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 = 0.928 for volume, r = 0.929 for hemoglobin concentration, r = 0.972 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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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and have precision, linearity, and carry-over features equal to or better than the manufacturer's specifications (unpublished data, May 1993).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 in- cluding 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, below 15 ng/mL) before iron treatment and 40 with heterozygous β-thalassemia and no iron deficiency (hemoglobin level, 8.9 to 12.4 g/dL; A3 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 hemollogenic 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 = .768 for MCHC v CHCM; r = .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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>All Subjects (n = 64)</th>
<th>Males (n = 32)</th>
<th>Females (n = 32)</th>
<th>P Value of t-Test (df)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Reference Range</td>
<td>Mean</td>
<td>Reference Range</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>14.0</td>
<td>12.9-15.7</td>
<td>14.5</td>
<td>13.3-15.7</td>
</tr>
<tr>
<td>Red blood cells (x10^12/L)</td>
<td>4.66</td>
<td>4.22-5.26</td>
<td>4.86</td>
<td>4.44-5.35</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>89.9</td>
<td>83.4-97.0</td>
<td>89.3</td>
<td>83.4-97.0</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>32.6</td>
<td>31.1-34.0</td>
<td>32.7</td>
<td>31.5-34.0</td>
</tr>
<tr>
<td>CHCM (g/dL)</td>
<td>31.6</td>
<td>30.1-33.2</td>
<td>31.7</td>
<td>30.9-33.2</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>30.3</td>
<td>27.9-32.0</td>
<td>30.1</td>
<td>27.9-31.9</td>
</tr>
<tr>
<td>CH (pg)</td>
<td>27.7</td>
<td>25.9-29.6</td>
<td>27.6</td>
<td>25.6-29.5</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>0.9</td>
<td>0.5-1.4</td>
<td>0.9</td>
<td>0.5-1.3</td>
</tr>
<tr>
<td>Reticulocytes (x10^12/L)</td>
<td>41.0</td>
<td>22.7-86.9</td>
<td>41.5</td>
<td>24.4-85.8</td>
</tr>
<tr>
<td>MCVr (fL)</td>
<td>111.7</td>
<td>103.2-126.3</td>
<td>112.2</td>
<td>104.0-123.8</td>
</tr>
<tr>
<td>CHCMr (g/dL)</td>
<td>28.3</td>
<td>25.9-30.6</td>
<td>28.6</td>
<td>26.3-30.2</td>
</tr>
<tr>
<td>CHr (pg)</td>
<td>26.1</td>
<td>25.9-30.6</td>
<td>26.6</td>
<td>26.3-30.2</td>
</tr>
</tbody>
</table>

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>66.8 (3.56)</td>
<td>115.1 (6.55)</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>111.7 (6.77)</td>
<td>100.1 (6.78)</td>
<td>92.3 (6.87)</td>
<td>120.9 (11.00)</td>
</tr>
<tr>
<td>MCV – MCV (%)</td>
<td>24.3 (5.87)</td>
<td>34.9 (8.31)</td>
<td>34.5 (8.01)</td>
<td>24.6 (6.25)</td>
</tr>
<tr>
<td>MCVr/MCV ratio</td>
<td>1.24 (0.06)</td>
<td>1.35 (0.08)</td>
<td>1.36 (0.08)</td>
<td>1.29 (0.06)</td>
</tr>
<tr>
<td>CHCM (g/dL)</td>
<td>31.6 (1.02)</td>
<td>27.3 (1.48)</td>
<td>28.5 (1.28)</td>
<td>31.0 (1.12)</td>
</tr>
<tr>
<td>CHCMr (g/dL)</td>
<td>26.3 (1.50)</td>
<td>26.4 (0.29)</td>
<td>22.3 (1.74)</td>
<td>25.7 (1.16)</td>
</tr>
<tr>
<td>CHCMr – CHCM (%)</td>
<td>16.7 (2.80)</td>
<td>-25.4 (4.89)</td>
<td>-21.9 (3.70)</td>
<td>-17.0 (4.11)</td>
</tr>
<tr>
<td>CHCMr:CHCM 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>21.7 (1.42)</td>
<td>19.7 (2.05)</td>
<td>18.9 (1.84)</td>
<td>23.5 (2.12)</td>
</tr>
<tr>
<td>Chr (pg)</td>
<td>28.6 (1.66)</td>
<td>19.8 (2.46)</td>
<td>19.9 (2.94)</td>
<td>24.9 (3.87)</td>
</tr>
<tr>
<td>Chr – CH ratio (%)</td>
<td>3.04 (0.59)</td>
<td>-0.2 (5.94)</td>
<td>5.7 (4.4)</td>
<td>3.4 (7.0)</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>
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 .928 to .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 transient 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 total 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, MCV was 98.9 fl, and 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 his serum iron was 10 μg/dL and his 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 allogeneic 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 × 10^8/kg). Figure 3 shows the observed changes in absolute neutrophil count (ANC), hyperfluorescent reticulocytes (HFR), and absolute neutrophil count (ANC).}

**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, but contrast with previous reports of hemoglobin production or loss during reticulocyte maturation. Our data are very similar to those obtained by Clarkson and Moore using a planimetric method on microphotographs of wet preparations stained with bright 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., using a laser scatter flow cytometer, 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, β-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. 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 (MCV 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 β-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 cytostatic drug therapy led to a relative increase in MCVr, rapidly followed by the development of true or functional iron deficiency, as it was shown during experimental induction of iron deficiency in humans by repeated phlebotomies. 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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