

Reticulocytes and Reticulocyte Enumeration

Roger S. Riley,* Jonathan M. Ben-Ezra, Rajat Goel, and Ann Tidwell

Department of Pathology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia

Key words: reticulocytes; reticulocyte count; hematology automation; hematology instrumentation; blood counts; flow cytometry; anemia; erythropoiesis; erythrocytes; erythrocyte count; fluorescent dyes; bone marrow transplantation

INTRODUCTION

Reticulocytes are immature red blood cells (RBCs). Reticulocytes originate in the bone marrow from the orthochromatic normoblast through nuclear exclusion. They are released into the peripheral blood after a period of maturation in the bone marrow and undergo further differentiation into mature RBCs. The enumeration of peripheral blood reticulocytes (“reticulocyte counting”) is often performed to obtain information about the functional integrity of the bone marrow since the reticulocyte count reflects the erythropoietic activity of the bone marrow, the rate of reticulocyte delivery from the bone marrow into the peripheral blood, and the rate of reticulocyte maturation. Reticulocytosis (an increased number of peripheral blood reticulocytes) occurs in anemic patients with a functional bone marrow, whereas anemic patients with a dysfunctional bone marrow produce decreased numbers of reticulocytes, and have decreased numbers of peripheral blood reticulocytes (i.e., reticulocytopenia). In addition to the evaluation of anemic patients, reticulocyte enumeration is also of value in monitoring bone marrow regenerative activity after chemotherapy or bone marrow transplantation. In the laboratory, the differentiation of the reticulocyte from the mature RBC is based on the presence of RNA and other substances in the reticulocyte, which are lost during differentiation into the mature RBC. Manual counting of reticulocytes by light microscopy with supravital dyes for RNA was developed in the 1940s and remains the standard method of reticulocyte enumeration. However, automated methods of reticulocyte enumeration developed during the past decade are much more accurate, precise, and cost-effective than manual counting, and are increasingly being performed in the clinical laboratory. In addition, the newer techniques provide a variety of reticulocyte-related parameters, such as the reticulocyte maturation index and immature reticulocyte fraction, which are not available with light microscopy. These new parameters are under evaluation in the clinical diagnosis and monitoring of anemia and other diseases.

This article reviews the physiology and pathophysiology of the reticulocyte, laboratory techniques of reticulocyte enu-

meration, and the clinical applications of reticulocyte counting. Reticulocyte enumeration is the subject of several reviews (1–7).

ERYTHROPOIESIS AND RETICULOCYTE MATURATION

Blood cells (RBCs, white blood cells, and platelets) are continuously renewed in the bone marrow from the hematopoietic stem cell. Stem cells both self-renew and self-differentiate to form populations of progenitor cells from which the major marrow cell lines (erythroid, myeloid, megakaryocytic, and lymphoid) arise. Early progenitor cells are multipotent, but their progeny are committed to the formation of a limited number of cell lines. The “microenvironment” provided by the stromal matrix of the bone marrow is essential for the survival, growth, and replication of hematopoietic stem cells.

Hematopoietic growth factors are necessary for the self-renewal of stem cells and for the proliferation and differentiation of lineage-committed progenitor cells. Since many of these hormone-like substances stimulate *in vitro* colony formation of progenitor cells, they are termed colony-stimulating factors (CSFs). Erythropoietin, one of the hematopoietic growth factors, is the major chemical regulator of erythropoiesis. Erythropoietin is a glycoprotein (34,000–39,000 kDa) produced by the kidney in response to reduced renal oxygen tension. Although erythropoietin accelerates the division of erythroblasts at all stages of development, its major effect is to stimulate resting (G₀) committed erythroid stem cells (CFU-E) to enter the cell cycle (Fig. 1) (8). Because optimal oxygen delivery to the tissues occurs at a hematocrit of 40–45%, the production of RBCs is very tightly regulated.

*Correspondence to: Roger S. Riley, M.D., Ph.D., Department of Pathology, Medical College of Virginia, Virginia Commonwealth University, Clinical Support Building 665-E, 403 N. 13th St., Richmond, VA 23298-0250. E-mail: rsriley@hsc.vcu.edu

Received 19 January 2001; Accepted 9 April 2001

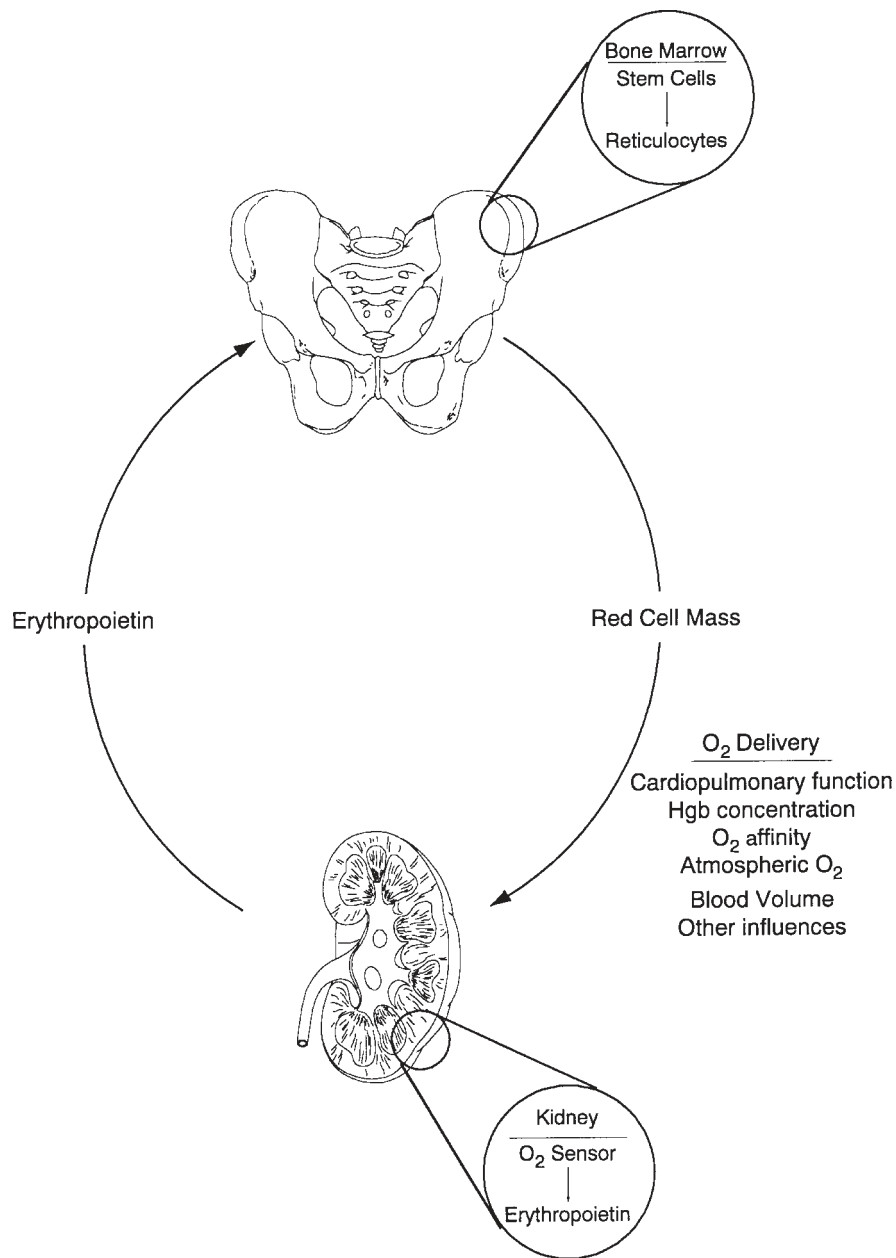


Fig. 1. The regulation of erythropoiesis.

A series of progressive structural and biochemical changes occur during the differentiation of immature RBC precursors into mature RBCs (9). The most important of these changes include:

1. Synthesis and cytoplasmic accumulation of hemoglobin.
2. Loss of protein-synthesizing apparatus and mitochondria.
3. Chromatin condensation, contraction, and extrusion of the nucleus.
4. Loss of cell-surface membrane receptor expression via exosome formation.
5. Changes in membrane cholesterol and phospholipid levels.
6. Changes in various intracellular enzyme levels, including glucose-6-phosphate dehydrogenase.

The morphological characteristics of the RBC precursors reflect these events. The pronormoblast results from cell division of the CFU-E, and is the first morphologically identifiable RBC. Subsequent cellular stages in erythropoiesis

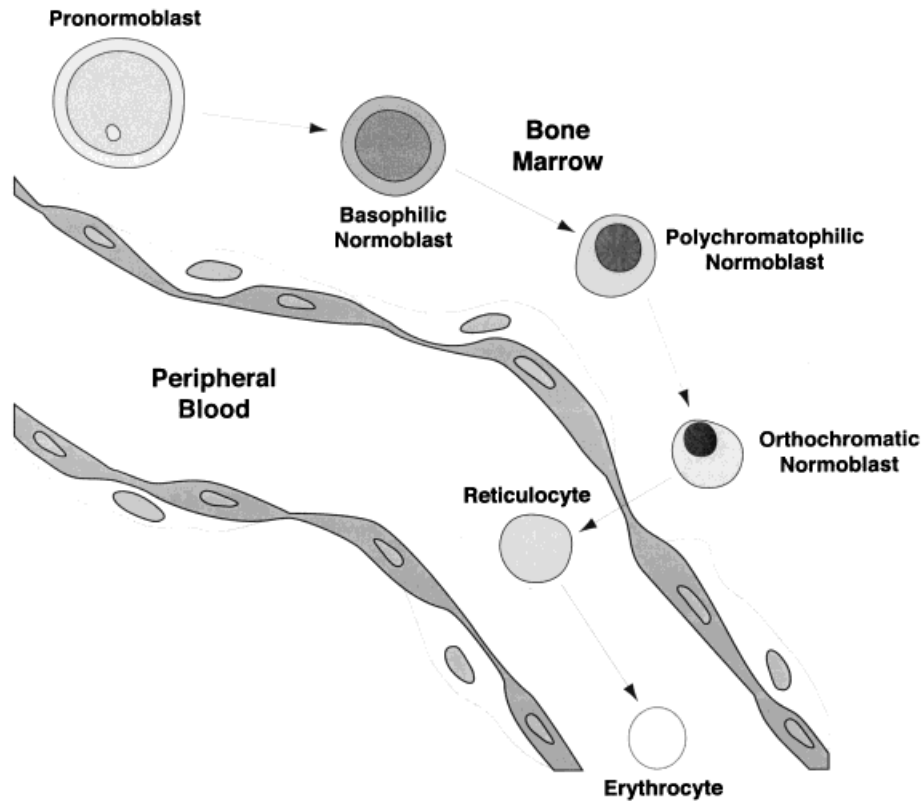


Fig. 2. Modern concept of erythropoiesis.

include the basophilic normoblast, polychromatophilic normoblast, orthochromatitic normoblast, reticulocyte, and mature RBC (Fig. 2) (10–12). Together, the RBCs and the various morphologically identifiable RBC precursors comprise the erythron. Complex changes in cellular biochemistry also occur during this time (13). The progression from a pronormoblast to a non-nucleated RBC requires 3–5 days.

The reticulocyte is an anucleate RBC, which is slightly larger than the mature RBC (10–15 μm vs. 6–8 μm). The early reticulocyte contains mitochondria, a small number of ribosomes, the centriole, and remnants of Golgi bodies. Early reticulocytes continue to synthesize hemoglobin, and approximately 20–30% of the total hemoglobin of the RBC is synthesized at this stage of RBC development. However, hemoglobin synthesis gradually decreases as cellular organelles are progressively lost and the reticulocyte becomes a mature RBC. Changes in the surface membrane of the reticulocyte, including the loss of the transferrin receptor, also occur during differentiation. After about 2 days in the bone marrow, reticulocytes are normally released into the peripheral blood and undergo final maturation. The term “reticulocyte” was derived from the deep blue precipitate seen in these cells after staining with new methylene blue and other tricyclic, heterochromatic, cationic dyes that bind and cross-link RNA and aggregate other organelles.

The “age” of a reticulocyte can be determined from its relative RNA content. For example, Heilmeyer and Westhäuser, using supravital stained blood preparations, classified reticulocytes into four groups (Groups I–IV) (14,15). In this classification scheme, nucleated RBCs with a dense perinuclear reticulum are classified into Group 0. Following extrusion of the nucleus, very young reticulocytes (Group I) have a dense, coherent mass of RNA and other organelles. The RNA becomes less dense with further maturation, and a reticular network appears in the region of the original mass (Group II). The RNA later scatters and decreases in amount (Group III). Only scattered remnants of RNA remain in the most mature reticulocytes (Group IV) and these are lost when the reticulocyte finally becomes a mature RBC. This classification system has been used to obtain information about reticulocyte maturity from the microscopic examination of blood smears. This provides information about bone marrow function beyond that provided by the relative or absolute number of reticulocytes in the peripheral blood (Fig. 3).

Stress reticulocytes are very young reticulocytes released into the blood stream in response to a severely anemic state. By phase contrast microscopy, stress reticulocytes are multilobular and motile, in contrast to the cup-shaped nonmotile characteristics of mature reticulocytes (16). Stress reticulocytes are found in the circulation only under conditions of

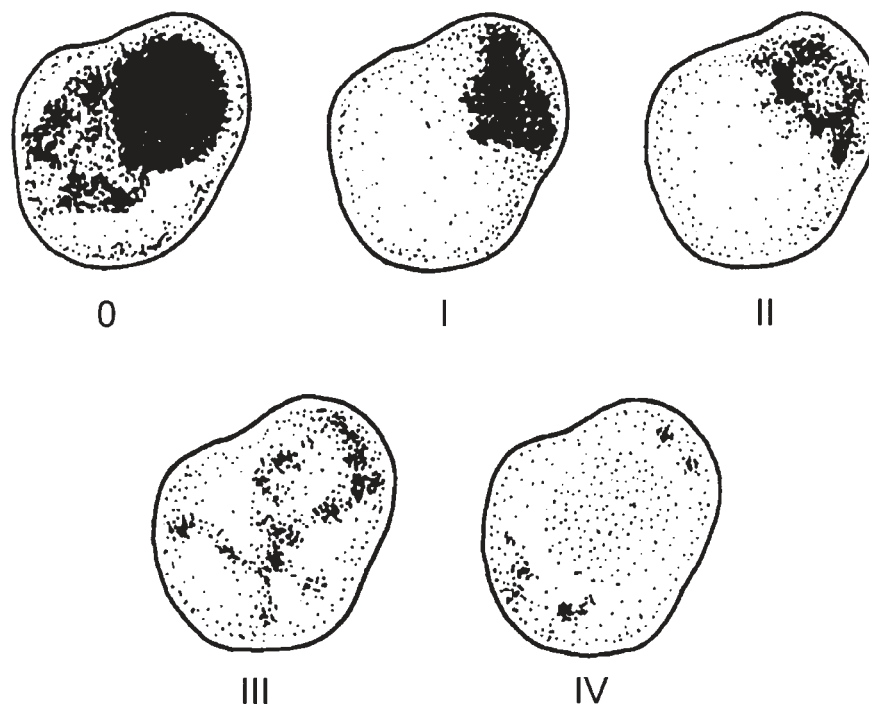


Fig. 3. A schematic illustration of reticulocyte maturation according to Heilmeyer. Group 0: nucleated RBC; Group I: most immature reticulocyte with dense reticulum; Group II: reticulocyte with extensive but loose reticu-

lar network; Group III: reticulocyte with scattered reticulum network; Group IV: most mature reticulocyte stage with scattered reticulum granules (237).

erythropoietic stress and are not present in nonanemic individuals (16). Although stress reticulocytes are rapidly removed from the circulation in hematologically normal individuals, their survival is increased in anemic individuals, apparently through by an adaptation of the spleen (17). Density-gradient fractionation is used to separate normal reticulocytes from stress reticulocytes.

LABORATORY TECHNIQUES OF RETICULOCYTE ENUMERATION

Most clinical laboratory techniques for reticulocyte enumeration are based on the detection of RNA (reticulin) in the cytoplasm of the reticulocyte. From the late 1940s until the early 1980s, reticulocyte enumeration was performed by microscopic examination of supravital stained peripheral smears. The development of RNA-specific fluorescent dyes in the 1980s provided a means by which to use the flow cytometer for reticulocyte enumeration, with greatly improved accuracy and precision. The development of flow cytometers dedicated to reticulocyte enumeration proved a popular alternative to light microscopy for laboratories performing high-volume reticulocyte enumeration. Flow cytometric analysis was also found to provide clinically valuable information not available by light microscopy. With the recent incorporation of flow cytometric technology (i.e., light scatter and immunofluorescence detection) into the hematology analyzer, reticu-

lyocyte counts and special reticulocyte parameters are becoming a routine part of the workup of hematologic specimens.

Reticulocyte analysis with fluorochrome-labeled monoclonal antibodies against reticulocyte surface receptors is available for research studies of the reticulocyte and diseases of the erythron. Techniques using density gradient fractionation and other phenomena have also been reported, but these have not achieved clinical utilization (Fig. 4).

Light Microscopy

The supravital staining technique described by Brecher (18) in 1949 remains the standard method for reticulocyte enumeration (19–21). In this technique, a few drops of the supravital dye solution (1.0% w/v of new methylene blue or brilliant cresyl blue) are mixed with an equal volume of anticoagulated (usually with EDTA) peripheral blood and incubated for several minutes. A thin smear of the stained blood preparation is made on a microscope slide, a counterstain (usually Wright's) is applied, and the slide is examined by light microscopy (22,23). An adequate number of erythrocytes (usually 1,000) in a well-stained area are examined, and the proportion of reticulocytes is determined. Reticulocytes possess a blue granular precipitate, which can vary from individual small blue granules to a network of blue reticular material (thus the name "reticulocyte"). Reticulocytes have a faint, diffuse basophilic hue (polychromasia) (Fig. 5).

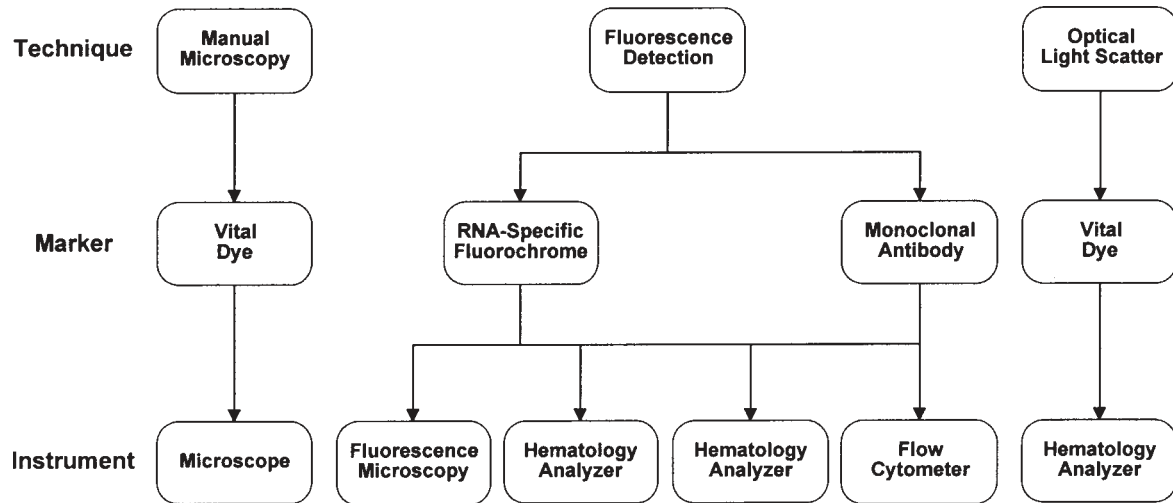


Fig. 4. Laboratory practices in reticulocyte enumeration. a: Present instrumentation and methodology.

