Erythrocyte Creatine Levels in Anemia

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ABSTRACT

The automated diacetyl-l-napthol procedure was found to be a simple and relatively quick method for the determination of erythrocyte creatine (EC), exhibiting both better precision and greater sensitivity than reticulocyte counting. A reference range of 1.8 to 5.0 mg per dl (mean, 3.0 ± 0.9 mg per dl) was established by measuring EC levels in 81 normal adults varying in ages from 20 to 47 years of age. Normal creatine values displayed a slight sex dependency, with females exhibiting somewhat higher levels than males. In evaluating the relative usefulness of EC versus reticulocyte counting in the assessment of anemia, both parameters were measured and compared in 41 patients with various hemolytic and nonhemolytic anemias. A direct relationship was noted between the two parameters (p < 0.0001); however, the actual data correlation was only fair (r = 0.49). In a serial study of an iron-deficiency anemic patient responding to iron administration, erythrocyte creatine levels were found to rise more slowly than the reticulocyte count and to remain elevated after the reticulocyte count had returned to normal.

Creatine is a physiologically important compound involved in muscle metabolism which acts as a biological reservoir of high-energy phosphate through formation of phosphocreatine. The molecule is synthesized in a two-step process occurring primarily in the liver and kidneys and is then distributed via the vasculature to the rest of the body. Creatine is metabolized by a spontaneous and irreversible non-enzymatic reaction to creatinine, which is then excreted by the kidneys.

While creatine is a constituent of all cells, it is present in highest concentrations in muscle (2.3 g creatine per kg of muscle) and is lowest in plasma or serum (0.2 to 1.0 mg per dl). Its presence in red blood cells was first documented in 1918 by Hunter and Campbell. Available data from different investigators show a reference range of erythrocyte creatine concentrations from 3.0 to 8.0 mg per dl of packed red blood cells.

In 1967, Griffiths and Fitzpatrick reported that erythrocyte creatine (EC) was a sensitive indicator of mean red cell age, with young cells exhibiting creatine levels six to nine times higher than old cells.
These authors found a significant correlation between the number of circulating reticulocytes and the creatine content of red cells in patients with iron deficiency, megaloblastic, and auto-immune hemolytic anemias. Subsequent reports by other investigators confirmed this correlation. That creatine is chiefly related to the mean age of the cell rather than to the degree of anemia was demonstrated by Opalinski and Beutler who found no correlation between levels of 2,3-diphosphoglycerate (2,3 DPG) (which generally increase with the severity of the anemia) and creatine content. More recently, Fehr and Knob found that in patients with various hemolytic anemias, there was a close correlation of EC with red cell survival times. In light of these findings, they proposed that creatine content might prove to be a sensitive indicator for the assessment of hemolytic disease.

Currently, the presence of hemolysis is detected primarily by an increase in red cell production, which in turn is estimated from the reticulocyte count. The high degree of statistical error associated with reticulocyte counting has hampered its diagnostic value and made desirable the development of a more precise index for monitoring the hemolytic process. The method used by Fehr and Knob has been evaluated for its suitability to the clinical laboratory. In addition, EC levels have been compared with the degree of reticulocytosis in patients with hemolytic and non-hemolytic disease and in an iron-deficiency anemic patient before and after the initiation of iron therapy.

**Materials and Methods**

Concentrations of red cell creatine, hematocrit percentages, and reticulocyte counts were obtained in 37 patients with various types of hemolytic and non-hemolytic anemias and in four patients with previous hemolytic disease presently in remission (table 1). Reference ranges for creatine were determined by measuring creatine levels in 81 normal adults.

Blood samples were collected in ethylenediamine tetraacetic acid (EDTA) for both hematological and creatine determinations. Reticulocytes were determined either by the Miller Disk method or by staining in methylene blue and calculating the percentage present based on 500 red cells counted. The reference range for reticulocytes in our laboratory is 0.5 to 1.5 percent. Hematocrit values were measured by the microcapillary method following centrifugation at 14,500 g for five minutes. Reference values for males and females are 42.0 to 52.0 percent and 37.0 to 47.0 percent, respectively.

