Physiologic Features of Hemolysis Associated With Altered Cation and 2,3-Diphosphoglycerate Content

By Maurice M. Albala, Normand L. Fortier, and Bertil E. Glader

A hemolytic disorder characterized by altered RBC cation composition (increased Na, decreased K), reduced monovalent cation content (decreased Na + K/liter RBC), and decreased levels of 2,3-diphosphoglycerate (2,3-DPG) is described. The etiology of these RBC abnormalities was not elucidated following extensive laboratory evaluation, although two important physiologic principles were manifested by this case: (1) Hemolysis was relatively well compensated (41% hematocrit) despite a significantly decreased RBC survival ($^{51}$Cr t1/2 = 10.5 days). This effect presumably was due to reduced 2,3-DPG content (1.9 µmol/ml RBC) and the associated increase in whole blood oxygen affinity (P50 = 19.6 mm Hg). (2) RBC size and water content were normal in spite of marked cation depletion. This anomaly was thought to reflect the osmotic effects of reduced polyvalent anion (2,3-DPG) content.

ALTERED ERYTHROCYTE cation content and membrane permeability have been observed in a variety of hemolytic disorders. In some cases these cation alterations are seen during incubation in vitro, while in others the membrane permeability abnormalities also are manifested in vivo. Hereditary hydrocytosis (frequently referred to as hereditary stomatocytosis) represents an RBC permeability defect (Na permeability > K permeability) associated with abnormal cation composition (high Na, low K), elevated total monovalent cation content, and increased cell water volume. Hereditary xerocytosis is a different type of RBC permeability defect (K permeability > Na permeability) characterized by monovalent cation depletion and cellular dehydration. [It should be noted that hereditary xerocytosis (Greek) previously was referred to as hereditary desiccocytosis (Latin), although the former terminology now is preferred in order to maintain linguistic consistency in describing these RBC abnormalities.]

We describe another hemolytic disorder associated with increased membrane permeability and reduced RBC cation content. In this case, however, monovalent cation depletion is associated with normal cellular hydration instead of dehydration. In addition, these RBC manifest a second abnormality, decreased levels of 2,3-diphosphoglycerate (2,3-DPG). We report this case because it illustrates two fundamental principles of red blood cell physiology, (1) the effects...
of reduced 2,3-DPG on the magnitude of anemia associated with hemolysis and (2) the effects of reduced 2,3-DPG content on cellular hydration.

CASE REPORT

D.C. was an Italian female first seen at age 11 yr for evaluation of jaundice. She was otherwise asymptomatic, and her past medical history was unremarkable. Abnormal physical findings included moderate icterus and splenomegaly. At age 14 yr she had a cholecystectomy, and bilirubin gallstones were found. Laboratory data were consistent with a well-compensated hemolytic process: normal hematocrit (41%), reticulocytosis (19%), and reduced RBC survival (t½ Cr RBC survival of 0.5 days) (Table 1). The following laboratory studies were normal: Coombs’ test, acid hemolysis test, sugar water test, test for Donath-Landsteiner antibody. There was no evidence of a hemoglobinopathy as indicated by normal hemoglobin (Hb) electrophoresis, normal fetal and HbA2 levels, negative Hb heat stability, and normal ratio of α to γ globin chain synthesis. The cyanide-ascorbate test was normal. The osmotic fragility test indicated a small population of RBC resistant to hypotonic lysis. Examination of the peripheral smear revealed occasional target cells, but otherwise RBC morphology was unremarkable. Plasma pH and inorganic phosphate concentration were normal.

MATERIALS AND METHODS

Collection and preparation of erythrocytes. Blood was obtained from patient D.C., healthy volunteers, and patients with reticulocytosis (autoimmune hemolytic anemia). Blood was collected in heparin (20 USP units/ml blood) or EDTA. RBC were separated by centrifugation and then washed three times in a HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid)-buffered salt solution (Na-HBS) with the following composition: 140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1 mM Na2HPO4, and 20 mM HEPES (pH 7.4, 37°C). Young and old RBC fractions were obtained by sedimenting washed RBC (45 min, 1000 g, room temperature) in a tabletop centrifuge (Sero-fuge, Clay-Adams, Parsippany, N.J.) and then removing the top tenth (young) and bottom tenth (old) of the cell column. Reticulocyte counts were performed on the separated fractions.