The reticulocyte count is usually reported as a percentage (i.e., reticulocytes per total RBCs examined). The normal mean percentage reticulocyte count by NMB light microscopy is 1.0% to 1.5%, with 3% being the upper limit of normal. Some studies have shown sex differences in the reticulocyte count, but this has not been universally confirmed (19).

The relative reticulocyte count is misleading when the RBC count is abnormal and/or erythropoietic stimulation to the bone marrow is taking place (i.e., in severe anemia). Under these circumstances, mathematical corrections must be applied to the relative count. As classified by Koepke and Koepke (23), these corrections include the following:

1. Packed cell volume (PCV) correction

$$\frac{\text{Reticulocyte index}}{\text{Absolute reticulocyte count}}$$

2. Shift correction
 Reticulocyte production (maturation) index

The PCV correction (reticulocyte index) is applied to specimens from anemic patients to compensate for the decrease in mature RBCs. The PCV corrects the percentage reticulocyte count to a normal PCV (0.45) using the following formula:

$$\text{Reticulocyte index} = \text{Reticulocyte count (\%)} \times \frac{\text{Patient PCV}}{0.45}$$

A more accurate method to correct for the effect of anemia is to calculate the absolute number of reticulocytes:

$$\text{Absolute reticulocyte count} = \text{Reticulocyte count (\%)} \times \text{RBC}(\text{RBC}/1 \text{ or } \text{RBC}/\text{mm}^3)$$

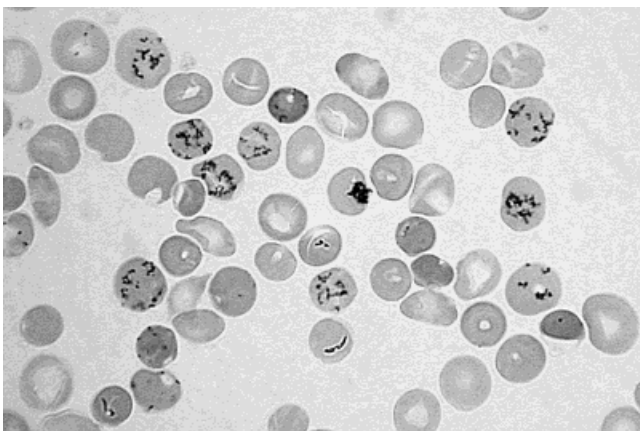


Fig. 5. Photomicrograph of peripheral blood smears stained with new methylene blue. Reticulocytes are differentiated from mature RBCs by the presence of an intracellular granular precipitate.

The absolute reticulocyte count is normally between 50,000 and 150,000 reticulocytes/mL (5×10^{10} and 1.5×10^{12} reticulocytes/L).

The “shift correction” is applied to percentage reticulocyte counts from patients undergoing intense erythropoietic stimulation. Under these circumstances, “young” basophilic macroreticulocytes (“shift cells”) from the bone marrow are released prematurely into the peripheral blood, causing a shortened reticulocyte maturation time in the bone marrow (sometimes to < 1 day), but a longer reticulocyte maturation time in the peripheral blood (23–27). Since shift cells have a cell diameter about 25% larger than that of normal cells, a correction for RBC maturation time and the PCV must be made when they comprise more than 5% of the total reticulocytes (23). The corrected count (“reticulocyte production index”) is calculated by the following formula (23):

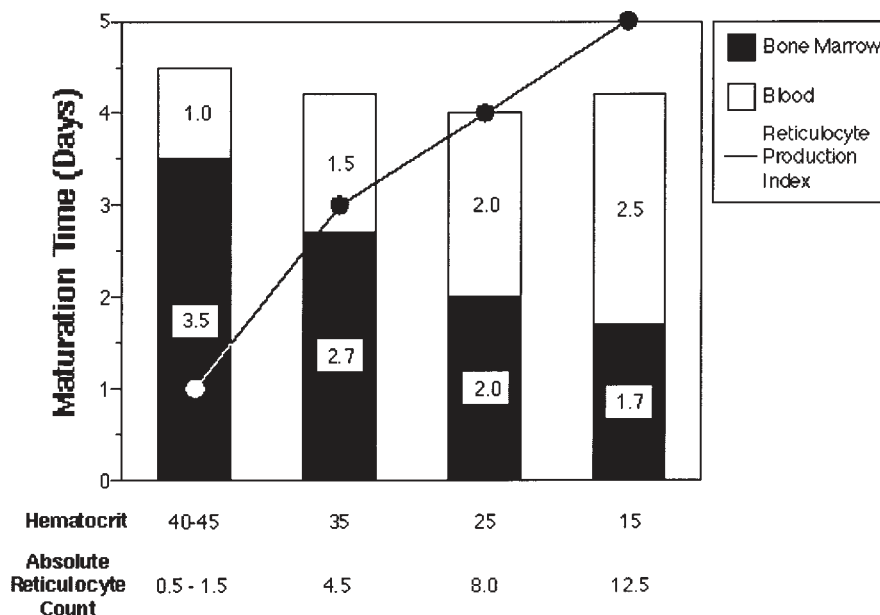


Fig. 6. The relationship between PCV (hematocrit) and blood maturation time. The total RBC maturation time is approximately 4.5 days. Normally, the maturation time of erythroid marrow normoblasts is about 3.5 days, and reticulocyte lifetime in the peripheral blood is approximately 1 day. How-

ever, in anemia the marrow maturation time progressively shortens, and reticulocytes circulate for a correspondingly longer period of time in the peripheral blood (238).

$$\text{Reticulocyte production index} = \frac{\text{observed reticulocyte count (\%)} \times \frac{\text{patient's PCV}}{0.45}}{\text{maturation time in peripheral blood}}$$

Unfortunately, the assumption of a linear relationship between the hematocrit and the reticulocyte maturation time is correct only in patients with an intact hematopoietic system. If erythropoietin production is impaired, or if the bone marrow does not respond appropriately to an increased erythropoietin level, the reticulocyte count will be inappropriately low and the reticulocyte production index will be incorrect (Fig. 6).

Technical Considerations

A number of factors besides pathophysiologic phenomena may compromise the accuracy of reticulocyte counts performed by manual counting (23). Of these factors, inter- and intralaboratory variability are the most important (19,23,28). Sources of laboratory variation include the following:

1. Specimen collection, transportation, and storage
2. Interobserver variability in morphological identification
3. Sample size (total number of cells counted)
4. Type and quality of blood film (distributional variability of reticulocytes)
5. Staining variations
6. The use of an ocular counting aid for standardized area reduction

Reticulocyte enumeration must be performed on whole blood collected into di/tripotassium or disodium EDTA at a final concentration of 3.7–5.4 mmol/mL (1.5–2.2 mg/mL) (29). Other anticoagulants, such as heparin or citrate, are not presently approved for reticulocyte enumeration, although one study found no statistical differences in the reticulocyte count or percentages of reticulocyte fractions between EDTA and trisodium citrate-anticoagulated samples, evaluated with the Sysmex R-3000 (30). Since reticulocytes in vitro undergo a time and temperature-dependent maturation, reticulocyte counting should be performed within 6 hr if the specimen is kept at room temperature, or up to 72 hr if the blood sample is refrigerated at 2–6°C (29). In one study, the relative proportion of reticulocytes decreased to around 80% of the initial value, and it reached 60% after 3 weeks of storage at 4°C. There was a strong negative correlation of the change in reticulocytes with time ($r = -0.9972$) (31).

Blood specimens for reticulocyte enumeration should not be visibly hemolyzed, and the patient should have a stable RBC count (29). An accurate RBC count should be determined on the same sample used for the reticulocyte count, by using a hematology analyzer or a flow cytometer (29).

Surveys by the College of American Pathologists (CAP) revealed the severity of the problem of interlaboratory variability in reticulocyte enumeration. For example, in the 1971, 1972, and 1974 CAP Comprehensive Hematology Surveys, the interlaboratory coefficient of variation (CV) on three stained, previously prepared reticulocyte smears (glass slides) varied from 25% to 48% (20). In 1984, a similar study conducted by

the CAP Reticulocyte Project found CVs ranging from 26.2% to 32.4% (28,32). CAP initially adopted the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS) for reticulocyte enumeration, and later encouraged utilization of the Miller ocular disc to remedy this situation. More recently, whole-blood proficiency testing was initiated by the CAP Hematology and Clinical Microscopy Resource Committee to assess sources of pre-analytic variation in reticulocyte enumeration (specimen mixing, staining, and slide preparation), in addition to slide interpretation (33). This survey material also provides a means to evaluate the increasing variety of instrument-assisted methods of reticulocyte enumeration now available or in development (Table 1). The 1994–1995 CAP Reticulocyte Survey revealed much better precision for the automated methods, with CVs at or below 15% (33).

Interobserver variability in the morphological identification of reticulocytes remains the major cause of laboratory imprecision in reticulocyte enumeration (23). For example, Peebles and colleagues (34) found that inconsistent application of morphologic criteria for reticulocyte identification caused proportional errors that could exceed 30% for each technologist. These problems pertain mostly to reticulocytes in Heilmeyer group IV, which normally comprise the majority of those in the circulation (~60%). This problem has been addressed by the establishment of more standardized criteria for morphologic identification. In this regard, Gilmer and Koepke (20) and Koepke and Koepke (23) concluded that Heilmeyer group IV cells should be identified by the presence of at least two clumps or granules of reticulium, while other definitions require three clumps of reticulium or the presence of a reticulium network.

Crouch and Kaplow (35) found that Heilmeyer group I and II erythrocytes are not normally present in the circulation, and proposed that the term “shift reticulocyte” should be restricted to these cells. These investigators also proposed the routine calculation and clinical use of the “shift reticulocyte count” (percentage of shift cells per 100 reticulocytes).

Cytoplasmic particles other than RNA (i.e., debris, Heinz bodies, Howell-Jolly bodies, nuclear remnants, siderotic granules, etc.), are another source of counting inaccuracy, since they can be stained by supravital dyes and confused with reticulum granules (36). Heinz bodies are particles of denatured hemoglobin that occur in patients with a variety of hematological disorders (i.e., hemolytic anemia, thalassemia major, congenital Heinz-body anemia, postsplenectomy disorders, etc.). Although adequate technologist training can usually alleviate problems caused by the misidentification of these particles, special stains are sometimes used to confirm their presence. For example, the presence of Heinz bodies can be confirmed by staining with acetylphenylhydralazine (APH), while nuclear material can be identified by the Feulgen or methyl green pyronin stains, and siderocytes by an iron stain (37).

The type and quality of the blood film and the number of cells counted are additional considerations in manual reticulocyte enumeration. The total number of cells counted determines the precision of reticulocyte enumeration. The 95% confidence limits are quite high (0.03–5.45%) in a count of 100 cells with 1% reticulocytes, but markedly decrease (0.46–1.84%) in a 1,000-cell count. Therefore, a minimum of 1,000 cells should be counted for optimal analytical precision (23,38). Ideally, blood films for reticulocyte enumeration

TABLE 1. Laboratory techniques of reticulocyte enumeration^a

Instrument/company	Dye	Technique	Present utilization
Light microscopy	New methylene blue	Manual counting	30.4%
Light microscopy with Miller ocular	New methylene blue	Manual counting	25.2%
Flow cytometry (Becton Dickinson, Beckman Coulter)	Thiazole orange	Fluorescence detection	1.2%
ABX PENTRA 120 Retic, ABX Montpellier, France)	Thiazole orange	Fluorescence detection	<1%
Cell-Dyn 4000 Hematology Analyzer (Abbott Diagnostics, Santa Clara, CA)	CD4K530	Fluorescence detection	4.0%
Sysmex R Series (TOA Medical Electronics, Kobe, Japan)	Auramine O	Fluorescence detection	4.1%
XL Hematology Analyzer (Beckman Coulter Inc., Fullerton, CA)	Coriphosphine-O	Fluorescence detection	<1%
Bayer/Miles ADVIA 120 Hematology System (Bayer/Miles Diagnostics Division, Tarrytown, NY)	Oxazine	Optical light scatter detection	4.0%
Bayer/Miles Technicon H-3 Hematology Analyzer (Bayer/Miles Diagnostics Division, Tarrytown, NY)	Oxazine	Optical light scatter detection	<1%
Cell-Dyn 3500 Hematology Analyzer (Abbott Diagnostics, Santa Clara, CA)	New methylene blue	Optical light scatter detection	<1%
Coulter GEN-S System (Beckman Coulter Inc., Fullerton, CA)	New methylene blue	Optical light scatter detection	16.1%
STKS/MAXM Hematology Analyzers (Beckman Coulter Inc., Fullerton, CA)	New methylene blue	Optical light scatter detection	17.8%

^aData from College of American Pathologists Survey RT, RT-04, 2000. Data from 3443 participating laboratories.

should be evenly stained with a uniform cellular distribution, but there is disagreement about the relative merits of wedge smears and spun smears, and the smear type has not been standardized among laboratories (34,39). On the other hand, ruled ocular devices that standardize the area to be counted, such as the Miller ocular reticle, theoretically reduce counting variability and are widely utilized in the clinical laboratory (23). The Miller ocular, developed by Dr. J.W. Miller of the National Institutes of Health (NIH), contains a small square set within a larger square, such that the area of the small square is one tenth that of the area of the large square (40–42). The number of erythrocytes is counted in the smaller square, while the number of reticulocytes is counted in the larger square. Since seven or eight erythrocytes are normally found in small square in the feathered edge of a blood smear, a total of about 1,500 erythrocytes can be counted from the examination of 20 ruled fields (42). The percentage reticulocyte count is determined from the following formula:

$$\% \text{ Reticulocytes} = \frac{\text{Total reticulocytes in larger squares}}{\text{Total RBCs in smaller squares} \times 9} \times 100$$

Reticulocyte counts with the Miller disc are significantly lower than conventional manual counts unless “edge rules” are observed (43). These require that only red cells lying within the small square and on two of its four edge lines (usually the right and lower lines) are counted. Cells touching the other two edges of the small square are not included in the count (43). Beginning in 1994, the Miller disc was included in the CAP accreditation inspection, and failure to utilize this device was considered a phase II deficiency. However, later CAP surveys did not confirm the beneficial nature of the Miller disc, and the requirement for its utilization was later revoked (Fig. 7).

Few investigators have considered the effect of age and various physiologic effects on reticulocyte counts. Reference intervals for reticulocyte counts and the reticulocyte maturity index in infancy, childhood, and during the puerperium have been reported (44–47). In one study on the effect of maternal smoking on neonatal cellular blood components, cord blood reticulocytes were found to be significantly lower in neonates from smoking mothers, as compared with those who did not smoke. However, there was no significant difference in whole-blood cell count, leukocyte differential count, and thrombocyte counts between the two groups (48). Sex- and age-specific reticulocyte reference limits for fully automated flow cytometric analysis have also been determined (49). The within-subject CV for the reticulocyte count was reported as 11%, while the mean reticulocyte volume, mean reticulocyte hemoglobin content, and mean reticulocyte hemoglobin concentration have a CV of between 1% and 2% (50).

Immunofluorescence

RNA-specific fluorochromes. Reticulocyte enumeration by immunofluorescence microscopy was first reported

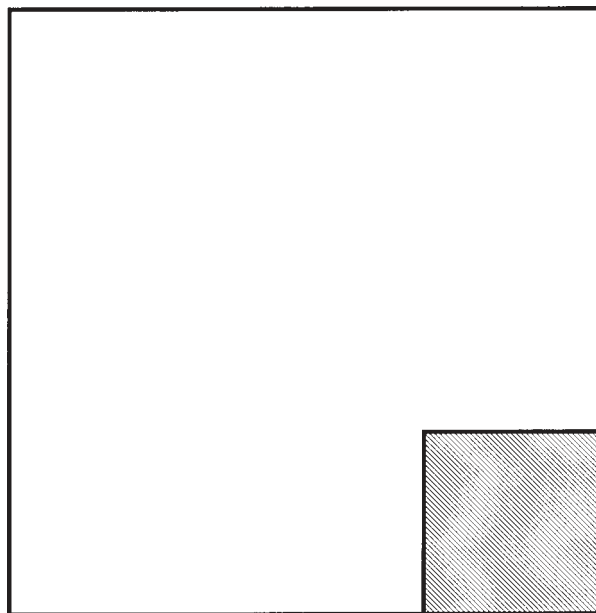


Fig. 7. The Miller ocular disk reticle. The small square occupies one-tenth the area of the large square. The number of erythrocytes is counted in the small square, and the number of reticulocytes is counted in the large square. The percentage reticulocyte count is determined from the formula given above.

by Kozenow and Mai (51) in the early 1950s, using an RNA/DNA-specific fluorochrome (acridine orange). Subsequent investigators used the UV-fluorescing dyes pyronin Y, thioflavin T, DiOC1(3), and proflavine for reticulocyte enumeration (52–57). Although similar results were obtained by light and fluorescence microscopy, fluorescence techniques offered few practical advantages over light microscopy, and were not widely used. In contrast to fluorescence microscopy, reticulocyte enumeration of peripheral blood stained with RNA/DNA-specific fluorochromes by flow cytometry offers many advantages over conventional light microscopy. For example, it is rapid, objective, semiautomated, technically easy to perform, and requires less technical labor than manual slide-based techniques. Flow cytometry also offers several of advantages over slide-based, manual counting techniques, including the ability to provide accurate information on the age distribution of the reticulocyte population, the elimination of subjective technical inconsistencies, counting precision, and cost efficiency for the analysis of large numbers of specimens. The major disadvantages of reticulocyte enumeration by immunofluorescent techniques are spuriously high flow cytometric reticulocyte counts caused by interfering RBC inclusions, such as nucleated RBCs and Howell-Jolly bodies, and the need for expensive, complex instrumentation and highly trained technologists (3,58). Interferents that are known to, or may potentially, lead to erroneous results with fluorescent dyed-based techniques of reticulocyte enumeration are listed in Table 2.

TABLE 2. Potential sources of interference with automated methods of reticulocyte analysis^a

Cellular elements	Cellular inclusions	Miscellaneous
Platelet clumps	Howell-Jolly bodies	Autofluorescence
Giant platelets	Heinz bodies	(porphyria, drugs)
Leukocytes	Pappenheimer bodies	Paraproteins
Leukocyte fragments	Parasites (malaria, babesia)	Cold agglutinins
Nucleated RBCs	Basophilic stippling	Platelet/RBC coincidence
	Hemoglobin H inclusion bodies	Hemolysis

^aUpdated from (29).

A wide variety of fluorochromes have been used for reticulocyte enumeration by flow cytometry. These include pyronin Y, acridine orange (AO), propidium iodide (PI), ethidium bromide (EB), dimethyloxacarbocyanine (DiOC1), thioflavin T, auramine, and proflavine (56–63). However, in the mid-1980s the development of a thioflavin T analogue [thiazole orange (TO)] specifically for reticulocyte enumeration greatly improved counting accuracy and made flow cytometric reticulocyte enumeration practical for clinical laboratories with commercial clinical flow cytometers with relatively low-powered visible lasers (64). This was largely due to the fact that, unlike thioflavin T and other fluorochromes which require ultraviolet excitation from large, expensive lasers, TO excitation occurs in the visible region of the spectrum (488 nm). In addition, commercial, FDA-approved TO-based reagent kits became available for clinical laboratories.

Fluorescence microscopy. Reticulocyte enumeration with fluorescence microscopy can be performed with unfixed blood stained with DiOC1(3) or AO. The blood is mixed with a dilute, buffered solution of the dye and counted as a wet preparation using a glass slide or hemocytometer with a glass coverslip (52). Ultraviolet to blue light is required for the excitation of these dyes, and a green long-pass filter must be in place for detection of the fluorescence emission. Blood cells are fixed in formalin prior to staining with AO. A fluorescence microscope with green excitation filters and red, long-pass emission filters is required for examination of the stained cells. The use of fluorescence microscopy for routine reticulocyte enumeration in a clinical laboratory setting has not been described.

