Reticulocyte Size in Nutritional Anemias

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Alterations in reticulocyte size occur 2–3 days after the onset of iron deficient or megaloblastic erythropoiesis and precede, by several weeks, changes in mean corpuscular volume (MCV). Iron-deficiency anemia induced in a normal subject by repeated phlebotomies was characterized by the initial development of larger than normal reticulocytes followed by an abrupt decrease in reticulocyte size. Micro-reticulocytes appeared 3 days after the fall in percent iron saturation and antedated the decrease in MCV to below normal by 6 wk. Mean reticulocyte size was disproportionately smaller than normal in patients presenting with iron deficiency. In contrast, reticulocyte size increased abruptly in a patient (and rats) 2–3 days after administration of methotrexate. Mean reticulocyte size was disproportionately larger than normal in patients presenting with folate or vitamin B₁₂ deficiency. Specific replacement therapy with iron, folate, or vitamin B₁₂ was quickly followed by normalization of reticulocyte size.

The measurement of mean corpuscular volume (MCV) is often unsatisfactory for the evaluation of patients with anemia secondary to early iron, folate, or vitamin B₁₂ deficiency. The MCV may not be useful because the long survival of red cells allows the indices to remain in the normal range for 6–12 wk following the development of iron deficiency, as shown by the classic phlebotomy study of Conrad and Crosby. Likewise, the folate deprivation study of Herbert demonstrates that macrocytosis does not develop until 14 wk following the attainment of low serum folate levels.

The purpose of our study was to test the hypothesis that changes in reticulocyte size would precede changes in MCV in patients developing iron, folate, or B₁₂ deficiencies. Accordingly, mean reticulocyte size was determined in normals, in a normal subject undergoing repetitive phlebotomies, and in patients with iron, folate, and B₁₂ deficiencies. Selected patients were restudied after specific replacement therapy. Serial changes in the reticulocyte size of a patient with metastatic adenocarcinoma receiving high dose methotrexate with leucovorin factor rescue therapy were observed. Similar observations were also made in rats given methotrexate.

MATERIALS AND METHODS

Reticulocyte size was determined by an adaptation of the planimetric method reported by Killman, in which reticulocytes identified in brilliant cresyl blue wet preparations were photographed under oil immersion (100 x). The thickness of the wet preparation was adjusted so that reticulocytes and nonreticulated erythrocytes (NRE) were viewed en face rather than obliquely. The resultant Kodachromes were projected onto butcher paper at a distance of 14 ft, and 25–30 pairs of reticulocytes and adjacent NRE were circled and then traced with a planimeter. The mean
of these measurements was used to determine the reticulocyte area/NRE area ratio. The use of wet preparations avoided the artifacts associated with stained smears, and the measurement of adjacent reticulocyte-NRE pairs eliminated a possible bias due to local changes in physico-chemical conditions that might affect the area of the cells being measured. Reticulocyte size, serum iron, serum and whole blood folates were performed on patients admitted with anemia to the University of South Alabama Medical Center, as well as 17 healthy members of the faculty and staff. Serum iron (SI), total iron-binding capacity (TIBC), serum and whole blood folates, and serum vitamin B12 were determined by previously described techniques. Hematocrit values and red cell indices were obtained on all subjects, using a Coulter S Electronic Cell Counter. A healthy 32-yr-old volunteer, after giving informed consent, underwent nine successive 500-ml phlebotomies with determinations of reticulocyte size, SI/TIBC, reticulocyte count, and MCV at frequent intervals. Iron deficiency was diagnosed in 18 patients with a SI/TIBC < 10\(^{-6}\), all of whom subsequently obtained a hematopoietic response to oral iron supplementation. Seven folate-deficient patients with serum and blood folate values less than 3 ng/ml and 30 ng/ml, respectively, and two B12-deficient patients with serum B12 values less than 160 ng/ml, constituted the megaloblastic study group. The Schilling’s test was abnormal and a hematopoietic response to vitamin B12 treatment occurred in both B12-deficient patients. Patients receiving antibiotics at the time of determination of serum and blood folates, patients with low SI/TIBC who subsequently failed to respond to oral iron supplementation, and patients with combined iron and folate deficiency were excluded from the study.

