Simultaneous Measurement of Reticulocyte and Red Blood Cell Indices in Healthy Subjects and Patients With Microcytic and Macrocytic Anemia

By Giuseppe d'Onofrio, Rosario Chirillo, Gina Zini, Gianfranco Caenaro, Maria Tommasi, and Giulia Micciulli

Using the new Bayer H*3 hematology analyzer (Leverkusen, Germany), we have determined red blood cell and reticulocyte indices in 64 healthy subjects, in patients with microcytosis due to iron deficiency (58 patients) and heterozygous β-thalassemia (40 patients), and in patients with macrocytosis (28 patients). We found in all cases that reticulocytes were larger than mature red cells by 24% to 35%, with a hemoglobin concentration 16% to 25% lower and a similar hemoglobin content. The correlation between red cell and reticulocyte indices was strikingly tight (r = .928 for volume, r = .929 for hemoglobin concentration, r = .972 for hemoglobin content) in all four groups, regardless of red blood cell size. The ratio of reticulocyte to red blood cell mean corpuscular volume (MCV ratio) was constantly above 1. Inversion of the MCV ratio was observed only in four patients. It was always abrupt and transitory and was associated with erythropoietic changes leading to the production of red blood cells of a different volume (treatment of megaloblastic anemia, functional iron deficiency, bone marrow transplantation). In two cases of marrow transplantation, reticulocyte volume fell during the aplastic phase after conditioning chemotherapy and then rapidly increased up to values higher than before; this production of macroreticulocytes was the earliest sign of engraftment.

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The introduction of flow cytometric methods, based on the measurement of fluorescence after staining with RNA-binding fluorochromes, has greatly improved the precision and accuracy of reticulocyte counting. The increased sensitivity in the low reticulocyte range and the availability of reticulocyte maturation indices based on the measurement of RNA content have recently extended the diagnostic applications of reticulocyte counting to new clinical settings, such as monitoring of erythroid regeneration after chemotherapy or bone marrow transplantation (BMT) and of erythropoietic response to erythropoietin administration.

The latest achievement in automatic reticulocyte counting is the simultaneous flow-cytometric measurement of volume and hemoglobin concentration on red blood cells and reticulocytes by the Bayer H*3 hematology analyzer (Leverkusen, Germany). We report the results of a two-institution study of the H*3 reticulocyte method, including the definition of the distribution ranges of reticulocyte and red blood cell indices in healthy subjects and in anemic patients with red cells of abnormal size, as well as our first observations on the clinical utility of such indices in the monitoring of erythropoietic function.

MATERIALS AND METHODS

The H*3 hematology analyzer. The Bayer H*3 is an automated blood cell counter that performs complete blood and reticulocyte counting using an optical method based on the measurement of scatter and absorption of helium-neon laser light, associated with automated peroxidase cytochemical white blood cell differential counting. The H*3 red blood cell counting method, in particular, is identical to that of the previous H*1 and H*2 systems. After isovolumetric red blood cell sphering, measurements of monochromatic light scattered at two different angular intervals are electronically processed to derive their volume and refractive index, which is a linear function of hemoglobin concentration. Thus, besides directly measuring mean corpuscular volume (MCV) and calculating mean corpuscular hemoglobin concentration (MCHC) and content (MCH), the Bayer H series instruments also provide a direct measure of cell hemoglobin concentration mean (HCCHM), which is compared in each analyzed sample with MCHC for quality control purposes and for detection of red blood cell abnormalities. The instruments also provide the percentage of red blood cells with volume less than 60 fl (microcytes) and with hemoglobin concentration lower than 28 g/dl (hypochromic red blood cells).

The H*3 reticulocyte method. Reticulocyte counting requires a preliminary manual mixing of 3 µl of whole blood with 3 ml of reticulocyte reagent, containing a surfactant, which spheres RBCs and reticulocytes, and the nucleic acid-binding dye oxazine 750, which selectively stains reticulocytes by complexion with cytoplasmic RNA. After a 15-minute incubation, the prepared sample is aspirated through the H*3 red blood cell wellcell, where three detectors measure laser light scatter at low angle (2° to 3°) and high angle (5° to 15°), and absorption. On a two-dimensional cytogram of absorption versus low-angle scatter, the stained reticulocytes are separated from unstained erythrocytes, platelets, and leukocytes by appropriate thresholds. Moreover, because the amount of light absorbed by reticulocytes is proportional to the intensity of staining and RNA content, reticulocytes are subdivided into three populations with low, medium, and high RNA content. From the amount of the light scattered at two different angles, the H*3 is capable of separately measuring reticulocyte mean corpuscular volume (MCVR) in femtoliters and corpuscular hemoglobin concentration mean (CHCM) in grams per deciliter, together with MCV and CHCM of mature erythrocytes. Mean hemoglobin content of reticulocytes (CHr) and red blood cells (CH) is calculated from the product of volume times hemoglobin concentration of single cells.