Measurements. RBC counts, hemoglobin concentration, and MCH were determined in a Coulter Model-S electronic counter. Hematocrit was determined in an International microcentrifuge and the MCV calculated from the Coulter RBC count and manual hematocrit (MCV = hematocrit x 10/RBC x 106). The MCHC was derived from the MCH and calculated MCV. Adenosinetriphosphate (ATP), 2,3-DPG, glycolytic intermediates, glycolytic enzymes, and Na-K concentration were measured by established techniques. The method of Schrotter and Kalinowsky was utilized for assay of 2,3-DPG mutase. Intracellular pH was measured according to the method of Astrup et al. Whole blood P50 (the partial pressure of oxygen at which Hb is 50% oxygenated) was measured by tonometry.

Incubation studies. Glucose consumption and lactate production were measured over a 4-hr period in RBC suspended in Na-HBS (20%, hematocrit) containing 5 mM glucose; these suspensions were incubated in a shaking water bath (100 oscillations/min) at 37°C. Identical incubation conditions were used to measure cation transport. Cell Na and medium K were measured every 30 min. Changes in cell K were determined indirectly from the hematocrit and
medium K concentration. There was no hemolysis during these studies, and thus more precise measurements of net K efflux could be obtained by measuring medium K changes (increased values above a small number) compared to cell K changes (decreased values from a large number). Passive cation permeability was estimated by the rate of Na-K change in RBC incubated with ouabain ($10^{-4} M$). Active transport was defined as the net cation differences between cells incubated in the presence and absence of ouabain.

RESULTS

Evaluation of erythrocyte metabolism indicated glucose consumption (4.4 $\mu$mol/ml RBC/hr) and lactate production (7.4 $\mu$mol/ml RBC/hr) were appropriately increased for cell age (Table 2). The adenine nucleotide content and levels of glycolytic intermediates both were normal, although the concentration of 2,3-DPG (1.9 $\mu$mol/ml RBC) was markedly reduced. Consistent with the decreased content of 2,3-DPG, whole blood $P50$ also was reduced (19.6 mm Hg). Intracellular pH was normal (7.2). Measurement of several glycolytic enzymes (hexokinase, glucose phosphate isomerase, phosphofructokinase, aldolase, triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, pyruvate kinase, and 2,3-DPG mutase) failed to show any quantitative or qualitative enzyme defect.

Measurement of RBC cations indicated Na was slightly increased (17.0 meq/liter RBC), while K was reduced (73.0 meq/liter RBC); consequently the total cation content (90.0 meq/liter RBC) was decreased (Table 3). In spite of this cation depletion, the MCV of the proband’s erythrocytes (110 $\mu$m$^3$) was similar to that of a comparable reticuloocyte population (107 $\mu$m$^3$) with a normal

<table>
<thead>
<tr>
<th>RBC Cation Content (meq/liter RBC)</th>
<th>MCV ($\mu$m$^3$)</th>
<th>MCHC (g/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na</td>
<td>K</td>
<td>Na + K</td>
</tr>
<tr>
<td>Patient (D.C.)</td>
<td>19</td>
<td>17.0</td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
<td>9.8</td>
</tr>
<tr>
<td>Normal values</td>
<td>0-2</td>
<td>7-10</td>
</tr>
</tbody>
</table>
Table 4. RBC Cation Changes in vivo

