Red Cell Calcium Leak in Congenital Hemolytic Anemia With Extreme Microcytosis

By James S. Wiley and Frances M. Gill

A child with congenital hemolytic anemia, extreme microcytosis and bizarre red cell morphology has been studied. Splenectomy at the age of 21 mo greatly improved the hemolytic anemia, although red cell morphology was unchanged. Aniso- and poikilocytosis were marked on a stained smear, and there were many small hyperchromatic cells of irregular shape. The MCV of 25 cu μ was very low and the MCHC was normal. Osmotic fragility of fresh blood was increased, and postsplenectomy blood showed a fraction of extremely fragile cells. Concentration and fluxes of Na+ and K+ were normal, except K+ efflux, which was stimulated by external Ca2+. Inward Ca2+ movement into the patient's red cells was elevated three-to-fourfold above red cells of the same mean age. Red cell Ca2+ concentration was raised 2.5 times normal and most of the Ca2+ was localized in the stroma. Red cell lipid, sialic acid, and ouabain-binding sites, all per milliliter of cells, were increased by 16%-23%, and, since these substances estimate the amount of membrane, it was likely that Ca2+ content per unit of membrane area was at least twice normal. Deformability of the cells, as judged by their filterability was markedly impaired. It was concluded that the red cell membrane was defective, and an increased membrane Ca2+ content was associated with reduced deformability, hemolysis, and distorted red cell morphology in this syndrome.

Fragmentation and distortion of red cell shape is often of diagnostic value in a microangiopathic hemolytic process.1 Extreme aniso- and poikilocytosis have also been described in thalassemia,2,3 while many contracted, distorted, and spiculated cells have been observed in isolated cases of pyruvate kinase deficiency4 and unstable hemoglobin hemolytic anemia (e.g., hemoglobin Hammersmith),5 although the latter two conditions usually lack a distinctive red cell morphology. The present study describes a case of congenital hemolytic anemia with extreme microcytosis and striking distortion of red cell shape in which there is no evidence for the above disorders. Cation permeability studies have established that the cell membrane is abnormal in this condition and that an increased influx of calcium ions and a raised cell calcium concentration are associated with the hemolytic process.

CASE REPORT

T.N., a 5-yr-old black male has had congenital hemolytic anemia of severe degree. He required an exchange transfusion on the third day of life for hyperbilirubinemia. The mother's and baby's blood types were both B positive, direct Coombs test was negative, G6PD was not decreased on a screening test, and a sickle cell preparation was negative. The infant was dis-
charged with a diagnosis of hyperbilirubinemia of unknown etiology. At 5 mo of age, the hemoglobin was 5 g/dl, despite iron therapy for the preceding month, and the child was hospitalized for studies. Physical examination showed a liver palpable 5 cm below the right costal margin and a spleen palpable 5 cm below the left costal margin. The hemoglobin was 5.4 g/dl, reticulocytes 31.3%, white blood cells 20,800/μl, nucleated red cells 77/100 white cells, and platelets 108,000/μl. The smear showed severe microcytosis, aniso- and poikilocytosis, fragmented red cells, basophilic stippling, and polychromasia. Bilirubin was 2.4 mg/dl, haptoglobin was absent, and red cell osmotic fragility was increased. The child was transfused. When the hemoglobin dropped to 5 g/dl again, he was referred to the Children's Hospital of Philadelphia for further evaluation. Height and weight were at the third percentile for a child of 9 mo, and examination showed frontal bossing, slight scleral icterus, a liver palpable 8 cm below the right costal margin, and a spleen palpable 11 cm below the left costal margin. Hemoglobin was 4 g/dl. Uric acid was 8.2 mg/dl; folic acid and B12 levels were normal. Radiographic studies showed mild cardiomegaly and an increased diploic space in the skull. An autohemolysis study showed 45.8% hemolysis at 48 hr, and glucose supplementation reduced the hemolysis only to 38.6%. The mother and father had normal values for blood counts, morphology on peripheral smear, reticulocyte counts, and osmotic fragility. Red cell enzyme and metabolic studies on the child and parents performed by Dr. Frank Oski and Dr. Susan Travis included glycolytic and hexosemonophosphate shunt enzymes, red cell ATP content, glycolytic intermediates, glutathione stability, glucose consumption, and lactate production. No deficiencies were found. The child continued to have a severe anemia which required periodic blood transfusions. Administration of prednisone (2 mg/kg for 2 wk) raised the hemoglobin level to about 7 g/dl and decreased the reticulocyte count to about 20%. Because of the severity of the hemolysis, his spleen was removed when he was 21 mo old. Following this, his hemoglobin level rose to 11 g/dl, and his reticulocyte count fell to about 3%.

