Erythrocyte Volume Distribution in Normal and Abnormal Subjects

By J. David Bessman and Randall K. Johnson

Size-frequency distribution curves of erythrocytes were generated with the Coulter Counter in 73 normal subjects and patients. Mean corpuscular volume (MCV) determined by routine calculation and MCV determined by size-frequency distribution were similar in all normal subjects and in patients with a single population of erythrocytes. Some patients with iron-deficiency anemia, folate deficiency, and vitamin B₁₂ deficiency had two discrete erythrocyte populations. Some patients with microcytic anemia were shown to have a population of normocytes in addition to the predominant microcytic population. Reticulocytes and normocytes were identified in two patients recovering from macrocytic anemia. Transfused blood was identified as a separate population in a patient with microcytic anemia. In cases with two erythrocyte populations, the MCV of the principal population, as determined from size-distribution curves, differed from the MCV of the entire erythrocyte pool, as was determined by routine methods. Analysis of sequential erythrocyte size distributions in patients under treatment demonstrated the dynamics of erythrocyte subpopulations. Anisocytosis was quantified and shown to be associated frequently with hospitalized patients.

A NUMBER OF DISEASES cause alteration in erythrocyte size, and measurements of this hematologic value have been useful in establishing diagnosis and monitoring treatment. Erythrocyte size is reported as mean corpuscular volume (MCV). Manually, division of erythrocyte volume (hematocrit) by erythrocyte concentration yields this value. However, in the past 15 yr, automated quantitation of the formed elements of blood, as well as the red cell indices, hemoglobin, and hematocrit, has supplemented and often supplanted routine manual microscopic determination. The instrument most commonly used is the Coulter Counter, and sophisticated interpretation has been made of the values it generates. On the Coulter Counter, each erythrocyte passing through an electric sensor causes a resistance change proportional to its volume; the cumulative resistance change divided by the number of erythrocyte samples is reported as MCV. Whether determined manually or by the Coulter method, MCV represents the average of all cells measured and as such will not reveal any heterogeneity in the cell population sampled. As an example, the degree of microcytosis is often underestimated because of inclusion of normocytes, macrocytes, and reticulocytes in the calculation of MCV. Direct microscopy has remained crucial to evaluate cell morphology and heterogeneity.

The Coulter Counter has been modified to display the size distribution of particles counted. Beginning with Brecher et al., a number of workers have

From the Laboratory of Experimental Chemotherapy, Drug Research and Development, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. 20014.
Submitted March 11, 1975; accepted April 5, 1975.
Address for reprint requests: J. David Bessman, M.D., Laboratory of Experimental Chemotherapy, National Cancer Institute, Bethesda, Md. 20014.
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analyzed the size distribution of erythrocytes. Recently the Coulter Counter has been further refined to avoid the technical difficulties and artifacts that formerly hindered its usefulness. Now it is capable of generating reproducible and useful erythrocyte size-distribution histograms. We have generated these histograms in normal subjects and a variety of patients. Full exploitation of this automated system can now include serial size-distribution analyses during disease progression and therapy and can provide clinical insight into the dynamics of erythropoiesis with speed and clarity. Additionally, any unusual heterogeneity of the erythrocytes can be quickly and quantitatively identified.

MATERIALS AND METHODS

Blood samples were obtained from patients and staff at the following hospitals: National Institutes of Health Clinical Center; Veterans Administration Hospital, Washington, D.C.; Walter Reed Army Medical Center, Washington, D.C.; and the District of Columbia General Hospital.

Blood samples were divided into two equal portions for Channelyzer analysis and for routine Coulter analysis. The Coulter Counter, Model ZBI (Coulter Electronics, Hialeah, Fla.) with a 70-μ aperture, modified by the Channelyzer attachment, was used. Instrument settings were: amplification, 4; aperture current, 1 mA; base channel threshold, 5; window width, 100; and the editing function was on.

For heterogeneous erythrocyte populations, the proportion of cells in the separate populations was found by determination of the area under each peak by integration.

