hemolysis |
| **Mean corpuscular HGB concentration (MCHC) (g/dL)** | Amount of HGB per cell relative to RBC size =HGB/HCT × 100 | 31–36 | **Decreased:** lead poisoning, vitamin B6 deficiency, copper deficiency<br>**Normal:** iron deficiency<br>**Increased:** hereditary spherocytosis, sickle cell disease, and homozygous hemoglobin C disease |
| **Red cell distribution width (RDW) (%)** | Variability in RBC size = coefficient of variation or standard deviation of MCV × 100% | 12–15 | **Increased:** recent transfusion, iron deficiency, vitamin B12 or folate deficiency, RBC fragmentation or agglutination, sideroblastic anemia, MDS<br>**Decreased:** Not clinically relevant |

*HCT* hematocrit, *MDS* myelodysplastic syndrome, *HGB* hemoglobin
[a]May vary by patient population and between laboratories

passing through a narrow aperture across which an electrical current is maintained cause changes in resistance, which can be measured in terms of pulse amplitude (corresponding to cell volume and size) and number of pulses (corresponding to the number of cells in a solution of known volume). Other instruments utilize purely optical-based methods of light scatter (allowing for determination of cell size and other physical properties) to enumerate RBCs, but regardless of the exact method used, an RBC volume distribution curve, histogram, or scatter plot is typically generated (Figs. 1.1a and 1.2a).

HCT, MCV, and RDW can be derived from the RBC volume histogram, depending on the type of technology used by the instrument. For example, some impedance-based instruments directly measure the HCT by summing the heights of all individual RBC pulses as each RBC passes through the aperture, a process known as cumulative pulse height detection. Once the HGB, RBC count, and HCT are known, the remaining RBC parameters, including MCV, can be calculated using the following formulae:

- MCV = HCT/RBC × 10
- MCH = HGB/RBC × 10
- MCHC = HGB/HCT × 100

Alternatively, other instruments may directly measure the MCV using an optical-based method, allowing for calculation of the HCT and other RBC parameters based on the MCV, RBC count, and HGB, as shown above. The RDW is another parameter calculated from the RBC volume distribution curve as either the coefficient of variation (CV) or the standard deviation (SD) of red cell size, thereby reflecting the degree of anisocytosis in an RBC population. It is helpful in the evaluation of anemia, particularly in distinguishing common causes of microcytic anemia from one another, as it is typically markedly elevated in the setting of recent transfusion or iron deficiency but only slightly elevated in thalassemia trait or anemia of chronic inflammation (Table 1.1). Knowledge of which RBC parameters are directly measured vs. calculated by a given analyzer is important, since factors causing

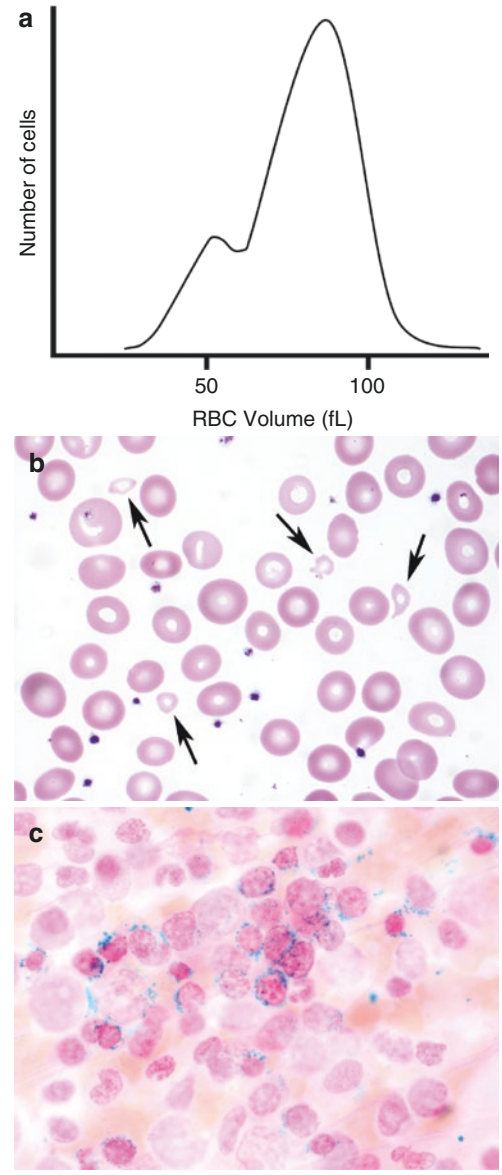


**Fig. 1.1** Sideroblastic anemia with bimodal RBC volume distribution curve. (**a**) In this case of myelodysplastic syndrome with ring sideroblasts, the RBC volume distribution curve demonstrates a classic bimodal distribution with two distinct RBC populations, one that is microcytic and a second that is normocytic to macrocytic. (**b**) The corresponding peripheral blood smear demonstrates significant anisocytosis with a population of microcytic poikilocytes (arrows). This image also illustrates the ideal area of the blood smear for performing morphologic evaluation and platelet count estimates, where RBCs are evenly spaced and barely touching one other, although overlapping of two to three cells is acceptable. (**c**) Perls' iron stain of the corresponding bone marrow aspirate smear contains numerous ring sideroblasts, accounting for greater than 50% of erythroid precursors.