He was initially seen by us at 3½ yr of age in good health. His only medication was oral penicillin. On physical examination, his height and weight were normal, there was mild frontal bossing, a grade 1/6 systolic murmur, and a palpable liver 2 cm below the right costal margin. A blood count revealed a hemoglobin of 11.5 g/dl, reticulocytes 4.8%, and white cells 22,200/μl. A 3-mo-old half-brother was examined and found to be normal. Further studies were done to exclude other causes of a congenital hemolytic anemia. Unstable hemoglobin hemolytic anemia was excluded by three tests; a normal brilliant cresyl blue preparation6 at 1, 4, and 24 hr, a negative isopropanol test,7 and a normal stability of hemolysate when heated at 65°C for 2 hr. The oxygen affinity of a hemolysate at pH 7 was measured by Dr. T. Asakura and was in the normal range. Hemoglobin electrophoresis on cellulose acetate and on starch gel at pH 8.6 revealed mainly HbA with normal quantitative levels of HbA2. Fetal hemoglobin, quantitated as alkali-resistant hemoglobin,8 was also normal. Thalassemia was excluded by globin synthesis studies in the peripheral blood,9 since the β/α-chain ratio of the patient was 1.05, compared with normal values of 0.99 ± 0.05.

MATERIALS AND METHODS

Reticulocytes and osmotic fragility measurements were performed by standard techniques.6 Cell water was measured by desiccation of a known weight of red cells. Red cells were packed at 28,000g for 1 hr at 4°C, and quadruplicate samples containing a known weight of packed cells were dried to constant weight at 95°C. No correction was made for the trapped extracellular space since this was shown to be less than 1% by use of 14C-sucrose. For cation studies, venous blood was collected into heparin, and the red cells were washed three times in a medium of composition 145 mM NaCl, 5 mM KCl, and 20 mM imidazole Cl, pH 7.5. To measure cell Na+ and K+, fresh red cells were separated and rapidly washed four times in cold 110 mM MgCl2 to remove extracellular Na+ ions. The packed cells were hemolyzed in 0.01 N NH4OH and the Na+ and K+ concentrations measured by flame photometry. Sodium influx was measured from the uptake of radioactivity by cells incubated in media containing 22NaCl.10 Glucose (10 mM) and ouabain (50 μM) were always added to the influx media. Sodium efflux and K+ influx were measured as previously described.11 Calcium influx was measured from the uptake of 45Ca by cells which had been preincubated so as to deplete ATP and thereby inhibit the activity of the outwardly directed Ca2+ pump.
Red cells were washed in a medium of composition 145 mM NaCl, 5 mM KCl, and 20 mM imidazole Cl, pH 7.5, and preincubated for 90 min in the same medium plus 1 mM sodium iodoacetate and 5 mM inosine. It was demonstrated, using the assay for Ca\(^{2+}\) efflux described below, that no measurable outward Ca\(^{2+}\) pumping could be detected in these ATP-depleted cells. The cells were then washed twice and added to prewarmed media of composition 145 mM NaCl, 5 mM KCl, 20 mM imidazole Cl, pH 7.5, plus 1.5 mM \(^{45}\)Ca\(_{2+}\) (1 µCi/ml). Samples were taken after 15 min, 1, 2, 3, and 4 hr, and washed four times in cold 150 mM NaCl plus 1 mM NaEGTA. Each cell pellet was hemolyzed in 0.01 M NH₄OH, and part of the hemolysate was deproteinized with 6% (w/v) perchloric acid plus 1 mM NaEGTA and \(^{45}\)Ca\(^{2+}\) in the supernatant was measured by liquid scintillation counting. To convert the uptake of radioactive calcium from nmole/mumole Hb into nmole/ml cells, the MCHC (µmole Hb/ml cells) was measured in each experiment both for the abnormal and normal cells. Na\(^{+}\) influx was measured in both normal and the patient's red cells depleted of ATP by the above procedure to find if the iodoacetate treatment had induced a generalized permeability increase. No increase in Na\(^{+}\) influx was found.

Calcium efflux was measured in cells which were preloaded with Ca\(^{2+}\) by use of an anion, salicylate, which absorbs strongly to fixed positive charges in the membrane and greatly increases transmembrane cation movements. Washing red cells were added to a medium of composition 150 mM Na salicylate, 5 mM imidazole Cl, pH 7.5, plus 2 mM \(^{45}\)Ca\(_{2+}\) (2 µCi/ml) at 0°C and incubated for 3 hr. The cells were then washed five times at 0°C in a medium of composition 145 mM NaCl, 5 mM KCl plus 20 mM imidazole Cl, pH 7.5, and, after the fifth wash, 1 ml of packed cells was added to 10 ml of the same medium plus 10 mM glucose which was prewarmed to 37°C. Samples were taken at 0.5, 1, 2, 3, and 4 min into tubes standing in an ice bath, the tubes were spun at 0°C and a sample of the supernatant was taken for liquid scintillation counting the radioactivity present outside the cells (\(N_f\)). No correction was made for counts released by hemolysis, since this was always less than 1.5% after the 4 min incubation. A portion of the whole cell suspension was also taken for counting to determine the radioactivity initially present inside the cells (\(N_0\)). The rate constant (\(k\)) for Ca\(^{2+}\) efflux was derived from the slope of the line between 0.5 and 4 min when log \((1 - N_f/N_0)\) was plotted against time.

