Increased Heat Sensitivity of Red Blood Cells in Hereditary Elliptocytosis With Acquired Cobalamin (Vitamin B₁₂) Deficiency

By Eric B. Schoomaker, William M. Butler, and Louis F. Diehl

Structural membrane proteins were studied from erythrocytes (RBC) of a patient with a nonhemolytic form of hereditary elliptocytosis (HE) who developed a microcytic anemia with fragmented RBC while cobalamin (B₁₂) deficient. Evidence is presented for qualitative changes in the patient’s RBC membranes not related to a loss of structural proteins. Sensitivity of RBC to heat treatment was studied as well as quantitative changes in proteins by densitometry of 1% SDS-10% PAGE gels. Fractions of RBC of various sizes from the patient while B₁₂ deficient all possessed a marked degree of heat sensitivity when compared to RBC from the patient after B₁₂ repletion, normal family members, HE controls, B₁₂-deficient controls, anemic controls, and normal controls. Because loss of spectrin (bands 1 + 2) from heat-sensitive RBC membranes in hereditary pyropoikilocytosis has been reported, the amount of spectrin relative to band 3 was measured. No decrease in the ratio of bands (1 + 2)/3 was found. In addition, no chromatographically abnormal membrane proteins were found by SDS-PAGE of the patient’s RBC while B₁₂ deficient. Our findings indicate that B₁₂ deficiency results in an abnormal membrane with enhanced instability in some forms of HE. Since protein loss was not found, we conclude that an alteration in membrane protein interaction may be involved.

E LLIPTICAL or oval-shaped erythrocytes (RBC) in the circulation are a common finding in various acquired anemias. However, elliptocytosis may also be inherited. There are at least five separate heritable membranopathies in which more than 25% of the circulating erythrocytes are elliptical or oval, including several hemolytic forms. Although significant hemolysis has been found to occur in only 12% of cases of hereditary elliptocytosis (HE), all affected family members of patients with the hemolytic forms of HE also have hemolytic HE, usually beginning in infancy. Conversely, in the nonhemolytic form(s) of HE, the absence of hemolytic disease usually remains throughout the lifetime of the patient and of all similarly affected family members.

The biochemical bases for the membranopathy in HE are not well understood. The bases for hemolysis are also unclear, although the spleen is known in some cases to play an important role. Nonetheless, several studies suggest that an abnormality of the major structural membrane protein, spectrin, may underlie the process. Detergent extraction of RBC ghosts produces a protein skeleton that retains the normal discocyte shape when prepared from normal erythrocytes, whereas an elliptical shape is seen when membrane skeletons are prepared from HE cells. Furthermore, heat instability of purified spectrin has been found in some families with HE either with or without an attendant heat sensitivity of intact whole RBC.

We recently encountered a patient with a nonhemolytic form of HE who presented with a microcytic, normochromic anemia and fragmented RBC on her smear. Because she and her father were previously known to have nonhemolytic HE, she was further examined for the superimposition of a second disorder leading to hemolysis. Cobalamin (B₁₂) deficiency was discovered and treated, with resolution of her anemia and RBC fragmentation. This patient provided us with a unique opportunity to investigate characteristics of RBC in HE that may underlie hemolysis in this disorder. She also provided an opportunity to study the possibility that RBC structural membrane proteins are altered in cobalamin deficiency and that such alterations promote RBC fragility. This report describes the clinical course of this patient and the studies done with her RBC in an effort to explain the anemia and fragmented cells. We found that the patient’s RBC had increased heat sensitivity during the period of cobalamin deficiency without identifiable qualitative or quantitative alterations in the major structural proteins of the RBC membranes.

MATERIALS AND METHODS

Case Report

The patient, L.G., is a 30-yr-old black woman who presented in February 1980, with malaise and hyperpigmentation of her palms. Her only drug ingestion consisted of oral iron preparations that she had received for 8 yr prior to our initial evaluation of her anemia. She was without hepatosplenomegaly or jaundice. Initial laboratory studies included a hemoglobin level of 9.3 g/dl, hematocrit 27.5%, MCV 78 fl, MCHC 38%, WBC 2.6 x 10⁹/liter with a normal differential count, and platelet count 330 x 10⁹/liter. A reticulocyte count was 3.4%. The peripheral blood film was dimorphic with many elliptocytes, poikilocytic microcytes (Fig. 1), and multi-lobed polymorphonuclear cells (PMNs). A Coulter Model ZH Channelizer
trophil.