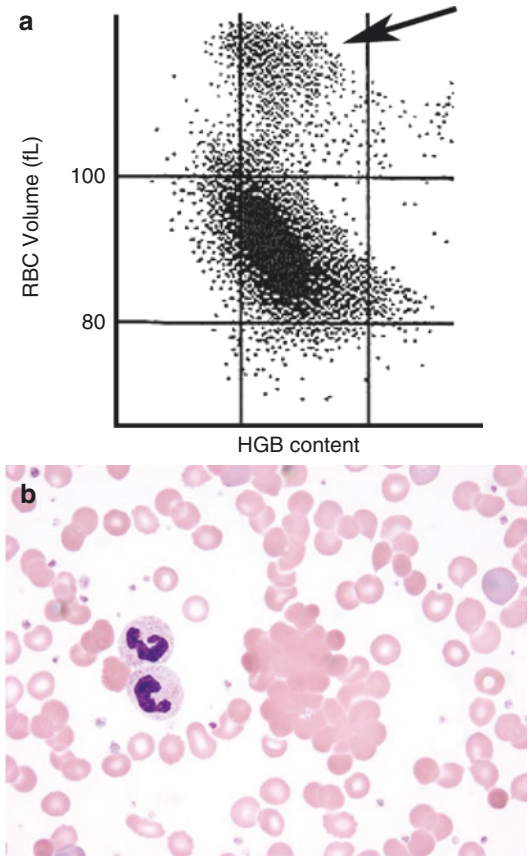


**Fig. 1.2** RBC agglutination in cold agglutinin disease. (**a**) This scatter plot is taken from an optical hematology analyzer that measures RBC indices using light scatter following isovolumetric sphering of RBCs. Each dot represents a single RBC on a plot of volume vs. HGB content. In this example of cold agglutinin disease, RBC agglutinates appear as a separate cell population with very high volumes (arrow). (**b**) The corresponding blood smear shows prominent RBC agglutination

spuriously elevated or low counts can potentially affect the measured parameter, as well as indices deriving from it (Table 1.2).

The shape of the RBC volume histogram is usually Gaussian in nature, and changes in its symmetry can provide information about potential abnormalities present in a blood sample, prompting additional analysis and/or microscopic smear review. For example, a left-sided extension of the curve, or failure of the histogram to reach baseline on the left side between the platelet and RBC channels, may indicate the presence of a population of small RBCs, such as microspherocytes or RBC fragments, or may reflect the presence of large or giant platelets. A bimodal RBC volume distribution, in which two RBC populations of distinct sizes are present, may reflect prior transfusion or an underlying sideroblastic anemia (Fig. 1.1). A right-sided extension of the histogram may be seen in the setting of macrocytic anemia or reticulocytosis secondary to hemolysis. Such findings will often trigger an instrument flag indicating an abnormality in the RBC volume distribution, and laboratories should have mechanisms in place to review blood smears for underlying morphologic findings that may be clinically significant, such as schistocytes, microcytic spherocytosis, macrothrombocytes, or marked polychromasia. By contrast, low or high MCV values in the absence of RBC volume distribution abnormalities likely do not need to be confirmed by microscopic review. Another situation requiring additional investigation by the laboratory is that of RBC agglutination, which will result in extension of the RBC volume histogram to the extreme right, yielding an apparent population of very large RBCs and spuriously high MCV and MCHC instrument measurements (Table 1.2 and Fig. 1.2). In this circumstance, repeat analysis after sample warming should be undertaken; if the abnormalities do not correct after incubation at 37 °C, HCT may be determined manually via high-speed column centrifugation, but other RBC parameters are not considered reliable and should not be reported.

Measurement of reticulocytes, nonnucleated immature red cells recently produced by the bone marrow that contain high amounts of RNA, is a highly useful test in the diagnostic work-up of anemia by helping to distinguish causes related to impaired bone marrow production of erythrocyte precursors (associated with a low reticulocyte count) from those related to blood loss or hemolysis (associated with a high reticulocyte count). Traditionally, it is determined manually by incubating a peripheral blood smear with a supravital dye, either new methylene blue or brilliant cresyl blue, allowing for dark blue staining of the filamentous ribonucleoprotein complex contained within reticulocytes followed by their microscopic

**Table 1.2** Causes of spurious results with automated hematology analyzers [1]

| Parameter | Spurious increase | Spurious decrease |
|---|---|---|
| **WBC count** | • Cryoglobulins<br>• Monoclonal proteins<br>• Nucleated RBCs<br>• Platelet clumps<br>• Unlysed RBCs | • Clotted sample |
| **RBC count**[a] | • Cryoglobulins<br>• Macrothrombocytes<br>• Markedly high WBC count | • Clotted sample<br>• In vitro hemolysis<br>• Microcytic or fragmented RBCs<br>• RBC agglutination |
| **HGB**[a] | • Cryoglobulins<br>• Hyperbilirubinemia<br>• In vitro hemolysis<br>• Lipemia<br>• Monoclonal proteins<br>• Markedly high WBC count | • Clotted sample |
| **HCT**[a] | • Cryoglobulins<br>• Hyperglycemia<br>• Macrothrombocytes<br>• Markedly high WBC count | • Clotted sample<br>• In vitro hemolysis<br>• Microcytic or fragmented RBCs<br>• RBC agglutination |
| **MCV**[a] | • Hyperglycemia<br>• Markedly high WBC count<br>• Old sample (swollen RBCs)<br>• RBC agglutination | • Cryoglobulins<br>• In vitro hemolysis<br>• Macrothrombocytes<br>• Microcytic or fragmented RBCs |
| **Platelets** | • Cryoglobulins<br>• In vitro hemolysis<br>• Fragmented WBCs<br>• Microcytic or fragmented RBCs<br>• RBC inclusions | • Clotted sample<br>• Macrothrombocytes<br>• Platelet clumps or satellitosis |