Conventional flow cytometry. Flow cytometric reticulocyte enumeration with AO can be performed on fixed or unfixed RBCs. For fixed-cell analysis, a mixture of AO and formaldehyde in citrate buffer is added to the specimen. Flow cytometric analysis is performed at an excitation wavelength of 488 nm. Forward angle light scatter (FALS) is used to discriminate reticulocytes from platelets, while RNA is quantitated by red fluorescence emission (> 650 nm). Unfixed cell analysis is performed by mixing the specimen with a solution of AO in buffered saline. Both green and red emission and

FALS are measured immediately. Blood is prepared for reticulocyte enumeration with thioflavin T by incubation with a buffered solution of the dye; after dilution with buffer, flow cytometric analysis is performed immediately with an excitation wavelength of 457 nm and an emission wavelength of >500 nm.

Reticulocyte enumeration with commercial flow cytometers provides automated gating and data analysis, occasionally in conjunction with automated sample preparation and sampling. Reticulocyte enumeration is usually performed with TO, and is technically simple to achieve. Fresh, anticoagulated blood is mixed with a solution of TO dye and incubated in the dark at room temperature for a brief period of time. Flow cytometric analysis is then performed, with the detection of FALS, right angle light scatter (RALS), and green fluorescence. Accurate data analysis is critical, but is relatively operator-independent with the recent advent of sophisticated commercial analytic software programs. The first requirement for data analysis is the accurate discrimination of RBCs from platelets and white blood cells by FALS, or by a combination of FALS and RALS; this process is termed “gating.” Gating is possible because platelets are small, while mature RBCs and reticulocytes are intermediate in size between platelets and leukocytes. Unfortunately, size alone is insufficient to discriminate reticulocytes from mature RBCs, even though reticulocytes gradually decrease in size during maturation. However, the quantitation of TO incorporation by measurement of the intensity of green fluorescence reveals young, immature reticulocytes as brightly fluorescent (high RNA content), while maturing reticulocytes show an intermediate fluorescence intensity (intermediate RNA content), and older reticulocytes show dim fluorescence (low RNA content). Therefore, reticulocytes are identified and enumerated within the gated RBC population on the basis of fluorescence intensity.

Since flow cytometric green fluorescence intensity is a continuum from the immature reticulocyte to the mature erythrocyte, accurate analysis of the green fluorescence histogram is critical for reticulocyte enumeration. In curve fitting analysis, distribution functions are fitted to a single parameter fluorescence histogram and area determinations are made in a manner analogous to cell cycle fraction analysis (56,65). Since curve fitting is applied to the overlapping region of reticulocytes and mature erythrocytes, this technique is theoretically accurate. Therefore, the easier discriminator method is usually applied. In this technique, a threshold value (“discriminator”) is set at the reticulocyte/mature erythrocyte region of a single parameter histogram (55,66). The cell populations with a fluorescence intensity greater than the threshold value are considered reticulocytes, and the reticulocyte frequency in the total population is determined from the following formula:

$$\% \text{ Reticulocytes} = \frac{\text{Counts above threshold value}}{\text{Total counts}}$$

In the discriminator technique, fluorescence intensity values (“thresholds”) representing the reticulocyte population are determined from normal blood specimens with known reticulocyte counts, or by the use of mathematical analysis (55). For this purpose, Tanke et al. (55) described a technique using the mode + full width at half-maximum of the RBC population as the discriminator setting. Although discrimination analysis is easily performed by a computer, its accuracy is compromised by the lack of consideration of the reticulocyte/mature erythrocyte interface. Some of the re-

cent clinical flow cytometric systems provide automated, computerized data acquisition and analysis with the discriminator method, with rapid determination of the reticulocyte percentage, absolute reticulocyte count, and reticulocyte maturation data. Furthermore, the results are usually printed in a detailed format suitable for submission to the patient’s chart and requesting physician, or can be interfaced to a laboratory information system. Detailed technical standards for reticulocyte enumeration by flow cytometry have been published (29) (Fig. 8).

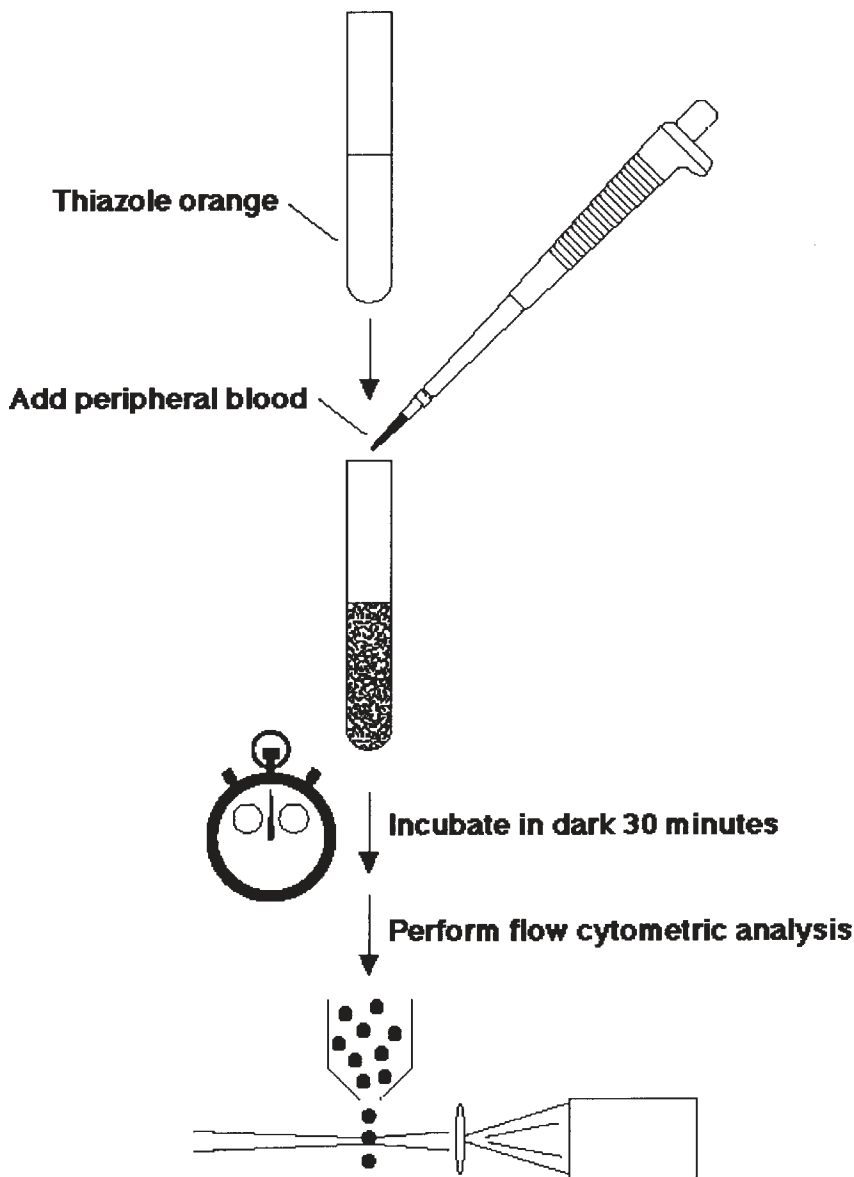


Fig. 8. Steps in reticulocyte enumeration with TO (Retic-COUNT™, Becton Dickinson Immunocytometry Systems, San Jose, CA). This procedure requires peripheral blood collected by venipuncture and anticoagulated with EDTA. The TO reagent (1 ml) is placed in a 12 × 75 mm test tube, followed by 5 ml of a well-mixed sample of peripheral blood. The specimen is incubated in the dark for 30 min, gently vortexed, and then analyzed on a flow cytometer optimized for green fluorescence quantitation. Data acquisi-

tion and analysis can be performed manually or automatically. Manual analysis involves gating the RBC population on a scattergram to exclude debris, platelets, and leukocytes, acquiring 10,000 or more fluorescence events in the RBC gate, setting a threshold cursor using an unstained sample, and determining the percentage of positive events (reticulocytes). Calculation of the absolute reticulocyte count can be performed if the RBC count is known.

Van Hove and collaborators (67) reported a detailed evaluation of the quality assurance issues of flow cytometric enumeration of reticulocytes utilizing TO and commercial (Retic-COUNT™) software. Although good linearity, precision, and accuracy for normal, low, and high reticulocyte counts were demonstrated, interference by artifacts, such as RBC autofluorescence, nucleated RBC, Howell-Jolly bodies, high leucocyte count, high platelet count, and giant platelets, falsely increased the number of reticulocytes unless software gate corrections were made. With appropriate corrections for artifact, fewer than 5% of the specimens were rejected for flow cytometric analysis, necessitating light microscopic counting. Peripheral blood anticoagulated with dipotassium EDTA provided stable reticulocyte counts for up to 30 hr after acquisition. Variations in the incubation time with TO from 2 to 7 hr at 25°C had no effect on the results, nor did changes in the incubation temperature up to 30°C. (67). Reference ranges for flow cytometric reticulocyte analysis are critical, since there is a tendency of flow cytometric analysis with TO to yield relative reticulocyte values approximately 1.5 times higher than the manual technique (68). In this regard, TO has been documented to be more sensitive than methylene blue (69). Detailed technical standards for reticulocyte enumeration by flow cytometry have been recently published (29).

Metzger and Charache (62) were among the first to report the clinical utilization of flow cytometric reticulocyte enumeration. In the large hematology laboratory at the Johns Hopkins Hospital (Baltimore, MD), where more than 60 reticulocyte counts/day were performed, these investigators evaluated the use of a thioflavin T fluorochrome and an EPICS C flow cytometer, and compared the results with manual reticulocyte counts performed on new methylene blue-stained films of each blood specimen. Excellent correlation was obtained between the manual and flow cytometric reticulocyte counts ($r = 0.984$). For manual counts, the CV varied from 17.1% in smears with markedly elevated reticulocyte counts, to 47.3% for smears with normal reticulocyte counts, with corresponding CVs for the same flow cytometric determinations of 1.3% to 6.4%. No fixation of the RBCs was required, and specimens were stored at room temperature for as long as 4 days with no change in the reticulocyte count. The introduction of semiautomated reticulocyte counting by flow cytometry resulted in greater laboratory efficiency, and the reduction of the technical staff by one full-time equivalent. Although flow cytometric counts were much more precise than those obtained by manual counting, rare cases of discrepancy were identified. For example, in one child with transient erythroblastopenia of childhood, repeated flow cytometric analysis showed a reticulocyte count of 0.3% to 0.4%, in spite of an absence of reticulocytes by conventional counts and a bone marrow devoid of hematopoietic precursors.

In similar studies using thioflavin T, EB, and TO, Corash et al. (58,70) and Carter et al. (71) later found that all methods produced comparative data and well-defined normal ranges. However, the values obtained with the fluorescent dyes

were slightly greater than those achieved with the manual counts; this observation was subsequently confirmed by many other investigators. Rabbit blood was found to be an excellent quality control material for flow cytometric reticulocyte enumeration, since the reticulocyte concentration and reticulocyte fluorescence intensity distribution were similar to those of human blood.

Several investigators evaluated the clinical utilization of TO for reticulocyte enumeration. For example, Carter et al. (71) found an excellent correlation between manual reticulocyte counts and reticulocyte counts performed by flow cytometry ($r = 0.98$). However, the flow cytometric counts were much more precise (CV = 3.1 for flow cytometric determinations, 11.9% for manual single-observer determinations, and 20.8% for multiple-observer determinations). After staining with TO, the reticulocyte count of peripheral blood specimens stored at 4°C did not change over a period of 6 hr; however, the counts were significantly different in specimens analyzed at 24 hr. The mean (mean % \pm 2 SD) reticulocyte counts for 78 male and 76 female blood donors was 0.74 ± 0.48 and 0.84 ± 0.56 , respectively ($P < 0.005$) but there were no sex differences in absolute counts ($36 \pm 24 \times 10^9/L$). These investigators did not address the financial considerations of reticulocyte enumeration. Similarly, Nobes and Carter (72) reported flow cytometric reticulocyte enumeration with TO to show a correlation of 0.99 with manual counting using brilliant cresyl blue. Good precision was found for specimens with an elevated reticulocyte count (1.4% CV with a reticulocyte count of 14.3%). However, the precision decreased with lower reticulocyte counts (7.3% with a reticulocyte count of 1.84%, and 33.3% with a reticulocyte count of 0.12%) (Fig. 9).

The advent of commercial reagents and software in the late 1980s greatly reduced the technical difficulty of flow cytometric reticulocyte enumeration, and this procedure became increasingly utilized in the clinical laboratory. Ferguson and collaborators (73) were among the first to report the use of a commercial TO reagent and software system (Retic-COUNT™, Retic-COUNT Enumeration Software, Becton Dickinson Immunocytometry, San Jose, CA) in a routine hematology laboratory. The precision of flow cytometric reticulocyte counts (mean CV: 4.3%, range 1.7: 6.6%) was significantly better than that achieved with manual determinations (mean CV: 22.4%, range: 8.3–44.2%), and flow cytometric determinations were linear over the range of 1.8–30.1% ($r = 0.99$). There was no difference between the flow cytometric adult male ($1.69\% \pm 0.52$) and female ($1.66\% \pm 0.48$) reference ranges, although flow cytometric counts were slightly higher than those obtained by light microscopy.

Dedicated reticulocyte analyzers. Dedicated, fully automated flow cytometers specifically designed for reticulocyte enumeration were developed and are produced by two companies: TOA (Kobe, Japan) and ABX (Montpellier, France) (63). The TOA instruments (Sysmex R-1000, Sysmex R-3000) are similar to conventional benchtop analytical flow

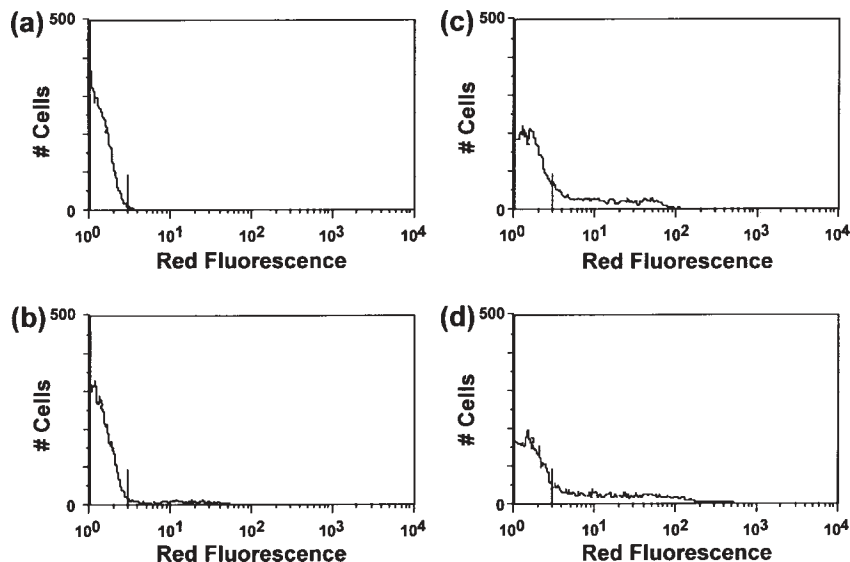


Fig. 9. Single-parameter histograms (green fluorescence vs. cell number) of peripheral blood specimen from a patient with reticulopenia ((a) 0.05% reticulocytes) and from patients with reticulocytosis ((b) 4.3% reticulocytes,

(c) 15.6% reticulocytes, and (d) 24.3% reticulocytes). Specimens were analyzed as described above with Retic-COUNT™ reagents and software (Becton Dickinson Immunocytometry Systems, Mountain View, CA) (239).

cytometers in the use of an argon ion laser and a sheath flow system; they utilize auramine-O as the fluorochrome for nucleic acid detection. Reticulocyte analysis is performed automatically, with minimal operator intervention. In the instrument, a small (100 mL) aliquot of EDTA-anticoagulated whole blood is mixed with auramine-O, followed by flow cytometric analysis. Platelets and nucleated cells are excluded from consideration by gating (forward scatter vs. side scatter), and both the RBC count and the absolute reticulocyte count ($10^9/L$) are reported. In addition, the reticulocyte population is automatically divided into fractions showing low, intermediate, and high fluorescence, and the percentage of cells in each fraction is calculated.

The ABX PENTRA 120 RETIC (VEGA RETIC) utilizes TO as a reticulocyte detection agent. An aliquot of whole blood (0.8 mL) is mixed with 2.5 mL of a proprietary formulation of TO and incubated at 35°C for 25 sec. An aliquot is analyzed with a 20 mW argon ion laser to determine fluorescence and true volume by resistivity of a maximum of 32,000 cells. Customized gating is used to separate reticulocytes from mature RBCs, WBCs, and platelets. The results are displayed on a reticulocyte matrix with RNA content (y-axis) plotted against cell volume (x-axis). Parameters determined by the ABX PENTRA 120 Retic include the proportional and absolute reticulocyte count, mean fluorescence index (MFI), and reticulocyte maturation fractions (low RNA content, RetL; medium RNA content, RetM; and high RNA content, RetH).

The Sysmex R-1000 system and related automated flow cytometric reticulocyte counters has been evaluated by several investigators. For example, Oyamatsu et al. (74,75) compared the Sysmex R-1000 with conventional light microscopy

for routine reticulocyte enumeration of 150 peripheral blood specimens. Although an excellent overall correlation was found between the two methodologies ($r = 0.97$), the within-run precision of the R-1000 was significantly better than that obtained by light microscopy. The presence of Jolly bodies, Pappenheimer bodies, and basophilic stippling did not influence the automated reticulocyte count, while specimens with cold-agglutinated RBCs and giant platelets produced abnormal flags. Nucleated RBCs (NRBCs) were not counted as reticulocytes by the instrument, but they were accompanied by increased numbers of young reticulocytes that were not delineated by manual counts. The authors concluded that the R-1000 could safely perform reticulocyte enumeration in blood containing abnormal RBCs or platelets if the abnormal flag is taken into consideration and a manual recount is performed when this flag occurs.

More recently, Bowen et al. (76) compared a modified TO technique and the R-1000 system for reticulocyte enumeration in whole-blood specimens obtained from 89 normal controls and 43 patients. A FACS 440 (Becton Dickinson) was used for the quantitation of green fluorescence. There was a good correlation between the two techniques for the determination of the percentage reticulocyte count ($r_s = 0.805$, $P < 0.01$) and absolute reticulocyte count ($r_s = 0.797$, $P < 0.01$). However, although blood samples stored at room temperature over a period of 5 days showed no significant change in the reticulocyte count as determined by the Sysmex R-1000 system, a small but significant ($P < 0.05$) decrease was noted by TO analysis. A reticulocyte reference range of $20\text{--}70 \times 10^9/L$ was established in the normal controls. The authors concluded that since both techniques provide accurate reticu-

lyocyte enumeration, economic considerations should be taken into account. The Sysmex R-1000 system is fully automated, so in a smaller clinical setting without an existing flow cytometer it can be fully integrated into the hematology laboratory with minimal expansion of the technical staff. On the other hand, reticulocyte enumeration by conventional flow cytometry is more economical for laboratories with existing instrumentation and personnel. In a similar manner to conventional flow cytometry, Davies et al. (77) found that automated reticulocyte analysis with the Sysmex R-1000 provided reliable quantitation of reticulocytes to very low levels in undergoing autologous or allogeneic bone marrow transplantation. Furthermore, the highly fluorescent reticulocyte (HFR) provided useful clinical information not available through manual counting.