Distinct populations, one with an MCV of 35.5 fl and one 82.8 fl (Fig. 1). A bone marrow aspirate was markedly megaloblastic. Serum B₁₂ was <50 pg/ml on two occasions (normal, 140~750 pg/ml). A Schilling test was compatible with the absence of intrinsic factor (Coulter Electronics, Hialeah, Fla.) profile of RBC showed two consistent with a pattern of Rh-linked HE. Intramuscular hydroxy-

Cobalamin was administered with subsequent cure of her anemia and relief of her symptoms.

Blood was first obtained for study 12 days after the cobalamin injection for the Schilling test and while she remained anemic with approximately 35% of her RBC represented by cell fragments and extreme microcytes.

**Control Subjects**

Blood samples were obtained for the studies outlined below from the patient and from several control groups. Normal control subjects were nonanemic laboratory personnel with normal RBC morpholo-

Five normal family members and one HE family member were studied; none was anemic. Three cobalamin-deficient patients were studied; all had isolated cobalamin deficiency with low serum B₁₂ levels and positive Schilling tests. One unrelated nonanemic nonhe-

molytic HE patient served as an HE control. Finally, three anemic patients served as anemic controls, one with widespread metastatic carcinoma and a microangiopathic hemolytic anemia, one with cold agglutinin-induced hemolytic anemia, and one with folate-deficient megaloblastic anemia. Blood from the patient and controls was handled in exactly the same manner. All studies were performed with informed consent and approval of the Human Investigations Committee of the Walter Reed Army Medical Center.

**Preparation of Cells and Membranes**

Blood from the patient and control subjects was obtained by venipuncture using EDTA anticoagulant. Following centrifugation, the plasma anduffy coat were promptly removed and the remaining cells were washed 3 times in Hanks' balanced salt solution at 4°C (Microbiological Associates, Walkerville, Md.) with removal of any remaining buffy coat after each wash. Granulocytes were further removed by filtration over nylon wool and the remaining RBC were washed in pH 7.4 phosphate-buffered saline (PBS). Packed RBC were frozen at −70°C after glycerolization in 40% glycerol–5% citrate until sufficient samples had been accumulated to study blood from several controls and the patient at one time.

Following serial deglycerolization in 12%–9%–5%–3% glycerol solutions, erythrocyte ghosts were prepared from the remaining PBS-washed whole RBC by the method of Dodge et al. Following the final wash and centrifugation, the ghost membranes were dissolved in sample buffer containing 1% SDS–40 mM dithiothreital and electrophoresed on 10% polyacrylamide discontinuous slab gels by the Laemml method as modified by Weber and Osborn. Samples were applied in wells measuring 2 mm in width. The resulting narrow electrophorograms provided highly reproducible densitometric scans on the scanner described below. Gels were

stained with Coomassie blue and the resultant bands numbered in accordance with the scheme developed by Fairbanks et al. Quantification of protein contained in the major bands 1, 2, and 3 was performed by scanning the gels using an ISCO Model 1310 gel scanner (Instrumentation Specialties Co., Lincoln, Neb.). Peaks on the densitometric tracings representing spectrin bands 1 + 2 and the anion channel band 3 were cut out and weighed on an analytical balance. From 2 to 5 replicate gels were used to provide data for Table 1. The coefficient of variation of bands (1 + 2)/3 ratios obtained from these replicates was 0.5%–19.3%.

The patient's RBC were separated into populations of varying sizes by unit gravity sedimentation in a Sta-put Velocity Sedimentation Cell Separator (Johns Scientific, Toronto, Canada). With this technique, cells of different sizes are isolated by permitting a thin layer of purified RBC to sediment at 4°C for 2–3 hr in an albumin density gradient. We collected fractions and the cells were sized on a Model ZH Coulter Channelizer (Coulter Electronics, Hialeah, Fla.). The cells were pooled into groups representing very small cells (MCV = 35 fl), intermediate cells (MCV = 80 fl), and large cells (MCV = 95 fl). These cells were washed free of albumin, glycerolized, and frozen at −70°C. With the exception of the sedimentation step, the processing of this blood and the preparation of membranes was identical to that of the controls. This sedimentation process does not alter the heat sensitivity of normal cells (data not shown).
population corresponds to cell fragments and extreme microcytes while the larger population are more typical elliptocytes. That portion of the total hemoglobin contained within each peak of the cell size distribution curve was computed from the whole blood hemoglobin.