*WBC* white blood cell, *RBC* red blood cell, *HGB* hemoglobin, *HCT* hematocrit, *MCV* mean corpuscular volume
[a]Depending on the analyzer and the parameters that are directly measured vs. calculated, factors that affect these parameters may also affect other RBC parameters listed here, as well as the mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC)

identification and enumeration [1]. The reticulocyte count is subsequently reported as a percentage of total RBCs, based on a count of at least 1000 red cells, and typically falls in the range of approximately 1–2%. The absolute reticulocyte count is calculated by multiplying this percentage by the RBC count. More recently, automated cell counters have used optical methods employing fluorescent RNA-specific binding dyes to measure reticulocytes, precluding the need for more time-consuming microscopic evaluation and allowing for increased precision and accuracy. Regardless of whether the reticulocyte count is determined manually or via an automated method, it is important to note that the value reported by the laboratory is uncorrected for the degree of anemia and the longer circulating lifespan of reticulocytes in anemic states because of accelerated release from the bone marrow. Therefore, when evaluating an anemic patient, the reported reticulocyte count must be corrected for these two factors to yield a reticulocyte production index that can be used to help determine the cause underlying the anemia [2]. In order to calculate the reticulocyte production index from the reported reticulocyte count, first multiply the reported reticulocyte count by a correction factor derived from the patient's hematocrit divided by the expected normal hematocrit (typically 40% for women and 45% for men) to yield a corrected reticulocyte count. Then, adjust the corrected reticulocyte to account for the longer circulating lifespan of early reticulocytes. The exact adjustment depends on the degree of anemia, but a simple way is to divide the corrected reticulocyte

count by two (which presumes that in anemic states, reticulocytes circulate for about 48 hours, compared to about 24 hours in non-anemic states). For example, a male patient with a hematocrit of 15% and a reported reticulocyte count of 6% would have a corrected reticulocyte count of 2% (6%×15%/45%) and a reticulocyte production index of 1 (2%/2). In general, anemia due to impaired marrow production is associated with a reticulocyte production index of <2, while anemia related to blood loss or hemolysis has a reticulocyte production index of >2.

Automation of reticulocyte counts using optical methods has allowed for the development of additional, novel laboratory parameters that may have utility in various clinical circumstances. For example, the HGB content of reticulocytes (CHr or Ret-He) has been reported to be a useful screening test for early iron deficiency in both children and adults and has been identified as beneficial in monitoring the adequacy of iron therapy in iron deficiency anemia [3–8]. The immature reticulocyte fraction, indicating the proportion of reticulocytes with highest RNA content, has been heralded as potential laboratory indicator of bone marrow response to erythropoietin or exogenous iron therapy [9, 10]. More complex studies of RBC population dynamics enabled by optical hematology instrumentation have identified characteristics associated with certain anemic conditions and pre-anemic states, allowing for predictive modeling prior to the clinical development of anemia [11–13]; while such strategies have yet to be employed routinely, they illustrate the potential utility of systems-based approaches to diagnose and monitor hematologic diseases.

## Analysis of Platelets and Associated Parameters

Although numerous methods exist for platelet enumeration, including manual, immunologic, and digital image analysis, most automated cell counters rely on impedance, optical light scatter, or a combination of the two. Impedance-based analysis is analogous to that described above for RBC analysis, where particles within a certain size range generate a platelet count, as well as volume distribution curve or histogram based on which possible impediments in count accuracy are flagged by the instrument. For example, failure of the platelet histogram to reach baseline at the low end or left side of the curve is an indicator of cytoplasmic fragments or electronic noise, while a similar pattern on the opposite side implies the presence of macrothrombocytes or microcytic RBCs. In these circumstances, microscopic smear review to estimate the platelet count (see the following section on Peripheral Blood Smear Evaluation) and compare it with that generated by the instrument is warranted. Optical counts rely on the light scatter properties of platelets and may incorporate a platelet-specific fluorescent dye, thereby limiting interference from cytoplasmic fragments or microcytic RBCs. Some platforms employ both an impedance and optical count, such that the latter is performed in instances when there is sufficient doubt about the accuracy of the impedance count. Immunologic counting of platelets using light scatter and platelet-specific antibodies, such as anti-CD41 or anti-CD61, is considered the reference method of platelet enumeration and demonstrates high accuracy, particularly in cases of severe thrombocytopenia [14–17].