Total cell calcium content was measured by atomic absorption spectroscopy of a dry ashed sample of red cells. Red cells, which had been carefully freed of buffy coat by repeated washing in saline, were further washed four times at 0°C in 150 mM NaCl plus 1 mM NaEGTA. The final pellet of washed cells was hemolyzed with 6 ml of 0.01 M NH₄OH (verified Ca\(^{2+}\)-free), and 5 ml of the hemolysate was added to a silica crucible (Vitreosil high-form 5-ml silica crucibles, Thermal American Corp.), slowly evaporated to dryness under an infrared lamp, and combusted at 500°C overnight in a muffle furnace. It was important that the sample was completely dry before combustion, since any trace of water led to uncontrollable bubbling when the sample was heated toward 500°C. The residue in each crucible was extracted with dilute acid by adding 3 ml of 0.1 N HCl plus 10 mM SrCl₂ to the crucible and shaking for 2 hr at room temperature. The contents of the crucible were decanted into polypropylene tubes, and the iron oxide was allowed to settle. Calcium in the supernatant was analyzed on a Varian Techtron Atomic Absorption Spectrophotometer at 422.6 nm using acetylene fuel plus compressed air. Standard solutions (5-50 \(\mu\)M Ca\(^{2+}\)) were prepared by dilution of commercial standard calcium nitrate (1000 ppm or 25 mM; Fisher Scientific Company, Fair Lawn, N.J.) in 10 mM SrCl₂. A 1-ml aliquot of the hemolysate was taken for measurement of hemoglobin concentration, so that the Ca\(^{2+}\) present in each crucible could be measured in terms of nmoles/ml cells. The red cell Ca\(^{2+}\) for each subject was the mean value of four or eight replicate crucibles.

Several precautions were observed to prevent introduction of Ca\(^{2+}\) contamination. All solutions used in the procedure were stored in polypropylene bottles since both glass and polystyrene containers contributed small and variable amounts of Ca\(^{2+}\) to a solution. When Pyrex glassware was used, it was acid washed and kept immersed in deionized water until immediately before use, when it was again rinsed in deionized water. All crucibles had close-fitting lids, and following combustion the lids were dusted free of refractory brick dust before opening. Recovery studies of Ca\(^{2+}\) added to crucibles and dry ashed at 500°C established that no significant amounts of Ca\(^{2+}\) were retained by the silica crucibles. \(^{45}\)CaCl₂ (1.25, 2.5, 3.75, 5, and 74 nmoles) was added to crucibles, 5 ml of a red cell hemolysate added, and the contents were dry ashed and extracted in dilute acid as above. The recovery of \(^{45}\)Ca was esti-
mated by liquid scintillation counting and was 81%, 85%, 86%, 95%, and 101%, respectively, for each of the above amounts of added Ca²⁺.

For measurement of their lipid content, red cells were extracted with 80 volumes of isopropanol and chloroform for measurement of cholesterol and lipid phosphorus. Nonlipid phosphorus was removed from the extracts by thrice washing with 0.05 M KCl (1/5 volume). Total phospholipid of red cells was taken to equal lipid phosphorus multiplied by 25. The volume of red cells extracted was calculated from duplicate samples in which hemoglobin was measured spectrophotometrically using an E₃₄₀ (mM) for oxyhemoglobin of 57.0 or an E₃₄₀ (mM) for cyanmethemoglobin of 44.0.

The enumeration of ³H-ouabain binding sites was measured essentially as outlined by Hoffman and in full detail by our laboratory. As above, the volumes of cells were calculated from hemoglobin spectrophotometry.

Sialic acid was measured in stroma prepared from a known volume of red cells which were hemolyzed and washed five times in 5 mM Tris-HCl, pH 7.4, at room temperature. Stroma were hydrolyzed in 0.1 N H₂SO₄ for 1 hr at 80°C and the sialic acid separated on a Dowex-1 formate column, eluted with 0.3 M formic acid, and determined by the method of Warren.