Samples were prepared in the following way: an 0.5-μl aliquot of fresh blood, mixed with EDTA, was suspended in 15 ml of Isoton (Coulter Electronics, Hialeah, Fla.) and analyzed with the instrument. All samples were analyzed in duplicate from two discrete aliquots. Wright-stained smears were made of each blood sample and were examined by direct microscopy.

MCV, hematocrit, and erythrocyte count were determined for each specimen on the Coulter Counter without benefit of the Channelyzer attachment. The MCV so determined will be designated in this article as MCV_A (arithmetic).

The Channelyzer was used to generate cell-size distribution curves as shown in Fig. 1. The

Fig. 1. Typical erythrocyte size-distribution curve in normal subject. The two values for cell volume at 50% of maximum frequency are determined. Erythrocyte volume range (EVR) is the range between these two values. Distribution mean corpuscular volume (MCV_D) is the average of these two values.
ERYTHROCYTE VOLUME DISTRIBUTION

absissa represents cell volume from 0 cu µ (channel 0) to 187 cu µ (channel 100). The ordinate represents the frequency of counts in each channel relative to the peak channel, that is, the channel with most cells. In each curve generated, the peak value represented 4000 cells. All counts were performed in duplicate. At each point along Channelyzer-generated size-frequency distributions, duplicate specimens of blood were always within 5% of each other. The peaks of the distribution curves were rounded, so that in many instances the precise location of the maximum peak was not obvious. To avoid a subjective element in locating the peak value, the cell volume at half-maximum on either side of the peak was determined. As the curves were symmetrical, the peak cell volume was calculated as the mean of these two values. This peak cell volume will be designated MCV D (distributional) (Fig. 1). The range of cell volume between the two cell volumes at half-maximum is termed erythrocyte volume range (EVR). Its determination is shown in Fig. 1. When additional, smaller peaks were present in heterogeneous cell populations, their MCV D was determined in similar fashion by averaging their two half-maximum values. The MCV D thus generated represents the mean cell volume of discrete cell populations rather than the mean volume of all cells in the sample.

RESULTS

The MCV D determined from Channelyzer curves was compared with the MCV A determined by the Coulter Counter alone in blood samples from 71 subjects. These values are plotted in Fig. 2. In all except six subjects, the discrepancy that was found between MCV D and MCV A was less than 6 cu µ. These cases are described in detail below. In three normal subjects, blood taken on two successive days was compared, and particle frequency over the spectrum of cell volumes varied no more than 4% from one day to the next. The MCV D s were unchanged.

In all but six subjects noted below, the distribution took the form of a single
Fig. 3. Erythrocyte size-frequency distribution in ten patients with microcytic anemia.

peak, approximately symmetrical. The distribution curve shown in Fig. 1 was characteristic of a normal subject. Peaks were consistently narrow in normal subjects. Wider peaks were common in patients and will be described below.

Ten untreated patients with severe hypochromic microcytosis (MCV_A less than 75 cu μ) were studied. All were ethanol abusers and had demonstrated
gastrointestinal blood loss with decreased serum iron and increased iron-binding capacity. Patient A had received 2 U of whole blood 3 days before our sampling; no other patient had received transfusion in the previous 3 mo. The erythrocyte-size distribution in these patients is shown in Fig. 3. In all patients, microcytes comprised the predominant population and appeared as a discrete peak. A second peak of larger cells was present in patient a (Fig. 3). A plateau of larger cells was seen in eight patients (Figure 3). The peak of microcytes contained from 63% to 99% of the total erythrocytes in the various patients (Table 1). The remainder of the cells were present as a secondary peak or a plateau. Anisocytosis and microcytosis were evident on smear in the patients shown in Fig. 3. Anisocytosis was not seen in the microcytic patients shown in Fig. 3. The MCVD of the predominant erythrocyte population in nine cases was less than the MCVa (Table 1). Patients a-f represent the six patients in Fig. 2 in which MCVa varied greatly from MCVD.