An issue that interferes with all three of these methods is that of ethylenediaminetetraacetic (EDTA)-dependent agglutinins leading to platelet

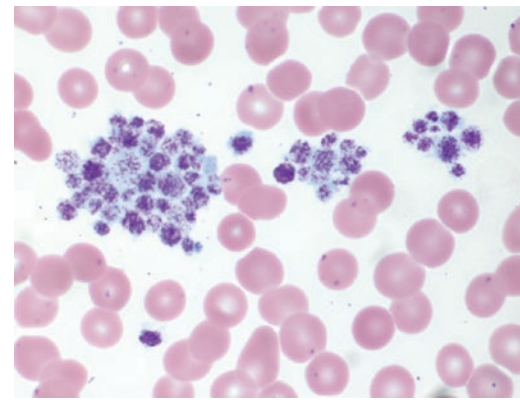

**Fig. 1.3** Pseudothrombocytopenia with platelet clumping. In this patient with presumed immune thrombocytopenic purpura, review of the peripheral blood smear was performed because of the low platelet count; this revealed prominent platelet clumps, indicating that the instrument platelet count is falsely low. The platelet clumps are much larger than individual platelets that they are typically counted as WBCs by automated instruments

clumping or satellitism and underestimation of the platelet count, a phenomenon known as pseudothrombocytopenia (Table 1.2 and Fig. 1.3). Although automated instruments can flag for the presence of platelet clumps (see the following section on Analysis of White Blood Cells), an initial low platelet count below a certain threshold that approaches clinical significance should elicit smear review to exclude for the presence of platelet clumps. In circumstances of severe clumping, the instrument count is deemed unreliable, and an estimate based on smear review may also be limited in terms of accurately quantifying platelets, particularly if platelet transfusion is being considered. In such scenarios, remeasuring the platelet count on a sample collected in citrate- or heparin-anticoagulated plasma may be an option; however, in some individuals, platelet clumps persist despite collection using alternative anticoagulants. In such cases of multianticoagulant-dependent pseudothrombocytopenia, treatment of the sample with amikacin has been reported to dissociate platelet clumps, allowing for accurate platelet enumeration without affecting other CBC parameters [18].

Additional platelet-related parameters generated by certain automated cell counters include the mean platelet volume (MPV) and immature platelet fraction (IPF), both of which may help determine the underlying cause of thrombocytopenia (Table 1.3). The MPV, measured in fL, represents the average platelet size and is analogous to the MCV for RBCs, but unlike the MCV, the MPV normal range varies depending on the underlying technology used for measurement (impedance vs. optical) and is therefore instrument-specific. Under normal conditions, when the marrow and spleen are functioning appropriately, platelet size correlates inversely with number, such that the MPV will be elevated in conditions of destructive thrombocytopenia (e.g., immune thrombocytopenic purpura and microangiopathy). Other conditions associated with a high MPV include certain congenital thrombocytopenias, such as Bernard-Soulier syndrome, May-Hegglin anomaly, and gray platelet syndrome, some cases of myelodysplastic syndrome (MDS), and hyposplenism or asplenia (due to the tendency of the spleen to sequester larger platelets). In contrast, a low MPV may be seen in thrombocytopenia secondary to marrow hypoplasia or aplasia, splenomegaly, and congenital disorders, such as Wiskott-Aldrich syndrome. An abnormal MPV in the absence of bleeding or thrombocytopenia is unlikely to be clinically significant. It should also be noted that MPV may be affected by preanalytical variables related to the decreased stability of platelet shape immediately after collection and the propensity for platelets to swell in EDTA after prolonged exposure. Therefore, appropriate evaluation of a persistently abnormal MPV includes peripheral smear review to look for morphological clues,

**Table 1.3** Mean platelet volume (MPV) and immature platelet fraction (IPF) in patients with thrombocytopenia

| Parameter | Definition | Normal range | Abnormal values and possible associations |
|---|---|---|---|
| Mean platelet volume (MPV) (fL) | Average platelet size | Instrument-specific | **Increased:** destructive thrombocytopenia (such as ITP and TTP); congenital thrombocytopenias, such as Bernard-Soulier syndrome, May-Hegglin anomaly, and gray platelet syndrome; some cases of MDS; hyposplenism or asplenia<br>**Decreased:** marrow hypoplasia or aplasia, splenomegaly, Wiskott-Aldrich syndrome |
| Immature platelet fraction (IPF) (%) | The fraction of reticulated platelets newly released by the bone marrow | 1–7 | **Increased:** peripheral platelet destruction (e.g., ITP, TTP, DIC); recovering marrow after chemotherapy and stem cell transplant<br>**Decreased:** bone marrow failure; MDS; bone marrow suppression due to infection, chemotherapy, or other drugs |

*ITP* immune thrombocytopenic purpura, *DIC* disseminated intravascular coagulation, *MDS* myelodysplastic syndrome, *TTP* thrombotic thrombocytopenic purpura

such as RBC fragments, abnormal leukocyte inclusions, hypogranular platelets, or dysplastic neutrophils, suggesting one of the above diagnoses. The MPV may also be used in conjunction with the IPF or the reticulated platelet count in distinguishing conditions associated with impaired platelet production from those related to accelerated destruction, as the latter typically show an elevated IPF [19, 20]. Analogous to reticulocytes, reticulated platelets represent young platelets recently released by the marrow with a higher RNA content that allows them to be identified optically on some platforms with the aid of a platelet-specific binding reagent.