In a later study, van Houte et al. (78) found a poor correlation between the microscopic count and both flow cytometric methods (FACScan $r = 0.61$; Sysmex $r = 0.57$), but the correlation between the flow cytometric methods was satisfactory ($r = 0.79$). Their reference ranges for the reticulocyte count, corresponding with the 2.5th and 97.5th percentile, were as follows: microscopic (8–30/1000), FACScan (11–27/1000) and Sysmex (8–18/1000). The Sysmex R-3000 methodology produced the lowest and narrowest reference range. Paterakis et al. (79) evaluated the performance of the Sysmex R-1000 for reticulocyte enumeration in patients with marked red cell heterogeneity (thalassemia and various sickle-cell syndromes). The technique was considered accurate in this patient population, although there was increased nonspecific fluorescence and a higher proportion of gating errors than in normal individuals. Patients with heterozygous beta-thalassemia were found to have slightly increased reticulocyte counts. Other evaluations of the Sysmex R-1000 have been reported by Laharrague et al. (80) and by Tichelli and associates (81). Automated reticulocyte analysis with the ABX PENTRA 120 Retic (Montpellier, France) was recently reported to show excellent precision and linearity (82). The ABX PENTRA 120 reticulocyte count showed good intraclass correlation with the Sysmex R-2000, flow cytometric, and manual counts, although measurements of the IRF were not concordant. Large platelets and erythrocytic inclusions will interfere with Sysmex analysis. There is one reported case of interference by falciparum malaria with a high level of intracellular parasitemia. Although the R-3000 reported a reticulocyte count of 21.6% ($918 \times 10^9/L$), light microscopy revealed 4% reticulocytes ($15.5 \times 10^9/L$) (83).

The comparative accuracy of flow cytometric and manual reticulocyte enumeration has been a subject of continuing interest. Most, but not all flow cytometric studies have demonstrated slightly higher absolute values for reticulocyte enumeration in comparison to light microscopy. Possible explanations include the higher sensitivity of fluorescence detection and differences in the binding of NMB and TO to RNA (84). Another concern is the effect of RBC inclusions

and other artifacts on flow cytometric reticulocyte enumeration. In this regard, Davis and Bigelow (3,85) found leukocytes and RBCs containing Howell-Jolly bodies to have no significant effect on reticulocyte quantitation, since they were excluded from the analysis by gating. However, Lofsness et al. (86) reported spuriously elevated automated reticulocyte counts in the presence of Howell-Jolly bodies, in proportion to the percentage of Howell-Jolly bodies observed on their Wright-Giemsa stained blood smears. Heinz bodies have been reported to interfere with automated reticulocyte enumeration in patients with an unstable hemoglobin hemolytic anemia. (87,88). Additional studies are needed to clarify these issues.

Hematology analyzers with immunofluorescence capability. Fluorescence reticulocyte enumeration using a recently developed hematology analyzer (Cell-Dyn(r)-4000; Abbott Diagnostika GmbH, Wiesbaden-Delkenheim, Germany) with an integrated argon ion laser light source and focused flow impedance has been reported (89–92). The instrument permits analysis of 26 hematologic parameters, including simultaneous determination of reticulocytes (% and absolute), reticulocyte quantitative maturational data (i.e., IRF, etc.), NRBCs, and a complete blood count (CBC) with a five-part differential on a peripheral blood specimen of <120 mL with a throughput of 115 samples/hr (93). The Cell-Dyn 4000 utilizes CD4K530 dye for reticulocyte enumeration, as discussed above. Blood specimens for reticulocyte enumeration can be refrigerated for up to 72 hr or stored at ambient temperature for up to 48 hr. Coefficient variations of 4.83%, 3.31%, and 2.02% were reported at reticulocyte levels of 1.09%, 4.31%, and 11.3%, respectively. The analysis is not affected by other cellular components, including NRBCs (90,91). The quantum efficiency of the CD4K530 was approximately an order of magnitude greater than TO, permitting the most immature reticulocytes to be separated from NRBCs and WBCs (90). An excellent correlation was achieved with manual reticulocyte counts ($R = 0.98$) (Fig. 10).

A comparative analysis of reticulocyte enumeration utilizing the CELL-DYN-4000, Sysmex R-3000, TO flow cytometry, and NMB visual microscopic counts showed the CELL-DYN-4000 to provide sensitive reticulocyte counts and IRFs comparable to those of TO flow cytometry, regardless of the flow cytometric analytic software utilized (94). In contrast, the Sysmex R-3000 and manual counts produced lower reticulocyte counts, which was attributed to a lower sensitivity for immature reticulocyte fractions. A later comparison of the CD4000 with manual, FACScan, and Sysmex R1000 methods also revealed good agreement between the CD4000 and the manual procedure, with no intermethod bias (89). There was excellent agreement between the CD4000 and Sysmex R1000 in the range of 0–6% reticulocytes, although there was some intermethod bias in the higher range. An exponential relationship ($r = 0.78$) between CD4000 IRF and Sysmex R-1000 HFR fraction values was found. A compari-

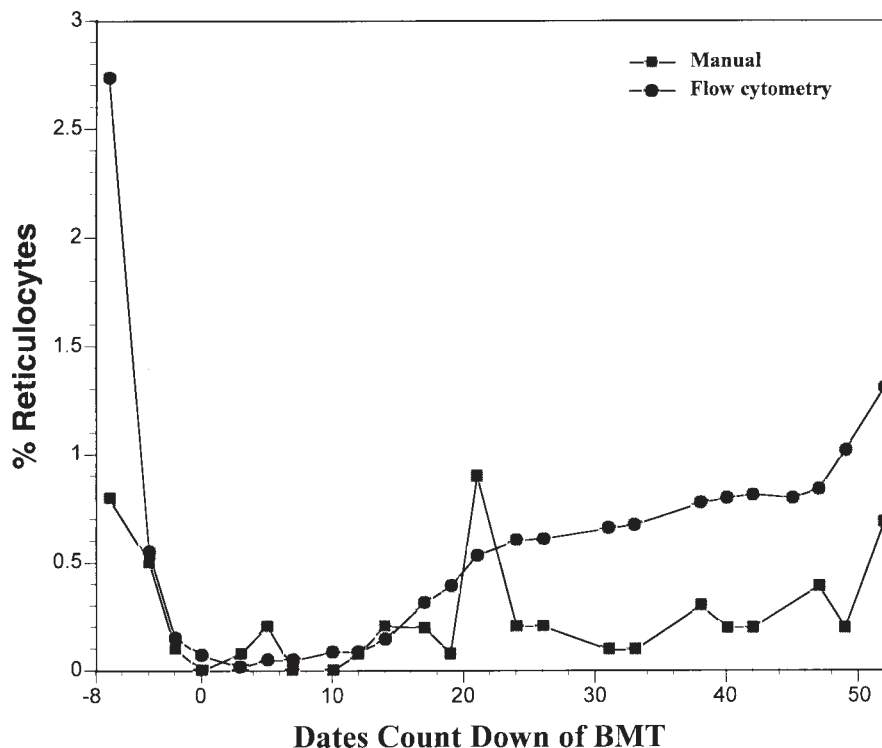


Fig. 10. Bone marrow transplantation. Flow cytometric and manual reticulocyte enumeration in a patient receiving allogeneic bone marrow trans-

plantation for malignant lymphoma. Graph shows serial reticulocyte assays obtained from 7 days pretransplantation to 52 days posttransplantation.

son of the CD4000 and FACScan indicated a distinct tendency for the FACScan to give higher reticulocyte estimates than the CD4000 (89). A recent comprehensive evaluation of five automated reticulocyte analyzers emphasized performance on samples with very low reticulocyte concentrations (95). Although all automated methods tended to overestimate the percentage and absolute reticulocyte count at low concentrations, the overestimation was statistically insufficient only for the SE 9500 RET. The SE 9500 RET and the CD 4000 produced the highest analytical sensitivity.

The CELL-DYN-4000 also provides precise ($CV < 6\%$) and accurate ($r_2 = 0.817$) counting of NRBCs in peripheral blood. In addition, bone marrow aspirates can be analyzed for total cellularity, erythroblasts, and granulocytes, while “nonviable” cells in the peripheral blood or bone marrow can be quantitated through the use of propidium iodide dye (96) (Fig. 11).

Reticulocyte maturation index. Reticulocyte RNA content, as determined by the quantitation of fluorescence intensity, has provided information previously unavailable with manual counts. Davis, Bigelow (3,4,85,98,100) and Davis et al. (97,99,101) were among the first to explore reticulocyte RNA content, and have extensively evaluated the mean fluorescence (reticulocyte maturation index, RMI, RNA index, and RNA content) of the TO-stained reticulocyte population as a clinical parameter since the late 1980s. The RMI is directly proportional to the amount

of reticulocyte intracellular RNA, but is also affected by iron stores and other factors.

Immature reticulocyte fraction. A new laboratory parameter, the immature reticulocyte fraction (IRF), was developed by Davis and the Spanish Multicentric Study Group for Hematopoietic Recovery, and other investigators (33,102–105) as a replacement for the reticulocyte maturity index. The IRF is the sum of reticulocyte fractions with medium and high fluorescence. They identified an elevation in the IRF as the first sign of hematologic recovery in the majority of patients receiving remission-induction chemotherapy and first sign of engraftment in those undergoing bone marrow transplantation. The clinical significance of the IFR is discussed below.

The International Society of Laboratory Hematology held a workshop in June 1997 to develop a consensus on the performance, standardization, and clinical utilization of the IRF. The workshop participants reached the following conclusions:

1. The IRF is a clinically useful parameter, with increased utilization and medical benefits anticipated.
2. The IRF is most effective when viewed in correlation with the absolute reticulocyte count.
3. The IRF should be expressed as a fraction (0.00–1.00).
4. Standardization of IRF methods is impractical at this time due to a lack of stable reference material and an accepted reference method.

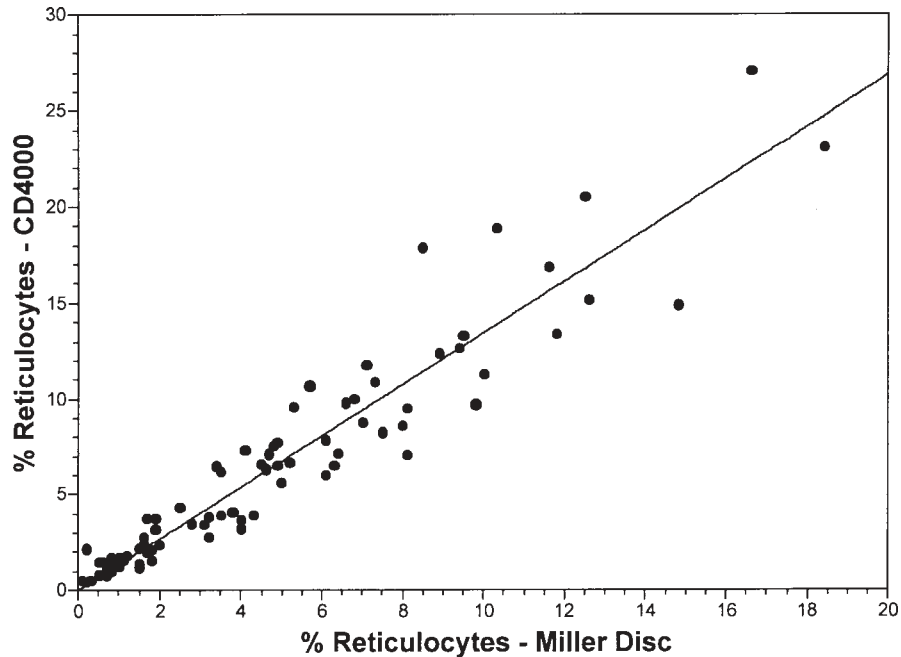


Fig. 11. Comparison of reticulocyte enumeration by light microscopy with Miller disc vs. CD4000. $n = 82$, $R = 0.9487$.

5. IRF (normal) reference ranges inherently differ between methods.
6. IRF measurements should allow for the detecting and reporting of decreased, normal, and increased values within the spectrum of clinical samples expected from patients with hematologic disorders. Manufacturers should report imprecision information (standard deviation and CV) for a given method at the low, median, and high values of the expected reference range.
7. Manufacturers should report intermethod comparisons over the entire IRF-reportable range.
8. IRF multilevel and stainable quality control material can and should be provided by manufacturers.
9. Sources of potential interference with IRF measurement should be evaluated and reported by manufacturers.

Reticulocyte-specific monoclonal antibodies. Fluorochrome-labeled monoclonal antibodies specific for several different reticulocyte antigens have been evaluated as reagents for reticulocyte enumeration. The antigens presently evaluated include the CD71 molecule (transferrin receptor, TfR, and T9 antigen), CD36, and other antigens.

CD71 is a homodimeric glycoprotein consisting of two 95 kDa chains which is expressed on early thymocytes, activated T and B lymphocytes, myelocytes, nucleated erythrocyte precursors, macrophages, and nonhematopoietic proliferating cells (106). The transferrin receptor functions in iron-dependent cell growth and proliferation. During these states, there is up-regulation of the TfR gene, with subsequent increases in mRNA and protein expression. CD71 receptors are abun-

dant on the surface of early reticulocytes, but gradually decrease during reticulocyte maturation, and are absent from mature RBCs (107,108). Single color flow cytometric enumeration of reticulocytes using a fluorochrome-labeled anti-CD71 antibody was first reported in 1983 (109,110). Although reproducible results were obtained, this method of analysis appears to offer few advantages over conventional methods of reticulocyte enumeration for routine clinical analysis, and is much more expensive to perform because of the relatively high cost of the monoclonal antibody. In addition, Shumak and Rachkewich (111) found a marked patient-to-patient variation in the number of receptors per reticulocyte (0–67,000), and were unable to correlate receptor density with the reticulocyte count, hemoglobin level, or other parameters. The clinicopathologic significance of these variations is unknown at the present time. A recently developed dual-color technique utilizing fluorochrome-labeled anti-CD71 and TO permits reticulocyte subset analysis (110).

The CD36 antigen (gpIV, gpIIIb) is a transmembrane glycoprotein and the receptor for thrombospondin and collagen, which is expressed on human thrombocytes, monocytes, macrophages, erythroblasts and B-cells (weak), epithelial cells, macrophages, and other cells. CD36 and the integrin alpha-4-beta-1 complex (a receptor for fibronectin and vascular cell adhesion molecule-1) are expressed on reticulocytes but not mature erythrocytes, and may mediate an abnormal adherence of red cells to the vascular endothelium, which contributes to the vascular occlusion characteristic of sickle cell disease (112–114). Utilizing density fractionation and flow cytometry, CD36-positive reticulocytes correlate with the

stress reticulocyte fraction in patients with chronic hemolytic diseases other than thalassemia (115). The significant elevation of CD36-positive stress reticulocytes in patients with sickle cell anemia, even after splenectomy, may contribute to the vasoocclusive episodes characteristic of this disease (115).

Optical light scatter

A new era in reticulocyte enumeration began with the recent development of methods for reticulocyte enumeration using existing hematology analyzers. This has enormous advantages for most laboratories, since it appears to offer the accuracy and sensitivity of flow cytometric reticulocyte enumeration without the need for separate instrumentation. In addition, this technology has the potential to eliminate the need for a technical staff trained in flow cytometry to perform reticulocyte enumeration, to greatly reduce laboratory operating expenses through elimination of manual reticulocyte counts, and to provide more rapid turnaround time. In addition, flow cytometric facilities operate only during weekdays in many institutions, which precludes the use of this instrumentation for reticulocyte counts, as they are required for patient care at nights and on weekends and holidays.

Abbott Diagnostika GmbH. The CELL-DYN 3500 (Abbott Diagnostika GmbH, Wiesbaden-Delkenheim, Germany) hematology analyzer has been recently utilized for reticulocyte enumeration. This instrument incorporates a 633 nm neon helium laser with optics and electronics for flow cytometric single cell analysis by electrical resistivity (impedance) and multi-angle light scatter at 0°, 10°, 90° polarized, and 90° unpolarized (116). With this technology, whole blood is stained in a single-step dilution procedure with a supravital dye solution, incubated for 5 min, and analyzed using 0°, 10°, and 90° light scatter (Fig. 12). Incubation time is not critical, and smears can be prepared from the stained blood for manual enumeration. Software-based data management provides the absolute and relative reticulocyte count, together with reticulocyte maturity information. The software also collates the hemogram, differential, and reticulocyte data.

Beckman Coulter, Inc. Beckman Coulter, Inc. (Fullerton, CA) was among the first to offer reticulocyte enumeration technology on their STKS™, MAXM™, and MAXM A/L instruments. The Coulter technique utilizes a new methylene blue stain and differentiates reticulocytes from mature RBCs, white cells, and platelets through measurement of impedance, radiofrequency, and laser light scatter (VCS technology). The computer software provides a reticulocyte percentage, absolute reticulocyte count, mean reticulocyte volume, and maturation index in 60–120 sec based on examination of 32,000 cells. Samples for analysis are prepared off-line. Whole blood is first incubated with a new methylene blue dye stain. An aliquot of the stained sample is then diluted with a hypotonic acid solution to clear hemoglobin from the cells. The instrument determines the volume, conductivity,

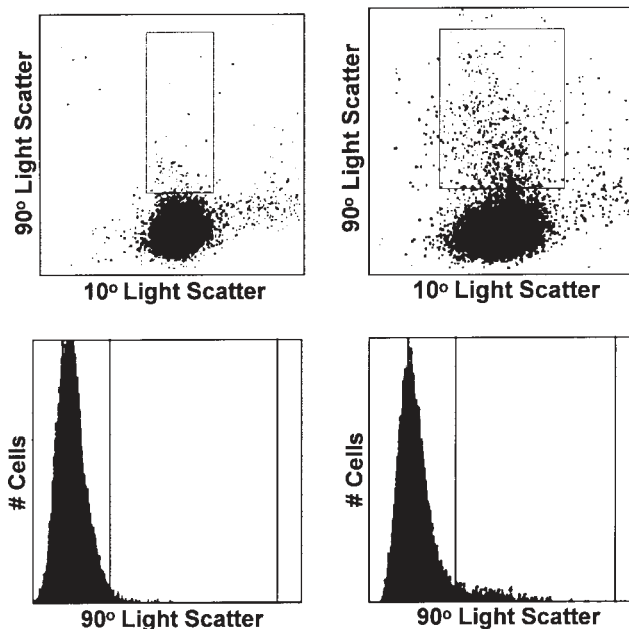


Fig. 12. Reticulocyte enumeration with optical light scatter analysis. **a:** Normal peripheral specimen stained with supravital dye. Reticulocytes (gated area, **top**) are separated from mature RBCs by dual-parameter light scatter at 10° and 90°. They appear in the area between the threshold limits on a single-parameter histogram of 90° light scatter (**bottom**). RBC: 3.65 MIL/mL; reticulocyte %: 1.65; reticulocyte #: 60.2 THO/mL. **b:** Peripheral blood specimen with increased reticulocyte count. RBC: 2.88 MIL/mL; reticulocyte %: 10.3; reticulocyte #: 296.6 THO/mL. Reproduced with the permission of Abbott Diagnostika GmbH.

and laser light scatter characteristics of each cell and plots the results within a three-dimensional matrix. The position of the reticulocytes within the matrix indicates their relative maturity. Less mature reticulocytes have more residual RNA and greater volume, scatter more laser light, and are larger than the more mature reticulocytes. With maturation, the reticulocyte loses RNA and moves toward the mature erythrocyte population, until finally it has no RNA, absorbs no stain, and merges with other mature RBCs. Both percent and absolute reticulocyte counts are clinically reportable.