Two patients with very prominent macrocytosis are described individually: VB, a 55-yr-old woman, had advanced undifferentiated adenocarcinoma (primary unknown). She had received methotrexate, 0.6 mg/kg weekly for 10 wk prior to and throughout the course of this study. Because of recurrent thrombophlebitis, she also received coumadin periodically; while receiving coumadin, she suffered a massive hemorrhage into the thigh. Serial blood measurements were taken, and their relation to her medication, hematocrit, and reticulocyte count are shown in Table 2. The serial erythrocyte-size distributions are shown in Fig. 4. The small secondary peak at 172 cu μ correlated well with separate, manually determined reticulocyte counts. It should be noted that her macrocytosis during methotrexate therapy remained unchanged after a brisk reticulocyte response to hemorrhage.

JM, a 69-yr-old man, had florid pernicious anemia, with congestive heart failure and an organic brain syndrome secondary to the anemia. Serial blood samples were examined during therapy with vitamin B12 and ferrous sulfate. The relation of his blood measurements to therapy, hematocrit, and reticulocyte count is shown in Table 3. The serial erythrocyte-size distributions are shown in Fig. 5. Of particular interest is the appearance and subsequent subsidence in

### Table 1. Erythrocyte Measurements in Ten Cases of Microcytic Anemia

<table>
<thead>
<tr>
<th>Case</th>
<th>MCVa</th>
<th>MCVD</th>
<th>Per Cent of Total Cells in Primary Peak</th>
<th>EVR in Primary Peak</th>
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</thead>
<tbody>
<tr>
<td>a</td>
<td>53</td>
<td>40</td>
<td>66</td>
<td>47</td>
</tr>
<tr>
<td>b</td>
<td>66</td>
<td>43</td>
<td>63</td>
<td>52</td>
</tr>
<tr>
<td>c</td>
<td>59</td>
<td>50</td>
<td>85</td>
<td>44</td>
</tr>
<tr>
<td>d</td>
<td>67</td>
<td>60</td>
<td>85</td>
<td>49</td>
</tr>
<tr>
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<td>g</td>
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<td>92</td>
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</tr>
<tr>
<td>i</td>
<td>60</td>
<td>55</td>
<td>94</td>
<td>36</td>
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<tr>
<td>j</td>
<td>69</td>
<td>69</td>
<td>99</td>
<td>32</td>
</tr>
</tbody>
</table>

MCVa, MCVD, and EVR in cubic microns.
Per cent of cells in total peak calculated by integration.
Table 2. Erythrocyte Measurements in Therapy of Patient (VB) With Adenocarcinoma Treated With Methotrexate

<table>
<thead>
<tr>
<th>Date</th>
<th>12/26</th>
<th>1/2</th>
<th>1/9</th>
<th>1/16</th>
<th>1/23</th>
<th>1/30</th>
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<td>25</td>
<td>28</td>
<td>31</td>
<td>33</td>
<td>36</td>
<td>37</td>
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<tr>
<td>Reticulocytes (%)</td>
<td>5.5</td>
<td>9.1</td>
<td>10.2</td>
<td>7.4</td>
<td>2.6</td>
<td>1.1</td>
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<tr>
<td>MCV&lt;sub&gt;A&lt;/sub&gt;</td>
<td>136</td>
<td>137</td>
<td>137</td>
<td>137</td>
<td>137</td>
<td>137</td>
<td></td>
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<tr>
<td>MCV&lt;sub&gt;D&lt;/sub&gt;</td>
<td>133</td>
<td>135</td>
<td>135</td>
<td>136</td>
<td>136</td>
<td></td>
<td></td>
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<tr>
<td>Mean reticulocyte volume</td>
<td>172</td>
<td>172</td>
<td>171</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Coumadin</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Hemorrhage</td>
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<td></td>
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</tbody>
</table>

MCV<sub>A</sub>, MCV<sub>D</sub>, and mean reticulocyte volume in cubic microns.

Fig. 4. Erythrocyte size-frequency distribution in a patient with adenocarcinoma treated with methotrexate (VB).