## Analysis of White Blood Cells and Nucleated Red Blood Cells

Most hematology analyzers provide automated WBC differential counts that include, at a minimum, the five main leukocyte subsets: neutrophils, lymphocytes, monocytes, eosinophils, and basophils. Depending on the platform, these are determined using a combination of technologies, including impedance (direct current and radiofrequency conductivity) and/or optical light scatter, in conjunction with various cell-specific lysing and staining reagents. Automated instruments provide both relative and absolute counts for WBC subsets; the latter are considered more clinically meaningful and should be included on CBC/differential reports issued by the laboratory [21]. Many newer analyzers also provide quantification of nucleated RBCs (in both relative [per 100 WBCs] and absolute units) with correction of total WBC count, since nucleated RBCs lead to spuriously high WBC counts when present in significant numbers (Table 1.2). Traditionally, the presence of any circulating nucleated RBCs has been considered to be an abnormal finding; however, the high sensitivity offered by modern automated cell counters has led to the recognition that small numbers of nucleated RBCs may be seen in healthy individuals without implying underlying pathology, particularly if other CBC parameters are normal [22]. Quantification of immature granulocytes (IGs) is another novel parameter offered by certain platforms; it represents the combined total number of circulating promyelocytes, myelocytes, and metamyelocytes. Studies have demonstrated good correlation with reference flow cytometry-based methods and suggest that elevated levels may be useful as an indicator of infection and other conditions related to bone marrow stress in both adult and pediatric populations [23–25]. At the very least, it likely represents a more objective and less time-consuming method of detecting a granulocytic "left shift" over traditional band neutrophil counts, which suffer from lack of reproducibility and limited sensitivity and specificity [26].

As with RBC and platelet analysis, cell counters act as screening devices for WBC abnormalities that fall out of expected parameters based on impedance, light scatter, and staining characteristics. Instrument flags are generated for the possible abnormalities that require peripheral blood smear review to confirm or exclude their presence. These include signal interference with the lymphocyte cluster by similarly sized cells or particles (e.g., nucleated RBC or platelet clump flags), interference at the interface of the lymphocyte and monocyte clusters (e.g., atypical lymphocytes or blast flags), interference at the interface of the monocyte and neutrophil clusters (e.g., blast flag), or altered positioning of the neutrophil cluster (e.g., left shift or immature granulocyte flags). Smears are typically reviewed by a medical technologist with training and expertise in peripheral blood smear morphology and cell classification. When abnormal cells such as blasts or atypical lymphocytes are identified, a traditional 100- or 200-cell manual differential count should be performed and resulted, supplanting the automated differential count. Smear review by the laboratory may also be considered when certain numerical thresholds are met or exceeded. Examples of the latter include absolute monocytosis or basophilia, moderate to severe pancytopenia that is new for a patient, or an absolute lymphocytosis in a child or adolescent to exclude the possibility of small lymphoblasts being erroneously categorized as lymphocytes by the analyzer. In addition, each laboratory should establish its own criteria for escalating a case further for pathologist review. Typically, this is performed in a subset of cases in which the findings are believed to represent a significant change from the patient's baseline that may be clinically significant (e.g., circulating blasts

or abnormal lymphoid cells suggesting the possibility of a previously undiagnosed or recurrent hematologic malignancy). However, these criteria vary depending on the complexity of the patient population served by a given laboratory.

While automated and manual differential analysis is useful in detecting abnormal circulating cell populations, immunophenotyping by flow cytometry is best used as an ancillary test to confirm CBC/differential findings and to determine the lineage of the abnormal cells [27]. For example, flow cytometry can help to establish a diagnosis of chronic lymphocytic leukemia in the setting of marked absolute lymphocytosis or can distinguish myeloid from lymphoid blasts in a case of previously undiagnosed acute leukemia. Flow cytometry is also helpful in situations requiring minimal residual disease evaluation, where neither automated cell counter-flagging nor morphological enumeration is sufficiently sensitive to detect small abnormal circulating populations. However, flow cytometry is unlikely to provide additional information over a manual differential count in cases of marked granulocytic hyperplasia with left shift, where the differential diagnosis includes a leukemoid reaction vs. chronic myeloid leukemia in chronic phase; in both instances, the small myeloid blast population is identifiable morphologically, and its neoplastic potential is not discernible by flow cytometry. Flow cytometry should also not be relied upon as a substitute for the morphologic blast count in the peripheral blood, since blasts may deviate from expected immunophenotypic characteristics (e.g., CD34 and CD117 negativity) [28].

## Peripheral Blood Smear Evaluation

The number of samples requiring manual slide review greatly influences laboratory costs, productivity, and turnaround time. At the very least, manual smear review should be undertaken when instrument flags are triggered by abnormalities in the distribution of cells and/or particles within the RBC, PLT, and WBC analysis channels, as described previously (Table 1.4). In addition, some suggested numerical thresholds prompting smear review are also provided in Table 1.4, based in part

**Table 1.4** Suggested criteria for peripheral blood smear review based on automated count and instrument flags

| Numerical abnormalities | Threshold |
|---|---|
| Pancytopenia | WBC $<4 \times 10^9$/L and/or ANC $<1 \times 10^9$/L and/or HGB <7 g/dL and/or PLT $<100 \times 10^9$/L |
| Initial platelet count | $<50 \times 10^9$/L (to rule out pseudothrombocytopenia due to platelet clumping or satellitism) |
| Lymphocytes | $>5 \times 10^9$/L |
| Monocytes | $>1.5 \times 10^9$/L |
| Eosinophils | $>2 \times 10^9$/L |
| Basophils | $>0.5 \times 10^9$/L |
| Immature granulocytes[a] | >5% |
| **Instrument flags** | |
| Dimorphic red blood cell population | |
| RBC fragments or abnormal distribution | |
| Nucleated RBCs[b] | |
| Platelet clumping | |
| Giant platelets or abnormal platelet distribution | |
| Immature granulocytes[b] | |
| Variant lymphocytes | |
| Blasts | |

*ANC* absolute neutrophil count, *HGB* hemoglobin, *PLT* platelet, *RBC* red blood cell, *WBC* white blood cell
[a]For analyzers that provide an automated immature granulocyte count
[b]May not be necessary for analyzers that can enumerate these cells, unless the count is flagged or exceeds a certain threshold

on consensus recommendations published by the International Society for Laboratory Hematology [29]. The latter may be modified by individual laboratories based on the automated platform in use, patient population served, clinician input, and following validation on patient samples.