A single dose of methotrexate (20 mg/kg) was given intraperitoneally to 14-wk-old Sprague-Dawley rats. Saline injected rats were used as controls. Several drops of tail vein blood were removed at each sampling for reticulocyte studies. A patient with metastatic adenocarcinoma of the colon receiving oral methotrexate (125 mg/sq m) at 6-hr intervals x 4 followed by leucovorin rescue was studied at varying intervals to determine effect of this treatment on reticulocyte size.

RESULTS

Estimation of Mean Reticulocyte Volume

The reticulocyte area/nonreticulated erythrocyte (NRE) area ratio of 17 normals was 1.125, indicating that the normal reticulocyte area was 13% greater than that of a NRE— a value identical to that of Killman. We assumed, as did Killman, that the cell thickness varied as the cell diameter in both reticulocytes and NRE. In normals, then, it follows that the ratio of the radii is \(\sqrt{1.13}\), and also that the reticulocyte volume/NRE volume ratio is \((1.13)^\frac{3}{2}\), or 1.20. We then defined the term mean reticulocyte volume (MRV) as \((\text{reticulocyte area}/\text{NRE area})^{\frac{3}{2}} \times \text{MCV}\). In normals, this would be the product 1.20 \(\times\) 88 fl or 106 fl. Thus in normals the reticulocyte was 13% larger by area (measured) and 20% larger by volume (estimated) than the NRE.

Serial Changes in MRV in a Phlebotomized Subject

The serial changes in MRV, hematocrit (Hct), reticulocyte count, MCV, and SI/TIBC were observed in a normal subject undergoing nine successive 500-ml phlebotomies to induce iron deficiency (Fig. 1). An abrupt fall in SI/TIBC occurred after the fifth 500-ml phlebotomy, which corresponded to the removal of approximately 1300 mg of iron or roughly equal to the iron stores of a normal man. An increase in MRV from a prephlebotomy value of 104 fl to a peak of 123 fl began concomitantly with the development of anemia and reticulocytosis. The measurement of the MRV thus permitted quantification of the phenomenon called “shift reticulocytosis,” which was confirmed by the identification of polychromatophilic macrocytes in the Wright-stained peripheral blood.
At a time when the reticulocyte count was still elevated, the SI/TIBC fell abruptly (fifth phlebotomy) and was followed two days later by a fall in MRV. These changes corresponded to a decrease in the reticulocyte volume/NRE volume ratio of 1.33 (Fig. 1 at A) to a value of almost unity within a week after the onset of a low SI/TIBC. Thus the production of microcytic erythrocytes commenced within days after the fall in SI/TIBC and antedated the fall in MCV by several weeks.

Temporally, the MRV fell two days after the fall in SI/TIBC and then reached a plateau (at B); the MCV was unchanged for several weeks before beginning a progressive decline. The MCV did not fall below the lower range of normal (79 fl) until day 63 (at C), or approximately 6 wk after the fall in MRV below its normal range (96 fl).

With iron supplementation the MRV increased rapidly, followed by the reticulocyte count and serum iron. The sequence of events occurring with iron deficiency was as follows: serum iron decreased (1st day) → decrease in MRV (3rd day) → decrease in reticulocyte count (7th day) → decrease in MCV (to 79 fl on 45th day). In contrast, iron replacement was associated with an increase in MRV followed by sequential increases in the reticulocyte count, serum iron, and MCV, respectively.

