Flow Cytometry of Reticulocytes Applied to Clinical Hematology

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Reticulocytes in fixed human blood samples were stained for RNA with the fluorescent dye pyronin Y and measured by flow cytometry. The resulting relative frequency distributions of the RNA fluorescence intensities conformed with the different stages in maturation from early reticulocytes to mature red cells. A computer program was written to calculate from these frequency distributions the relative number of reticulocytes, their relative RNA content, and the median of the reticulocyte population (RNA index). This method was applied to 30 healthy blood bank donors (control group), as well as to patients with various hematologic disorders showing abnormal erythropoietic activity. The measured percentage of reticulocytes, RNA content, and RNA index were found to correlate well with the various hematologic disorders. Changes in erythropoiesis could be clearly followed, as was demonstrated by analyzing blood samples from children with aplastic anemia or acute myeloid leukemia, who were treated with allogeneic bone marrow transplantation. Measurements on blood samples from healthy blood bank donors showed that with this method, small changes in the reticulocyte population, such as the appearance of polychromatic erythrocytes in the peripheral blood 5-8 hr after donation, can be detected. The statistical reliability and the information provided on the maturation stage of the entire reticulocyte population make flow cytometry of peripheral blood reticulocytes a more informative method for the study of hematologic abnormalities than conventional methods for reticulocyte counting and classification.

A NUMBER OF METHODS are presently available to evaluate the erythropoietic activity of the bone marrow. The rate of production of red cells can be calculated from independent measurements of erythrocyte lifespan and red cell volume using $^{51}$Cr or by measuring the turnover rate of red cell iron labeled with $^{59}$Fe. A disadvantage of these methods is that they are relatively complex and require steady-state conditions, which often cannot be reached.

A relatively simple and commonly used method to estimate the rate of erythropoiesis is the visual reticulocyte count. The limitations of this method in evaluating the rate of red cell production have been reviewed in the past. These visual methods for counting reticulocytes are hampered by the fact that they do not provide easily accessible information on the maturation stage. Furthermore, they are statistically unreliable for determining percentages of reticulocytes of less than 1% or detecting small but significant changes in reticulocyte populations as a consequence of blood loss, radiation, or chemotherapy.

Reticulocytes vitally stained with dyes like brilliant cresyl blue and methylene blue have been classified according to the morphology of the precipitation pattern of the ribosomal RNA in stages I, II, III, and IV by Heilmeyer. These studies have shown that under conditions of stimulated erythropoiesis, reticulocytes of stages I and II, which normally occur only in the bone marrow, appear in the peripheral blood (reticulocyte shift). The maturation of these reticulocytes of stages I and II proceeds under such conditions in the peripheral blood. This implies that the number of reticulocytes in the peripheral blood no longer reflects the situation of the bone marrow and cannot be used as an adequate parameter of the activity of the red cell production system. Hillman and Finch have attempted to correct the absolute reticulocyte count for this shift. Theoretically, it is possible to determine the degree of anemia by counting the morphologically distinguishable subclasses of reticulocytes. This method, however, is too laborious for a routine hematology laboratory.

For reasons, a relatively simple and rapid method to count the total number of reticulocytes with respect to maturation stage would be of clinical importance. Measurements of RNA in reticulocytes in blood smears by absorption scanning photometry and microfluorometry have been reported. These techniques presently are also too slow and laborious for routine purposes.

In a previous study we have shown that quantitative reticulocyte analysis is feasible with flow cytometry using a suitable fluorescent staining technique for RNA. This method is based on fluorescence measurements of cells in suspension that sequentially pass through a laser beam. Its results have been compared with the conventional technique for counting reticulocytes by parallel investigations of the same blood sample, as well as by visual examination of the reticulocytes, which were deposited onto microscopic slides after measurement by flow sorting. It could be shown...
that by using this technique the large statistical errors of manual reticulocyte counts as well as their subjective classification can be overcome, since about 10² red blood cells are readily measured within a few minutes.

