An Automated Optoelectronic Reticulocyte Counter

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Microscopic reticulocyte counting is time consuming and imprecise. A new reticulocyte counter has been developed, and the authors evaluated its utility for laboratory use. The counter, R-1000® of Sysmex-TOA Medical Electronics Company, Kobe, Japan, is based on the principles of flow cytometry. Reticulocytes are detected as fluorescent cells stained with a basic dye, auramine O, under argon-laser light. The automated count had high correlation to the manual count (r = 0.941). Linearity and reproducibility were both high. About 60 specimens were tested in one hour. Not only the reticulocyte percentage and count but also the maturity of reticulocytes was found from the intensity of the fluorescence, whether high, moderate, or slight. Normal reference values were 0.007 ± 0.0055 (0.70 ± 0.55%) for the reticulocytes, (4.63 ± 1.09) × 10⁹/L for the reticulocyte count, 2.3 ± 1.9% for highly fluorescent cells, 18.7 ± 5.1% for moderately fluorescent cells, and 78.8 ± 6.6% for cells with slight fluorescence.

In patients with suppressed bone marrow function, such as is caused by chemotherapy, the reticulocyte fraction and count were low, and cells with slight fluorescence increased. In patients in whom bone marrow function was stimulated, such as with hemolytic anemia, the reticulocyte percentage, reticulocyte count, and highly fluorescent cells were high. Patients with chronic renal failure being treated by hemodialysis had a similar reticulocyte pattern to that in hemolytic anemia except that the reticulocyte count was decreased. Results for elderly patients were not different from those of healthy young controls. Some patients with a normal reticulocyte count and percentage had numerous highly fluorescent cells, perhaps because of hemolytic anemia not yet identified. Automated reticulocyte counting provides reliable data, such measurement should be useful for analysis of the kinetics of red blood cells and for the study of the pathogenesis of anemia. (Key words: Reticulocytes; Automatic counter; Flow cytometry; Instrument evaluation) Am J Clin Pathol 1989;92:57-61

Materials and Methods

Instrument

The reticulocyte counter R-1000® (Sysmex-TOA Medical Electronics Co., Kobe, Japan) is a flow cytometer with a sheath flow system. One hundred microliters of blood is aspirated with edetate (EDTA), and the intracellular RNA is stained by a basic dye, auramine O, which fluoresces in argon-laser light. The forward scatter reflects the cell size and the lateral scatter, the RNA content. We examined the reticulocyte percentage in the total red blood cells, the reticulocyte count per microliter of blood, and the percentages of reticulocytes with different intensities of fluorescence: high, moderate, or slight, all displayed automatically by the machine. The rate of testing was 60 specimens per hour. The red blood cell count was checked simultaneously by an electronic method.

Correlation

The automated count found by the counter was compared with the manual count, which was measured with a blood film stained with brilliant cresyl blue. Preliminary studies with new methylene blue and brilliant cresyl blue gave similar results.

Reproducibility

The reproducibility was evaluated by the repetition of tests five times and expressed as the coefficient of variation (CV).
Carry-Over

Carry-over was measured by the method of Broughton and associates.²

Dilution Test

Blood samples were diluted with physiologic saline, and linearity with the dilution was examined.

Anticoagulants and Storage Time

Five fresh blood samples were put into five bottles containing disodium EDTA (15 mg/mL blood), heparin (9 IU/mL blood), or citrate-phosphate-dextrose (CPD; 0.14 mL/mL blood). The reticulocytes were stored at room temperature and measured 0, 24, and 48 hours after blood sampling. Blood in bottles with EDTA was also examined after being stored at 4 °C.

Interference by Drugs

Ascorbic acid (final concentrations of 0.2, 1, and 2 mg/mL blood), flavin adenine dinucleotide (2, 10, and 20 U/mL blood), cyanocobalamin (2, 10, and 20 mg/mL), deoxycycline hydrochloride (2, 10, and 20 mg/mL), and phenolsulfonphthalein (0.2, 1, and 2 mg/mL) were mixed with blood; the mixtures were left at room temperature for 20 minutes; and the reticulocytes were examined. The test was repeated five times, and the CV of the results was calculated.

Abnormal Specimens

To examine interference when there were large numbers of cells or by abnormal in vivo substances, five specimens or more of the following kinds were chosen: a leukocyte count over 20 × 10⁹/L, a red blood cell count over 6.0 × 10¹²/L, a platelet count over 1,000 × 10⁹/L, plasma bilirubin level over 50 μmol/L (2.9 mg/dL), plasma glucose level over 25 mmol/L (450 mg/dL), plasma triglyceride level over 3.5 mmol/L (310 mg/dL), partial coagulation, or partial hemolysis.

Healthy Subjects

We used the recommendations of the International Committee of Standardization in Hematology to choose 123 healthy donors aged 20–40. Their hemoglobin concentration was 12–16 g/dL, mean corpuscular volume (MCV) 80–100 fl, and mean corpuscular hemoglobin concentration (MCHC) 19.9–22.3 mmol/L (32.1–35.9 g/dL). They were the nonanemic group.