Several published studies show excellent correlation between the Beckman Coulter optical light scatter analysis and the manual and flow cytometric methods of reticulocyte enumeration. For example, Buttarello and collaborators (117) found that the MAXM analyzer provided reticulocyte counts with good overall correlation with manual and automated (Sysmex R-1000) methods, although there was evidence of a significant overestimation at low concentrations and a moderate underestimation at normal and high reticulocyte concentrations. Imprecision at low (16.1%), intermediate (16.9%), and high (9.5%) reticulocyte concentrations was much lower than the manual method (67%, 28.9%, and 13.0%, respectively) (117). In a similar study, Davies et al. (118) reported a correlation coefficient of 0.98 with conventional light micros-

copy. The CV with the manual technique was 16–22%, vs. 12.3% with automated analysis. Rudensky (119) reported between-run CVs of 4.8%, 6.9%, and 14.5% for reticulocyte counts of 9.5%, 2.4% and 0.7%, respectively, and within-run CVs of 8.6%, 8.1%, and 6.1% for reticulocyte counts of 0.9%, 5.3%, and 18.4%, respectively. In this study, there were no significant changes in the reticulocyte count with samples stored for up to 72 h at 4–8°C.

Reticulocyte enumeration with the Beckman Coulter STKS was evaluated in a children's hospital by Ghevaert and collaborators (120). An instrument flag (R flag) occurred in 12% of the total specimens analyzed, indicating a reticulocyte count out of the linear range of the instrument or the presence of interference. One-third of the flags resulted from a reticulocyte percentage < 0.5%, which was confirmed by light microscopy, while the other two-thirds resulted from the presence of sickle cells, spherocytes, or Howell-Jolly bodies. The instrument tended to overestimate the reticulocyte count in patients with beta-thalassemia major. This problem was attributed to the presence of Pappenheimer bodies, even though these specimens were not flagged. The correlation coefficient of the total population between manual and instrumental reticulocyte counts was highly significant, with an *r*-value of 0.862.

The Beckman Coulter GEN·S hematology system is a top-of-the-line hematology analyzer that offers standard fully automated, on-line reticulocyte analysis utilizing new methylene blue dye. In addition to the features of the STKS and MAXM, the Gen-S includes the IntelliKinetics™ application and AccuGate™ software. IntelliKinetics™ is a system of software and hardware that provides accurate control of timing, reagent reaction temperatures, and volumes. AccuGate software utilizes nonlinear separation techniques, multi-resolution analysis, and adaptive gating to accurately differentiate small red cell populations with overlapping features. As a result, the instrument has a linear range of 0–30%, or 0–750 reticulocytes × 10⁹/L.

Similar results were recently reported for the Beckman Coulter GEN·S hematology analyzer (121,122). Chiron et al. (123) found a unique parameter provided by the GEN·S, the mean spherized corpuscular volume (MSCV), to be potentially useful in the evaluation of patients with hereditary spherocytosis (HS). Under the hypoosmotic conditions of reticulocyte enumeration, the MSCV became smaller than the MCV, presumably because of increased osmotic fragility and cell fragmentation.

Bayer/Miles, Diagnostics Division. Reticulocyte enumeration utilizing the Bayer/Miles Technicon H*3 blood analyzer (Bayer/Miles, Diagnostics Division, Tarrytown, NY) has been developed. In this system, the nucleic acid-binding dye oxazine 750 is used, and reticulocyte enumeration is determined by helium-laser light absorption and light scatter at low angle (2°–3°) and high angle (5°–15°). An absorption threshold is used to separate stained reticulocytes from un-

stained RBCs. Since the amount of light absorbed by reticulocytes is proportional to the intensity of staining and their RNA content, three populations of low, medium, and high RNA content can be defined. In addition to the absolute and relative reticulocyte counts, this technology permits direct measurement or calculation of reticulocyte cellular indices, including the reticulocyte mean cell volume (MCV_r), reticulocyte mean cell hemoglobin concentration (CHCM_r), reticulocyte cell hemoglobin content (CHR), and their respective distribution widths (RDW_r, HDW_r, and CHDW_r) (6).

Several studies have shown the Bayer/Miles H*3 technique to be precise, with an acceptable intraclass correlation between it and other automated techniques. Buttarello and collaborators (124) reported a CV of 11.6% with the Bayer/Miles H*3, compared with 4.2% for the Sysmex R-1000 and 24.2% for manual microscopy. The Bayer/Miles H*3 tended to overestimate low reticulocyte values with respect to both the microscopic method and the R-1000, a tendency that was especially pronounced in patients with marrow aplasia. Brugnara et al. (125) compared the Bayer/Miles H*3 reticulocyte counting with manual counting, flow cytometry, and the Sysmex R-3000. There was acceptable intraclass correlation between the Bayer/Miles H*3 and the Sysmex R-3000 (0.952), and flow cytometry (0.922). Reticulocyte counts were stable after storing blood samples for 72 hr at 4°C. The direct cost/test, including depreciation, were as follows: manual counting: \$1.61; Sysmex R-3000: \$6.03; flow cytometry: \$3.34; and Bayer/Miles H*3: \$3.49. Cost/test for flow cytometry and Miles H*3 were \$3.34 and \$3.49, respectively (125). Reference ranges for reticulocytes on the Bayer/Miles Technicon H*3 have been reported (126).

Other Techniques

Techniques using density gradient fractionation and other phenomena have been reported, but these have not been accepted for clinical use (127).

Automated counting devices for NMB-stained peripheral blood smears ("image analysis") are under development to decrease the imprecision associated with manual reticulocyte enumeration. In particular, slide-based instruments developed for the automated determination of the white blood cell differential (Geometric Data Hematrak, Coulter Diff 3) have been utilized (128,129). These devices provide excellent counting reproducibility, but the reticulocyte count must be verified manually in many circumstances. In one study utilizing fluorescence image cytometry, *in vitro* changes in the degree of sickling of reticulocytes and nonreticulocytes were studied under conditions of complete (PO₂ = 0 mm Hg) and partial (PO₂ = 30 mm Hg) deoxygenation at pH 7.4. Reticulocytes were identified by AO staining, while the degree of sickling was quantitated by image analysis. A subset of reticulocytes was identified that was more susceptible to sickling than nonreticulocytes. Furthermore, sickling increased dramatically

with time, even under conditions of partial deoxygenation approximating those normally observed in the venous circulation. The effect of several metabolic inhibitors on sickling was studied using this methodology (130). In another application of image analysis, a two-stage staining technique was used to quantitate the proportion of reticulocytes containing fetal hemoglobin (HbF, F-reticulocytes). The results correlated well with the conventional F-reticulocyte immunofluorescence assay, but the technique was much more reliable and easier to perform (131).

CLINICAL UTILIZATION OF RETICULOCYTE ENUMERATION

The erythropoietic activity of the bone marrow and the rate of delivery of cells from the bone marrow into the peripheral blood determine the number of reticulocytes in the peripheral blood. Since reticulocyte enumeration provides information about the bone marrow activity and the effectiveness of RBC production, it is crucial in the diagnosis of anemic patients, and for monitoring bone marrow transplantation patients, patients undergoing therapy with marrow toxic drugs, and patients being treated for anemia (132).

General Considerations

Reticulocytosis (an increased number of circulating reticulocytes) normally occurs in anemic patients with functional bone marrow. This includes patients with blood loss or hemolytic anemias (sickle cell anemia, thalassemia, spherocytosis, glucose-6-phosphate dehydrogenase deficiency, immune hemolytic disease, and hypersplenism), and patients who have been successfully treated for other types of anemia. In contrast, patients with marrow ablative disorders, impaired erythropoiesis, or decreased erythropoietin production may show a normal or decreased reticulocyte count in spite of severe anemia. Such patients include those with iron, folate, or vitamin B₁₂ deficiency anemias, pernicious anemia, immunologic or drug-induced red cell aplasia, leukemia or

metastatic carcinoma, renal failure, idiopathic myelofibrosis, and other disorders (Table 3).

Accurate reticulocyte enumeration is critical for the diagnosis of many hematologic diseases and for the classification of patients with anemia. In addition to its diagnostic value, the reticulocyte count is also playing an increasingly important role in monitoring the progress of patients receiving conventional or experimental therapy for a variety of diseases. In this regard, a new era in clinical hematology began with the use of recombinant human erythropoietin (rhEPO) and other hematologic growth factors to stimulate erythron production. Recently, rhEPO used in conjunction with oral or parenteral iron administration has been used to stimulate erythropoiesis in anemia subjects, including those with refractory anemia associated with various malignant disorders, preterm infants, chronic renal failure, and other patients (133–140). In addition, the use of rhEPO in patients scheduled for major elective surgical procedures can facilitate preoperative autologous blood collection and minimize the need for allogeneic blood transfusions (141–143). Synthetic erythropoietin has also been successfully utilized in patients who refuse transfusion based on religious convictions (144). Finally, rhEPO and other hematologic growth factors are essential to promote bone marrow regeneration in patients receiving chemotherapy or bone marrow transplantation. The major clinical applications of the reticulocyte count and reticulocyte indices are listed in Table 4.

Reference intervals for reticulocyte analysis have been determined by several investigators. Sandberg and collaborators (50) performed a detailed study of 13 healthy adult individuals over a 7-week period utilizing the Sysmex R-1000 and the Technicon H*3. Mean values obtained in this population with the Sysmex were as follows: reticulocytes: $0.044 \times 10^{12}/L$, HFR 1.5%, MFR: 10.4%, LFR: 88%. The within-subject CV for the reticulocyte count was 11% (8.8–14) and the between-subject variation was 33% (24–55). Although Sandberg et al. (50) did not identify a sex-associated difference in the reticulocyte count, Tarallo et al. (49,145)

TABLE 3. Clinical significance of the reticulocyte count

Clinical disease	Mechanism	Cause
Reticulocytopenia		
Hypochromic anemias	Impaired hemoglobin synthesis	Iron deficiency, anemia of chronic disease, thalassemias, sideroblastic anemia
Aplastic anemias	Impaired erythropoiesis	Idiopathic, renal disease, metastatic infiltration of bone marrow, viral infections, immunologic-, drug-, or radiation-induced red cell aplasia
Megaloblastic anemia	Impaired DNA synthesis	Vitamin B ₁₂ , folate deficiency
Aplastic crisis in hemolytic anemia	Variable	Variable
Reticulocytosis		
Blood loss	Increased erythropoiesis	Covert blood loss, subacute overt blood loss
Hemolytic anemias	Increased RBC destruction	Hemoglobinopathies, disorders of red cell membranes, immune hemolytic disease, glycolytic enzyme defects, hypersplenism

TABLE 4. Clinical utilization of reticulocyte count and reticulocyte indices

	Reticulocyte count	Reticulocyte indices
Hematologic diagnosis		
Classification of anemic patients	•	•
Diagnosis and severity of hemolytic anemia	•	•
Assessment of bone marrow function	•	•
Aplastic crisis in hemolytic anemia	•	•
Myelodysplasia	•	•
Occult or compensated hemorrhage or hemolysis	•	•
Sickle cell crises and other complications in sickle cell anemia	•	•
Treatment monitoring		
EPO therapy in ESRD, AIDS, MPO, infants, etc.	•	•
Bone marrow regeneration post BMT or chemoRx	•	•
Renal transplantation engraftment	•	•
Anemia therapy (Fe, ruEPO, B ₁₂ , folate etc.)	•	•
Bone marrow toxic insults	•	•
Hydroxyurea therapy in sickle cell anemia	•	•
Neonatal transfusion requirements	•	•
Other applications		
Timing of stem cell harvest	•	•
Erythropoietin abuse in sport contestants	•	•

reported a significantly higher reticulocyte count in men than women using the Sysmex R-3000. There was, however, no statistical difference between boys and girls aged 4–19 years, nor was the reticulocyte count affected by menstrual cycle, smoking, oral contraceptives, or menopause. Bock and Herkner (44,146) evaluated automated reticulocyte enumeration in infants and children, and established age-dependent reference ranges. In serial studies of anemic newborn infants and pediatric patients recovering from chemotherapy-induced bone marrow suppression or ineffective erythropoiesis resulting from renal failure, these investigators found that the proportion of highly fluorescent reticulocytes significantly earlier than the RBC, Hb, or Hct. In children aged 3 months to 18 years, Turowski and co-workers (47) reported the following results, expressed as mean \pm 2 SD: reticulocyte count: $2.00 \pm 1.56\%$; absolute reticulocyte count: $88.8 \pm 68.94 \times 10^3/\text{mL}$; and IRF: 0.22 ± 0.16 .

Anemia

Anemias secondary to bone marrow aplasia, nutritional disorders, and bone marrow infiltration are characterized by very low reticulocyte counts ($<2\%$ corrected). In these patients, reticulocyte enumeration by manual supravital staining confirms the low reticulocyte count, but does not provide further diagnostic information. Lin and collaborators (147,148) evaluated automated reticulocyte evaluation in a large group of these

patients. Very low reticulocyte counts ($<0.03 \times 1,012/\text{L}$) and very low immature fractions ($<10\%$) were characteristic of patients with aplastic or megaloblastic anemias, while patients with marrow infiltrative diseases had nearly normal reticulocyte counts, but high immature fractions ($>30\%$). In patients with pancytopenia resulting from aplastic anemia, infiltrative marrow disorder, hypersplenism, or megaloblastic anemia, the absolute reticulocyte counts were lowest in patients with aplastic anemia or megaloblastic anemia and highest in patients with hypersplenism. The marrow reticulocyte counts and shift ratio to circulating blood did not add additional useful information for the classification of these anemias.

Hemolytic anemia without reticulocytosis is characteristic of pernicious anemia (PA), in which the reticulocyte count remains relatively low (2–3%) in spite of a florid hemolytic anemia, short RBC life span, and marked marrow erythroblastic hyperplasia (1). In PA this occurs because the severe nuclear-cytoplasmic dyssynchrony results in the loss of RNA prior to nuclear extrusion (149). This phenomenon also occurs in patients with folic acid deficiency or iron deficiency anemia with superimposed hemolytic disease (1,150). A different situation occurs in autoimmune hemolytic anemia, in which life-threatening aplastic crises may occur in spite of a striking erythroblastic response because of immune destruction of erythroblasts (151,152). Aplastic crises with reticulopenia are also seen in viral infections, hereditary spherocytosis, delayed hemolytic transfusion reactions, and chronic hemolytic diseases such as sickle cell anemia (1,153–156).

“Pseudoreticulocytosis” has been described in patients with myelodysplastic syndrome (157–159). Pseudoreticulocytes are mature RBCs that retain substantia reticulofilamentosa but lack the relatively high MCV and erythrocyte enzymes characteristic of normal reticulocytes. This unusual finding is caused by delayed reticulocyte maturation. Kinetic studies in one patient with pseudoreticulocytosis revealed an erythrocyte life span of 44 days and a calculated reticulocyte maturation time of >20 days (159).

In patients with sickle cell anemia, hydroxyurea therapy increases the production of fetal hemoglobin (Hgb F) and is beneficial in alleviating both the hemolytic and vasoocclusive manifestations of the disease. Reticulocyte enumeration is widely utilized with other laboratory and clinical parameters to monitor the safety and efficacy of hydroxyurea administration (160–166). Reticulocyte enumeration has also been used to predict the likelihood of sickle cell crises, cholelithiasis, and other complications in sickle cell anemia patients (167–169).

Major and minor surgery induces a state of hypoferrremia in the presence of adequate iron stores, resembling the anemia of chronic disease. This effect is reflected by a decrease the serum iron concentration, serum transferrin concentration, and transferrin saturation, while the ferritin concentration is elevated (170). In contrast, serum erythropoietin concentration and the reticulocyte count are raised after ma-

major surgery only (170). Preoperative treatment with recombinant human erythropoietin (rhEPO) has been utilized to stimulate erythropoiesis and partially overcome the postoperative anemic state (171). Under these circumstances, only an increase in the distribution widths of reticulocytes after the second postoperative day was reported to correlate with stimulation of erythropoiesis (172).

F-reticulocytes

Reticulocytes containing fetal hemoglobin (Hb F) are termed F-reticulocytes. Measurement of F-reticulocytes is used in patients with sickle cell disease and beta-thalassemia to assess the efficacy of hydroxyurea, butyrate, and other drugs given to elevate the levels of Hb F. Immunofluorescence microscopy and fluorescence image cytometry has been utilized for the simultaneous determination of F-hemoglobin and reticulocytes (131,173,174).

Reticulocyte maturation index

Wells and collaborators (175) found a significantly elevated reticulocyte mean channel fluorescence in iron-deficient or iron-depleted patients, compared to healthy controls, while the reticulocyte mean channel fluorescence in patients with the anemia of chronic disease was not significantly different from the controls. There was a significant correlation of the reticulocyte mean channel fluorescence with total iron-binding capacity ($P < 0.0001$, $r = 0.62$) and ferritin ($P < 0.0001$, $r = 0.40$). The clinical value of the flow cytometrically determined reticulocyte count and RMI was also proven for renal transplantation patients and patients with anemia from a variety of causes (101,134,135,176–178).

Immature reticulocyte fraction

Chang and co-workers (179) evaluated the IFR in patients with anemia. An IFR < 0.23 was found to correlate with an unresponsive or underresponsive bone marrow, whereas an IFR > 0.23 was found in patients showing an adequate erythroid response to the anemia. In this study, there was a weak but significantly positive correlation with the absolute reticulocyte count (ARC) and reticulocyte production index (RPI), indicating that IFR is an additional useful parameter to evaluate the erythropoietic activity in anemic patients and further classify the etiology of the anemia. Most patients with an increased IFR (> 0.23) and increased ARC had an adequate erythroid response to anemia, while the finding of a subnormal or normal ARC, an $RPI \geq 2$, and an IFR > 0.23 correlated with an underlying clinical condition, such as acute infection, iron deficiency anemia, human immunodeficiency virus infection, sickle disease with crisis, pregnancy, and myelodysplastic syndrome. Most patients with an IFR < 0.23 also showed an $RPI < 2$, and had clinical diseases with decreased erythropoietic activity—mainly chronic renal insufficiency (179).

Watanabe and collaborators reported similar results (180). A high IFR with an increased ARC was characteristic of an enhanced erythropoietic state, such as an acquired hemolytic anemia or acute blood loss, while an increased IFR with a normal or reduced ARC was found in patients with dyserythropoietic or ineffective erythropoietic conditions, such as acute myeloid leukemia, myelodysplastic syndrome, aplastic anemia, or megaloblastic anemia. Reticulocyte maturity was normal in patients with reduced erythropoiesis, including chronic renal failure and iron deficiency anemia (180).

Reticulocyte hemoglobin content

There is a great interest among hematologists and clinical pathologists in reticulocyte cell indices, especially the reticulocyte hemoglobin (CHr), as indicators of erythropoiesis in a variety of clinical situations (181,182). The mean hemoglobin content of reticulocytes (CHr) and RBCs (CH) is calculated as the product of the volume and the hemoglobin concentration of single cells. Brugnara and colleagues (183,184) have confirmed the clinical significance of CHr in a wide variety of disease states. For example, in children they found CHr and Hb levels to be the only significant predictors of iron deficiency, while the CHr was the only significant multivariate predictor of iron deficiency anemia. A CHr value of 26 PG provided the optimal cutoff for iron deficiency based on sensitivity and specificity analysis (183).

Reticulocyte hemoglobin

The reticulocyte hemoglobin (retHb) is a measure, in grams per liter, of the hemoglobin contained in the circulating reticulocyte compartment (185). It is a calculated value, obtained by multiplying the absolute reticulocyte count and the CHr. A related value, the hemoglobin contained in the RBC pool (rbcHb), can be calculated by subtracting retHb from the total hemoglobin. The ratio between the two pools (rbcHb/retHb) provides a rough estimate of RBC survival. In 50 normal control subjects, retHb values were 1.76 ± 0.59 g/L, while the rbcHb/retHb ratio had a normal value of 76.6 ± 21.9 (185). Among a group of patients with various hemoglobinopathies, the retHb values were highest in patients with SS disease with no alpha-thalassemia (6.47 ± 3.05 g/L, $n = 20$), while the rbcHb/retHb ratio was the lowest in these same patients. The retHb and rbcHb/retHb ratio were helpful in monitoring therapy with hydroxyurea, intravenous or oral iron, or rHuEPO (185).