### Table 1. Heat-Induced Budding of Erythrocytes at 47°C and Ratios of Protein Contained in Band 1 + 2 and Band 3 (see Methods) for Controls and Patient, Both B₁₂ Deficient and Following Repletion

<table>
<thead>
<tr>
<th>Group</th>
<th>Percent Budded Cells</th>
<th>Mean + SE</th>
<th>Ratio 1 + 2/3</th>
<th>Mean + SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal controls</td>
<td>0.7% ± 0.2%*</td>
<td>0.98</td>
<td>0.98 ± 0.03†</td>
<td></td>
</tr>
<tr>
<td>A.B.</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B.A.</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E.S.</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R.W.</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T.B.</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-HE family controls</td>
<td>0.8% ± 0.3%</td>
<td>1.25 ± 0.03†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B.H.</td>
<td>2</td>
<td>1.29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M.W.</td>
<td>1</td>
<td>1.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C.S.</td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E.V.</td>
<td>0.5</td>
<td>1.19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E.V., Jr</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HE controls</td>
<td>9.5% ± 3.0%*</td>
<td>0.92</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P.K.</td>
<td>12.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E.V., Sr</td>
<td>4.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B₁₂-Deficient controls</td>
<td>0.5% ± 0.0%</td>
<td>0.81</td>
<td>0.88 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>L.H.</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F.M.</td>
<td>0.5</td>
<td>0.82</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H.T.</td>
<td>0.5</td>
<td>1.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anemic controls</td>
<td>2.3% ± 1.6%</td>
<td>1.05 ± 0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T.S.</td>
<td>0.5</td>
<td>1.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B.B.</td>
<td>5.5</td>
<td>1.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F.N.</td>
<td>1.0</td>
<td>1.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient—B₁₂ deficient</td>
<td>57.0</td>
<td>1.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intermediate cells</td>
<td>19.5</td>
<td>1.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large cells</td>
<td>28.5</td>
<td>1.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient—B₁₂ replete</td>
<td>10.0</td>
<td>1.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L.G.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p < 0.001.
† p < 0.001.

Individual values for (1 + 2)/3 ratios are the averages of from 2 to 5 determinations on each subject. Shown also are selective p values for Student’s t tests of differences of means between groups. All other differences between group means are not statistically significant.

### Heat Treatment of Cells

To determine the sensitivity of RBC to heat, a modification of the method of Zarkowsky and coworkers was employed. Deglycerolized washed cells were suspended to a hematocrit of 1% in PBS. Aliquots of 0.2 ml were placed in a stainless steel cup with a water jacket in which temperature can be regulated to within 0.2°C. Samples were heated for 10 min at predetermined temperatures from 45 to 50°C. This is of sufficient duration to produce maximal demonstrable changes in deformability, the extractability of spectrin, and morphological changes in normal erythrocytes. After heating, the RBC were fixed for counting in 1% glutaraldehyde/PBS. The resulting fixed cells were examined under Nomarsky optics for the presence of budded forms (Fig. 3). All cells with distinct buds and grossly segmented cells were classified as budded. A total of 200 cells were counted, since this count gave the greatest replicability of percent budded cells for any given sample.

### RESULTS

Erythrocytes obtained from the patient on her first clinic visit and at the time of the present studies fell into two distinct size populations (Fig. 2). The smaller population corresponds to cell fragments and extreme microcytes while the larger population are more typical elliptocytes. That portion of the total hemoglobin contained within each peak of the cell size distribution curve was computed from the whole blood hemoglobin.

![Fig. 3. Morphological changes under Nomarsky optics for B₁₂-deficient (upper) and B₁₂-replete (lower) RBC from the patient that occur with heating at the temperatures shown for 10 min. Cells have been fixed in 1% glutaraldehyde-PBS.](image)
Separation of the entire RBC population into fractions of different sizes by unit gravity sedimentation resulted in the cell size distributions shown in Fig. 5.

Budding and fragmentation of normal erythrocytes was not noticeable below 49°C (Fig. 6). Above this temperature, over one-third of the cells became budded, segmented, or spherocytic. Erythrocytes from family controls behaved in a fashion identical to normal RBC. Because an insignificant degree of budding occurred at 47°C for normal controls and family controls, this was chosen as a convenient temperature to contrast the behavior of HE controls and the patient (Table 1).

In contrast to RBC from normals and family members, erythrocytes from the patient 4 mo after cobalamin replacement was begun and from two nonhemolytic HE subjects were mildly heat sensitive (Fig. 6 and Table 1). The mean degree of budding of cells at 47°C was 9.5% for HE controls versus 0.7%–0.8% for normal and family controls. Four months after B₁₂ repletion, 10% of the patient's RBC budded at 47°C, well within the expected range for HE controls. However, while B₁₂-deficient, the patient's erythrocytes ap-
appeared to be quite heat sensitive, with 57.0%, 19.5%, and 26.5% budding occurring among the small, intermediate, and large cells, respectively. Cells from cobalamin-deficient controls and other anemic controls demonstrated normal degrees of budding at 47°C and the higher temperatures studied.