Specimens from Patients

Patients with iron deficiency or hemolytic anemia were examined before and after iron treatment. Blood specimens from patients on chemotherapy, with bone marrow suppression, were also obtained. When the leukocyte count was 3 × 10⁹/L or less, and the platelet count was 50 × 10⁹/L or less, bone marrow function was judged to be suppressed. Patients with chronic renal insufficiency treated by hemodialysis for at least four years were also examined. Patients aged 70 or more were divided into two groups, those without anemia and those with anemia, and their reticulocytes were examined. Anemia in the elderly was mainly secondary anemia.

Complete Blood Count (CBC)

An automated blood counter, the E-4000® (Sysmex-TOA), was used to find the CBC.

Reagents

All chemicals for interference tests and the manual counting of reticulocytes were purchased from Sigma Chemical Company (St. Louis, MO). Brilliant cresyl blue was from Merck AG (Darmstadt, Federal Republic of Germany).

Results

Performance of the R-1000

The counting function of the R-1000 was examined first. Correlation between red blood cell counts found by
FIG. 2. Correlation of the reticulocyte percentage. The electronic method used the R-1000, and the manual method involved counting of a blood film stained with brilliant cresyl blue.

the E-4000 (x) and R-1000 (y) was high \( r = 0.991, n = 100, y = 1.00x - 1.812 \).

Results by the automated count (y) and the manual count (x) of reticulocytes were compared, and \( y = 0.965x + 0.025 \) was found \( r = 0.942, n = 70 \); Fig. 2).

Reproducibility was examined for 15 specimens each tested five times. Its CV was 5.84%.

**Results of the Dilution Test**

In 14 blood samples, correlation of the red blood cell count (y) with the reticulocyte count (x) was found to be \( r = 0.996 (y = 0.35 + 0.98x) \). In five trials, carry-over was not observed.

**Influences of Various Reagents and the Time and Temperature of Storage on the Results of Reticulocyte Counting**

Changes in the reticulocyte count and percentage were examined by the automated and manual methods under different conditions. With all anticoagulants used, the reticulocyte percentage decreased significantly, but the decrease was less in CPD solution. The red blood cell count did not decrease (Figs. 3 and 4). Blood treated with EDTA is routinely used in the clinical laboratory, so the effects of temperature on the reticulocyte percentage in EDTA-treated samples was examined. When specimens were kept at 4 °C, the decrease that occurred during storage was less than at other temperatures, especially when storage was for 24 hours.

Interference by various reagents was tested in five specimens for each reagent. The CV was 4.71%, 3.93%, 4.79%, 6.02%, and 5.79% with ascorbic acid, cyanocobalamin, flavine adenine dinucleotide, doxycycline hydrochloride, and phenolsulfonphtalein, respectively.

Leukocytosis, erythrocytosis, and thrombocytosis had no effect on the automated reticulocyte counting, nor did hyperbilirubinemia, hyperglycemia, lipemia, partial hemolysis, or partial coagulation.

**Reticulocyte Count in Healthy Subjects and Patients with Hematologic Disorders (Table 1)**

In 123 healthy subjects, the mean reticulocyte fraction was 0.007 ± 0.005 g (0.70 ± 0.55%) of the total red blood cells, and the mean reticulocyte count was 43.6 ± 19.0 × 10^9/L. Of the total reticulocytes, highly fluorescent cells accounted for 2.33 ± 1.95%; moderately fluorescent ones, 18.73 ± 5.07%; and slightly fluorescent ones, 78.80 ± 6.50%. Thus, most of the reticulocytes were of the slightly fluorescent type, and their RNA content was low.

In patients with iron deficiency anemia before iron treatment, the reticulocyte percentage and count were higher than in healthy controls. When iron was given and the reticulocytes were measured within one week, the reticulocyte count was increased, as was the reticulocyte percentage, although not as much. Patients with hemolytic anemia had very high reticulocyte percentages and counts, and the increase in highly or moderately fluorescent cells was significant. Healthy elderly subjects and patients with secondary anemia had a high percentage of slightly fluorescent reticulocytes. Patients in the nadir stage in chemotherapy had the highest percentage of slightly fluorescent reticulocytes. Patients treated by hemodialysis had a low retic-
ulocyte count, but a high reticulocyte percentage, high percentages of very or moderately fluorescent cells, and a low percentage of slightly fluorescent cells.

**Discussion**

Reticulocytes are immature red blood cells. In general, they mature for two days after leaving the bone marrow. Analysis of reticulocytes therefore provides much information on erythropoiesis. At present, besides the manual method, which gives rise to inaccuracy and imprecision of counting, other methods such as the image analysis method or the cytometric method, using dyes of the acridine orange family, are used. However, those methods require treatment of the specimens before measurement. Auramine O is a fluorescent dye for RNA staining, and its use in the preparation of reticulocyte samples facilitates the analysis of the reticulocyte count.