**MRV and MCV in Patients With Iron Deficiency Compared to Normals**

The means and standard deviations of the presenting MCV and MRV of 18 patients with iron-deficiency anemia were compared to those of 17 normal subjects (Fig. 2). The MCV of the iron-deficient patients (73 ± 13 fl) was statistically different ($t = 4.6, df = 33$) from the MCV of the normals (88 ± 4 fl). The MRV of the iron-deficient patients (79 ± 13 fl) was also significantly less ($t = 7, df = 33$) than the MRV of the normals (106 ± 9 fl). However, the MRV of iron-deficient patients appeared to be disproportionately smaller than the MCV, suggesting that the MRV was a better discriminant for iron deficiency than the MCV. Two subjects with iron deficiency were given oral iron supplementation and a rapid increase in reticulocyte size occurred (Fig. 6). Only a minimal change in MCV was observed.
Serial Changes in MRV in Megaloblastic Erythropoiesis

Because we were not able to study the onset of megaloblastic erythropoiesis in patients developing folate or vitamin B₁₂ deficiency, we administered methotrexate, a potent inhibitor of dihydrofolate reductase,⁷ to rats (Fig. 3). An increase in reticulocyte size, as indicated by a rise in the reticulocyte area/NRE area ratio, occurred over a 7-day period after the administration of methotrexate. In contrast, no change in the reticulocyte area/NRE area ratio of the controls occurred.

A patient receiving high dose methotrexate with leucovorin rescue had serial determinations of reticulocyte size (Fig. 4). An abrupt rise in MRV from a baseline of 92 fl to a maximum of 142 fl occurred by the 5th day after methotrexate followed by a return to normal on the 12th day. The MCV (79 fl) remained unaltered for the duration of the study.

MRV and MCV in Patients With Folate and B₁₂ Deficiencies Compared to Normals

The MCV of the folate/B₁₂-deficient patients (103 ± 11 fl) was statistically greater \( t = 5.1, df = 24 \) than the MCV of the normals (88 ± 4 fl) (Fig. 5). Similarly, the MRV of the folate/B₁₂-deficient group (139 ± 25 fl) was also significantly greater \( t = 4.9, df = 24 \) than the MRV of the normals (106 ± 9 fl).

Four patients with folate deficiency and two patients with B₁₂-deficiency were given specific replacement therapy and rapid normalization of reticulocyte size

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**Fig. 2.** MCV and MRV in patients with iron deficiency (SI/TIBC < 10%) compared to normals (means ± 1 SD). Patients with polycythemia rubra vera are indicated by open circles.

**Fig. 3.** Changes in reticulocyte area/NRE area ratio in rats injected intraperitoneally with methotrexate, 20 mg/kg (closed circles), or saline (open circles). Means ± 1 SD for three animals in each group are represented.
DISCUSSION

The onset of microcytosis does not commence with the onset of iron deficiency because large numbers of previously produced normocytic red cells greatly outnumber the relatively few microcytes released in the early phases of iron-deficient erythropoiesis. The infusion of a small number of macrocytic “shift reticulocytes” just prior to exhaustion of iron stores and the onset of reticulocytopenia are additional factors that offset the development of microcytic red cell indices. Similar arguments can be posed to explain the slow onset of macrocytosis in patients with megaloblastic anemia.

Our data suggest that, although some red cell remodeling must occur, red cell size will ultimately be determined by sustained alterations in reticulocyte size. Thus, in iron-deficient erythropoiesis, small reticulocytes will be produced.
in advance of microcytosis by indices, and, in megaloblastic erythropoiesis, large reticulocytes will be produced in advance of macrocytosis by indices.

Factors regulating red cell size include erythropoietin, iron, folate, and $B_12$; all presumably influence normoblast size prior to expulsion of the nucleus.8'14 “Remodeling” refers to those factors that influence erythrocyte size after enucleation. These factors include loss of membrane and cytoplasm by fragmentation15'20 and, in certain special circumstances, the premature destruction of an extreme population of reticulocytes (“reticulocytolysis”).16 For the purposes of this study, remodeling of red cells secondary to altered cholesterol/phospholipid ratios is probably unimportant.21

The most important information regarding the regulation of red cell size is derived from studies which use the technique of size distribution analysis.22'24 This technique is particularly suited for the investigation of those states that are associated with high reticulocyte counts. Although it has been used to study anemias of bone marrow failure,25 it has serious limitations in the presence of marked reticulocytopenia (< 1%). Hence, we advocate a technique in which individual reticulocytes are identified and sized.

