Reticulocyte Counting by Flow Cytometry

A Comparison with Manual Methods*

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ABSTRACT

The reticulocyte count (RC) is a key diagnostic test in the evaluation, classification, and response to therapy of anemia. The RC, as determined by manual methods, has a frustrating inherent imprecision owing to its binomial counting statistics (i.e., low counts/low precision) and inaccuracy because of inter- and intraobserver variability as to what indeed is a reticulocyte.

Fluorescent activated cytometric (FACS) analysis of reticulocytes by thiazole orange (TO) is a rapid, relatively simple, and precise method for counting reticulocytes. The automated method counts 10,000 cells or more vs. 1,000 cells counted by the manual method. Although inherently more precise, the FACS method may be inaccurate owing to the presence of Howell-Jolly bodies, nucleated red blood cells (RBCs), sickled cells, or giant platelets. The RC by FACS is well correlated with the manual method and the reference ranges are similar.

A new parameter by FACS, the reticulocyte maturation index (RMI), provides an independent measurement of reticulocyte RNA content. Although the RMI does not correlate with RC either by FACS or manual methods, it does provide an independent parameter of erythropoietic activity and may be useful in predicting bone marrow engraftment or further subclassifying anemias.

Determination by FACS of the RC offers significant advantages over manual methods in monitoring a patient's erythropoietic response. However, one must be cognizant of potential pitfalls in the method.

Normal Erythropoiesis

During the normal maturation of erythroid precursors in the bone marrow, there is gradual condensation of the nuclear chromatin with concomitant reduction of both the nuclear and total cell size. When the nucleus becomes pyknotic, it is extruded. Simultaneously, there is cytoplasmic synthesis of hemoglobin. The heme portion of hemoglobin is synthesized in the mitochondria, and
the globin chains separately on the polyribosomes. Covalent linkage of these two moieties then occurs in the mitochondria. The RNA containing polyribosomes can remain in the now anucleated erythrocyte for up to four days. Over this four day period of time, there is progressive diminution in the number of polyribosomes and hence hemoglobin synthesis. Approximately 25 percent of hemoglobin is synthesized in the reticulocyte or the equivalently polychromatophilic anucleate stage of erythroid development. These early anucleated polyribosome-rich erythrocytes have a greater cell volume than mature erythrocytes and thus appear macrocytic. These macrocytic erythrocytes will stain polychromatically with standard Wrights-Giemsa stains since hemoglobin synthesis has not been completed.

Primary reticulocyte maturation (three days) occurs in the bone marrow. The less than completely mature erythroid cell then traverses the marrow sinusoid and normally completes its final maturation (one day) in the peripheral blood. With the normal progressive loss of the protein synthesizing polyribosomes, there is a cessation of hemoglobin synthesis, and transformation from reticulocyte to normal mature RBC. In normal peripheral blood stained with Wrights-Giemsa, the more mature late reticulocytes are virtually indistinguishable from totally mature erythrocytes. However, the appearance of easily identifiable “polychromatophilic macrocytes” or “shift cells” by Wrights-Giemsa staining, generally reflects the premature release of reticulocytes from the marrow. The appearance of such “shift” reticulocytes indicates accelerated erythropoiesis and carries significant diagnostic importance.

Morphologic Definition of the Reticulocyte

When the polyribosome-rich red cells have just lost their nucleus, they can be stained with supravital stains such as New Methylene Blue (NMB) or Brilliant Cresyl Blue (BCB). If two or more particles of basophilic polyribosomal material are present, the cell is termed a reticulocyte. Normal young reticulocytes may contain much polyribosomal material, which is progressively lost as they continue to mature to normal red cells.

A morphologic classification scheme for such maturation has been developed, based on Heilmeyer’s original work, revealing five stages. This scheme is now the generally accepted morphologic standard for manual reticulocyte count measurement. A biochemical rather than a morphological definition of a reticulocyte could be that of an anucleated erythrocyte containing seven to 17 percent of the original erythroblast ribonucleic acid.