<table>
<thead>
<tr>
<th>Subject</th>
<th>Centrifuge Tenth</th>
<th>Retics (%)</th>
<th>Cation Content (meq/liter RBC)</th>
<th>Cation Content (meq/liter RBC)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Na</td>
<td>K</td>
</tr>
<tr>
<td>Control</td>
<td>Top</td>
<td>36</td>
<td>12.0</td>
<td>108.0</td>
</tr>
<tr>
<td></td>
<td>Bottom</td>
<td>5</td>
<td>8.6</td>
<td>103.0</td>
</tr>
<tr>
<td>Patient D.C.</td>
<td>Top</td>
<td>43</td>
<td>21.2</td>
<td>77.2</td>
</tr>
<tr>
<td></td>
<td>Bottom</td>
<td>4</td>
<td>15.2</td>
<td>68.6</td>
</tr>
</tbody>
</table>

Cation content was determined in young (top 10%) and old (bottom 10%) washed RBC centrifuged for 45 min (see Materials and Methods). Control reticulocytes were obtained from a patient with autoimmune hemolytic anemia.

cation content (105 meq/liter RBC). In addition, the patient’s MCHC (34 g/dl) was normal in spite of moderate cation depletion.

Separation of young (top) and old (bottom) centrifuged erythrocytes showed that the abnormal cation composition was present in young RBC (Na 21.2, K 77.2 meq/liter RBC) as well as in the older dense cells (Na 15.2, K 68.6 meq/liter RBC) (Table 4). No significant net cation changes were noted when the patient’s RBC were incubated 4 hr in a physiologic salt solution in the absence of ouabain (Table 5). In the presence of ouabain, however, both Na and K permeability were increased, and these passive cation movements were linear throughout the experimental period. Active Na-K transport was increased appropriately for the intracellular Na concentration.

**DISCUSSION**

The major detectable abnormalities associated with this hemolytic disorder were an altered RBC cation composition and a marked reduction in 2,3-DPG content. The known causes of decreased 2,3-DPG include intracellular acidosis and glycolytic enzyme deficiencies proximal to the formation of 2,3-DPG. No such defects were detected in this patient’s erythrocytes. The RBC cation alterations also defy easy explanation. Passive cation leaks were greater than normal, but the rate of active Na-K transport was correspondingly increased, and no net cation changes were seen when the cells were incubated in vitro without ouabain. Separation of young and old RBC indicated that the youngest RBC also had a grossly abnormal cation composition, although no significant

Table 5. RBC Cation Changes in vitro (meq/liter RBC/hr)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Patient D.C.</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Na</td>
<td>K</td>
<td>Na + K</td>
<td>Na</td>
</tr>
<tr>
<td>Without ouabain</td>
<td>-0.1</td>
<td>0</td>
<td>-0.1</td>
<td>-0.2</td>
</tr>
<tr>
<td>With ouabain</td>
<td>+1.4</td>
<td>-1.1</td>
<td>+0.3</td>
<td>+3.4</td>
</tr>
<tr>
<td>Active transport</td>
<td>1.5</td>
<td>1.1</td>
<td>3.6</td>
<td>2.4</td>
</tr>
</tbody>
</table>

RBC suspensions (20% hematocrit in Na-HBS) were incubated at 37°C in the presence and absence of ouabain (10^-4 M). Changes in cell Na (measured directly) and cell K (calculated from changes in medium K and hematocrit) were determined every 30 min over a 4-hr period. Active transport was calculated from difference in cation change in presence and absence of ouabain. Control RBC (20% reticulocytes) were obtained from a patient with autoimmune hemolytic anemia.
additional changes were associated with aging in vivo. In spite of our inability to define the cause of 2,3-DPG depletion and altered cation composition, it is tempting to relate these physiologic phenomena, since earlier studies suggested Na and K permeability were increased in 2,3-DPG-depleted RBC. More recently, however, it was shown that cation permeability alterations are not related to the concentration of this organic phosphate but rather as a function in vitro of the agents (e.g., bisulfite) used to deplete 2,3-DPG. Consistent with this finding, there is no evidence that abnormal RBC cation permeability is a feature of proximal glycolytic enzyme defects with reduced levels of 2,3-DPG.