Bone Marrow Regeneration

In addition to the diagnosis of hematological diseases, reticulocyte counting also provides useful information regarding the degree of regenerative activity that takes place after treatment of iron deficiency or vitamin B₁₂/folate deficiency

anemias, a course of chemotherapy, bone marrow transplantation, or malaria infestation (77,97,135,176,186–201). In patients with end stage renal disease on hemodialysis, reticulocyte enumeration has been utilized in the diagnosis of iron deficiency anemia and to follow the efficacy of treatment with oral iron supplementation, erythropoietin, and other agents (202–205).

Much of the interest in the clinical use of automated reticulocyte technology has focused on patients with chemotherapy-induced marrow aplasia. For example, Kuse (206) evaluated reticulocyte levels with the Sysmex-3000 in patients with acute myelogenous leukemia (AML) treated aplasiogenic cytostatic drug therapy. During therapy induction, the reticulocyte count reached an extreme nadir ($<0.01/\text{pL}$) and consisted only of cells with a low fluorescent ratio (LFR) (206). The reticulocyte fraction with medium fluorescence ratio (MFR) began to rise at a median interval of 16 days, followed by HFR reticulocytes a median of 1 day in all cases with partial or complete remission. Granulocytes levels reached the critical limit of $\geq 0.5/\text{nl}$ at a median of 7 days after MFR and 5 days after HFR. Davis and collaborators (97) found the RMI to be an independent parameter of erythropoiesis in autologous bone marrow transplantation patients. In these patients the RMI, but not the absolute number or percentage of reticulocytes, correlated temporally with absolute neutrophil counts in the posttransplant period, but was the earliest indicator of bone marrow engraftment in most patients. Three patterns of engraftment (early, delayed, and failed) were defined by the RMI. Davis et al. (207) later proposed criteria for the standardization of RMI measurements. A ratio of HFRs, defined using a normal adult population, and expressed as the total number of reticulocytes (HFR %), was superior for interlaboratory comparison and clinical utilization. A multi-institutional interlaboratory correlation study performed at eight sites with four different types of flow cytometers revealed interclass correlations among all sites ranged from 0.79 to 0.99 for the reticulocyte counts and from 0.41 to 0.88 for the reticulocyte maturity index (4). Later studies confirmed these reports. For example, Dalal and co-workers (208) found that the RMI predicted engraftment earlier (median day 17, range 10–63 days) than the absolute neutrophil count (ANC, median day 19, range 8–63 days), WBC (median day 19, 9–71), absolute reticulocyte count (median day 19, 11–125), or platelet count (median day 29, 11–237) in 37 patients undergoing autologous or allogeneic bone marrow transplantation. However, in all patients the combined use of the ANC and RMI (whichever increased first) predicted engraftment earlier (median 15 days) and more confidently (no false starts) than either used alone. In patients mobilized with chemotherapy and growth factors for peripheral blood stem cell harvest, Remacha et al. (209) found that an increase in immature reticulocyte fractions preceded the presence of circulating CD34⁺ cells by about 2 days, suggesting that flow cytometric reticulocyte enumeration might be helpful in monitoring the timing of stem cell harvesting.

The Spanish Multicentric Study Group for Hematopoietic Recovery evaluated automated flow cytometric reticulocyte enumeration with the Sysmex-2000 in the evaluation of hematopoietic recovery status postchemoradiotherapy-induced aplasia and autologous or allogeneic bone marrow transplantation (102). Serial automated flow cytometric quantitative reticulocyte counting provided a useful and early measure of erythropoiesis indicative of hematopoietic reconstitution or successful bone marrow engraftment following marrow transplantation. Similarly, in aplastic anemia patients receiving bone marrow or peripheral stem cell transplantation, the HFR counts provided the most reliable and sensitive index of hemopoietic recovery after bone marrow or peripheral blood stem cell transplantation (210). A difference in the clinical significance of the HFR counts autologous selected CD34⁺ peripheral blood stem cell transplantation and unfractionated PBPC transplantation has been noted (211). The HFR count gave advance notice of complete and stable hemopoietic engraftment in patients receiving unfractionated PBPC transplantation, while the HFR and ANC count showed almost simultaneous recovery in patients receiving the CD34⁺ selected transplants. In a series of patients receiving peripheral blood stem cell transplantation for non-Hodgkin's lymphoma, George and collaborators (212) found that recovery of the HFR to 2% of the total reticulocytes preceded an ANC of $0.1 \times 10^9/\text{L}$ in 92% of the patients, and preceded an ANC of $0.5 \times 10^9/\text{L}$ in 96% of cases.

In patients with lymphoma and myeloma, Gowans et al. (213) did not find Sysmex R2000 reticulocyte parameters useful in optimizing the timing of peripheral blood stem cell harvesting due to high interpatient variability. In this study, a rising immature myeloid index (IMI), determined on the Sysmex SE9000, was the most accurate predictor of early bone marrow regeneration.

Chronic Renal Failure

The recent introduction of recombinant human erythropoietin (rHuEPO, epoetin beta) revolutionized the treatment of anemia associated with chronic renal failure. In addition, rHuEPO therapy is under evaluation for anemia associated with many other diseases associated with relative erythropoietin deficiency or bone marrow suppression, including cancer, cancer chemotherapy and bone marrow transplantation, myelodysplasia, prematurity, perioperative state and preparation for elective surgery, zidovudine-induced anemia in HIV-infected patients, sickle cell anemia, and other diseases (214–226). Since rHuEPO-stimulated erythropoiesis depends on an adequate and continuous supply of iron, close laboratory monitoring of iron status is critical to achieving an optimal therapeutic response, and rHuEPO treatment is often accompanied by intravenous (IV) iron therapy. Of the conventional parameters, the reticulocyte count is the most useful laboratory monitor. More recently, several other parameters

have been shown to provide a more accurate measure of the marrow iron supply. These parameters are the CHr, percentage of hypochromic erythrocytes, and RBC ferritin (RBCFer) (182,203,204,227,228).

Reticulocyte count

In chronic renal failure patients receiving erythropoietin, Dalbak and associates (229), using the Sysmex-1000, reported an increase in reticulocytes within days, significantly prior to hemoglobin levels, which increased only after 2–3 weeks. Tarallo et al. (49) utilized the Sysmex R-3000 to perform reticulocyte enumeration in 1,219 healthy individuals 4 to >60 years of age. Although the reticulocyte counts in children were not statistically different, men >20 years old had a significantly higher reticulocyte count than women of the same age. The reticulocyte count was not affected by moderate smoking, menstrual cycle, oral contraceptives, or menopause (49). Automated flow cytometric reticulocyte analysis was used by Jeffrey et al. (202) to evaluate patient responses to low-dose erythropoietin. In the study, 16 adult dialysis patients were administered modest doses of recombinant human EPO (rHuEPO) subcutaneously thrice weekly (mean dose: 15.7; SD: 3.7 U/kg). Although there was a wide variation in response, the reticulocyte count increased in 14 patients during the first week, and reached a maximal response during the second week. Upon further investigation, five patients who exhibited a brisk reticulocyte response without a sustained increase in hemoglobin level had gastrointestinal bleeding. Other disease processes were found in the two patients who showed a poor reticulocyte response. The authors concluded that reticulocyte enumeration provides useful clinical information in renal dialysis patients receiving rHuEPO.

Immature reticulocyte fraction

Davis and coworkers (230) studied the clinical significance of the IRF in chronic renal failure and co-workers. Automated reticulocyte counts and the IRF were determined with the Cell-Dyn 4000 in 114 patients receiving chronic dialysis and recombinant erythropoietin therapy during a 6-month period. The IRF weakly correlated with other laboratory values, but was the only laboratory parameter to significantly vary with the degree of anemia. An IRF > 0.30 and either normal or increased reticulocyte counts correlated with a subsequent increase in hemoglobin levels, while most patients who did not develop an elevated IRF showed a subsequent worsening of their anemia.

Reticulocyte hemoglobin

In one of the largest studies of hemodialysis patients, Fishbane and co-workers (231) found that a CHr of less than 26 pg at baseline had a sensitivity of 100% and a specificity of 80% in predicting iron deficiency. The percentage of hy-

pochromic RBCs, serum ferritin, and transferrin saturation were less accurate. With intravenous iron dextran therapy, most iron-deficient patients responded with a correction of the mean CHr to > 26 pg within 48 hr. Cullen et al. (205) performed detailed laboratory studies of patients undergoing chronic dialysis and also concluded that the CHr is superior to the percentage of hypochromic red cells in detecting iron deficiency. In this study, CHr < 26 pg had a diagnostic sensitivity and specificity of 100% and 73%, respectively, compared with 91% and 54% for a percentage of hypochromic red cells > 2.5%. Sowade and collaborators (171) performed a large double-blind, randomized, placebo-controlled clinical trial of epoetin beta therapy in cardiac surgery patients, and concluded that the monitoring of reticulocyte parameters provided an objective analysis of erythropoietic activity.

Myelodysplasia provides a challenging situation for the evaluation of erythropoiesis, and was one of the first diseases to be studied with the Bayer/Miles H*3 analyzer. In 32 MDS patients and 10 elderly normal subjects, Bowen and collaborators (232) found that reticulocytes from MDS patients were larger and had a lower hemoglobin concentration than reticulocytes from the control group, and concentrated hemoglobin more during maturation to RBCs. Among individual subtypes of MDS, reticulocytes from sideroblastic patients had a lower hemoglobin content and concentration than refractory anemia patients. Although the pathogenesis of these findings is unclear, they may assist in determination of early therapeutic response in MDS patients (232).

During the postpartum period, Richter et al. (45,233) found no evidence of functional iron deficiency or increased erythropoiesis. The minor changes in a group of 82 postpartum patients included an increase in microcytic cells, from 0.9% prepartum to 1.4% on day 42 postpartum, a decrease in hypochromic cells from 4.3% to 1.9%, and a decrease in the reticulocyte mean corpuscular volume from 134 to 125 fL. The reticulocyte mean hemoglobin content was unchanged during this period (45,233).

Other Applications

Miscellaneous uses of flow cytometric reticulocyte enumeration include investigations of the persistence of reticulocytes in refrigerated blood storage and in vivo post-transfusion, the relationship between CD36 expression and stress reticulocytosis in patients with sickle cell anemia and other chronic hemolytic disorders, and the presence of reticulocytes reacting with the IgG fraction of an antiserum against cord RBC membranes (F-IgG reactive RBCs) (115,234,235).

A recent novel application of reticulocyte enumeration is to detect erythropoietin abuse in sports (236). In these circumstances, precise, accurate determinations of the reticulocyte count are needed.

SUMMARY AND FUTURE PROSPECTS

The final stage of RBC differentiation occurs in the peripheral blood. The immature RBCs (reticulocytes) that are released by the bone marrow still contain RNA and some cellular organelles. Reticulocytes gradually lose their protein synthesizing machinery, and normally become mature RBC (erythrocyte) after about 3 days in the peripheral blood. Since the number and characteristics of the reticulocytes in the peripheral blood reflect the activity of the bone marrow, reticulocyte counting has become a fundamental part of the evaluation of patients with hematopoietic disease.

Circulating reticulocytes are decreased in patients with impairment of bone marrow function, and are increased in cases of blood loss or destruction with normal bone marrow activity. Reticulocyte enumeration has been performed for several decades by light microscopy, with the use of a supravital dye (new methylene blue), which binds to the RNA in the reticulocyte. However, the accuracy and precision of this assay are greatly compromised by its subjective nature, and by the limited number of cells (200–1,000) that can be counted by a technologist in a reasonable length of time. In contrast, automated techniques of reticulocyte enumeration are more precise, accurate, objective, and cost-effective, since 30,000 or more cells can be accurately evaluated in a very short period of time. A variety of RNA-specific fluorescent dyes have been utilized for automated reticulocyte enumeration, and some hematology analyzers utilize optical light scatter analysis to perform reticulocyte analysis on specimens stained with new methylene blue or other dyes. Approximately 45% of laboratories recently participating in the CAP reticulocyte presently use some form of automated reticulocyte analysis. In addition to relative and absolute reticulocyte counts, automated techniques provide information regarding the age distribution of reticulocytes, which is not available by light microscopy. Measurements of the RMI, IRF, CHR, and other parameters are still under investigation, but there is extensive evidence that these parameters are useful in the accurate classification of anemia patients, and monitoring patients receiving ruEPO or recovering from chemotherapy or bone marrow transplantation. Although economic considerations limit dedicated reticulocyte analyzers to laboratories with a large reticulocyte sample volume, the recent trend to incorporate reticulocyte analysis into the routine capacity of the hematology analyzer will make automated reticulocyte analysis increasingly common. However, since the results of automated reticulocyte analysis are influenced by analytic methodology, dye staining sensitivity for RNA, and software discrimination of reticulocytes from erythrocytes and other cells, method-specific reference intervals are presently necessary, and most automated methods tend to overestimate the reticulocyte count, particularly at low reticulocyte concentrations (95). Perhaps in a few years the manual reticulocyte count will become a thing of the past.

REFERENCES

1. Crosby WH. Reticulocyte counts. *Arch Int Med* 1981;141:1747–1748.
2. Cavill I. The rejected reticulocyte. *Br J Haematol* 1993;84:563–565.
3. Davis BH, Bigelow NC. Clinical flow cytometric reticulocyte analysis. *Pathobiology* 1990;58:99–106.
4. Davis BH, Bigelow NC. Reticulocyte analysis and reticulocyte maturity index. *Methods Cell Biol* 1994;42:263–274.
5. Nielsen OJ, Kjaersgaard E, Karle H. Renaissance of the reticulocyte. *Ugeskr Laeger* 1994;156:5673–5675.
6. Brugnara C. Use of reticulocyte cellular indices in the diagnosis and treatment of hematological disorders. *Int J Clin Lab Res* 1998;28:1–11.
7. Koepke JA. Update on reticulocyte counting. *Lab Med* 1999;30:339–343.
8. Goodnough LT, Skikne B, Brugnara C. Erythropoietin, iron, and erythropoiesis. *Blood* 2000;96:823–833.
9. Palis J, Segel GB. Developmental biology of erythropoiesis. *Blood Rev* 1998;12:106–114.
10. Mel HC, Prenant M, Mohandas N. Reticulocyte motility and form: studies on maturation and classification. *Blood* 1977;49:1001–1009.
11. Castoldi GL, Beutler E. Erythrocytes. In: Zucker-Franklin D, Greaves MF, Grossi CE, et al., editors. *Atlas of blood cells. Function and pathology*, 2nd ed., vol. 1. Philadelphia: Lea & Febiger; 1988. p 47–156.
12. Dessypris E. Erythropoiesis. In: Lee GR, Foerster J, Lukens J, et al., editors. *Wintrobe's clinical hematology*, 10th ed., vol. 1. Baltimore: Williams and Wilkins; 1999. p 169–192.
13. Noble NA, Xu QP, Ward JH. Reticulocytes. I. Isolation and in vitro maturation of synchronized populations. *Blood* 1989;74:475–481.
14. Heilmeyer L. Blutfarbstoffwechselstudien: probleme, methoden, und Kritik der Whippleschen Theorie. *Dtsch. Arch Klin Med* 1931;171:123–153.
15. Heilmeyer L, Westhäuser R. Reifungsstadien an überlebenden reticulocyten in vitro und ihre bedeutung für die schätzung der täglichen hämoglobinproduktion in vivo. *Z Klin Med* 1932;121:361–655.
16. Coulombel L, Tchernia G, Mohandas N. Human reticulocyte maturation and its relevance to erythropoietic stress. *J Lab Clin Med* 1979;94:467–474.
17. Noble NA, Xu QP, Hoge LL. Reticulocytes II: reexamination of the in vivo survival of stress reticulocytes. *Blood* 1990;75:1877–1882.
18. Brecher G. New methylene blue as a reticulocyte stain. *Am J Clin Pathol* 1949;19:895–896.
19. Deiss A, Kurth D. Circulating reticulocytes in normal adults as determined by the new methylene blue method. *Am J Clin Pathol* 1970;53:481–484.
20. Gilmer PR, Koepke JA. The reticulocyte. An approach to definition. *Am J Clin Pathol* 1976;66:262–267.
21. Crosby S. A quick and easy method for the staining of reticulocytes. *Am J Clin Pathol* 1979;72:1011–1013.
22. Finch CA, Marshall PN, Trobaugh FE. Method for reticulocyte counting. NCCLS Proposed Standard H16-P. Villanova, PA: National Committee for Clinical Laboratory Standards, 1984.
23. Koepke JF, Koepke JA. Reticulocytes. *Clin Lab Haematol* 1986;8:169–179.
24. Brecher G. New methylene blue as a reticulocyte stain. *Am J Clin Pathol* 1949;19:895–896.
25. Brecher G, Stohman F. Reticulocyte size and erythropoietic stimulation. *Proc Soc Exp Biol Med* 1961;107:887.
26. Ganzoni A, Hillman R, Finch C. Maturation of the macroreticulocyte. *Br J Haematol* 1969;16:119–135.
27. Papayannopoulou T, Finch C. Radioiron measurements of red cell maturation. *Blood Cells* 1975;1:535–546.
28. Savage RA, Skoog DP, Rabinovitch A. Analytic inaccuracy and imprecision in reticulocyte counting: a preliminary report from the College of American Pathologists Reticulocyte Project. *Blood Cells* 1985;11:97–112.