The present study describes a number of clinical applications of flow cytometric analysis of reticulocytes in peripheral blood. A computer program has been written to derive from the frequency distributions the relative number of reticulocytes, their RNA content, and the median of the reticulocyte population (RNA index). Studies were carried out on two groups of persons: healthy blood bank donors and patients with various hematologic disorders, such as forms of primary and secondary anemia. The sensitivity of this method was assessed in healthy blood donors by determining the extent to which polychromatic erythrocytes (reticulocytes of stages I and II) were detectable in the peripheral blood as a consequence of blood donation. These cells are observed in blood smears after relatively severe chronic and acute blood loss. Their frequency, however, is considered too low for accurate measurements with conventional methods for counting reticulocytes.

The possibility of monitoring changes in erythropoietic activity by this method was investigated in patients with aplastic anemia (AA) or acute myeloid leukemia (AML) who were treated with bone marrow transplantation.

**MATERIALS AND METHODS**

Blood samples from 30 healthy blood bank donors were taken just before donation. From some volunteers, extra blood samples were obtained 5, 8, and 24 hr after donation to determine the appearance of polychromatic erythrocytes in the peripheral blood as a consequence of donation.

Blood samples of patients who were treated with allogeneic bone marrow transplantation were investigated by flow cytometry in a longitudinal study. Among these were 5 patients with aplastic anemia (AA) (1 Fanconi and 3 non-Fanconi anemias) and 3 patients with acute myeloid leukemia (AML). The treatment of these patients with bone marrow transplantation has been described elsewhere.

The last group studied were patients with hematologic abnormalities. Among them were patients with spherocytosis, β-thalassemia, kidney transplantation, and a premature birth.

**Fixation and Staining**

The blood samples, with EDTA as anticoagulant, were resuspended using a whirl mixer. A 50-μl sample was then taken and diluted with phosphate-buffered saline (PBS) (0.15 M NaCl, 1.5 mM phosphate, pH 7.2) to a total volume of 5.0 ml. The diluted samples were fixed in 7% formaldehyde. For this purpose, 2.0 ml of 25% formaldehyde was added to the 5.0 ml of blood cell suspension under continuous mixing. The 25% formaldehyde solution was prepared by adding PBS to formalin, which is 37% formaldehyde in water. Optimal results were obtained when the 2.0 ml 25% formaldehyde solution was added incrementally to the cells, e.g., 5 additions of 0.4 ml in approximately 15 min. The cells were fixed in this solution for at least 2 hr or, if necessary, stored in fixative at 4°C until staining and flow analysis were performed. It was found that fixed cells can be stored at 4°C for months without significant changes in the results obtained by flow analysis.

Prior to staining with pyronin Y, the cells were washed twice with PBS to remove the fixative (5-min centrifugation at 300 g). The supernatant was removed and the cell pellet was resuspended in 1.0 ml of 0.01% (w/v) solution of pyronin Y in sodium acetate buffer (1.0 M, pH 4.7). This dye solution was prepared by dissolving 1 g of pyronin Y (Gurr, High Wycombe, England) in 100 ml of distilled water. This solution was purified by chloroform extraction (3 fractions of 100 ml each) as described by Rothbarth. The purified pyronin Y solution was diluted with 1.0 M sodium acetate buffer, pH 4.7, to a concentration of 0.01%. Staining was carried out for 30 min at room temperature. After staining, the cell suspension was centrifuged (5 min at 300 g) and the supernatant was removed. The cells were washed once with 1.0 ml PBS (5-min centrifugation at 300 g) and again resuspended in 1.0 ml of PBS.