Filterability of red cells was assessed on washed red cells suspended at a hematocrit of 2.0% in a medium of composition 145 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 20 mM imidazole Cl, pH 7.5, plus 0.25 g/dl human serum albumin. The filtration time was then measured for passage of 2.0 ml of suspension through a polycarbonate filter (Nuclepore Membrane Filters, Pleasanton, Calif.) of 3-μm pore size at a negative pressure of 10 in. of water. Mean values are shown ± 1 SD and differences between sample means were analyzed by a Student's t test for significance.

RESULTS

Red Cell Indices and Morphology

Despite the elevated reticulocyte count, microcytosis was evident in stained smears. Aniso- and poikilocytosis were marked, and there were numerous small hyperchromatic cells with one or two blunt projections or filaments projecting from the surface (Fig. 1). Wet preparations of blood showed dumbbell-shaped red cells to be present as well as the other forms noted above. Morphology of the red cells was the same before and after splenectomy. Mean corpuscular
FIG. 2. Osmotic fragility curve of the patient's red cells after splenectomy.

Volume was 25 cu μ when measured on the Electrozone Celloscope (Particle Data Corporation, Elmhurst, Ill.) with a 30-μm orifice. Machine settings were adjusted so that the entire volume distribution of red cells could be visualized on the oscilloscope. The mean corpuscular hemoglobin concentration was measured from manual determinations of hemoglobin and hematocrit and was 35.0 g/dl immediately after venesection and was unaltered in blood which had stood 3 hr at room temperature. Red cell indices measured on a standard Coulter Model “S” counter gave spuriously high values for mean corpuscular hemoglobin concentration, presumably because the smallest cells were excluded from the red cell count.

Osmotic Fragility

Osmotic fragility both pre- and postsplenectomy was markedly increased and 50% hemolysis occurred at a NaCl concentration of 0.60 g/dl, compared with 0.40-0.44 g NaCl/dl for normals. The osmotic fragility curve of post-splenectomy blood showed a “tail” of very fragile cells which accounted for over half of the total population (Fig. 2). Analysis of red cell membrane components, described below, showed no deficiency of lipid to account for the susceptibility to hypotonic lysis. Moreover, the high fragility was not due to an increase in cell water content.

Cell Cations and Water Content

The water content of red cells was analyzed on three separate occasions within 2 hr of venesection and was 633 ± 10 mg/g cells which was significantly below the normal mean of 658 ± 6 mg/g cells (p < 0.01). Red cell Na⁺ and K⁺ were measured on three separate occasions immediately after venesection and
Table 1. Red Cell Sodium and Potassium Concentrations and Fluxes*

<table>
<thead>
<tr>
<th></th>
<th>Na⁺⁺</th>
<th>K⁺⁺</th>
<th>Na⁺⁺ + K⁺⁺</th>
<th>Na⁺⁺ Influx</th>
<th>Glucose</th>
<th>Glucose + Ouabain</th>
<th>Active Flux</th>
<th>Glucose</th>
<th>Glucose + Ouabain</th>
<th>Active Flux</th>
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<tr>
<td>Patient</td>
<td>9.8t</td>
<td>93.8t</td>
<td>103t</td>
<td>2.35</td>
<td>3.1</td>
<td>0.9</td>
<td>2.2</td>
<td>2.6</td>
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<td>2.0</td>
</tr>
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<td>99.0</td>
<td>107</td>
<td>2.0</td>
<td>2.9</td>
<td>0.8</td>
<td>2.1</td>
<td>2.0</td>
<td>0.5</td>
<td>1.5</td>
</tr>
<tr>
<td>±SD</td>
<td>±1.5</td>
<td>±5</td>
<td>±5</td>
<td>±0.2</td>
<td>±0.4</td>
<td>±0.2</td>
<td>±0.2</td>
<td>±0.3</td>
<td>±0.1</td>
<td>±0.2</td>
</tr>
</tbody>
</table>

*Concentrations are in μEq/ml cells and fluxes are in μEq/ml cells/hr measured in a medium of composition 145 mM NaCl, 5 mM KCl plus 20 mM imidazole Cl. pH 7.5.
†Mean value of three separate determinations.

always lay within the normal range whether expressed per milliliter of cells or per milliliter of cell water (Table 1).

Sodium and Potassium Fluxes

Both the influx and efflux of Na⁺ ions were normal. Sodium influx was 2.35 μEq/ml cells/hr for the patient compared with 2.0 ± 0.2 μEq/ml cells/hr for normals, and addition of 1.5 or 10 mM CaCl₂ to the incubation media did not change the Na⁺ influx into the patient's cells. Sodium efflux was measured both in the absence and the presence of ouabain to inhibit the active pumping of Na⁺ ions. Both the active and passive components of Na⁺ efflux were normal (Table 1).