The most commonly used stain for morphologic evaluation of the peripheral blood smear is the Wright or Wright-Giemsa stain. This staining protocol contains methanol, which fixes the cells to the glass slide, and both acidic and basic dyes that are variably taken up by cellular constituents at a pH of 6.4 maintained by a buffer, allowing for recognition of distinct cell and particle types at different stages of maturation. Best staining results are obtained from slides prepared within a few hours of sample collection [30]. Proper blood smear preparation and staining are essential to accurate assessment of cellular morphology and detection of abnormalities, particularly when evaluating neutrophil and platelet granularity (Fig. 1.4) [28]. Table 1.5 lists artifacts related to poorly prepared or stained smears and possible causes.

Peripheral smear examination should occur sequentially at low (×10 objective, ×100 total), intermediate (×40 high dry or ×50 oil immersion objective, ×400 or ×500 total), and high (×100 oil immersion objective, ×1000 total) magnifications. Low magnification allows for assessment of overall smear quality, including identification of artifacts (Table 1.5), as well as abnormalities in the distribution of RBCs and platelets such as rouleaux or clumps, while higher magnifications allow for assessment of individual cellular morphology and estimation of the platelet count in situations where the count provided by the instrument requires verification (see previous section on Analysis of Platelets and Associated Parameters) [30]. WBC abnormalities can be suspected at intermediate magnification but should be confirmed at high magnification. In addition, if a formal manual leukocyte differential is undertaken because abnormal WBCs are identified, this should generally be performed at high power using the ×100 objective (×1000 total magnification). The appropriate area for leukocyte evaluation and classification resides in the region of the smear where the RBCs are arranged as a monolayer, evenly spaced, and are close to one another but not quite touching (Fig. 1.1b); in a sample with a normal RBC count, this area contains about 200–250 RBCs per ×100 oil immersion field. This ensures that WBC evaluation is performed in an area of the smear where leukocyte morphology is optimal and devoid of artifacts seen in thick (dark, shrunken cells) or thin (fragmented cells) areas of the smear. To allow for systematic differential analysis and minimize slide distribution error (the phenomenon whereby larger cells, such as monocytes, granulocytes, and blasts, tend to localize at the edges of the smear), the smear should be examined from one edge to another in a sweeping up and down pattern without skipping areas, and consecutive WBCs should be counted [30]. Typically, 100 WBCs are counted, but a count of 200 cells is recommended if the WBC count is moderately elevated (>40 × $10^9$/L) or in patients with a myeloid neoplasm [28]. Preparation of buffy coat smears from the peripheral blood [31] or digital image analysis (see below) can permit adequate cell enumeration in leukopenic samples. Nucleated red blood cells should be counted separately as a relative count (per 100 WBCs); however, if the instrument can report a valid numerical nucleated RBC count, then repeating the count manually is unnecessary.

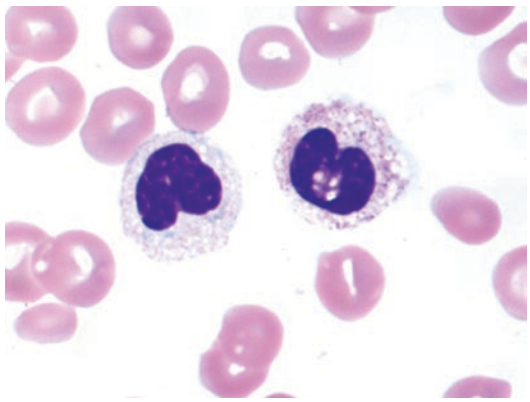

**Fig. 1.4** Dysplastic neutrophils in a case of myelodysplastic syndrome. In this image, the cytoplasm of the neutrophil on the left is hypogranular compared to that on the right. Other fields demonstrated nuclear lobation abnormalities within neutrophils. RBCs are well-stained, indicating that the hypogranular cytoplasm is not simply an artifact of suboptimal, pale staining

The platelet count estimate, if needed, may be performed in the same region of the smear where leukocytes are evaluated, also under ×1000 total magnification. The average number of platelets,