**Flow Cytometry**

Pyronin-Y-stained blood cells were passed through a 50-μm nylon mesh filter prior to analysis. The cell suspension was then diluted with PBS to an appropriate concentration for flow cytometry (generally 1:10). Flow cytometry was performed with a FACS IV cell sorter (Becton Dickinson FACS systems, Sunnyvale, CA). A Spectra Physics (Mountain View, CA) 5W argon-ion laser (model 164-05), operating at 514.5 nm with an intensity of 1.0W was used as excitation light source for pyronin Y. Since the fluorescence emission maximum of pyronin Y is at 580 nm, the standard band pass filter for red light of the FACS (600 nm) was replaced by a long wave pass filter KV 550 (Schott, Mainz, W. Germany). The red pyronin Y fluorescence was detected by an EM1 photomultiplier tube equipped with a red sensitive S-20 cathode. In all experiments, a 50 or 70 μm orifice was used. The sheath fluid consisted of PBS containing 0.01% sodium azide and was filtered through 0.2-μm Millipore filters prior to use. Generally, cell suspensions were analyzed for two parameters: forward light scatter and red pyronin Y fluorescence. Typical sample rates were 500–1,000 cells/sec. The forward light scatter parameter, which is a measure of size, was used to electronically eliminate small particles such as platelets and cellular debris, which were below the threshold size for measurements. Histograms of fluorescence intensity and forward light scatter were collected for 100,000 cells per blood sample in 256 or 1,024 channels.

For the demonstration of polychromatic erythrocytes in blood after donation, all blood samples were stained and

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*Recently we have investigated the use of glutaraldehyde as a fixative. Though the method has to be evaluated on a larger material, so far good results were obtained. The procedure is as follows. Erythrocytes (10 μl whole blood) were washed 3 times with Good's buffer (140 mM NaCl, 10 mM HEPES, 1 mM MgCl₂, pH 7.5) and fixed in suspension with a 0.1% glutaraldehyde solution (Merck, Darmstadt, W. Germany) in Good's buffer for 15 min under continuous agitation. Cells were then washed 3 times with 1 ml 5% hydroxyammonium chloride (Merck, Darmstadt, W. Germany) in PBS (adjusted to pH 7.4) to block unreacted aldehyde groups, and subsequently stained with pyronin Y. It should be noted that fixation and staining procedure are readily carried out in micro test tubes (Eppendorf, Hamburg, W. Germany), using an Eppendorf centrifuge (type 3200). In such a way, the entire preparation procedure can be completed within 1 hr.*
FLOW CYTOMETRY OF RETICULOCYTES

Subsequently, three parameters are calculated for the reticulocyte population: the relative number, their RNA content (relative to the mature red cells), and the median value (relative to LEVEL) of the population. The last two parameters describe the maturity of the reticulocyte population and their shift toward immature stages. Mathematical definitions of the calculated functions and abbreviations used are given in Fig. 1.

After analysis, univariate histograms were plotted using a matrix type printer (Print Swiss, Basel, Switzerland). In all cases the number of cells on the ordinate was plotted on a logarithmic scale.

RESULTS

The reticulocyte distributions obtained by flow cytometry shown in Fig. 2 are representative for healthy adult persons. A main peak of mature red blood cells is seen in the lower fluorescence channels. The cells in the channels at the right of this peak represent reticulocytes with increasing RNA content toward the higher channels. The average number of reticulocytes in blood of healthy blood bank donors was 2.7% (SD ± 1.2%), the average RNA content was 20 relative units (SD ± 7), and the RNA index was found 1.48 (SD ± 0.06).

As already mentioned, reticulocytes with relatively low RNA content (Heilmeyer stages III and IV) are seen in the peripheral blood of healthy persons under normal conditions (see Fig. 1). During increased erythropoietic activity of the bone marrow, younger reticulocytes with relatively high RNA content also appear in the peripheral blood (Heilmeyer stages I and II).

The results of the studies of the effect of blood donation in healthy donors are shown in Fig. 3. It is clear that 5–8 hr after donation of 500 ml of blood, a significant increase of relatively strongly fluorescing cells can be observed. These cells represent cells with relatively high RNA content (polychromatic erythrocytes). Counts in the indicated windows (channels 320–1,000) were 232, 582, 479, and 253 after 0, 5, 8, and 24 hr, respectively, for a total number of 100,000 erythrocytes analyzed. In order to determine the statistical significance of this increase, every blood sample taken at different times after donation was stained and analyzed by flow cytometry in quadruplicate. The average number of reticulocytes within the indicated window was significantly increased 5 and 8 hr after
Table 1. Number of Relatively Strongly Fluorescing Reticulocytes (See Window in Fig. 3) at Different Time Intervals After Blood Donation

<table>
<thead>
<tr>
<th>Time Intervals After Blood Donation</th>
<th>0 hr</th>
<th>5 hr</th>
<th>8 hr</th>
<th>24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor 1</td>
<td>232</td>
<td>582</td>
<td>479</td>
<td>253</td>
</tr>
<tr>
<td>Donor 2</td>
<td>417</td>
<td>481</td>
<td>580</td>
<td>449</td>
</tr>
<tr>
<td>Donor 3</td>
<td>380</td>
<td>477</td>
<td>625</td>
<td>438</td>
</tr>
</tbody>
</table>

Total number of red blood cells analyzed was 100,000. Given numbers are averages of 4 experiments.