Manual Reticulocyte Count Measurement

The standard 1,000 cell manual reticulocyte count has a notoriously high coefficient of variation (CV) because of its inherent imprecision and inaccuracy. Contributing factors include: interobserver variation in the definition of a reticulocyte, staining variability, variable distribution of cells on the slide, and statistical sampling error. The reticulocyte count statistics follow a binomial distribution, which results in unacceptable precision for reticulocyte counts less than approximately one percent, i.e., within the normal range. Further, this range of measurement is where detection of early erythroid activity would be made, such as just after a bone marrow transplant, just after chemotherapy, correction of iron, B12 or folate deficiency, or in a sickle patient recovering from an erythroid crisis. This statistical deficiency can be corrected by counting more cells (as noted in table I). However, the counting of the required 10,000 red cells by manual
TABLE I
Flow Cytometric Versus Manual Reticulocyte Count
95 Percent Confidence Limits

Expected Reticulocyte Counts and CV's

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<thead>
<tr>
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<tbody>
<tr>
<td>0.1</td>
<td>0-3</td>
<td>100</td>
<td>0.04-0.16</td>
<td>22</td>
<td>0.07-0.14</td>
<td>18</td>
</tr>
<tr>
<td>1</td>
<td>0.4-1.6</td>
<td>32</td>
<td>0.8-1.2</td>
<td>10</td>
<td>0.9-1.1</td>
<td>5.7</td>
</tr>
<tr>
<td>5</td>
<td>3.6-6.4</td>
<td>14</td>
<td>4.6-5.4</td>
<td>4.2</td>
<td>4.8-5.2</td>
<td>2.8</td>
</tr>
<tr>
<td>10</td>
<td>8.2-11.9</td>
<td>9.5</td>
<td>9.4-10.6</td>
<td>3.0</td>
<td>9.6-10.3</td>
<td>1.7</td>
</tr>
<tr>
<td>15</td>
<td>12.8-17.4</td>
<td>7.5</td>
<td>14.3-15.7</td>
<td>2.4</td>
<td>14.6-15.4</td>
<td>1.4</td>
</tr>
</tbody>
</table>

*Normal range 0.5 - 1.5 % (Adults)


methods would require a nearly superhuman effort. Other inaccuracies in the manual method include potential errors from miscounting Howell-Jolly bodies (deoxyribonucleic acid remnants), Heinz bodies (oxidized or denatured hemoglobin), Pappenheimer bodies (siderotic granules), or Hemoglobin H inclusions as precipitated reticulum.

Clinical Interpretation of the Reticulocyte Count

An increased peripheral blood reticulocyte count is a sensitive and reliable indicator of increased hematopoietic activity and is an essential element in the diagnostic workup of the various anemias (figure 1). For clinical purposes, reticulocytes are commonly reported as a percentage of the erythrocytes. For adults, the reticulocyte count is commonly given as 0.5 percent to 1.5 percent with a slightly higher range for women, and 2.5 percent to 6.5 percent for newborns.

An inherent feature of the reticulocyte count percentage is that it is made relative to the mature red cell population and, consequently, may appear increased when there is a significant reduction in the number of mature erythrocytes. Inexperienced physicians may not know that an apparently elevated reticulocyte (percentage) count may not be elevated when correction is made for a low red cell count owing to severe anemia. This problem is fairly common, as the patients most closely examined for erythroid recovery are often severely anemic. In such cases,
or in periods of intense erythropoiesis, the percentage reticulocyte count is inaccurate and a "correction" can be made (table II). First, an absolute reticulocyte count can be calculated by multiplying the percentage reticulocyte count by the RBC count. Secondly, the reticulocyte count may be "corrected" for the degree of anemia by comparing the patient’s hematocrit to a normal hematocrit.