**Table 1.5** Peripheral blood smear artifacts and their causes [30]

| Issue(s) | Possible cause(s) |
|---|---|
| Dark stain:<br>• RBCs appear gray in color<br>• WBCs too dark in color<br>• Eosinophil granules appear gray instead of orange | • Stain or buffer too alkaline<br>• Inadequate rinsing<br>• Prolonged staining<br>• Heparinized blood sample |
| Pale stain:<br>• RBCs pale or red in color instead of pink<br>• WBCs barely visible | • Stain or buffer too acidic<br>• Over-rinsing<br>• Inadequate buffering |
| Insufficient air-drying artifact:<br>• RBCs contain refractile or punched-out central area | • Slide not allowed to dry sufficiently prior to staining<br>• Equipment used to make slide is wet or contains condensation |
| Stain precipitate on slide | • Old or contaminated staining reagents<br>• Equipment used to make slide is dirty or contains staining reagent residue |
| Smear too short; covers less than two-thirds of slide length | • Size of blood drop used to make smear too small<br>• Elevated HCT in blood sample |
| Feathered edge not smoothed and rounded but irregular or bullet-shaped | • Spreader slide pushed too quickly |
| Smear contains irregularities, holes, or streaks | • Spreader slide chipped or rough-edged<br>• Hesitation or uneven pressure in forward motion of spreader slide<br>• Dirt on slide used for smear |
| Smear too narrow; does not span nearly the whole width of the slide | • Drop of blood not allowed to spread across width of spreader slide |
| Poor leukocyte distribution (monocytes and granulocytes mostly at edges of smear) | • Spreader slide pushed too slowly |

*RBC* red blood cell, *WBC* white blood cell, *HCT* hematocrit

based on counting five to ten ×100 oil immersion fields, is multiplied by 20,000 to provide an estimate of the total number of platelets per microliter [30]. This result can then be compared to the instrument count, and if the instrument count is deemed inaccurate, the estimated platelet count may be reported as decreased, normal, or elevated per the laboratory's reference range. Caution should be exerted in reporting platelet count estimates numerically, particularly in cases of severe thrombocytopenia where platelet transfusion may be a consideration.

As with CBC and WBC differential analysis, peripheral smear preparation and evaluation have entered the age of automation. Many automated hematology platforms now offer the convenience and advantages of automated slide preparation and staining systems. These so-called auto slide maker stainers aspirate an aliquot of EDTA-anticoagulated whole blood and prepare a wedge-type blood smear similar to a manual smear prepared using the traditional push/pull method. In addition, instrument software systems, known as "middleware," allow for optimization of smear preparation using HCT information from the blood sample, thereby minimizing smearing artifacts resulting from abnormalities inherent to a sample (Table 1.5). Once dried, the smear is automatically advanced to the staining area of the instrument where stain and buffer are dispensed onto the slide per the staining protocol. In many instances, the slide maker stainer is physically connected to the main cell counter on a robotic track or line, allowing for smears to be prepared automatically on only those samples requiring microscopic review according to predetermined numerical or instrument flagging criteria and minimizing the need for operator intervention. These instruments are also flexible, allowing for staining of previously made blood smears or other sample types, such as body fluid cytospin and bone marrow aspirate smears, depending on the preferences and needs of the laboratory.

More recently, digital image analysis of prepared, stained blood smears via platforms such as CellaVision (CellaVision AB, Lund, Sweden) provides an opportunity for laboratories to ensure interobserver consistency in reporting practices and potentially to improve sensitivity in the detection of rare cell types [32]. These automated slide scanning devices help to locate and preclassify (via an artificial neural network) WBCs and nucleated RBCs and allow for evaluation of larger fields of view for characterization of RBC morphology and estimation of platelet counts. Images of pre-classified WBCs require confirmation by a trained morphologist (usually a medical technologist) prior to finalization of the manual differential count. The instrument software allows for side-by-side morphologic comparison of different groups of leukocytes present in the same smear, making it relatively easy to distinguish two or more populations with overlapping characteristics from one another (e.g., monocytes and variant lymphocytes). Laboratories can also create reference libraries of images illustrating typical or unusual findings, underscoring the potential educational utility of such platforms. Studies of this technology have demonstrated very good correlation with traditional 100-cell manual differentials, with superior accuracy depending on cell type and operator experience [33, 34]. Digital image analysis also allows for counting a larger number of cells in leukopenic samples, a frequent issue in posttreatment settings, improving sensitivity and turnaround times [34, 35].

Despite these advances in hematology automation, laboratory personnel and pathologists need to be cognizant of situations requiring procedural adjustments. For example, we have found in our laboratory that samples with extremely high WBC counts exceeding $400 \times 10^9$/L are poorly stained, making it difficult to evaluate cellular stage of maturation and cytoplasmic granularity and leading to a high rate of WBC misclassification on digital image analysis and traditional microscopic review. In such cases, our laboratory manually prepares the smear following dilution of the sample, allowing leukocytes to take up sufficient amounts of stain on the automated stainer and more accurate cell classification. Another example relates to the presence of large numbers of smudge cells, a characteristic feature of chronic lymphocytic leukemia, but also seen in other conditions associated with leukocytosis. Since certain cell types have greater fragility and propensity to rupture during the smearing process, basing a leukocyte differential on a sample with many smudge cells can lead to erroneous counts. Manual preparation of the blood smear following sample dilution with bovine serum albumin can minimize cell fragmentation and ensure evaluation of sufficient numbers of intact cells for a more accurate differential [36].