Total K⁺ influx for the patient, 2.6 μEq/ml cells/hr, was not significantly different from the influx for controls 2.0 ± 0.3 μEq/ml cells/hr and both the active and passive components of influx were within the normal range (Table 1). Moreover, the magnitude and affinities of a cotransport system for Na⁺ plus K⁺ ions which has recently been described in the red cell11 were also normal. Potassium efflux was then measured for cells incubated in the absence or presence of Ca²⁺ ions. Efflux of K⁺ into Ca²⁺-free media was identical for the patient and normals, with values between 1.6 and 3.4 μEq/ml cells/hr. However, K⁺ efflux was increased from the patient's cells into media containing 10 mM Ca²⁺ ions, since K⁺ efflux was 2.75 and 18.5 μEq/ml cells/hr for the patient on two occasions compared with 1.25 and 2.3 μEq/ml cells/hr for normals (Table 2). This abnormal effect of Ca²⁺ on the patient's cells led us to study their Ca²⁺ permeability.

Table 2. Effect of Calcium on K⁺ Efflux*

<table>
<thead>
<tr>
<th></th>
<th>K⁺⁺ Efflux (μEq/ml cells/hr)</th>
<th>Na Ca²⁺</th>
<th>1.5 mM Ca²⁺</th>
<th>10 mM Ca²⁺</th>
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<tbody>
<tr>
<td>T.N. (1)</td>
<td>1.65</td>
<td>—</td>
<td>2.75</td>
<td></td>
</tr>
<tr>
<td>(2)</td>
<td>3.4</td>
<td>2.8</td>
<td>18.5</td>
<td></td>
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<tr>
<td>Normals(1)</td>
<td>1.95</td>
<td>1.65</td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td>(2)</td>
<td>2.2</td>
<td>2.2</td>
<td>2.3</td>
<td></td>
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</tbody>
</table>

*Media contained 145 mM NaCl, 5 mM KCl, 20 mM imidazole Cl, pH 7.5, plus 0, 1.5, or 10 mM CaCl₂.
Calcium Influx

Calcium uptake was measured in washed red cells which had been depleted of ATP by initial preincubation with metabolic inhibitors for 90 min.\textsuperscript{12,13} Normal red cells showed a biphasic $\text{Ca}^{2+}$ uptake, with a rapid initial uptake of $2.3 \pm 0.4$ nmole/ml cells within 15 min, followed by a slower linear uptake of $0.8$ nmole/ml cells/hr reaching a total uptake of $5.4 \pm 1.5$ nmole/ml cells after 4 hr of incubation. Calcium uptake into the patient's red cells was increased almost tenfold above normal. On two separate occasions there was a rapid uptake of 7–8 nmole/ml cells followed by linear uptakes reaching values of 50 and 64 nmole/ml cells after 4 hr (Fig. 3). On the two occasions, the patient had a reticulocytosis of 2.0\% and 2.3\% and, although reticulocytes have an increased $\text{Ca}^{2+}$ influx, control erythrocytes from a patient responding to folic acid therapy with 3.7\% reticulocytes showed a $\text{Ca}^{2+}$ uptake of only 20 nmole/ml cells/4 hr. Thus $\text{Ca}^{2+}$ influx into the patient's cells was elevated three- to fourfold above red cells of the same mean age.

Calcium Content

Measurement of red cell $\text{Ca}^{2+}$ by atomic absorption spectroscopy showed that the abnormal cells contained increased amounts of this cation. Normal red cells contained $5.4 \pm 2.4$ nanomole $\text{Ca}^{2+}$/ml cells ($n = 11$). On four separate occasions, the patient's red cells contained 13.5, 10.0, 16.4, and 16.9 nmole

Fig. 3. Calcium uptake by patient's red cells. Patient had 2.0\% and 2.3\% reticulocytes when tested on two occasions (●), while control cells had 3.7\% reticulocytes (▲). Mean values for normal cells (○) show ±1 SD by the vertical bars.
Ca\(^{2+}\)/ml cells so that the mean cell Ca\(^{2+}\) of 14.2 ± 3.1 nmole/ml cells was significantly higher than normal (p < 0.01).

The location of the red cell Ca\(^{2+}\) was studied by hemolyzing washed red cells in 10 volumes of 5 mM Tris–HCl buffer, pH 7.4, and separating the stroma by centrifugation. Stroma from the patient contained 55% of the total cell Ca\(^{2+}\), normal stroma contained 35%.