For creatine measurements, blood samples were stored at 4°C and analyzed within seven days of collection. Under these conditions no decrease in EC concentrations was observed. Hemolysed blood samples were not used. Immediately before analysis, red cells were concentrated by centrifugation twice at 2000 to 3000 RPM for 10 minutes, followed each time by removal of the serum and buffy coat. Packed cells were vigorously suspended by a vortex mixer to ensure homogeneity and then lysed by diluting 0.1 ml of the packed cells into 0.9 ml of 0.1 percent saponin. The creatine content of the hemolysate was then determined by the diacetyl-l-napthol reaction adapted to the Technicon Autoanalyzer and expressed as mg of creatine per dl of packed red cells.

The autoanalyzer system used was assembled according to the flow diagram described by Griffiths. A sampling rate of 30 per hour with a 1:1 sample to wash ratio was found to give reasonable peak separation with a minimal amount (2.8 percent) of carryover.

Reagents required for the procedure were 0.04 percent diacetyl, 0.8 percent l-naphthol in alkali, and 0.9 percent saline; their preparation has been described elsewhere. Aqueous solutions of creatine sul-
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CREATINE (mg/dL)

Figure 1. Erythrocyte creatine standard curve.

Fate*, varying in concentration from 0.02 to 0.15 mmol per L (0.25 to 2.0 mg per dl) were used as standards.

The regression equation for the relationship between blood reticulocyte counts expressed in percent of red cells and erythrocyte creatine was calculated by the least squares method.

Results

LINEARITY

A typical standard curve obtained with the aqueous creatine solutions is shown in

* Sigma Chemical, St. Louis, MO 63178.

Figure 1. The automated method proved to be sensitive to 0.25 mg per dl and linear up to 10 mg per dl. Since the hemolysates were a 1:10 dilution of red cells, their creatine concentrations were usually quite low. For that reason, a reduced working range of 0.25 to 2.0 mg per dl was used to provide greater sensitivity.

PRECISION

The within-run precision of the creatine assay was assessed by measuring the creatine concentration in separate hemolysates prepared from the same red cell sample (table II). For comparison is listed the within-run precision of reticulocyte measurements performed by different technologists on separate slide preparations of the same blood sample.

For a normal blood sample, the EC measurements exhibited a 5.6 percent relative standard deviation (RSD) which is much lower than the variation of 20.0 percent observed in reticulocyte counting. In a hemolytic sample, which yielded higher values for both parameters, creatine precision increased slightly; reticulocyte precision, however, decreased sharply, exhibiting an R.S.D. of 25.9 percent.

REFERENCE RANGE

To establish a reference range for EC as determined by the automated diacetyl-L-napthol reaction, creatine levels were measured in 81 normal adults varying in ages from 20 to 47 years of age. The distribution graph (figure 2) illustrates that while the overall range observed in red cell creatines was 1.4 to 5.1 mg per dl, the majority of values fell between two and three mg per dl. The reference interval determined by non-parametric statistics was 1.8 to 5.0 mg per dl (with a mean value of 3.0 ± 0.9 mg per dl) which is in excellent agreement with that reported by Opalinski and Beutler.10 It is, however,
### TABLE I

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Number of Patients</th>
<th>Number of Determinations</th>
<th>HCT (Percent)</th>
<th>Reticulocyte (Percent)</th>
<th>Erythrocytic Creatine (mg/dl)</th>
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<td>Patients with clinical hemolytic anemia</td>
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<td>1.7 - 22.4</td>
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<td>3.4 - 8.0</td>
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<td>1</td>
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<td>3.1</td>
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<td>24</td>
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<td>6.8</td>
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<td>1.2 - 2.2</td>
<td>1.4 - 1.8</td>
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<td>G6PD deficiency</td>
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<td>3.2 - 3.6</td>
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<td>1</td>
<td>37</td>
<td>0.6</td>
<td>3.6</td>
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</tbody>
</table>

HCT = Hematocrit  
DCT = Direct coombs test  
G6PD = Glucose-6-phosphate dehydrogenase  
TTP = Thrombotic thrombocytopenia purpura

slightly lower than the values noted by some investigators.\(^1,2,6,10\)

Several researchers\(^6,8,10\) have reported a sex dependency of EC concentrations, with females exhibiting somewhat higher levels than males. To determine if this dependency was reflected in our data, the normal values shown in figure 2 were separated by sex and are plotted in figure 3. The upper plot (figure 3a) illustrates the EC distribution for 36 males and the lower plot (figure 3b) that for 45 females. Although the majority of individuals still fall in the two to three mg per dl bracket, females do appear to exhibit a broader overall range of values as compared to a somewhat smaller range found in males. This finding is reflected in the reference intervals determined for each group: 1.9 to 4.0 for males and 1.7 to 5.0 for females. A statistical analysis of the data indicated that the variance seen in the two groups was significantly different (p < 0.01). Although the mean creatine value for females (3.1 ± 1.0 mg per dl) was also higher than that for males (2.8 ± 0.6 mg per dl), the difference was not statistically significant.