Table 3. Erythrocyte Measurements During Hematonic Therapy of Patient JM

<table>
<thead>
<tr>
<th>Date</th>
<th>1/31</th>
<th>2/1</th>
<th>2/4</th>
<th>2/6</th>
<th>2/7</th>
<th>2/8</th>
<th>2/11</th>
<th>2/12</th>
<th>2/19</th>
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<td>16</td>
<td>17</td>
<td>17</td>
<td>19</td>
<td>24</td>
<td>21</td>
<td>27</td>
<td>25</td>
<td>35</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>0.1</td>
<td>0.1</td>
<td>16.5</td>
<td>22.7</td>
<td>20.3</td>
<td>2.8</td>
<td>1.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCV&lt;sub&gt;A&lt;/sub&gt;</td>
<td>137</td>
<td>139</td>
<td>144</td>
<td>142</td>
<td>127</td>
<td>124</td>
<td>117</td>
<td>107</td>
<td></td>
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<tr>
<td>MCV&lt;sub&gt;D&lt;/sub&gt;</td>
<td>140</td>
<td>137</td>
<td>121</td>
<td>110</td>
<td>103</td>
<td></td>
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</tr>
<tr>
<td>Mean reticulocyte volume</td>
<td>172</td>
<td>172</td>
<td>166</td>
<td>162</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MCV<sub>A</sub>, MCV<sub>D</sub>, and mean reticulocyte volume in cubic microns.
Therapy with vitamin B<sub>12</sub>, folate, and FeSO<sub>4</sub> was given from 1/29 through 2/21.
reticulocytes during hematinic therapy. At the beginning of therapy, the major peak was made up solely of macrocytes (Fig. 5). This peak progressively widened as reticulocytes matured to normocytes (Fig. 5) and macrocytes progressively decreased (Fig. 5). The reticulocytosis also was reflected in the increasing hematocrit shown in Table 3.

The presence and degree of anisocytosis were determined by measuring the EVR, as described above. The EVR was not determined in VB and JM, cases with two distinct yet confluent populations. Figure 6 shows the relationship between MCV₂ and EVR. The EVR was between 34 and 40 cu µ in ten normal subjects. Among 59 hospitalized subjects, 19 had EVR ≤ 40 cu µ, and 40 had EVRs from 42 to 57 cu µ. Of the 40 patients with increased EVR, only nine had smears that were routinely identified as slightly or moderately anisocytic. Five of five patients with aortic valve prostheses and six of ten patients with ethanol...
abuse and gastrointestinal blood loss had increased EVR. Of the other 44 patients, 25 with various diseases had increased EVR.

**DISCUSSION**

Erythrocyte size-frequency distribution, formerly generated only by arduous microscopic development of Price-Jones curves, can now be readily and accurately determined. Since relatively large numbers of cells are measured and counted quickly with minimum effort, the dynamics of cell populations, previously unclear, now can be studied easily. When this technique was first investigated, limitations of aperture size and electric current resulted in artificially skewed curves, and subpopulations could be obscured in the skew. With the present aperture size and the instrument settings used, the distribution curves were symmetrical, without skew. This study confirms the reproducibility and precision of the method. The data presented here strongly suggest that deviation in the character of distribution curves may be an early indication of disturbance, especially when all of the usual hematologic measurements are still within normal limits.

The patient whose erythrocytes are distributed over a wider than normal range has precisely the condition termed anisocytosis, whether the MCV is normal or not. A number of clinical situations, especially when chronic and unmodified (e.g., iron, folate, or vitamin B12 deficiency; thalassemia; valvular prosthesis), may result in a single anisocytic erythrocyte population. In such cases, the MCVa and MCVb will be the same, but the distribution curve will reveal any alteration of EVR. Anisocytosis was first quantitated by Price-Jones. In our study, while all five patients with valvular prostheses had normal or near normal MCV (Fig. 6), all had increased EVR. Probably the characteristic helmet cells, along with the increased rate of erythropoiesis, contribute to this increase. England and Down similarly identified one patient with thalassemia. Whether the range taken as the criterion is that between half-maximum and half-maximum, including about 70% of cells counted, or the range...
that includes 95% of cells,\textsuperscript{14} the same populations are likely to be labeled abnormal. The half-maximum method has the advantage of ease and of avoiding a possible misleading conclusion by excluding small discrete populations such as reticulocytes. Results from our relatively small group of subjects suggest that anisocytosis is very common among hospitalized patients. Much more extensive study is required to identify subgroups of patients most often associated with anisocytosis and to determine the significance, if any, of anisocytosis as an isolated finding.