**Calcium Efflux**

When red cells were loaded with 0.6–0.7 μmole Ca\(^{2+}\)/ml cells at 0°C and subsequently reincubated at 37°C, a rapid extrusion of Ca\(^{2+}\) occurred by an ATP-dependent process. This outward flux could occur against a concentration gradient of Ca\(^{2+}\) and thus represented an active transport mediated by the Ca\(^{2+}\) pump. Calcium efflux in normal red cells consisted of at least two kinetic components. An initial component was lost within the first 30 sec with kinetics that were too rapid to study by conventional sampling techniques. The magnitude of this rapidly extruded component could be estimated graphically by extrapolating the time course of Ca\(^{2+}\) extrusion back beyond the 0.5-min point to zero time (dotted lines in Fig. 4). The value of cell Ca\(^{2+}\) where this extrapolation

![Fig. 4. Calcium extrusion from red cells. Cells were preloaded with Ca\(^{2+}\) at 0°C and reincubated at 37°C in media containing no Ca\(^{2+}\) (○, ●) or 1.5 mM Ca\(^{2+}\) (□, ■). Initial cell calcium was 0.7 and 0.6 μmoles/ml cells for normal and patient, respectively.](image-url)
interacted zero time was then subtracted from initial cell Ca²⁺ to give the amount of rapidly extruded Ca²⁺. For normal red cells, the rapid component comprised 19% to 26% of the initial cell Ca²⁺ concentration. Extrusion of the major fraction of cell Ca²⁺ was less rapid and followed first-order kinetics with a half-time of 2.1 min and rate constant (k) of 0.33 min⁻¹ (Fig. 4). The kinetics of Ca²⁺ extrusion were identical whether the medium contained no Ca²⁺ or 1.5 mM Ca²⁺.

Calcium extrusion from the patient’s red cells showed one important difference from the normal—the rapid initial Ca²⁺ loss which occurred within the first 30 sec was increased to between 35% and 42% of the initial cell Ca²⁺ concentration (Fig. 4). Thus, the patient’s cells contained twice the normal amount of rapidly exchanging cell Ca²⁺ which could be lost within seconds to the medium. The major fraction of cell Ca²⁺ was extruded with kinetics almost identical to normal. Half-time for the efflux was 1.9 min, and the rate constant (k) was 0.36 min⁻¹; again, it made little difference to the kinetics whether the medium contained no Ca²⁺ or 1.5 mM Ca²⁺ (Fig. 4). The behavior of this second component of Ca²⁺ efflux was attributed to the calcium pump, which clearly operated at a normal rate in the patient’s cells.

Membrane Lipid, Sialic Acid, and Cation Pump Sites

The results above showed an increased Ca²⁺ concentration per milliliter of abnormal cells and also showed that the bulk of this Ca²⁺ was localized in the membrane. To assess the significance of this high Ca²⁺ concentration, it was necessary to know the amount of membrane per milliliter of cells relative to normal. Although membrane area cannot be directly measured, an alternative approach is to compare the quantities of lipid and sialic acid per milliliter of cells, since, in the red cell, these substances are found only in the stroma. Moreover, quantitation of cation pump sites by the ³H-ouabain binding technique might also provide another estimate of relative membrane area in the two types of cells. Table 3 shows values for red cell cholesterol, phospholipid, sialic acid, and ouabain-binding sites all expressed per milliliter of red cells. Values for the abnormal cells were increased in every case by 23% for cho-

| Table 3. Analysis of Membrane Composition and Cation Pump Sites as an Estimate of Relative Surface Area |
|-------------------------------------------------|-------------------------------------------------|-----------------|----------------|-----------------|
| Red Cell Cholesterol (mg/ml cells) | Red Cell Phospholipid (mg/ml cells) | Molar Ratio Cholesterol: Phospholipid | Red Cell Sialic Acid (µmole/ml cells) | Red Cell Ouabain-binding Sites (molecules/ml cells) |
| Normal subjects (n = 2) | 1.52* | 3.78† | 0.81 | 0.43† | 396 x 10⁶§ |
| Patient T.N. (n = 2) | 1.87 | 4.42 | 0.85 | 0.50 | 458 x 10⁶ |
| Increment for patient T.N. (%) | +23 | +17 | +16 | +16 | 396 x 10⁶§ |

* Equivalent to 12.9 µg cholesterol per 10⁸ cells.
† Equivalent to 31.9 µg phospholipid per 10⁸ cells.
§ Equivalent to 30 x 10⁶ molecules per cell.
§§ Equivalent to 336 ouabain-binding sites per cell.
lesterol, 17\% for phospholipid, 16\% for sialic acid, and 16\% for ouabain-binding sites. The control cells were from normal subjects who had not been splenectomized, and since red cell lipid increased by some 15\% after splenectomy\textsuperscript{26} it was likely that the lipid content of cells from postsplenectomy controls would be almost the same as the splenectomized patient. Whichever analysis is taken for comparison, it is clear that the amount of membrane per milliliter of abnormal cells cannot be more than 15\%-20\% above normal. It follows that the 2.5-fold increases in Ca\textsuperscript{2+} concentration and three- to fourfold increase in Ca\textsuperscript{2+} influx expressed per milliliter of abnormal cells would hold even if Ca\textsuperscript{2+} values were expressed per unit of membrane area.