It is not the purpose of this report to describe another interesting hemolytic anemia with unexplainable RBC findings. Rather, we present this case because it illustrates two important physiologic principles. The first is related to the fact this patient was not anemic (hematocrit 41%) in spite of significant hemolysis (shortened RBC survival, reticulocytosis). The explanation for this compensated hemolysis is related to decreased 2,3-DPG content and the corresponding increase in whole blood oxygen affinity ($P_{50} = 19.6$ mm Hg). Increased oxygen affinity for hemoglobin impairs oxygen release to peripheral tissues and thereby stimulates erythropoietin production and RBC formation. These phenomena are known to produce polycythemia in individuals with high oxygen affinity hemoglobins. In conjunction with the hemolytic disorder described in this report, however, increased whole blood oxygen affinity partially protects against “anemia” and leads to a normal RBC mass. Nevertheless, it is likely that hemolysis in our patient was only partially compensated.
since active erythropoiesis persisted (19% reticulocytes). The RBC mass was normal, but O\textsubscript{2} delivery presumably was less than optimal.

Travis et al. reported a similar case of well-compensated hemolysis (Hb 16.6 g/dl, reticulocytes 4.6\%), reduced 2,3-DPG content (1.9 \mu mol/ml RBC), and increased oxygen affinity for hemoglobin (P\textsubscript{50} = 18.5 mm Hg).\textsuperscript{20} In their study the reduced 2,3-DPG concentration was due to a partial deficiency of 2,3-DPG mutase. More recently, however, Rosa et al. described an individual with complete absence of 2,3-DPG mutase activity and no detectable 2,3-DPG.\textsuperscript{21} In contrast to the patient with hemolysis and partial 2,3-DPG mutase deficiency, the intriguing patient described by Rosa et al. had no evidence of hemolysis. In fact, the patient was moderately polycythemic (hematocrit 55\%); this presumably was related to the increased P\textsubscript{50} for Hb (17.3 mm Hg). Taken together, these cases clearly indicate the relative importance of 2,3-DPG to Hb oxygen affinity, oxygen delivery, and the steady-state Hb concentration. What remains to be resolved, however, is the relationship between reduced 2,3-DPG content, the presence or absence of hemolysis, and the regulation of RBC cation composition.

The second physiologic principle demonstrated in this report relates to regulation of RBC hydration and cell size. Water freely crosses the RBC membrane, and normal cell water content is determined by the difference between intracellular and extracellular osmolality. RBC oncotic pressure is regulated by the concentration of relatively impermeant solutes: monovalent cations and polyvalent anions. Under conditions where monovalent cation content decreases, RBC lose water and decrease in size and the MCHC increases. Examples of these effects of cation depletion are seen with irreversibly sickled cells,\textsuperscript{22} and also with RBC that manifest a specific increase in K efflux.\textsuperscript{7} RBC from our patient also had a decreased total cation content but the cells were larger than expected for the degree of cation depletion. In addition, the MCHC (34 g/dl) indicated that the water content of these RBC was normal. (Although data are not shown, two direct measurements of erythrocyte water content also failed to show differences between control and patient RBC.) This discrepancy between cation and water content relates to a second factor regulating intracellular osmolality, the polyvalent anion concentration. Hb and 2,3-DPG are the main intracellular polyvalent anions (accounting for 65 meq anionic charges/liter RBC),\textsuperscript{23} and both have negative charges far in excess of their molar concentration. Under conditions of reduced polyvalent anion content (such as the decreased 2,3-DPG in our patient), RBC monovalent anion content is increased as a requisite for maintaining electrical neutrality. The larger the fraction of anionic charge due to monovalent anions, the greater the intracellular osmolality, and hence the greater the water content and cell volume.\textsuperscript{24} Thus as a consequence of these two abnormalities (decreased cation content, which tends to decrease cell water, and reduced 2,3-DPG content, which tends to increase cell water), the RBC of our patient had a relatively normal water content and cell size for the degree of reticulocytosis.

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REFERENCES

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