29. Koepke JA, Broden PN, Corash L, et al. Methods for reticulocyte counting (flow cytometry and supravital dyes): approved guideline. NCCLS document H44-A. Wayne, PA: NCCLS, 1997.
30. Lippi G, Nicoli M, Solero P. Reticulocyte analysis on sodium citrate-anticoagulated samples. *Lab Hematol* 1999;5:67-69.
31. Tsuda I, Tatsumi N. Reticulocytes in human preserved blood as control material for automated reticulocyte counters. *Am J Clin Pathol* 1990;93:109-110.
32. Rabinovitch A. Reticulocyte count. *CAP Today* 1993;7:42.
33. Davis BH. Clinical practice in reticulocyte testing. *CAP Today* 1996;10:25-28.
34. Peebles DA, Hochberg A, Clarke TD. Analysis of manual reticulocyte counting. *Am J Clin Pathol* 1981;76:713-717.
35. Crouch JY, Kaplow LS. Relationship of reticulocyte age to polychromasia, shift cells, and shift reticulocytes. *Arch Pathol Lab Med* 1985;109:325-329.
36. Schmitt RG. Heinz-bodies and reticulocyte counts. *Am J Med Technol* 1972;38:109-110.
37. Schwab ML, Lewis AE. An improved stain for Heinz bodies. *Am J Clin Pathol* 1969;51:673-675.
38. Greenberg ER, Beck JR. The effects of sample size on reticulocyte counting and stool examination. The binomial and poisson distributions in laboratory medicine. *Arch Pathol Lab Med* 1984;108:396-398.
39. May JA, Sage BH. Spinner films for reticulocyte counts. *Am J Med Technol* 1976;42:357-360.
40. Brecher G, Schneiderman M. A time saving device for the counting of reticulocytes. *Am J Clin Pathol* 1950;20:1079-1083.
41. Nazi KM. The Miller disk: an improvement in the performance of manual reticulocyte counts. *Lab Med* 1986;17:742-744.
42. McKenzie SB, Gauger CA. Analysis of reticulocyte counts using various methods. *Clin Lab Sci* 1991;4:37-41.
43. Koepke JF, Koepke JA. Reticulocytes. *Clin Lab Haematol* 1986;8:169-179.
44. Bock A, Herkner KR. Reticulocyte maturity pattern analysis as a predictive marker of erythropoiesis in paediatrics. Part I. Evaluation of age-dependent reference values. *Clin Lab Haematol* 1994;16:247-251.
45. Richter C, Huch A, Huch R. Erythropoiesis in the postpartum period. *J Perinat Med* 1995;23:51-59.
46. Kling PJ, Schmidt RL, Roberts RA, Widness JA. Serum erythropoietin levels during infancy: associations with erythropoiesis. *J Pediatr* 1996;128:791-796.
47. Turowski D, Wysocka J, Butkiewicz AM. Peripheral blood reticulocytes and their reference range values for percentage, absolute count, and immature fraction in children, measured with flow cytometry. *Folia Histochem Cytobiol* 2000;38:31-36.
48. Mercelina-Roumans PE, Ubachs JM, van-Wersch JW. The reticulocyte count and its subfractions in smoking and non-smoking pregnant women. *Eur J Clin Chem Clin Biochem* 1995;33:263-265.
49. Tarallo P, Humbert JC, Mahassen P, Fournier B, Henny J. Reticulocytes: biological variations and reference limits. *Eur J Haematol* 1994;53:11-15.
50. Sandberg S, Rustad P, Johannesen B, Stolsnes B. Within-subject biological variation of reticulocytes and reticulocyte-derived parameters. *Eur J Haematol* 1998;61:42-48.
51. Kozenow W, Mai H. Fluorescence microscopy of blood cells. *Ztschr. Kinderheilk* 1952;70:552.
52. Vander JB, Harris CA, Ellis SR. Reticulocyte counts by means of fluorescence microscopy. *J Lab Clin Med* 1963;62:132.
53. Thær A, Becker H. Microscope fluorometric investigations on the reticulocytic maturation distribution as diagnostic criterion of disordered erythropoiesis. *Blut* 1975;30:339-348.
54. Tanke HJ, Nieuwenhuis IA, Koper GJ, Slats JC, Ploem JS. Flow cytometry of human reticulocytes based on RNA fluorescence. *Cytometry* 1981;1:313-320.
55. Tanke HJ, Rothbarth PH, Vossen JM, Koper GJ, Ploem JS. Flow cytometry of reticulocytes applied to clinical hematology. *Blood* 1983;61:1091-1097.
56. Jacobberger JW, Horan PK, Hare JD. Flow cytometric analysis of blood cells stained with the cyanine dye DiOC1[3]: reticulocyte quantification. *Cytometry* 1984;5:589-600.
57. Sagawa H, Tatsumi N. Flow cytometric analysis of leukocytes and reticulocytes stained with proflavine. *Osaka City Med J* 1997;43:199-208.
58. Corash L, Rheinschmidt M, Lieu S, Meers P, Brew E. Fluorescence-activated flow cytometry in the hematology clinical laboratory. *Cytometry Suppl* 1988;3:60-64.
59. Tanke H. Current developments in flow cytometry and fluorescent labels. *Anal Cell Pathol* 1989;2:63-65.
60. Hackney JR, Cembrowski GS, Prystosky MB, Kant JA. Automated reticulocyte counting by image analysis and flow cytometry. *Lab Med* 1989;20:551-555.
61. Wallen CA, Higashikubo R, Dethlefsen LA. Comparison of two flow cytometric assays for cellular RNA—acridine orange and propidium iodide. *Cytometry* 1982;3:155-160.
62. Metzger DK, Charache S. Flow cytometric reticulocyte counting with thioflavin T in a clinical hematology laboratory. *Arch Pathol Lab Med* 1987;111:540-544.
63. Kojima K, Niri M, Setoguchi K, Tsuda I, Tatsumi N. An automated optoelectronic reticulocyte counter. *Am J Clin Pathol* 1989;92:57-61.
64. Lee LG, Chen CH, Chiu LA. Thiazole orange: a new dye for reticulocyte analysis. *Cytometry* 1986;7:508-517.
65. Tanke HJ, van Ingen EM. A reliable Feulgen-acriflavine-SO₂ staining procedure for quantitative DNA measurements. *J Histochem Cytochem* 1980;28:1007-1013.
66. Sage BJ, O'Connell JP, Mercolino TJ. A rapid, vital staining procedure for flow cytometric analysis of human reticulocytes. *Cytometry* 1983;4:222-227.
67. Van Hove L, Goossens W, Van Duppen V, Verwilghen RL. Reticulocyte count using thiazole orange. A flow cytometry method. *Clin Lab Haematol* 1990;12:287-299.
68. Hansson GK, Andersson M, Jarl H, Stemme S. Flow cytometric analysis of reticulocytes using an RNA-binding fluorochrome. *Scand J Clin Lab Invest* 1992;52:35-41.
69. Schimenti KJ, Lacerna K, Wamble A, et al. Reticulocyte quantification by flow cytometry, image analysis, and manual counting. *Cytometry* 1992;13:853-862.
70. Corash L, Rheinschmidt M, Lieu S, Meers P, Brew E. Enumeration of reticulocytes using fluorescence-activated flow cytometry. *Pathol Immunopathol Res* 1988;7:381-394.
71. Carter JM, McSweeney PA, Wakem PJ, Nemet AM. Counting reticulocytes by flow cytometry: use of thiazole orange. *Clin Lab Haematol* 1989;11:267-271.
72. Nobes PR, Carter AB. Reticulocyte counting using flow cytometry. *J Clin Pathol* 1990;43:675-678.
73. Ferguson DJ, Lee SF, Gordon PA. Evaluation of reticulocyte counts by flow cytometry in a routine laboratory. *Am J Hematol* 1990;33:13-17.
74. Oyamatsu T, Shimizu N, Takeuchi K, et al. Automated measurement of reticulocyte count by flow cytometry. II. Analysis of the blood containing abnormal erythrocytes or giant platelets. *Jpn J Clin Pathol* 1989;37:807-812.
75. Oyamatsu T, Shimizu N, Takeuchi K, et al. Automated measurement of reticulocyte count by flow cytometry. I. Basic study. *Jpn J Clin Pathol* 1989;37:697-701.
76. Bowen D, Bentley N, Hoy T, Cavill I. Comparison of a modified thiazole orange technique with a fully automated analyser for reticulocyte counting. *J Clin Pathol* 1991;44:130-133.
77. Davies SV, Cavill I, Bentley N, Fegan CD, Poynton CH, Whittaker JA. Evaluation of erythropoiesis after bone marrow transplantation: quantitative reticulocyte counting. *Br J Haematol* 1992;81:12-17.

78. van Houte AJ, Bartels PC, Schoorl M, Mulder C. Methodology-dependent variations in reticulocyte counts using a manual and two different flow cytometric procedures. *Eur J Clin Chem Clin Biochem* 1994;32:859–863.
79. Paterakis GS, Voskaridou E, Loutradi A, Rombos J, Loukopoulos D. Reticulocyte counting in thalassemic and other conditions with the R-1000 Sysmex analyzer. *Ann Hematol* 1991;63:218–222.
80. Laharrague P, Corberand JX, Fillola G, Marcelino N. Evaluation of an automatic analyzer of reticulocytes: the Sysmex R-1 000. *Annales Biologie Clinique* 1990;48:253–258.
81. Tichelli A, Gratwohl A, Driessen A, et al. Evaluation of the Sysmex R-1000. An automated reticulocyte analyzer. *Am J Clin Pathol* 1990;93:70–78.
82. Lacombe F, Lacoste L, Vial JP, et al. Automated reticulocyte counting and immature reticulocyte fraction measurement. Comparison of ABX PENTRA 120 Retic, Sysmex R-2000, flow cytometry, and manual counts. *Am J Clin Pathol* 1999;112:677–686.
83. Laurencet FM, Martinez T, Beris P. Spurious extreme reticulocytosis with an automated reticulocyte analyzer. *N Engl J Med* 1997;337:1922–1923.
84. Koepke JF. Reticulocyte counting. In: Koepke JA, editor. *Practical laboratory hematology*. New York: Churchill Livingstone; 1990. p 99–108.
85. Davis BH, Bigelow NC. Flow cytometric reticulocyte quantification using thiazole orange provides clinically useful reticulocyte maturity index. *Arch Pathol Lab Med* 1989;113:684–689.
86. Lofsness KG, Kohnke ML, Geier NA. Evaluation of automated reticulocyte counts and their reliability in the presence of Howell-Jolly bodies. *Am J Clin Pathol* 1994;101:85–90.
87. Hinchliffe RF. Errors in automated reticulocyte counts due to Heinz bodies. *J Clin Pathol* 1993;46:878–879.
88. Espanol I, Pedro C, Remacha AF. Heinz bodies interfere with automated reticulocyte counts. *Haematologica* 1999;84:373–374.
89. d'Onofrio G, Kim YR, Schulze S, et al. Evaluation of the Abbott Cell Dyn 4000 automated fluorescent reticulocyte measurements: comparison with manual, FACScan and Sysmex R1000 methods. *Clin Lab Haematol* 1997;19:253–260.
90. Kim YR, Kantor J, Chupp V. A rapid and sensitive automated reticulocyte method and stable reagent system. *Lab Hematol* 1996;2:57 (abstract).
91. Van Hove L, Janik J, Becker R, Schisano T, Kim YR, Chupp V. Cell-Dyn® 4000, the next generation hematology analyzer. *Lab Hematol* 1996;2:71.
92. Grimaldi E, Scopacasa F. Evaluation of the Abbott CELL-DYN 4000 hematology analyzer. *Am J Clin Pathol* 2000;113:497–505.
93. Goossens W, Scott CS, Walsh A, Vandekerckhove PH, Van Orshoven A, Van Hove L. First basic performance evaluation of the CELL-DYN® CD4000. *Lab Hematol* 1996;2:151–156.
94. Davis BH, Bigelow NC, Van Hove L. Immature reticulocyte fraction (IRF) and reticulocyte counts: comparison of CELL-DYN® 4000, Sysmex R-3000, thiazole flow cytometry, and manual counts. *Lab Hematol* 1996;2:144–150.
95. Buttarello M, Bulian P, Farina G, et al. Flow cytometric reticulocyte counting. Parallel evaluation of five fully automated analyzers: an NCCLS-ICSH approach. *Am J Clin Pathol* 2001;115:100–111.
96. d'Onofrio G, Zini G, Tommasin M, Van Hove L. Integration of fluorescence and hemocytometry in the CELL-DYN® 4000: reticulocyte, nucleated red blood cell, and white blood cell viability study. *Lab Hematol* 1996;2:131–138.
97. Davis BH, Bigelow NC, Ball ED, Mills L, Cornwell III GG. Utility of flow cytometric reticulocyte quantification as a predictor of engraftment in autologous bone marrow transplantation. *Am J Hematol* 1989;32:81–87.
98. Davis BH, Bigelow NC. Flow cytometric reticulocyte analysis and the reticulocyte maturity index. *Ann N Y Acad Sci* 1993;677:281–292.
99. Davis BH, Bigelow NC, Koepke JA, et al. Flow cytometric reticulocyte analysis. Multiinstitutional interlaboratory correlation study. *Am J Clin Pathol* 1994;102:468–477.
100. Davis BH, Bigelow NC. Automated reticulocyte analysis. Clinical practice and associated new parameters. *Hematol Oncol Clin North Am* 1994;8:617–630.
101. Davis BH, Ornvold K, Bigelow NC. Flow cytometric reticulocyte maturity index: a useful laboratory parameter of erythropoietic activity in anemia. *Cytometry* 1995;22:35–39.
102. Spanish Multicentric Study Group for Hematopoietic Recovery. Flow cytometric reticulocyte quantification in the evaluation of hematologic recovery. *Eur J Haematol* 1994;53:293–297.
103. Davis BH. Reticulocyte maturation: a proposal for consensus on nomenclature. *Lab Hematol* 1996;2:48 (Abst).
104. Davis BH. Immature reticulocyte fraction (IRF): by any name, a useful clinical parameter of erythropoietic activity. *Lab Hematol* 1996;2:2–8.
105. Procopio N, Wystepek E, Mattson JC, Bigelow NC, Davis BH. An instrument comparison of immature reticulocyte fraction (IRF). *Lab Hematol* 1996;2:76.
106. Schwarting R, Stein H. Cluster report: CD71. In: Knapp W, Dorken B, Rieber EP, et al., editors. *Leucocyte typing IV. White cell differentiation antigens*. Oxford: Oxford University Press; 1989. p 455–460.
107. van der Heul C, Kroos MJ, van Eijk HG. Characterization and localization of the transferrin receptor on rat reticulocytes. *Int J Biochem* 1982;14:467–476.
108. Horton MA. Expression of transferrin receptors during erythroid maturation. *Exp Cell Res* 1983;144:361–366.
109. Seligman PA, Allen RH, Kirchanski SJ, Natale PJ. Automated analysis of reticulocytes using fluorescent staining with both acridine orange and an immunofluorescence technique. *Am J Hematol* 1983;14:57–66.
110. Serke S, Huhn D. Identification of CD71 (transferrin receptor) expressing erythrocytes by multiparameter-flow-cytometry (MP-FCM): correlation to the quantitation of reticulocytes as determined by conventional microscopy and by MP-FCM using a RNA-staining dye. *Br J Haematol* 1992;81:432–439.
111. Shumak KH, Rachkewich RA. Transferrin receptors on human reticulocytes: variation in site number in hematologic disorders. *Am J Hematol* 1984;16:23–32.
112. Sugihara K, Sugihara T, Mohandas N, Hebbel RP. Thrombospondin mediates adherence of CD36+ sickle reticulocytes to endothelial cells. *Blood* 1992;80:2634–2642.
113. Joneckis CC, Ackley RL, Orringer EP, Wayner EA, Parise LV. Integrin alpha 4 beta 1 and glycoprotein IV (CD36) are expressed on circulating reticulocytes in sickle cell anemia. *Blood* 1993;82:3548–3555.
114. Wick TM, Eckman JR. Molecular basis of sickle cell-endothelial cell interactions. *Curr Opin Hematol* 1996;3:118–124.
115. Browne PV, Hebbel RP. CD36-positive stress reticulocytosis in sickle cell anemia [see comments]. *J Lab Clin Med* 1996;127:340–347.
116. Dorner K, Schulze S, Reinhardt M, Seeger H, Van Hove L. Improved automated leucocyte counting and differential in newborns achieved by the haematology analyser CELL-DYN 3500. *Clin Lab Haematol* 1995;17:23–30.
117. Buttarello M, Bulian P, Pra MD, Barbera P, Rizzotti P. Reticulocyte quantification by Coulter MAXM VCS (volume, conductivity, light scatter) technology. *Clin Chem* 1996;42:1930–1937.
118. Davies JI, Smyth MS, Martin JH. Automated reticulocyte counting: evaluation of the Coulter STKS haematology analyser reticulocyte counting function. *Clin Lab Haematol* 1997;19:89–92.
119. Rudensky B. Comparison of a semi-automated new Coulter methylene blue method with fluorescence flow cytometry in reticulocyte counting. *Scand J Clin Lab Invest* 1997;57:291–296.
120. Ghevaert C, Fournier M, Reade V, Mehiaoui L, Goudemand J. Laboratory evaluation of the Coulter STKS reticulocyte method in a children's hospital. *Lab Hematol* 1997;3:92–97.

121. Kessler C, Machin SJ, Pollard Y, et al. Reticulocyte performance on the Coulter® GEN'S System. *Lab Hematol* 1997;3:41–47.
122. Picard F, Gicquel C, Marnet L, Guesnu M, Levy JP. Preliminary evaluation of the new hematology analyzer Coulter GEN-S in a university hospital. *Clin Chem Lab Med* 1999;37:681–686.
123. Chiron M, Cynober T, Mielot F, Tchernia G, Croisille L. The GEN.S: a fortuitous finding of a routine screening test for hereditary spherocytosis. *Hematol Cell Ther* 1999;41:113–116.
124. Buttarello M, Bulian P, Venudo A, Rizzotti P. Laboratory evaluation of the Miles H.3 automated reticulocyte counter. A comparative study with manual reference method and Sysmex R-1000. *Arch Pathol Lab Med* 1995;119:1141–1148.
125. Brugnara C, Hipp MJ, Irving PJ, et al. Automated reticulocyte counting and measurement of reticulocyte cellular indices. Evaluation of the Miles H*3 blood analyzer. *Am J Clin Pathol* 1994;102:623–632.
126. Charuruks N, Limpanasithikul W, Voravud N, Virochpoka T, Nuchprayoon C. Reference ranges of reticulocytes in adults. *J Med Assoc Thai* 1998;81:357–364.
127. Burns ER, Goldberg SN, Wenz B. A new assay for quantifying human reticulocytes. *Am J Clin Pathol* 1987;88:338–342.
128. Perel I, Herrmann A, Clark T. Automated differential leukocyte counting by the Geometric Data Hematrak system: eighteen months experience in a private pathology laboratory. *Am J Clin Pathol* 1980;12:449–460.
129. Kaplow LF, Crouch JY. Automated reticulocyte counts. *Lab Med* 1982;13:167–169.
130. Horiuchi K, Onyike AE, Osterhout ML. Sickling in vitro of reticulocytes from patients with sickle cell disease at venous oxygen tension. *Exp Hematol* 1996;24:68–76.
131. Osterhout ML, Ohene-Frempong K, Horiuchi K. Identification of F-reticulocytes by two-stage fluorescence image cytometry. *J Histochem Cytochem* 1996;44:393–397.
132. Cavill I. Iron and erythropoiesis in normal subjects and in pregnancy. *J Perinat Med* 1995;23:47–50.
133. Murphy PT, Hutchinson RM. Identification and treatment of anaemia in older patients. *Drugs Aging* 1994;4:113–127.
134. Breymann C, Zimmermann R, Huch R, Huch A. Use of recombinant human erythropoietin in combination with parenteral iron in the treatment of postpartum anaemia. *Eur J Clin Invest* 1996;26:123–130.
135. Breymann C, Major A, Richter C, Huch R, Huch A. Recombinant human erythropoietin and parenteral iron in the treatment of pregnancy anemia: a pilot study. *J Perinat Med* 1995;23:89–98.
136. Cazzola M, Ponchio L, Pedrotti C, et al. Prediction of response to recombinant human erythropoietin (rHuEpo) in anemia of malignancy. *Haematologica* 1996;81:434–441.
137. Meyer MP, Haworth C, Meyer JH, Commerford A. A comparison of oral and intravenous iron supplementation in preterm infants receiving recombinant erythropoietin. *J Pediatr* 1996;129:258–263.
138. Ohls RK, Veerman MW, Christensen RD. Pharmacokinetics and effectiveness of recombinant erythropoietin administered to preterm infants by continuous infusion in total parenteral nutrition solution. *J Pediatr* 1996;128:518–523.
139. Samanci N, Ovali F, Dagoglu T. Effects of recombinant human erythropoietin in infants with very low birth weights. *J Int Med Res* 1996;24:190–198.
140. Polenakovic M, Sikole A. Is erythropoietin a survival factor for red blood cells? *J Am Soc Nephrol* 1996;7:1178–1182.
141. Faris PM, Ritter MA, Abels RI. The effects of recombinant human erythropoietin on perioperative transfusion requirements in patients having a major orthopaedic operation. The American Erythropoietin Study Group. *J Bone Joint Surg Am* 1996;78:62–72.
142. Heiss MM, Tarabichi A, Delanoff C, et al. Perisurgical erythropoietin application in anemic patients with colorectal cancer: a double-blind randomized study. *Surgery* 1996;119:523–527.
143. Sans T, Bofil C, Joven J, et al. Effectiveness of very low doses of subcutaneous recombinant human erythropoietin in facilitating autologous blood donation before orthopedic surgery. *Transfusion* 1996;36:822–826.
144. Soukup J, Menzel M, Roth S, Radke J. The perioperative use of recombinant erythropoietin (rhEPO) in Jehovah's Witnesses. *Anaesthetist* 1996;45:745–749.
145. Tarallo P, Humbert JC, Fournier B, Mahassen P, Henny J. Reticulocytes: reference limits. *Clin Lab Haematol* 1996;18(Suppl 1):13–14.
146. Bock A, Herkner KR. Reticulocyte maturity pattern analysis as a predictive marker of erythropoiesis in paediatrics. Part II. Pilot study for clinical application. *Clin Lab Haematol* 1994;16:343–348.
147. Lin CK, Chiu CF, Kuo BI, Lin FM, Jiang ML, Chow MP. Do the reticulocyte maturation fractions and bone marrow reticulocyte count further help the classification of anemias? *Chung Hua I Hsueh Tsa Chih Taipei* 1994;53:270–275.
148. Lin CK, Hsu HC, Chau WK, Jiang ML, Chiu CF. Reticulocyte count with maturation fractions in pancytopenic evaluation by a fully automated counter. *J Clin Lab Anal* 1993;7:371–375.
149. Paolino W. La questione della scarsa reticulocitozi degli anemici perniciosi. *Minerva Med* 1945;2:181–184.
150. Crosby WH, Conrad ME. Hereditary spherocytosis: observations on hemolytic mechanisms and iron metabolism. *Blood* 1960;15:662–674.
151. Conley CL, Lippman SM, Ness PM, Petz LD, Branch DR, Gallagher MT. Autoimmune hemolytic anemia with reticulocytopenia and erythroid marrow. *N Engl J Med* 1982;306:281–286.
152. Conley CL, Lippman SM, Ness P. Autoimmune hemolytic anemia with reticulocytopenia. A medical emergency. *JAMA* 1980;244:1688–1690.
153. Brownell AI, McSwiggan DA, Cubitt WD, Anderson MJ. Aplastic and hypoplastic episodes in sickle cell disease and thalassaemia intermedia. *J Clin Pathol* 1986;39:121–124.
154. King KE, Shirey RS, Lankiewicz MW, Young-Ramsaran J, Ness PM. Delayed hemolytic transfusion reactions in sickle cell disease: simultaneous destruction of recipients' red cells [see comments]. *Transfusion* 1997;37:376–381.
155. Petz LD, Calhoun L, Shulman IA, Johnson C, Herron RM. The sickle cell hemolytic transfusion reaction syndrome. *Transfusion* 1997;37:382–392.
156. Owren PA. Congenital hemolytic jaundice: the pathogenesis of the hemolytic crisis. *Blood* 1948;3:231–248.
157. Carulli G, Marini A, Azzara A, Lucchetti A, Petrini M. Pseudoreticulocytosis in a case of myelodysplastic syndrome. *Acta Haematol* 1998;100:156–158.
158. de-Pree C, Cabrol C, Frossard JL, Beris P. Pseudoreticulocytosis in a case of myelodysplastic syndrome with translocation t(1;14)(q42;q32). *Semin Hematol* 1995;32:232–236.
159. Hertenstein B, Kurrle E, Redenbacher M, Arnold R, Heimpele H. Pseudoreticulocytosis in a patient with myelodysplasia. *Ann Hematol* 1993;67:127–128.
160. Dover GJ, Humphries RK, Moore JG, et al. Hydroxyurea induction of hemoglobin F production in sickle cell disease: relationship between cytotoxicity and F cell production. *Blood* 1986;67:735–738.
161. Goldberg MA, Brugnara C, Dover GJ, Schapira L, Charache S, Bunn HF. Treatment of sickle cell anemia with hydroxyurea and erythropoietin. *N Engl J Med* 1990;323:366–372.
162. el-Hazmi MA, al-Momen A, Kandaswamy S, et al. On the use of hydroxyurea/erythropoietin combination therapy for sickle cell disease. *Acta Haematol* 1995;94:128–134.
163. Steinberg MH, Lu ZH, Barton FB, Terrin ML, Charache S, Dover GJ. Fetal hemoglobin in sickle cell anemia: determinants of response to hydroxyurea. Multicenter Study of Hydroxyurea. *Blood* 1997;89:1078–1088.
164. Ballas SK, Marcolina MJ, Dover GJ, Barton FB. Erythropoietic activity in patients with sickle cell anaemia before and after treatment with hydroxyurea. *Br J Haematol* 1999;105:491–496.
165. Kinney TR, Helms RW, O'Branski EE, et al. Safety of hydroxyurea in