**TABLE II**
Various Corrections Used in Reporting Reticulocytes

<table>
<thead>
<tr>
<th>Method</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute Count</td>
<td>Reticulocytes (%) x RBC Count</td>
</tr>
<tr>
<td>Reticulocyte Index</td>
<td>Reticulocytes (%) x Patient’s Hct 0.45</td>
</tr>
<tr>
<td>Reticulocyte Production Index</td>
<td>Reticulocytes (%) x Patient’s Hct 0.45</td>
</tr>
</tbody>
</table>

Maturation Time (days)

- 1.0 days when Hct = 45
- 1.5 days when Hct = 35
- 2.0 days when Hct = 25
- 2.5 days when Hct = 20

The various reticulocyte "corrections" can be used to categorize anemia on a kinetic basis.
crit: namely the reticulocyte index. Finally, since the percentage of reticulocytes can be altered by the presence of "shift" reticulocytes indicating premature release from the marrow, the reticulocyte production index (RPI) has been proposed to correct for both the "shift" and anemia.

Flow Cytometric Reticulocyte Count Measurements

Automated methods for counting reticulocytes, which can overcome many of the previously-mentioned limitations of the manual methods, include either image analysis of blood films using automated microscopy or fluorescent activated flow cytometric analysis (FACS) methods. In the FACS methods, a fluorescent dye, such as thiazole orange (TO), acridine orange (AO), or pyronin Y, combines with ribosomal RNA in reticulocytes. The degree of fluorescent emission caused by the excitation of the bound dye is proportional to the amount of RNA in an erythrocyte and hence inversely to its maturity. Because it is a simple staining procedure and good resolution is obtained, TO is becoming the fluorescent dye of choice for reticulocyte counting in flow cytometry. The acquisition and analysis of the stained samples by FACS are relatively simple. By setting the forward-scatter (FSC) and side-scatter (SSC) amplifier gains to the "LOG" mode, the white blood cell (WBC) population, which will also stain with the TO, is "off scale" and therefore not included in the data acquisition. The RBC/reticulocyte population will be the major population "on scale." After acquisition, a gate is drawn around the RBC/retic population which further excludes any small WBCs, platelets, and debris seen. This gated population can then be assessed for percent positive reticulocytes (FL-1, green fluorescence) based on a paired control sample for each patient (figure 2-A, C, E).

There are now software programs available that further simplify the statistical analysis used in FACS reticulocyte counting. After appropriate instrument calibration, such as with CaliBRITE Beads, the reticulocyte software uses those calibrations as a starting point and automatically sets up the appropriate instrument settings and gains. Using the FSC vs. SSC dot plot, the RBC/reticulocyte population is adjusted to the upper right hand corner of the dot plot and a FSC threshold is set. After acquisition of 10,000 to 50,000 events (counts), the RBC/reticulocyte gate is established. This gate should be greater than 85 percent of the total population in the dot plot. Using an internal algorithm, the software establishes a break point on the right side of the baseline of the main negative cell population for each patient. From this point the positive cell population can be determined, which results in a histogram display similar to figure 2-E. This automatic calculation eliminates the need for a negative control tube to be run with each patient. A FL-1 green fluorescence histogram, with a marker drawn and the percent positive and the mean fluorescent channel stated, is then printed. If the RBC count is provided, an absolute reticulocyte count can automatically be obtained.

The automated FACS method eliminates much of the statistical variability in reticulocyte counting; however, some erythrocyte elements, such as Howell-Jolly bodies or nucleated erythrocytes, may cause false positive results. These cells usually do not interfere if the window gates and fluorescence thresholds are properly set. Some nonerythroid conditions, such as chronic lymphocytic leukemia or conditions with giant platelets, may also cause spurious results.
operator must be alert to abnormal histogram distributions and gate appropriately to avoid potentially erroneous results from patients with these conditions (figure 2-B, D, F).

A review of a Wrights-Giemsa stained slide and/or a manual reticulocyte smear evaluation should be performed on all suspicious FACS histograms. Once a patient has been determined to give discrepant reticulocyte results by flow cytometry, which cannot be corrected by operational gate changes, then only a manual reticulocyte count should be performed. The incidence of patients with discrepant results between FACS and manual reticulocyte counting methods will depend on the patient population of each respective institution.