### TABLE II

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean ± S.D.</th>
<th>R.S.D.</th>
<th>N</th>
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<td>Creatine</td>
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<td>5.6(^*)</td>
<td>10</td>
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<tr>
<td>Reticulocyte counts</td>
<td>1.0 ± 0.2(^*)</td>
<td>20.0</td>
<td>10</td>
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<tr>
<td>Hemolytic sample</td>
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<tr>
<td>Creatine</td>
<td>6.2 ± 0.26 mg/dl</td>
<td>3.9</td>
<td>10</td>
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<tr>
<td>Reticulocyte counts</td>
<td>5.4 ± 1.4(^*)</td>
<td>25.9</td>
<td>7</td>
</tr>
</tbody>
</table>

R.S.D. = Relative standard deviation  
\(^*\)Percent
assessment of anemia, both parameters were measured and compared in 37 patients with various hemolytic and non-hemolytic anemias and in four patients with previous hemolytic anemia (table I).

Seven patients had multiple determinations while the remainder had only one sample analyzed. The large amount of variation seen in the hematological parameters for patients with sickle cell anemia, sickle cell disease, sickle cell thalassemia, and idiopathic hemolytic anemia is due to the blood transfusions these patients received during the course of the study.

A linear regression analysis of the data (figure 4) illustrates that while there certainly is a direct relationship between the percentage of circulating reticulocytes and the EC level \( (p < 0.0001) \), the actual data correlation is only fair \( (r = 0.49) \). Correcting the crude reticulocyte percentage for hematocrit variation (male values were normalized to 45.0 percent; female values to 42.0 percent) showed no improvement in correlation \( (r = 0.42, p < 0.001) \) (data not shown).

The relationship between reticulocytosis and red cell creatine content is more clearly discernible in the serial study of a female patient with iron-deficiency anemia presented in figure 5. Prior to iron therapy the reticulocyte count was normal, but the creatine level was elevated to almost three times the normal mean value. After iron administration was begun, the reticulocyte count rose sharply and then decreased gradually over a period of 40 to 50 days to a normal level. Creatine levels, on the other hand, rose more slowly and remained elevated for a longer period of time, declining after 45 days to a level almost within the normal range.

Discussion

The automated diacetyl-l-napthol procedure has proven to be a simple and relatively quick method for the determination of EC. In comparison with manual reticulocyte counting, creatine measurements offer a much higher level of precision. The inherent limitations of the reticulocyte count have been attributed to a variety of problems, the most important of which appears to be the large variations in morphologic identification of reticulocytes by different technologists.\(^{12}\) These variations critically compromise counting accuracy and hamper the diagnostic utility of the reticulocyte count. The automated creatine assay, by virtue of its simplicity, can provide rapid and reliable
Figure 5. Effect of iron administration on reticulocytosis and erythrocyte creatine in a female patient with iron-deficiency anemia. Iron treatment was begun on day 1 after the first blood sample was drawn.

Information which can be used to supplement the often inaccurate reticulocyte count or provide additional insights in the appraisal of anemia.

An interesting finding of this study was that females exhibit higher levels of creatine than do males, an observation also noted by other investigators.6,8,10 Since the majority of the women sampled in these studies were still in the menstrual epoch, it seems likely that the elevated creatine levels are due to menstrual loss, a hypothesis first proposed by Griffiths and Fitzpatrick.6 This would also explain the broader range of creatine levels which were found in females in this study since blood samples were drawn from women at different stages of the menstrual cycle. That this finding has not been reported by other researchers may be due to the smaller number of women sampled in other studies.

In the assessment of anemia, EC appears to be a more sensitive indicator of mean cell age than the number of circulating reticulocytes. This observation is best illustrated in the hematological profile of the iron-deficiency anemic patient, in which creatine levels were not only consistently higher than reticulocytes, but were also elevated when the reticulocyte count had returned to normal. Similar findings were noted by Griffiths and Lothian,7 Griffiths and Fitzpatrick,6 Opalsinski and Beutler,11 and Bowen et al1 in patients with hemolytic and non-hemolytic anemia.