- children with sickle cell anemia: results of the HUG-KIDS study, a phase I/II trial. *Pediatric Hydroxyurea Group. Blood* 1999;94:1550–1554.
166. Koren A, Segal-Kupershmit D, Zalman L, et al. Effect of hydroxyurea in sickle cell anemia: a clinical trial in children and teenagers with severe sickle cell anemia and sickle cell beta-thalassemia. *Pediatr Hematol Oncol* 1999;16:221–232.
 167. Baum KF, Dunn DT, Maude GH, Serjeant GR. The painful crisis of homozygous sickle cell disease. A study of the risk factors. *Arch Intern Med* 1987;147:1231–1234.
 168. Losek JD, Hellmich TR, Hoffman GM. Diagnostic value of anemia, red blood cell morphology, and reticulocyte count for sickle cell disease. *Ann Emerg Med* 1992;21:915–918.
 169. Omonge E, Ogutu EO, Aluoch JR. Clinical and laboratory predictors of cholelithiasis in patients with sickle cell anaemia. *East Afr Med J* 1998;75:347–350.
 170. van Iperen CE, Kraaijenhagen RJ, Biesma DH, Beguin Y, Marx JJ, van de Wiel A. Iron metabolism and erythropoiesis after surgery. *Br J Surg* 1998;85:41–45.
 171. Sowade O, Sowade B, Brilla K, et al. Kinetics of reticulocyte maturity fractions and indices and iron status during therapy with epoetin beta (recombinant human erythropoietin) in cardiac surgery patients. *Am J Hematol* 1997;55:89–96.
 172. Sowade O, Sowade B, Gross J, et al. Evaluation of erythropoietic activity on the basis of the red cell and reticulocyte distribution widths during epoetin beta therapy in patients undergoing cardiac surgery. *Acta Haematol* 1998;99:1–7.
 173. Horiuchi K, Osterhout ML, Ohene-Frempong K. Survival of F-reticulocytes in sickle cell disease. *Biochem Biophys Res Commun* 1995;217:924–930.
 174. Maier-Redelsperger M, Elion J, Girot R. F reticulocytes assay: a method to evaluate fetal hemoglobin production. *Hemoglobin* 1998;22:419–425.
 175. Wells DA, Daigneault-Creech CA, Simrell CR. Effect of iron status on reticulocyte mean channel fluorescence. *Am J Clin Pathol* 1992;97:130–134.
 176. Noguero P, Perez J, et al. High fluorescence reticulocytes increase in peripheral blood is the first sign of circulating stem cells after aplasia-induced chemotherapy. *Exp Hematol* 1993;21:1138.
 177. Moulin B, Ollier J, George F, et al. Serum erythropoietin and reticulocyte maturity index after renal transplantation: a prospective longitudinal study. *Nephron* 1995;69:259–266.
 178. Breymann C, Bauer C, Major A, et al. Optimal timing of repeated r-erythropoietin administration improves its effectiveness in stimulating erythropoiesis in healthy volunteers. *Br J Haematol* 1996;92:295–301.
 179. Chang CC, Kass L. Clinical significance of immature reticulocyte fraction determined by automated reticulocyte counting. *Am J Clin Pathol* 1997;108:69–73.
 180. Watanabe K, Kawai Y, Takeuchi K, et al. Reticulocyte maturity as an indicator for estimating qualitative abnormality of erythropoiesis. *J Clin Pathol* 1994;47:736–739.
 181. d'Onofrio G, Zini G, Caenaro G, Tommasi M, Micciulli G. Simultaneous measurement of reticulocyte and red blood cell indices in healthy subjects and patients with microcytic and macrocytic anemia. *Blood* 1995;85:818–823.
 182. Schaefer RM, Schaefer L. Hypochromic red blood cells and reticulocytes. *Kidney Int Suppl* 1999;69:S44–48.
 183. Brugnara C, Zurakowski D, DiCanzio J, Boyd T, Platt O. Reticulocyte hemoglobin content to diagnose iron deficiency in children. *JAMA* 1999;281:2225–2230.
 184. Brugnara C. Reticulocyte cellular indices: a new approach in the diagnosis of anemias and monitoring of erythropoietic function. *Crit Rev Clin Lab Sci* 2000;37:93–130.
 185. Brugnara C, Zelmanovic D, Sorette M, Ballas SK, Platt O. Reticulocyte hemoglobin: an integrated parameter for evaluation of erythropoietic activity. *Am J Clin Pathol* 1997;108:133–142.
 186. Tanke HJ, van Vianen PH, Emiliani FM, et al. Changes in erythropoiesis due to radiation or chemotherapy as studied by flow cytometric determination of peripheral blood reticulocytes. *Histochemistry* 1986;84:544–548.
 187. Inoue T, Tatsumi N. Evaluation of erythropoiesis by new methylene blue staining to establish reticulocyte maturity in bone marrow aspirates and peripheral blood. *Acta Cytol* 1991;35:479–480.
 188. Greinix HT, Linkesch W, Keil F, et al. Early detection of hematopoietic engraftment after bone marrow and peripheral blood stem cell transplantation by highly fluorescent reticulocyte counts. *Bone Marrow Transplant* 1994;14:307–313.
 189. Davies SV, Fegan CD, Kendall R, Beguin Y, Cavill I. Serum erythropoietin during autologous bone marrow transplantation: relationship to measures of erythroid activity. *Clin Lab Haematol* 1995;17:139–144.
 190. Lesesve JF, Lacombe F, Marit G, Bernard P, Belloc F, Reiffers J. High fluorescence reticulocytes are an indicator of bone marrow recovery after chemotherapy. *Eur J Haematol* 1995;54:61–63.
 191. Burns DL, Mascioli EA, Bistrrian BR. Effect of iron-supplemented total parenteral nutrition in patients with iron deficiency anemia. *Nutrition* 1996;12:411–415.
 192. d'Onofrio G, Tichelli A, Foures C, Theodorsen L. Indicators of haematopoietic recovery after bone marrow transplantation: the role of reticulocyte measurements. *Clin Lab Haematol* 1996;18(Suppl 1):45–53.
 193. Kuse R, Foures C, Jou JM, d'Onofrio G, Paterakis G. Automated reticulocyte counting for monitoring patients on chemotherapy for acute leukaemias and malignant lymphomas. *Clin Lab Haematol* 1996;18(Suppl 1):39–43.
 194. Paterakis GS, Tsavaris N, Loukopoulos D. The effect of GM-CSF on reticulocytes, haemoglobin and haematocrit in patients receiving chemotherapy for solid tumours. *Clin Lab Haematol* 1996;18:7–12.
 195. Sawabe Y, Kikuno K, Iseki T, Lida S, Tabata Y, Yonemitsu H. Changes in serum erythropoietin and the reticulocyte count during chemotherapy for leukemias. *Eur J Haematol* 1996;57:384–388.
 196. Vannucchi AM, Bosi A, Ieri A, et al. Combination therapy with G-CSF and erythropoietin after autologous bone marrow transplantation for lymphoid malignancies: a randomized trial. *Bone Marrow Transplant* 1996;17:527–531.
 197. Remacha AF, Bellido M, Garcia-Die F, Marco N, Ubeda J, Gimferrer E. Serum erythropoietin and erythroid activity in vitamin B12 deficiency. *Haematologica* 1997;82:67–68.
 198. Santamaria A, Martino R, Bellido M, Remacha AF. Reticulocyte recovery is faster in allogeneic and autologous peripheral blood stem cell transplantation than in bone marrow transplantation. *Eur J Haematol* 1997;58:362–364.
 199. Camacho LH, Gordeuk VR, Wilairatana P, Pootrakul P, Brittenham GM, Looareesuwan S. The course of anaemia after the treatment of acute, falciparum malaria. *Ann Trop Med Parasitol* 1998;92:525–537.
 200. Ashenden MJ, Pyne DB, Parisotto R, Dobson GP, Hahn AG. Can reticulocyte parameters be of use in detecting iron deficient erythropoiesis in female athletes? *J Sports Med Phys Fitness* 1999;39:140–146.
 201. Worel N, Greinix HT, Schneider B, et al. Regeneration of erythropoiesis after related- and unrelated-donor BMT or peripheral blood HPC transplantation: a major ABO mismatch means problems. *Transfusion* 2000;40:543–550.
 202. Jeffrey RF, Khan AA, Kendall RG, Norfolk DR, Will EJ, Davison AM. Quantitative reticulocyte analysis may be of benefit in monitoring erythropoietin treatment in dialysis patients. *Artif Organs* 1995;19:821–826.
 203. Bhandari S, Norfolk D, Brownjohn A, Turney J. Evaluation of RBC ferritin and reticulocyte measurements in monitoring response to intravenous iron therapy. *Am J Kidney Dis* 1997;30:814–821.
 204. Bhandari S, Turney JH, Brownjohn AM, Norfolk D. Reticulocyte in-

- dices in patients with end stage renal disease on hemodialysis. *J Nephrol* 1998;11:78–82.
205. Cullen P, Soffker J, Hopfl M, et al. Hypochromic red cells and reticulocyte haemoglobin content as markers of iron-deficient erythropoiesis in patients undergoing chronic haemodialysis. *Nephrol Dial Transplant* 1999;14:659–665.
 206. Kuse R. The appearance of reticulocytes with medium or high RNA content is a sensitive indicator of beginning granulocyte recovery after aplasiogenic cytostatic drug therapy in patients with AML. *Ann Hematol* 1993;66:213–214.
 207. Davis BH, DiCorato M, Bigelow NC, Langweiler MH. Proposal for standardization of flow cytometric reticulocyte maturity index (RMI) measurements. *Cytometry* 1993;14:318–326.
 208. Dalal BI, Stockford GR, Naiman SC, Spinelli JJ, Phillips GL. Criteria for marrow engraftment: comparison of reticulocyte maturity index with conventional parameters. *Bone Marrow Transplant* 1996;17:91–92.
 209. Remacha AF, Martino R, Sureda A, et al. Changes in reticulocyte fractions during peripheral stem cell harvesting: role in monitoring stem cell collection. *Bone Marrow Transplant* 1996;17:163–168.
 210. Sica S, Sora F, Laurenti L, et al. Highly fluorescent reticulocyte count predicts haemopoietic recovery after immunosuppression for severe aplastic anaemia. *Clin Lab Haematol* 1999;21:387–389.
 211. Sica S, Salutari P, Laurenti L, et al. Highly fluorescent reticulocytes after CD34+ peripheral blood progenitor cell transplantation. *Bone Marrow Transplant* 1998;21:361–364.
 212. George P, Wyre RM, Bruty SJ, Sweetenham JW, Duncombe AS. Automated immature reticulocyte counts are early markers of engraftment following autologous PBSC transplantation in patients with lymphoma. *J Hematother Stem Cell Res* 2000;9:219–223.
 213. Gowans ID, Hepburn MD, Clark DM, Patterson G, Rawlinson PS, Bowen DT. The role of the Sysmex SE9000 immature myeloid index and Sysmex R2000 reticulocyte parameters in optimizing the timing of peripheral blood stem cell harvesting in patients with lymphoma and myeloma. *Clin Lab Haematol* 1999;21:331–336.
 214. Beguin Y. Erythropoietin and the anemia of cancer. *Acta Clin Belg* 1996;51:36–52.
 215. Mittelman M. Anemia of cancer: pathogenesis and treatment with recombinant erythropoietin. *Isr J Med Sci* 1996;32:1201–1206.
 216. Pradella M, Cavill I, d'Onofrio G. Assessing erythropoiesis and the effect of erythropoietin therapy in renal disease by reticulocyte counting. *Clin Lab Haematol* 1996;18(Suppl 1):35–37.
 217. Sulkova S, Krajhanzl A. Erythropoietin and its use in renal failure. *Edtna Erca J* 1996;22(suppl):1–7.
 218. Aboulafia DM. Use of hematopoietic hormones for bone marrow defects in AIDS. *Oncology (Huntingt)* 1997;11:1827–1834, 1839, 1843–1824.
 219. Muirhead N. Renal disease. II. The clinical impact of recombinant human erythropoietin. *J R Coll Phys Lond* 1997;31:125–130.
 220. Casadevall N. Treatment of anaemia with rHuEpo in patients with MDS. *Med Oncol* 1998;15(Suppl 1):S35–S37.
 221. Cazzola M. How and when to use erythropoietin. *Curr Opin Hematol* 1998;5:103–108.
 222. Osterborg A. Recombinant human erythropoietin (rHuEPO) therapy in patients with cancer-related anaemia: what have we learned? *Med Oncol* 1998;15(Suppl 1):S47–S49.
 223. Rachmilewitz EA, Aker M. The role of recombinant human erythropoietin in the treatment of thalassemia. *Ann N Y Acad Sci* 1998;850:129–138.
 224. Biesma DH. Erythropoietin treatment for non-uremic patients: a personal view. *Neth J Med* 1999;54:10–15.
 225. Klaesson S. Clinical use of rHuEPO in bone marrow transplantation. *Med Oncol* 1999;16:2–7.
 226. Muirhead N. Erythropoietin and renal transplantation. *Kidney Int Suppl* 1999;69:S86–S92.
 227. Mittman N, Sreedhara R, Mushnick R, et al. Reticulocyte hemoglobin content predicts functional iron deficiency in hemodialysis patients receiving rHuEPO. *Am J Kidney Dis* 1997;30:912–922.
 228. Sunder-Plassmann G, Horl WH. Laboratory diagnosis of anaemia in dialysis patients: use of common laboratory tests. *Curr Opin Nephrol Hypertens* 1997;6:566–569.
 229. Dalbak LG, Theodorsen L, Aune MW, Sandberg S. Reticulocytes—new possibilities with automated counting. *Tidsskr Nor Laegeforen* 1993;113:709–712.
 230. Davis BH, MBigelow NC, van Hove L, Dumler F. Evaluation of automated reticulocyte analysis with immature reticulocyte fraction as a potential outcomes indicator of anemia in chronic renal failure patients. *Lab Hematol* 1998;4:169–175.
 231. Fishbane S, Galgano C, Langley Jr RC, Canfield W, Maesaka JK. Reticulocyte hemoglobin content in the evaluation of iron status of hemodialysis patients. *Kidney Int* 1997;52:217–222.
 232. Bowen D, Williams K, Phillips I, Cavill I. Cytometric analysis and maturation characteristics of reticulocytes from myelodysplastic patients. *Clin Lab Haematol* 1996;18:155–160.
 233. Richter C, Weitzel H, Huch A, Huch R. Failure to demonstrate postpartum functional iron deficiency using quantitative red blood cell analysis. *Eur J Obstet Gynecol Reprod Biol* 1999;84:69–74.
 234. Perry ES, Moore RH, Berger TA, et al. In vitro and in vivo persistence of reticulocytes from donor red cells. *Transfusion* 1996;36:318–321.
 235. Lunghi M, Giuliani AL, Torboli M, et al. Red blood cells expressing fetal antigens: their presence in adults with certain forms of anemia. *Acta Haematol* 1996;96:29–35.
 236. Lippi G, Guidi G. Laboratory screening for erythropoietin abuse in sport: an emerging challenge. *Clin Chem Lab Med* 2000;38:13–19.
 237. Friedman E. Reticulocyte counts: how to use them, what they mean. *Diagn Med* 1984;7:33.
 238. Hillman R, Finch C. Erythropoiesis: normal and abnormal. *Semin Hematol* 1985;4:327–358.
 239. Riley RS, Mahin EJ, Ross W. Clinical applications of flow cytometry. New York: Igaku-Shoin Medical Publishers; 1993. p 604.