Fig. 3. Fluorescence histograms obtained by flow cytometry of pyronin-Y-stained red blood cells. Abscissa and ordinate as in Fig. 2. Distributions obtained for blood of a healthy blood donor taken at different time intervals after donation. Indicated windows contained 232, 582, 479, and 253 cells for 0, 5, 8, and 24 hr after donation, respectively.

donation ($p < 0.001$, Student's t test). Comparable results were obtained in two other donors (see Table 1).

Typical examples of the results of the longitudinal studies of patients after bone marrow transplantation are given in Fig. 4A (patients with aplastic anemia) and Fig. 4B (patients with acute myeloid leukemia). Changes in the reticulocyte population due to erythropoietic activity of the accepted graft appear 7–14 days after transplantation. It is seen that not only the percentage of reticulocytes increases with time, but also that the RNA content and the RNA index strongly increase, suggesting the appearance of younger reticulocytes (stages I and II) in the peripheral blood. Similar results were obtained for 5 other patients who accepted the graft. Generally, a take of the graft could be predicted about 2 days earlier on the basis of the observed reticulocyte populations than with conventional visual counts. One patient with a Fanconi type of aplastic anemia rejected the graft. Neither an increase in number of reticulocytes nor increasing RNA content or RNA index could be observed, indicating lack of renewed erythropoiesis (data not shown). Another patient with aplastic anemia treated with bone marrow transplantation initially accepted the bone marrow and showed slowly increasing erythropoietic activity. However, on day 43, where most patients with transplants have abundant reticulocytes, reticulocytes of relatively high RNA content were no longer present. Ten days later (day 53), the patient died of acute graft-versus-host disease, which may account for this observation (data not shown).

In Fig. 5, some examples of fluorescence distributions are given that were obtained for patients with other hematologic abnormalities. Figure 5A represents the reticulocyte distribution of a patient with β-thalassemia. Reticulocytes with relatively high RNA content can be observed. Figure 5B shows the distribution obtained for a patient with spherocytosis. The number of reticulocytes with a relatively low amount of RNA is especially increased compared to normal blood samples. Figure 5C represents the measured reticulocyte distribution in blood from a prematurely born child, and Fig. 5D shows a distribution of reticulocytes in blood from a patient 2 mo after successful kidney transplantation. Patients shown in Fig. 5 C and D had conventional manual reticulocyte counts of 7.9% and 8.0%, respectively. Sensitive quantitative measurements of the RNA fluorescence by flow cytometry, however, show differences in the average fluorescence values of the mature red cells for these two patients. The population of “mature” red blood cells of the prematurely born child has a higher average fluorescence intensity (Fig. 5C) than the population of mature red cells in the blood of the kidney patient (Fig. 5D). Note that all patients shown in Fig. 5, besides an increased percentage of reticulocytes, had high RNA content values and a high RNA index compared to the healthy blood bank donors (see Fig. 2).

DISCUSSION

Compared to conventional methods for counting reticulocytes, flow cytometry has advantages. Since 100,000 blood cells are easily analyzed within minutes by flow cytometry, statistically reliable results are obtained. The sensitivity of the method is clearly shown in the studies of healthy blood bank donors after blood donation. When a window is chosen by which the relatively immature reticulocytes are selected (see Fig. 3), the changes in percentage of strongly fluorescing reticulocytes after donation of blood are significant, even though they are in the order of less than 1% of the total number of cells analyzed (increase of 0.23%–0.58% 5 hr after donation; $p < 0.001$).
FLOW CYTOMETRY OF RETICULOCYTES