Study samples. K3EDTA-anticoagulated peripheral blood samples were analyzed with the H*3 within 2 to 76 hours of phlebotomy. They were refrigerated at 4°C if the analysis had to be delayed more than 4 hours. A small number of the study samples were mailed in refrigerated bags from Rome to Treviso using an express daily courier. These procedures were defined after a stability study, which

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had shown that K3EDTA specimens do not show significant changes in reticulocyte percentage, absolute values, or indices after 8 hours at room temperature and 72 hours at 4°C, or after 12 hours in refrigerated bags. The study of H*3 stability was performed as a part of a complete instrument performance evaluation, which proved that all H*3 measurements are highly comparable with the reference methods and have precision, linearity, and carry-over features equal or better than the manufacturer’s specifications (unpublished data, May 1993).

Reference ranges. The reference ranges for the H*3 hemoglobin, red blood cell, and reticulocyte measurements and indices were obtained from 32 male and 32 female healthy adult subjects, with no clinical symptoms and normal results at a screening blood test including complete blood count, differential count, and serum ferritin level. Reference ranges were calculated as the 95 central percentiles of the distribution.

Patient study. We also investigated the reticulocyte and red blood cell indices in anemic patients with microcytosis or macrocytosis. The microcytic anemia group included 58 patients with overt iron deficiency anemia (hemoglobin level, 5.4 to 10.7 g/dL; serum ferritin level, 8.9 to 12.4 g/dL; A2 hemoglobin level, above 5%; serum ferritin level, above 15 ng/mL). The macrocytic anemia group included 28 patients with anemia of different etiology (hemoglobin level, 4.5 to 11.2 g/dL; MCV; above 100 fL): myelodysplastic syndromes, eight patients; chronic liver diseases, seven; AIDS treated with AZT, nine; folate deficiency, two; and vitamin B12 deficiency, two. Moreover, during our H*3 evaluation, we were able to follow with daily H*3 analysis a small number of hematologic patients, including two patients undergoing bone marrow transplantation.

RESULTS

Reference ranges. Table 1 shows the mean values and 95 central percentile distributions of red blood cell and reticulocyte parameters provided by the H*3 in 64 healthy subjects. Results of hemoglobin measurement and red blood cell count showed a lognormal distribution, while all other parameters, including reticulocyte percentage, absolute number, and indices, were normally distributed. With the exception of hemoglobin and red blood cell count, we did not find any statistically significant difference between sexes. The calculated red cell indices MCHC and MCH were somewhat higher than the corresponding directly measured parameters CHCM and CH, but the correlation between them was as good as expected (r = 0.768 for MCH v CHCM; r = 0.881 for MCH v CH).

Reticulocyte and red blood cell indices. Table 2 shows the mean values and standard deviations of the red blood cell and reticulocyte indices generated in the H*3 reticulocyte method. It also shows the mean percentage differences and ratios between the value of red blood cell MCV, CHCM, and CH and the corresponding reticulocyte indices MCVR, CHCMr, and CHr, both in healthy subjects and in patients with microcytic and macrocytic anemia. The MCVR of reticulocytes is consistently higher than red blood cell MCV in the four groups we have studied, with a mean difference of about 24% for normocytic and macrocytic subjects and about 34% for patients with microcytic anemia. The frequency histograms of MCVR and MCV in healthy subjects show a

| Table 1. Mean Values and Reference Ranges of Red Blood Cell and Erythrocyte Parameters Measured With the H*3 on 64 Healthy Subjects |
|-----------------|-----------------|-----------------|-----------------|
| Parameter       | Mean Reference Range | Mean Reference Range | P Value of t-Test (m/f) |
| Hemoglobin (g/dL) | 14.0 (12.5-15.7) | 14.5 (13.5-15.7) | <.01 |
| Red blood cells (x10^12/L) | 4.66 (4.22-5.26) | 4.66 (4.45-5.35) | .466 (4.20-4.78) |
| MCV (fL) | 89.9 (83.4-97.9) | 89.3 (83.4-97.0) | 90.5 (84.9-95.0) |
| MCHC (g/dL) | 32.6 (31.0-34.0) | 32.7 (31.5-34.0) | 32.7 (31.1-33.6) |
| CHCM (pg) | 31.6 (30.1-33.2) | 31.7 (30.9-33.2) | 31.5 (30.1-32.9) |
| CH (pg) | 30.3 (27.6-32.0) | 30.1 (27.9-31.9) | 30.4 (27.9-31.4) |
| Reticulocytes (%) | 27.7 (25.6-29.6) | 27.6 (25.7-29.5) | 27.8 (25.6-30.5) |

Hemoglobin, red blood cells, MCHC, and MCH were obtained as a part of the H*3 complete blood count. All remaining red blood cell and reticulocyte parameters were generated in the H*3 reticulocyte method.