**Table 1. Reticulocyte Count in Healthy Subjects and Patients with Hematologic Disorders**

<table>
<thead>
<tr>
<th></th>
<th>Reticulocyte (%)</th>
<th>Reticulocyte Count (×10⁹/L)</th>
<th>Highly Fluorescent Cells (%)</th>
<th>Moderately Fluorescent Cells (%)</th>
<th>Slightly Fluorescent Cells (%)</th>
<th>Hemoglobin (g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy subjects (n = 123)</td>
<td>0.70 ± 0.55</td>
<td>43.6 ± 19.0</td>
<td>2.33 ± 1.95</td>
<td>18.73 ± 5.07</td>
<td>78.80 ± 6.50</td>
<td>13.62 ± 1.01</td>
</tr>
<tr>
<td>Bone marrow suppression (n = 27)</td>
<td>0.10 ± 0.06</td>
<td>2.3 ± 1.4</td>
<td>1.78 ± 0.36</td>
<td>6.53 ± 6.16</td>
<td>92.39 ± 8.84</td>
<td>7.09 ± 1.43</td>
</tr>
<tr>
<td>Hemolytic anemia (n = 13)</td>
<td>5.48 ± 1.54</td>
<td>145.9 ± 38.8</td>
<td>12.84 ± 2.75</td>
<td>30.50 ± 3.95</td>
<td>56.57 ± 6.10</td>
<td>8.97 ± 1.68</td>
</tr>
<tr>
<td>Iron deficiency anemia* (n = 8)</td>
<td>1.85 ± 1.15</td>
<td>62.4 ± 31.3</td>
<td>2.88 ± 1.29</td>
<td>11.30 ± 3.62</td>
<td>85.68 ± 4.68</td>
<td>7.64 ± 2.32</td>
</tr>
<tr>
<td>Iron deficiency anemia† (n = 8)</td>
<td>1.90 ± 0.82</td>
<td>82.5 ± 26.5</td>
<td>4.83 ± 2.15</td>
<td>20.36 ± 3.54</td>
<td>74.85 ± 5.47</td>
<td>10.61 ± 1.48</td>
</tr>
<tr>
<td>Secondary anemia (n = 38)</td>
<td>2.05 ± 1.28</td>
<td>73.1 ± 59.0</td>
<td>5.91 ± 4.01</td>
<td>23.75 ± 6.23</td>
<td>70.16 ± 9.43</td>
<td>9.42 ± 1.59</td>
</tr>
<tr>
<td>Hemodialysis (n = 87)</td>
<td>1.23 ± 0.44</td>
<td>31.9 ± 14.2</td>
<td>6.24 ± 4.43</td>
<td>27.53 ± 7.78</td>
<td>66.10 ± 11.16</td>
<td>7.88 ± 1.40</td>
</tr>
<tr>
<td>Aged nonanemic patients (n = 15)</td>
<td>1.04 ± 1.54</td>
<td>46.6 ± 12.8</td>
<td>1.75 ± 1.39</td>
<td>14.44 ± 5.12</td>
<td>83.67 ± 6.21</td>
<td>13.68 ± 1.41</td>
</tr>
<tr>
<td>Aged, with secondary anemia (n = 18)</td>
<td>1.07 ± 0.44</td>
<td>32.7 ± 11.6</td>
<td>2.50 ± 1.73</td>
<td>14.38 ± 4.47</td>
<td>83.04 ± 5.66</td>
<td>9.05 ± 1.72</td>
</tr>
</tbody>
</table>

*Before treatment. †After treatment.

SI units: reticulocyte % × 0.01 = number fraction reticulocytes; hemoglobin (g/dL) × 10 = hemoglobin (g/L).

**Fig. 3.** Effects of reagents and storage. After 24 and 48 hours of storage, the reticulocyte count of samples (n = 5 at each time) was found as a percentage, with the results at 0 hours as 100% (O, with CPD; ●, with heparin).

**Fig. 4.** Effects of storage and temperature. Blood samples (n = 5 at each time) were treated with EDTA. After 24 and 48 hours of storage, the reticulocyte count was found as a percentage, with results at 0 hours as 100% (●, room temperature; O, at 4 °C).
similar to acridine orange, but not widely used. The R-1000 has a sheath flow system, which helps provide accurate counting, sizing, and fluorometry. The most important question about automated apparatus is the correlation to the manual count. A high correlation coefficient and high reproducibility are required. No carry-over and the lack of interference by other kinds of cells or by reagents are also desirable. One problem here was that specimens with a high reticulocyte fraction of over 0.10 (10%) were often counted as being lower by the R-1000. Probably cells with a high RNA content (type O or I cells in Heilmeyer's classification) would be missed in the leukocyte region.

Another function of the R-1000 is its grading of reticulocytes into three subtypes: highly, moderately, and slightly fluorescent cells. About 78% of the total reticulocytes were slightly fluorescent cells in healthy adults; in the elderly, this increased to about 84%. When bone marrow was suppressed, the category of slightly fluorescent cells reached 92%. When bone marrow function was activated, percentages of highly or moderately fluorescent reticulocytes increased. Iron deficiency anemia caused a decrease and secondary anemia an increase in slightly fluorescent cells. With hemodialysis, the reticulocyte percentage was normal, but the count was increased. We concluded that this new reticulocyte counter was useful and reliable for routine laboratory work and should make possible further expansion of the field of red blood cell research.

References