The longitudinal studies of patients after bone marrow transplantation indicate that the frequency distributions of the fluorescence intensities of the reticulocytes do reflect changes in erythropoietic activity of the bone marrow. In these distributions, reticulocytes with different RNA content can be distinguished. Unlike the morphological stages of Heilmeyer, they are not separated into distinct populations, but show distributions that reflect the continuous loss of RNA during the development of the immature reticulocyte toward the mature red cell. The degree of maturation of the reticulocyte population is well described by the parameters “RNA content” and “RNA index” calculated from the frequency distributions. Theoretically, it is to be expected that an increase of only relatively mature reticulocytes (stages III and IV) will cause higher RNA content values, but will not greatly influence the RNA index. In situations where a reticulocyte shift occurs, the appearance of reticulocytes of stages I and II in the peripheral blood will lead to a higher RNA index measured. It should be noted that this RNA index is rather constant for healthy persons (average value 1.48 ± 0.06), but rapidly increases for the type of hematology patients seen in Fig. 5.

Therefore, it can be concluded that flow cytometry of reticulocytes offers the possibility of overcoming the deficiencies of currently used manual methods for counting reticulocytes: namely, absence of information on the maturation stage and insufficient statistical reliability for measuring low percentage of reticulo-
cytes (1% and lower) and small changes, particularly when the percentage of reticulocytes is low.

These drawbacks of the routine test are one reason why reticulocyte counts were only used as rough indicators of erythropoietic activity. Flow cytometry of peripheral reticulocytes offers the hematologist a relatively simple and useful test to study the erythropoietic activity of the bone marrow. If necessary, additional kinetic information of erythropoiesis can be obtained by radioactive iron labeling procedures.

Flow cytometric analysis of reticulocytes also offers a sensitive method of measuring blood loss. Since the normal hematocrit value, which is used as a parameter for blood loss, has 20% variability among healthy individuals, chronic losses of 0.5 liter of blood or less are difficult to diagnose. The diagnosis of such losses may now be possible with the more reliable flow cytometric method of counting polychromatic erythrocytes, as is shown in this study for acute blood loss of 0.5 liter.

The investigations of blood from the prematurely born child and the patient with a kidney transplant illustrate the usefulness of the information that may be provided to the clinician by this technique. Figure 5, for instance, shows two different reticulocyte distributions at a time when conventional visual reticulocyte counts were essentially the same (7.9% and 8.0%). Figure 5C was obtained with blood from a prematurely born child. The average fluorescence intensity of the mature red cells is higher than obtained for the patient shown in Fig. 5D (patient with transplanted kidney). This is probably due to the presence of many nearly mature cells in the blood of the premature child. This observation illustrates that, besides the measured parameters (percentage, RNA content, and index), additional relevant information can sometimes be derived from these distributions by visual interpretation. It should be noted that only distributions within one experiment were compared because of the dependence of the measured fluorescence intensity of the instrument conditions.

In a routine laboratory, flow cytometric analysis of reticulocytes could probably be performed in a cost-effective way. Important to this respect is that the method can be automated to a high degree. Assuming that a short fixation procedure can be applied (such as the described procedure in which glutaraldehyde is used), fixation and staining of the cells could be done in less than 1 hr using an automated staining and washing instrument developed for the staining of cells in microtiter plates. Flow cytometers can be equipped with special devices that automatically sample from these microtiter plates. Measurements and calculations would then take no more than 1 hr for 30 samples. Though commercial flow systems are still relatively expensive (high costs of laser operation), a special purpose instrument may be built for a much lower price. A recent pilot study with the new Becton Dickinson Research Analyzer (which is a mercury arc lamp-based flow system and therefore cheaper in use) has shown that these systems provide enough sensitivity to perform this type of reticulocyte measurement.

We believe that quantitative measurements of reticulocyte populations as described in this study should be carried out primarily in an experimental laboratory. It may initially confuse the hematologist, since the results obtained are on occasion different from the results achieved with the old methods. However, because of the important advantages discussed here, flow cytometry has the potentiality to become an accepted and useful tool for assessing reticulocyte counts in routine clinical hematology.

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Flow cytometry of reticulocytes applied to clinical hematology

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