There are obvious limitations to measurement of only one cell dimension (albeit the largest). The observation that cell thickness diminishes more rapidly than cell diameter as microcytosis develops in patients with iron deficiency26 does not necessarily invalidate our assumption that the cell thickness varies as the cell diameter in both reticulocytes and NRE. Electron micrographs often depict the reticulocyte as having a rather complex, puckered shape,27 making our estimations of cell volume less secure. Furthermore, our hypothesis assumes that the rate of remodeling is not accelerated or retarded in iron, folate, or $B_12$ deficiency. These objections aside, data from the phlebotomy, methotrexate, and specific replacement therapy experiments (Figs. 1, 3, 4, and 6) indicate that the reticulocyte area/NRE area ratio accurately reflects day-to-day
changes in erythropoiesis in patients developing or recovering from iron-deficient or megaloblastic erythropoiesis.

Erythropoietin has been demonstrated to increase the life span of the reticulocyte in the circulation at the expense of the time spent in the marrow by causing early release.28,29 The increase in reticulocyte size after phlebotomy (before iron deficiency) (Fig. 1) most probably results from the effect of erythropoietin, causing an acceleration of hemoglobin synthesis and thus increasing cytoplasmic volume as well as stimulating the premature release of immature macroreticulocytes from the marrow compartment.3 The initial slight rise in MCV from 89 to 93 fl after phlebotomy can be explained by the increase in reticulocyte volume (132 fl - 106 fl) x the increase in reticulocyte count (0.06 - 0.01), or 26 fl x 0.05 = 1.3 fl. Much greater increments may occur with sustained erythropoietin stimulus in the absence of iron deficiency.23,30

The abrupt decrease in reticulocyte size after the fall in SI/TIBC (Fig. 1) suggests that the tendency of erythropoietin to produce macroreticulocytes is overridden by the more peremptory effect of iron deficiency in causing the production of microreticulocytes. Similarly, specific replacement therapy with folate or B12 leads to an abrupt decrease in reticulocyte size in patients with megaloblastic anemia (Fig. 6), indicating that the vitamin deficiency is more important than erythropoietin in determining reticulocyte size.

In the phlebotomy experiment, the MCV continued to decline after the MRV had reached a plateau (Fig. 1 at B) due to the culling out of senescent normocytic red cells and their replacement by more recently manufactured microcytes. This experiment suggests that in early iron deficiency the MRV is more sensitive than the MCV, whereas later, when long-standing iron deficiency has led to the development of microcytosis (Fig. 1 at C), the converse is true. Thus, the clinical role of determination of reticulocyte size may actually lie in corroborating the serum iron measurement in reticulocytopenic patients who have developed exhaustion of iron stores but who are not yet microcytic by indices.31 Patients with early iron deficiency probably constitute a significant percentage of patients with normocytic anemia and justify further evaluation of this technique. The divergence of the MRV and MCV curves (from B to C) after most of the normocytes produced in the pre-iron-deficiency period have been culled from the circulation leads us to speculate that remodeling of microreticulocytes may be proportional to that of normal reticulocytes (i.e., a 15%–20% reduction in volume).

The production of macroreticulocytes in patients with folate deficiency appears to be less useful as a discriminant than in patients with B12 deficiency. This may have been due to the fact that several days elapsed between the determination of folate value and the measurement of reticulocyte size, allowing an opportunity for partial correction of the deficiency by ingestion of a hospital diet. The rapid normalization of reticulocyte size occurring with specific replacement therapy (Fig. 6) supports this conclusion. Additional studies using the technique for determination of reticulocyte size might yield information on the relative importance of the serum folate measurement vis-a-vis the red cell folate measurement in determining the production of macroreticulocytes.
example, if a low serum folate (in the presence of a normal red cell folate) is associated with macroreticulocytosis, then one might conclude that low serum folate alone is sufficient to produce megaloblastic erythropoiesis.

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