Table 2. Average Values (standard deviation), Percentage Differences, and Ratios of Red Blood Cell and Reticulocyte Indices Obtained With the H*3 Reticulocyte Method in Normal and Abnormal Subjects

<table>
<thead>
<tr>
<th>Index</th>
<th>Healthy Subjects (n = 64)</th>
<th>Iron Deficiency (n = 58)</th>
<th>β-Thalassemia Trait (n = 40)</th>
<th>Macrocytosis (n = 27)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCV (fL)</td>
<td>89.9 (4.03)</td>
<td>74.3 (4.89)</td>
<td>68.6 (3.56)</td>
<td>111.5 (5.55)</td>
</tr>
<tr>
<td>MCVR (fL)</td>
<td>111.7 (6.77)</td>
<td>100.1 (8.78)</td>
<td>92.3 (6.67)</td>
<td>139.0 (11.00)</td>
</tr>
<tr>
<td>MCV - MCVr (%)</td>
<td>24.3 (5.81)</td>
<td>34.9 (8.31)</td>
<td>34.6 (8.01)</td>
<td>24.6 (6.25)</td>
</tr>
<tr>
<td>CHCM/MCV ratio</td>
<td>1.24 (0.08)</td>
<td>1.35 (0.08)</td>
<td>1.38 (0.08)</td>
<td>1.25 (0.08)</td>
</tr>
<tr>
<td>CHCMr (pg)</td>
<td>31.6 (0.02)</td>
<td>27.3 (1.48)</td>
<td>28.6 (1.28)</td>
<td>31.0 (1.12)</td>
</tr>
<tr>
<td>CHCMr - CHCM (%)</td>
<td>26.3 (1.50)</td>
<td>26.4 (2.09)</td>
<td>22.9 (1.74)</td>
<td>25.7 (1.16)</td>
</tr>
<tr>
<td>CHr/MCHM ratio</td>
<td>0.83 (0.04)</td>
<td>0.76 (0.05)</td>
<td>0.78 (0.04)</td>
<td>0.83 (0.04)</td>
</tr>
<tr>
<td>CH (pg)</td>
<td>27.1 (1.42)</td>
<td>19.7 (2.05)</td>
<td>18.9 (1.84)</td>
<td>33.7 (2.12)</td>
</tr>
<tr>
<td>CHr (pg)</td>
<td>28.6 (1.60)</td>
<td>19.6 (2.46)</td>
<td>19.9 (2.04)</td>
<td>34.9 (3.87)</td>
</tr>
<tr>
<td>CHr - CH (%/100)</td>
<td>3.04 (0.59)</td>
<td>-0.2 (0.54)</td>
<td>5.7 (4.04)</td>
<td>3.4 (7.70)</td>
</tr>
<tr>
<td>CHr/CH ratio</td>
<td>1.03 (0.04)</td>
<td>1.00 (0.07)</td>
<td>1.06 (0.04)</td>
<td>1.03 (0.08)</td>
</tr>
</tbody>
</table>

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Fig 1. Frequency distributions of reticulocyte and red blood cell indices obtained with the H*3 reticulocyte method in 64 healthy subjects.

normal distribution and are well separated one from the other, with only minimal overlap (Fig 1). An MCV ratio was calculated as the ratio of MCVr to MCV generated in the H*3 reticulocyte method; this simple index describes and quantifies the size difference between mature red blood cells and reticulocytes. In all subjects, regardless of red blood cell size, the MCV ratio was higher than 1. The behavior of H*3 CHCM and CHCMr in all our groups was almost specular to that of the volume. In healthy subjects we found a mean CHCMr of 26.3 g/dL, compared with a CHCM of 31.6 g/dL, with a mean negative difference of 16.7%. The frequency histograms of CHCM and CHCMr (Fig 1) show normal distribution and no overlap, similarly to those of the volume, although their position along the X axis is inverted. Similar differences were found in patients with microcytic or macrocytic anemia. The mean values of the ratio of CHCMr to CHCM were consistently lower than 1, ranging from 0.83 to 0.75 in the four groups. The hemoglobin content per cell, on the other hand, was very similar in reticulocytes and mature erythrocytes in all the subjects that we have studied, with small differences ranging from −0.2% in iron deficiency to +5.7% in β-thalassemia, so that frequency histograms of CHr and CH were widely overlapped (Fig 1), and the CHr:CH ratio was very close to 1.