**Filtration Time**

The deformability of red cells was assessed by the filtration time of cell suspensions through polycarbonate filters of 3-\textmu m pore size. Filtration time of normal red cells was 125 ± 45 sec (n = 6) while the patient's cells failed to pass the filter in every test (all times > 10 min).

**DISCUSSION**

The hematologic findings in this case are unusual, and there seems little doubt that this syndrome forms a distinct entity. The clinical features of the case were that of a congenital hemolytic anemia which was greatly improved by splenectomy. Blood smears showed marked microcytosis and severe aniso- and poikilocytosis with a normal MCHC. Laboratory tests showed increased osmotic fragility of fresh blood and increased autohemolysis with only partial correction by glucose supplementation. Unstable hemoglobin hemolytic anemia was excluded by normal results for brilliant cresyl blue incubation, isopropanol precipitation, heat precipitation at 65°C, and oxygen affinity of the hemolysate. Although severe aniso- and poikilocytosis with microcytosis have been described in thalassemia, this diagnosis was eliminated by the increased osmotic fragility, normal MCHC, and a normal \( \beta/\alpha \)-chain ratio in the globin synthetic studies. The literature contains only two reports of a similar syndrome. Dacie has described a child with congenital hemolytic anemia\textsuperscript{27} whose blood smear, as in the present case, showed microcytosis, aniso- and poikilocytosis, and the presence of dense hyperchromatic cells which differ from microspherocytes only in their lack of symmetry. Splenectomy in Dacie's case was performed in infancy and produced remission of the anemia, so that 25 yr later the hemoglobin was 13.5 g/dl with reticulocytes 6\%.* Thus, in both cases, splenectomy improved the anemia, although it did not completely prevent the hemolytic process. Other cases with peripheral blood findings similar to that above have been reported recently.\textsuperscript{28}

In our case, cation flux studies indicated a membrane abnormality which was manifested as an increased Ca\textsuperscript{2+} influx and a raised cell Ca\textsuperscript{2+} concentration. Human red cells are almost 1000-fold less permeable to Ca\textsuperscript{2+} ions than to Na\textsuperscript{+} ions, and, moreover, the intracellular concentration of this divalent ion is kept extremely low by the activity of a Ca\textsuperscript{2+} pump located in the cell membrane.\textsuperscript{29}

* Dacie JV: Personal communication.
This Ca\textsuperscript{2+} pump is an ATP-ase\textsuperscript{29,30} and the time course of inward Ca\textsuperscript{2+} movement can only be studied under conditions in which the pump is inactive, such as in cells which have been depleted of ATP.\textsuperscript{12,13} Studies of Ca\textsuperscript{2+} uptake by ATP-depleted cells have shown that the Ca\textsuperscript{2+} permeability of the reticulocyte is about 70-fold greater than the mature cell.\textsuperscript{31} However, the 2.0%-2.3% reticulocytosis in the patient's blood did not account for the observed Ca\textsuperscript{2+} influx, which was three- to fourfold greater than red cells of the same mean age. The increased permeability of these abnormal cells to Ca\textsuperscript{2+} probably led to their higher Ca\textsuperscript{2+} concentration, which was raised 2.5-fold above normal. The slight reticulocytosis of the patient's red cells cannot explain their higher Ca\textsuperscript{2+} levels, since reticulocyte-rich red cells have a normal Ca\textsuperscript{2+} concentration.* The Ca\textsuperscript{2+} concentration of normal red cells is extremely low, and mean values of 16 and 15 nmole/ml cells were obtained by Harrison and Long\textsuperscript{32} and by Lichtman and Weed.\textsuperscript{33} The former value was obtained after washing cells in isotonic saline containing approximately 2 \textmu M Ca\textsuperscript{2+} as contaminant and even lower values for red cell Ca\textsuperscript{2+}, around 3 nmole/ml cells, were observed when the washing medium contained EDTA or EGTA to chelate Ca\textsuperscript{2+} ions.\textsuperscript{32} This latter value is not different from the Ca\textsuperscript{2+} concentration found for normal cells in this study (5.4 ± 2.4 nmole/ml cells). Whatever the true value for red cell Ca\textsuperscript{2+} concentration, the results in this study were obtained by the same method with paired observations, which validates the major conclusion that the patient's red cells have a higher Ca\textsuperscript{2+} concentration relative to normals.