Since the flow cytometric method counts 10,000 or more cells (now 30,000 cells in our laboratory) in contrast to a 1,000 cell manual reticulocyte count, the improved statistical precision of the FACS method is well documented (table I). The accuracy of the FACS method for correctly identifying reticulocytes, however, may be questioned relative to manual methods. In the manual NMB methods, two particles of blue staining reticulum must be seen before a cell is counted as a reticulocyte, whereas the sensitivity of the FACS method is such that cells with much less reticulum would be detected. It is also possible that the amount of reticulum precipitated by NMB may not be fully equivalent to the RNA bound by the various fluorescent dyes.

The Reticulocyte Maturation Index

The fluorescence intensity of the TO-stained reticulocyte is proportional to
Comparison of CAP Workload Recording of Manual Versus Fluorescent Activated Cytometric Reticulocyte Counting

<table>
<thead>
<tr>
<th>Method</th>
<th>CAP Units</th>
<th>CAP Technical Time</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MANUAL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mix with NMB = 5-10 min.</td>
<td>9</td>
<td>9 min/1 sample</td>
</tr>
<tr>
<td>Prepare Smear</td>
<td></td>
<td>90 min/10 samples</td>
</tr>
<tr>
<td>Dry = 5-10 min.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Count - 1,000 CELLS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Report</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FLOW</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calibrate Instrument = 7 min.</td>
<td>11</td>
<td>11 min/1 sample</td>
</tr>
<tr>
<td>Load Program &amp;</td>
<td></td>
<td>22 min/10 samples</td>
</tr>
<tr>
<td>Patient List = 3 min.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>5ul Blood + 1ml TO = 30 sec.</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incubate 30 min.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acquire and analyze</td>
<td></td>
<td></td>
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<tr>
<td>1 sample = 45 sec.</td>
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<tr>
<td>10 samples = 4.5 min.</td>
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Comparison of time and effort of manual versus automated reticulocyte counting. The automated method clearly reduces technical time for batch analysis of reticulocytes and is not advantageous for single analysis.

The amount of RNA within the cell, which in turn is a function of the maturity of that cell. Measurement of this fluorescence intensity has been termed as the reticulocyte maturation index (RMI).4,5 The RMI is derived from the fluorescence histogram by quantifying the geometric mean fluorescence intensity of the positive cells. There is no correlation between the RMI and reticulocyte percentage by FACS in healthy adults or hospitalized patients. The RMI appears to be an independent and sensitive indicator of erythroid activity.4,5

The RMI measurement provides an independent objective measurement of bone marrow engraftment following transplantation. Engraftment is currently determined principally by the rise in the absolute neutrophil count (ANC). The ANC and RMI rise in parallel in early uncomplicated bone marrow transplant recovery. Although the ANC is a sensitive indicator of bone marrow engraftment, the ANC can drop during episodes of clinical or subclinical infection and could be misleading as to the success of transplantation. In contrast, the RMI is essentially independent of an infectious process. The RMI can also be used to subclassify anemias, particularly the hypoproliferative anemias where the reticulocyte count is low, or as an indicator of erythropoietic activity following therapy for anemia.5

The reticulocyte count by FACS does not lend itself to random individual samples since the procedure is more labor
intensive with respect to instrument set-up time and is best suited for batch analysis (table III). The instrumentation is relatively expensive at this time and is not suitable for low volume laboratories.

**Conclusions**

In summary, the flow cytometric approach to the reticulocyte (percentage) count greatly expands the utility of the test by expanding the useful operational range of the test to well below the normal range. The limitations of the manual method in measuring normal or low reticulocyte counts are essentially eliminated, permitting detection of early erythroid bone marrow recovery in oncology patients, and increasing the sensitivity of the detection of early erythroid recovery in a variety of other hematologic disorders. The RMI appears to be a separate, clinically useful indicator of bone marrow recovery following transplantation or chemotherapy and in the evaluation of erythroid activity in anemic conditions.

**References**

1. Becton Dickenson Immunocytometry Systems: FACScan™ AutoCOMP™ Software.