These results indicate that, regardless of the final red blood cell size, reticulocytes are consistently larger than the mature erythrocytes that originate from them, whereas their hemoglobin concentration is consistently lower: as a consequence of these changes, hemoglobin content is almost the same. The constancy of this general pattern of reticulocyte maturation is clearly demonstrated by the comparison of red cell and reticulocyte indices using linear regression (Fig 2), which shows very high coefficients of correlations (r range from 0.928 to 0.972) with tight distribution of measurements around the regression line. The regression lines have slopes very close to 1 for all three indices, whereas the Y intercept
has a positive value for MCV:MCVR and a negative value for CHCM:CHCMr and is close to 0 for CH:CHr.

Inversion of the MCV ratio. During the 8 weeks of our study, we were able to discover only four cases of abrupt and transitory inversion of the MCV ratio, that is, of subjects having reticulocytes smaller than mature erythrocytes. Case 1 was a 51-year-old male patient who was being treated for megaloblastic anemia developed 7 years after gastrectomy for peptic ulcer. At the time of clinical diagnosis, his hemoglobin concentration was 4.5 g/dL and his MCV was 140 fL. He was treated with daily intramuscular vitamin B12 administration for the first week and then once a week. We did not have the H*3 system during the first treatment period, but we observed a rapid reticulocyte response (up to 13.4%) using the fluorescence reticulocyte counter Sysmex-Toa R-1000 (Kobe, Japan). When we analyzed his blood with the H*3 after 17 days of treatment, his hemoglobin concentration had risen to 8.5 g/dL, and his MCV was lowered to 109.8 fL, while his MCVr was 108.8 fL, so that the MCV ratio was 0.95. Eleven days later, hemoglobin concentration was 10.2 g/dL, and MCVr was 98.9 fL, whereas the MCVr was 116.7 fL, with an MCV ratio of 1.18, within the normal range. It is probable that in this case the inversion of the MCV ratio was consequent to the delivery from bone marrow of new normocytic reticulocytes, produced in the presence of restored vitamin B12 availability. They were smaller than the circulating, still macrocytic population of mature red blood cells, most of which had been formed before the vitamin administration.

Case 2 was a 45-year-old male patient with acquired immunohemolytic anemia of the warm antibody type, which was monitored with the H*3 for 20 days while he was being treated with intermediate-dose prednisone and not transfused. He had an almost constant hemoglobin concentration ranging from 8.1 to 9.7 g/dL, whereas the reticulocyte percentage, which had been above 8% for the first 15 days, showed a progressive decline to less than 1%, preceded by 2 days by a gradual rapid decrease of MCVr, from 130 to 91.5 fL, and the MCV was 93.2. The MCV ratio was 0.98 and stayed close to 1 for the 2 successive days, after which we had to stop the follow-up for the end of the H*3 evaluation. Similarly, the CHr decreased from a normal value of 29.0 pg at the time of reticulocytosis down to 19.8 pg, a value typically found in iron deficiency, while red blood cell CH was still normal (27.8 pg). Concomitantly with the decrease in reticulocyte percentage, MCVr, and CHr, the H*3 showed a progressive increase of the hypochromic red blood cells up to 23.0%, without any increase in the percentage of microcytes. Bone marrow iron stores of the patient were normal, but its serum iron was 10 μg/dL and its iron-binding capacity was 66 μg/dL, with a transferrin saturation of 15%. Thus, in this case the decrease in reticulocyte number and size was probably related to the acute onset of severe functional iron deficiency, similar to that recently reported in patients treated with erythropoietin for anemia of chronic renal failure or autologous blood donation.

Case 3 was a 22-year-old female patient in complete remission from Ph1-positive acute lymphoblastic leukemia who underwent autologous bone marrow transplantation from her ABO-incompatible sister. Conditioning treatment with busulphan and cyclophosphamide was started 7 days before infusion of donor bone marrow cells (3 × 10^6/kg). Figure 3 shows the observed changes in absolute neutrophil count (ANC), hyperfluorescent reticulocytes (HFR), and absolute neutrophil count (ANC), packed red blood cells.