Calcium in normal red cells is mostly present in the membranes;\textsuperscript{32,33} and the location of Ca\textsuperscript{2+} in the patient’s cells was also shown to be predominantly in the membrane. Analysis of membrane components showed that the patient’s cells had slightly increased (16%-23%) membrane area per milliliter of cells. It follows that the 2.5-fold increase in Ca\textsuperscript{2+} concentration per milliliter of cells represented at least a twofold increase in Ca\textsuperscript{2+} content per unit of membrane area. The kinetics of Ca\textsuperscript{2+} efflux also were consistent with an increased membrane Ca\textsuperscript{2+} content since the initial rapid component of Ca\textsuperscript{2+} loss from the patient’s cells was almost twice normal. The magnitude of this rapid initial component is unaffected by depleting the red cell of ATP prior to Ca\textsuperscript{2+} loading,* so that this component may represent a fraction of cell Ca\textsuperscript{2+} which is bound to the plasma membrane in a superficial and readily exchangeable location. Other evidence suggests that intramembranous Ca\textsuperscript{2+} is not regulated by the calcium pump,\textsuperscript{33} so that the demonstration of normal Ca\textsuperscript{2+} pumping by the abnormal cells is consistent with a membrane location for the increased red cell Ca\textsuperscript{2+}.

Despite an increased Ca\textsuperscript{2+} permeability, the patient's cells showed a normal influx and efflux of Na\textsuperscript{+} as well as a normal K\textsuperscript{+} influx. In addition, a cotransport system for Na\textsuperscript{+} plus K\textsuperscript{+} ions, recently described in our laboratory, was also normal.\textsuperscript{11} Potassium efflux from the patient's cells, although normal in a Ca\textsuperscript{2+}-free medium, was increased into a medium containing 10 mM Ca\textsuperscript{2+} ions. The ability of Ca\textsuperscript{2+} to induce an outward K\textsuperscript{+} leak is well known in human red cells, although Ca\textsuperscript{2+} must enter the cell before it can exert this effect.\textsuperscript{12,13,36} The increased K\textsuperscript{+} efflux in media containing 10 mM Ca\textsuperscript{2+} offers further support for

\*Wiley JS, Shaller CC: Unpublished observations
the increased Ca\(^{2+}\) permeability of these abnormal cells. Only one K\(^+\) efflux measurement was performed into media containing 1.5 mM Ca\(^{2+}\) (which is equal to the free ionized plasma Ca\(^{2+}\) concentration),\(^{37}\) and it was shown that K\(^+\) efflux was not increased, a finding which is consistent with the normal K\(^+\) concentration of the patient's cells.

Why are the patient's cells osmotically fragile? Membrane lipid per milliliter of abnormal cells is increased by 17%-23%, which would tend to produce osmotic resistance.\(^{21,38}\) Moreover, cell water content is slightly decreased by 4%, and this change could also cause osmotic resistance. Clearly, some third factor, perhaps related to abnormal membrane structure, is responsible for the very high osmotic fragility of the patient's cells.

The association of hemolytic anemia with increased red cell Ca\(^{2+}\) content may suggest a deleterious effect of this cation on the survival of the cell. Accumulation of intracellular Ca\(^{2+}\) markedly reduces filterability and increases viscosity of the human red cell. Since the same effect is apparent in hemoglobin-poor ghosts, it appears that Ca\(^{2+}\) reduces the deformability of the cell membrane.\(^{39}\) The reduced filterability of the abnormal cells in the present study is consistent with their increased membrane Ca\(^{2+}\) content and points to membrane rigidity as a cause of the hemolysis. While it seemed possible that hemolysis resulted from reduced deformability of the cell membrane, a second factor which must be considered is the increased osmotic fragility of these abnormal cells. Studies both in hereditary spherocytosis and in autoimmune hemolytic anemia have demonstrated an inverse relation between osmotic fragility and cell survival in vivo.\(^{26,40,41}\) However, in both these spherocytic hemolytic anemias, the increased osmotic fragility is a direct result of loss of membrane lipid with reduction in the surface area of the red cell.\(^{26,41,42}\) Such a mechanism does not apply in the present case since the lipids per unit volume of red cells were normal or even slightly increased (Table 3). Whatever the cause of the reduced deformability of the patient's red cells, it clearly had an adverse effect on their ability to traverse narrow segments of the microvasculature in bone marrow and spleen. Red cells must pass between endothelial cells lining the walls of the splenic sinuses, and marked distortion of the red cell occurs as it passes from splenic cord into sinus.\(^{43-46}\) Impaired deformation of the patient's cells would lead to their sequestration in the spleen, and it may be inferred from the beneficial effect of splenectomy that this organ was the major site of cell destruction.

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Red cell calcium leak in congenital hemolytic anemia with extreme microcytosis

JS Wiley and FM Gill