## Bone Marrow Aspirate Evaluation

Unlike the situation described in the previous sections, preparation, staining, and evaluation of bone marrow aspirate are largely manual processes. Air-dried bone marrow aspirate smears may be prepared at the bedside or from the buffy coat layer of an EDTA-anticoagulated aspirate sample, in a manner similar to peripheral smears. Alternatively, marrow particles can be identified grossly and crushed between two slides (crush preparation), or touch imprints of marrow particles or of the core biopsy specimen can be performed; the latter is particularly helpful in the setting of bone marrow fibrosis eliciting a "dry tap" with collection of limited aspirate material. Smeared marrow particles (spicules) and fat droplets can be visualized on an unstained slide with the naked eye, providing assurance of bone marrow, rather than peripheral blood, sampling [1]. A standard Romanowsky-type staining method, such Wright-Giemsa or May-Grünwald-Giemsa, should be employed for routine morphologic analysis. As with peripheral smears, well-controlled staining protocols are essential for accurate morphological evaluation, particularly in cases where a diagnosis of myelodysplastic syndrome is suspected. A differential count of 500 nucleated cells (excluding macrophages and megakaryocytes) is recommended. This should be performed in areas of the smear adjacent to,

but not overlying, spicules, thereby limiting the effects of smear thickness and hemodilution. Additional stains that can be performed on bone marrow aspirate smears include cytochemical stains for enzyme constituents, such as myeloperoxidase (indicative of myeloid differentiation) and nonspecific esterases (indicative of monocytic differentiation) to help in determining blast lineage in cases of acute myeloid leukemia; however, some laboratories have replaced these tests with the use of flow cytometric immunophenotyping [28]. Perls' iron stain (Prussian blue reaction) to assess for storage iron in bone marrow macrophages and dendritic cells, sideroblasts (iron-containing erythroid precursors), and ring sideroblasts (erythroid precursors with five or more iron-containing granules encircling at least one-third of the nucleus) remains a standard part of morphologic assessment and particularly critical in evaluating cases of myelodysplastic syndrome with fewer than 5% blasts (Fig. 1.1c) [1, 28, 37].

## Bone Marrow Trephine Biopsy Evaluation

Proper evaluation of the bone marrow biopsy mandates proper application of several preanalytic techniques: (1) obtaining an adequate core biopsy, which should be at least 1.5 cm in length and be taken at a right angle to the cortical bone surface so as to sample deeply into the hematopoietic marrow space, (2) adequate fixation, (3) proper decalcification, (4) adequate processing, and (5) careful histologic sectioning, generating intact 2–3 μm sections [38]. Neutral-buffered formalin is an adequate fixative for bone marrow, provided fixation time is sufficient and processing and sectioning are done properly, but many labs use fixatives containing metals, such as Zenker's, Bouin's, B5, aceto-zinc formalin, and B-plus, which improve nuclear detail [39, 40]; issues with toxicity and disposal of mercury have led to less frequent use of mercury-containing fixatives (Zenker's and B5). Decalcification agents include strong acids, buffered acids, and agents containing calcium chelators, often combined with acids. Decalcification time should be as short as possible, since acids have deleterious effects on the morphology and immunogenicity; agitation of the decalcification solution can help speed the decalcification process. Clot sections, representing either loose portions of the biopsy specimen that lack bone or clotted aspirated marrow, do not require decalcification. While clot specimens often are not optimal for evaluating marrow cellularity and may miss pathology associated with the bone trabeculae, they often provide superior immunostaining results and are also more amenable for molecular genetic testing than the decalcified bone marrow biopsy. Formalin-based fixation, paraffin processing, and decalcification all leach iron from the bone marrow, and thus iron stains on bone marrow biopsies (and to a lesser extent, clot sections) are less sensitive than iron stain on aspirate smears. While many labs perform a routine iron stain on the bone marrow biopsy, when assessment of storage iron or ring sideroblasts is essential for diagnosis, an iron stain on the bone marrow aspirate smear should always be performed [41].

In addition to at least one level stained with hematoxylin and eosin, many laboratories perform additional stains on the bone marrow sections, either routinely or as on an as-needed basis depending on the clinical suspicion and findings on routine histology. These stains are listed in Table 1.6. Because of the small size of the bone marrow core and tissue wastage between sectioning, when special stains and/or immunostains are ordered, additional blank slides may be cut up front for use if additional staining is needed. Of note, some immunostains on decalcified material may give different results from non-decalcified material. Thus, demonstration of staining of internal control cells in the bone is preferable to external non-decalcified control tissue in verifying a successful stain; alternatively, a decalcified bone marrow external control may be used. In some cases, different antigen retrieval or staining techniques may be needed for some immunostains (e.g., CD34) in bone marrow to ensure adequate sensitivity for detecting the cells of interest.

**Table 1.6** Stains performed on bone marrow biopsies

| Stain | Usage | Role |
|---|---|---|
| Hematoxylin and eosin | Routine: one or two levels | Evaluates routine histologic features; screens for the need for additional stains |
| Giemsa | Routine in many labs | Enhances the visualization of eosinophils, plasma cells, mast cells, and early erythroid forms |
| Iron | Routine in many labs | Evaluates storage iron but less sensitive than iron stain on the aspirate. Ring sideroblasts may sometimes be visualized in non-decalcified clot sections |
| Reticulin | Routine in many labs | Evaluates reticulin fibers in the marrow; essential for evaluating myeloproliferative neoplasms and also helpful in evaluating all myeloid neoplasms and some nonneoplastic conditions |
| Periodic acid Schiff | Routine in some labs | Enhances the visualization of megakaryocytes; detects fungal organisms |
| Trichrome | Typically only if Grade 2–3/3 fibrosis is seen on reticulin stain | Evaluates collagen fibrosis in the marrow; positivity usually indicates an advanced state of fibrosis |
| Other special stains | As needed, depending on clinical and histologic findings | Includes stains for microorganisms and Congo red |
| Immunohistochemistry | As needed, depending on clinical and histologic findings | Results may be different from non-decalcified tissue |

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