DISCUSSION

We have determined with the H*3 hematologic analyzer the mean values and distribution ranges of reticulocyte and red blood cell indices in healthy subjects and in patients with abnormal red blood cells. In healthy subjects we have found that, both in men and in women, reticulocytes are on average...
24.3% larger than mature erythrocytes, their hemoglobin content is on average 16.7% lower, and their hemoglobin content is almost the same, with a mean difference of 3.0%. These results confirm the classical concept that maturation of the reticulocyte is associated with loss of size and increase in density,\textsuperscript{17} but contrast with previous reports of hemoglobin production\textsuperscript{18} or loss\textsuperscript{19} during reticulocyte maturation. Our data are very similar to those obtained by Clarkson and Moore\textsuperscript{20} using a planimetric method on microphotographs of wet preparations stained with brilliant cresyl blue: they calculated, in fact, a mean reticulocyte volume of 106 fL in 17 normal subjects, compared with our MCVr of 111.7 fL, and a difference between reticulocyte and red blood cell volume of -20%. Colella et al.\textsuperscript{18} using a laser scatter flowcytometer, obtained in 33 normal blood samples an MCVr of 15% higher than that of mature red blood cells, whereas the CHCMr was 5% lower and the CHr 9% higher.

The study of patients with iron deficiency, \(\beta\)-thalassemia trait, and macrocytic anemia of various origin showed that changes associated with reticulocyte maturation in abnormal erythropoiesis are similar to those of normal subjects. In patients with macrocytosis, the mean MCVr was 139 fL, a value identical to that found by Clarkson and Moore.\textsuperscript{20} In contrast with the wide range of red cell size in our groups of patients, we found that a strikingly tight relationship does exist between reticulocyte indices and the corresponding red cell indices (Fig 2). We chose the ratio of MCVr to MCV (MCVR ratio) as the simplest index that quantifies the size differences between reticulocytes and mature erythrocytes. An MCV ratio above 1.0 was a universal finding in our experience: in men and women, normal and abnormal erythropoiesis, and normocytic, microcytic, and macrocytic red blood cells. Its mean values ranged from 1.24 in healthy subjects and in patients with macrocytosis to 1.35 in microcytic patients with untreated iron deficiency or heterozygous \(\beta\)-thalassemia.

We observed rare exceptions to this general rule of the constancy of the MCV ratio, such as in a patient receiving vitamin B12 treatment for megaloblastic anemia, whose new normal-sized reticulocytes were smaller than the preexisting macrocytic erythrocytes, and in a case of functional iron deficiency developed during the clinical course of autoimmune hemolytic anemia. In this patient, a relative insufficiency of iron supply to erythroblasts caused by prolonged erythropoietic stimulation was shown by low transferrin saturation in the presence of normal iron stores\textsuperscript{14} and increased percentage of hypochromic erythrocytes.\textsuperscript{15,16} It caused a dramatic decrease in both reticulocyte percentage and MCVr, with inversion of the MCV ratio, as well as a sudden decrease of CHr, which has recently been reported as an early indicator of iron deficiency.\textsuperscript{13} During the follow-up of two bone marrow transplantation patients, the succession of suppression and regeneration of erythropoiesis was associated with important changes in MCVr and MCV ratio. The decrease in reticulocyte percentage that occurred after conditioning chemotherapy, in fact, was associated with progressive decrease of MCVr and transitory inversion of the MCV ratio. In both cases erythroid regeneration was heralded by an abrupt increase of MCVr, which in a few days reached values above normal. The first transitory increase in MCVr of our first bone marrow transplant patient immediately after transfusion was probably caused by the presence of normal-sized reticulocytes in refrigerated blood (unpublished observation, May 1993); alternatively, it could represent the first sign of erythroid regeneration and its suppression caused by the transfusion.

Our results confirm that, in steady conditions, intravascular reticulocyte maturation is characterized by a decrease in size from reticulocytes to mature erythrocytes, expressed by an MCV ratio higher than 1.0. However, when a sudden change in erythropoiesis takes place and leads to the production of reticulocytes of different size, a change of MCVr occurs much earlier than the MCV change, due to the long red blood cell survival in peripheral blood. A rapid MCVr increase, for instance, caused by the production of macroreticulocytes with high RNA content,\textsuperscript{4,5,7,8} characterizes the brisk erythropoietic stimulation that occurs after chemotherapy, bone marrow transplantation, or erythropoietin treatment. On the other hand, the production of microreticulocytes, leading to rapid MCVr decrease with inversion of the MCV ratio, rapidly follows the development of true or functional iron deficiency, as it was shown during experimental induction of iron deficiency in humans by repeated phlebotomies.\textsuperscript{20} On these bases, simultaneous measurement of reticulocyte and red blood cell indices, which is now available on automated routine hematology systems, seems to be a much more sensitive indicator of sudden erythropoietic changes than conventional MCV, thus representing a powerful tool for the real-time monitoring of erythropoiesis.

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