Although a relationship certainly exists between EC and the degree of reticulocytosis (p < 0.0001), the actual data correlation was only fair (r = 0.49). Since two different entities are being measured—one an index of red blood cell (RBC) production, the other an index of cell age—one would not expect excellent agreement. However, other researchers have reported much better correlation between the two parameters.2,11 The major factors contributing to the large variations found in this study were felt to be the inherent errors in reticulocyte counting (as discussed) and the time at which the blood sample was drawn. In the iron-deficiency anemic patient, it was noted that the relationship between the two parameters varied considerably over the time course of the study. Initial samples drawn when the patient first began producing reticulocytes exhibited creatine levels which were generally lower than expected based on the percentage of reticulocytes measured. However, later samples taken as the reticulocyte count began decreasing to normal demonstrated quite the reverse relationship, with creatine levels appearing appropriately high. This observation can be explained by the fact that young red cells retain their initial high levels of creatine well into the post-reticulocyte phase.6 While the maturation time of the reticulocyte to a mature RBC in the peripheral blood is only one or two days, creatine levels in young cells are elevated for as long as 20 days.

Increases in serum creatine have been noted in a variety of disease states including renal failure, progressive muscular dystrophy, and systemic lupus erythema-
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While no direct evidence is available on the effect of these serum creatine elevations on EC, data from in vitro studies of creatine transport suggest that there is probably little, if any, effect. Early reports by Griffiths indicated that the red cell was impermeable to creatine; however, more recent studies by Rapoport et al. and Ku and Passow using [14C]-creatine have demonstrated that the molecule is actively transported into the cell. Simultaneous measurements of RBC creatine during active transport, however, showed a striking degree of constancy, presumably owing to a facilitated diffusion of creatine out of the cell which balances the influx.

Exactly how and when the red cell acquires its creatine is unknown. Presumably, it is taken up by immature precursors of the red cell and slowly diffuses out of the cell as it ages. That EC content correlates well with red cell survival time and does not appear to be affected by increases in plasma creatine suggests that RBC creatine levels may be independent of disease processes which do not effect cellular production or destruction rates. This, plus its high sensitivity to changes in cell age, make creatine particularly attractive for use as an aging parameter. Currently, the most widely used criterion for estimating mean cell age is the reticulocyte count; however, it is notoriously imprecise and the reticulocyte too short lived to be a sensitive indicator. A number of red cell enzymes including glutamic oxaloacetic transaminase, hexokinase, glucose-6-phosphate dehydrogenase, and cholinesterase are known to decrease in activity with cell age and have been explored as potential parameters of aging.

Various drawbacks encountered with the use of some of these enzymes, however, include instability on storage, low sensitivity to changes in cell age, possible contamination from white blood cells and platelets, and erroneous results owing to possible interferences from non-RBC age-dependent disease processes. The most accurate method of determining mean cell age, the Tvi51Cr, is a tedious and time-consuming procedure which involves the injection of radioactive material into the patient. Erythrocyte creatine measurements offer a simple and sensitive method for determining mean red cell age and because the assay is not subject to contamination from white cells or platelets is inherently more specific than enzymatic methods.

Determination of EC may well prove to be particularly useful in screening for the possibility of hemolytic anemia since it is a more sensitive indicator of decreased mean RBC age than the reticulocyte count and may be increased in value when the reticulocyte count is normal. For example, approximately 25 percent of patients with autoimmune hemolytic anemia have normal reticulocyte counts even though they are actively hemolyzing and an elevated EC may well detect most of those patients. Fehr and Knob demonstrated that an elevated EC was frequently present in patients with mild hemolysis (as shown in 51Cr survival studies) while in many of these patients the reticulocyte count was normal.

On the other hand, it will be important to study other categories of anemia in which decreased RBC production is the main mechanism of the anemia and to determine what role, if any, increased RBC destruction plays in elevating EC levels in such patients (as occurred in our patient with preleukemia). It is evident that if EC is frequently elevated in this type of patient, its usefulness as a screen for hemolytic anemia will be seriously impaired. However, EC may well provide other insights into RBC metabolism and production in all types of anemia.

References

2. Fehr, J. and Knob, M.: Comparison of red cell creatine level and reticulocyte count in apprais-