The two cases of macrocytosis described above demonstrate the usefulness of serial measurement of abnormal erythrocyte size-frequency distributions (Figs. 4 and 5). The patient with pernicious anemia demonstrated very well the dynamics of hematologic recovery (Fig. 5). An initially macrocytic cell population was first augmented by reticulocytes; the reticulocytes then matured into normocytes, adding to the mature erythrocyte pool. As erythrocyte mass increased, the reticulocytosis decreased, and the newly formed normocytes increasingly predominated in the entire erythrocyte population. In sharp contrast is the static distribution of the patient with methotrexate-induced macrocytosis (Fig. 4). The reticulocytes were called forth from the marrow by blood loss, not by any change in the folate-deprived environment. Therefore, they continued to reflect a folate deficiency and continued to mature into macrocytes. Thus the peripheral mature erythrocyte mass was increased, but its size distribution was unaltered.

Of considerable interest is that reticulocyte size correlated well in these two cases with the hematinic balance. The patient whose vitamin B\textsubscript{12} deficiency was reversed had reticulocytes that decreased in average size as vitamin B\textsubscript{12} balance returned. The patient who remained folate deprived had reticulocytes that remained large throughout the reticulocytosis. Brecher and Stohlman\textsuperscript{21} showed that stress reticulocytes, reticulocytes in response to severe erythropoietic stimulation, initially were larger than normal and that, progressively, subsequent reticulocytes returned to normal. These stress reticulocytes may themselves cause a temporary macrocytosis. However, in the two patients described above, the vitamin B\textsubscript{12} or folate deficiency seemed sufficient to explain the increased reticulocyte size, especially in view of the two patients' divergent subsequent courses. The alteration of reticulocyte size, difficult to appreciate on a blood smear, may be of importance, as it may afford a quantitative and serial measurement of the bone marrow erythropoietic status. We have not determined if the same is true for iron balance.

When more than one erythrocyte population exists, the MCV\textsubscript{A} may distort the true values. In patient JM, the large number of reticulocytes were averaged with the erythrocytes, falsely elevating the MCV\textsubscript{A} (Table 3). The MCV\textsubscript{D} more accurately depicted the actual rapid fall in the size distribution of the mature erythrocytes.

Equally striking is the distortion in patients with iron-deficient microcytosis. Relatively few large erythrocytes, either from the anisocytosis common in iron-deficiency anemia\textsuperscript{15,16} or from reticulocytosis, could raise the MCV\textsubscript{A} to lessen the apparent severity of the iron deficiency. This severity would be revealed by the MCV\textsubscript{D}. The patients whose erythrocyte distribution showed a major peak
of microcytic cells and a substantial secondary peak or plateau (Fig. 3) are examples. The size of the secondary component corresponded with the divergence of MCV_A from MCV_D; normal subjects had no such second component. Thus it is unlikely to represent a technical artifact. The secondary component may reflect a mixed deficiency of both iron and either folate or vitamin B₁₂, or may reflect partial repletion of the iron deficiency. A similar profile in a patient with treated microcytic anemia has been reported,¹⁴ and a single microcytic peak has been reported in a patient with iron deficiency.⁷

A complex interaction of a variety of factors determines the size and number of reticulocytes and, subsequently, mature erythrocytes. Therefore, the existence of a stable erythrocyte size-frequency distribution may be considered evidence of a stable pattern of marrow erythropoiesis, whether normal or abnormal. These distributions are more readily obtained and evaluated than are marrow specimens. Thus they may be a valuable means to monitor marrow response, either to replacement therapy or to drugs with known potential for marrow damage such as antineoplastic agents²²,²³ or chloramphenicol.²⁵ While size-frequency distribution analysis cannot substitute completely for careful marrow analysis, it provides a means of almost continuous evaluation of marrow response between bone marrow aspirations.

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REFERENCES

17. Sullivan LW: The megaloblastic anemias,
Erythrocyte volume distribution in normal and abnormal subjects

JD Bessman and RK Johnson