No electrophoretically abnormal proteins on SDS-PAGE gels were found in RBC from the patient while B₁₂ deficient (Fig. 7). Similarly, there were no apparent differences in the RBC membrane proteins of normal controls, family controls, HE controls, anemic patients, and B₁₂-deficient controls.

Because variations in the quantities of important structural membrane proteins could exist without any difference in the pattern of proteins on polyacrylamide gels, we examined the concentration of the major structural membrane proteins, spectrin, bands 1 and 2, in relation to the transmembrane anion channel, protein band 3 (1 + 2/3). The bands (1 + 2)/3 ratio of RBC from the patient while cobalamin deficient were identical to the ratio following cobalamin replacement (Table 1). Furthermore, when the patient was B₁₂ deficient, there was no difference between her bands (1 + 2)/3 ratio and the mean value of family controls. We did observe, however, a statistically significant elevation of the mean of the (1 + 2)/3 ratios of three non-HE family members studied over the mean value of normal controls, anemic controls, and cobalamin-deficient controls. These latter three groups did not differ from one another in this regard. The apparent elevation of spectrin (or depression of band 3) in the RBC membranes of family members may reflect the genetic control of the concentrations of membrane proteins. However, as (1 + 2)/3 ratios may be altered by nonuniform dissolution of membrane proteins, nonuniform dye binding to proteins, inaccuracy of densitometry, and other technical errors, observed differences must be interpreted cautiously.

**DISCUSSION**

This patient presented a diagnostic dilemma: having previously been found to have nonhemolytic HE with a first-degree relative (father) with the same autosomal dominant condition, the discovery of a fragmentary microcytic anemia suggested a second superimposed disease. The population of very small RBC fragments and the overall microcytosis of her RBC may represent two different processes and will be discussed individually.

By bone marrow examination and chemical tests, a diagnosis was made of pernicious anemia without iron deficiency, a condition expected to produce macrocytosis. It is worth noting, however, that the patient has HbC trait, a hemoglobinopathy in which microcytosis has been noted alone, in combination with α-thalassemia

Fig. 7. Erythrocyte membrane protein pattern in 1% SDS-10% PAGE for (A, F, and H) normal controls, (B) non-HE family member, (C) patient L.G.—B₁₂ deficient, (D) HE control, (E) patient L.G.—B₁₂ replete, (G) B₁₂-deficient control. Gels were stained with Coomassie blue. These gels were made with conventional 1-cm wide sample wells and are slightly overloaded so as to clearly demonstrate minor protein bands.

In contrast to our patient, however, this patient developed a macrocytic anemia without microcytes, fragments, or the dimorphism evident in the present case.

Hereditary elliptocytosis is a heterogeneous group of disorders. One form of nonhemolytic HE is linked to the Rh locus on chromosome 1.² The non-Rh-linked form or forms includes a minority of cases with overt hemolysis. Independent of the hemolytic potential of the trait, Tomaselli and coworkers have reported an apparent alteration of the conformational stability of membrane spectrin in HE.³ This is reflected in a
decreased solubility of spectrin from RBC membranes of certain kindreds following heat treatment of the protein. The temperature at which spectrin denaturation occurs in these HE cells (48.0 ± 0.1°C) differs significantly from normal RBC spectrin and non-heat-sensitive HE spectrin (49.0 ± 0.3°C). Furthermore, for one subgroup of HE patients, this temperature corresponds to the temperature at which RBC fragmentation and spherocytosis was observed by these authors.

Overall, we observed a similar pattern of heat-induced budding, fragmentation, and spherocytosis. Normal RBC and RBC from family members began to undergo morphological changes at 49°C, while the HE control erythrocytes and those from the patient following B₁₂ replacement fragmented at 48°C. These observations do not, however, explain the formation of small and fragmented cells with cobalamin deficiency.

It is conceivable that the loss of a critical structural component resulted from the patient's pernicious anemia. Feo and colleagues reported a deficiency of membrane protein band 4.1 in association with a hemolytic form of HE. However, our patient's PAGE electrophorogram was unaltered by cobalamin deficiency and demonstrated no loss of a major protein component (Fig. 7).

Sensitivity of RBC membrane spectrin to heat treatment at temperatures within the physiologic range has also been observed in hereditary pyropoikilocytosis (HPP). This disorder results in a congenital hemolytic anemia and budded, fragmented RBC quite similar to our patient's B₁₂-deficient cells. This morphological similarity and the decreased conformational stability of spectrin from some HE RBC led us to hypothesize that cobalamin deficiency imposed an even greater destabilizing effect on the cells of this HE patient, which resulted in fragmentation of her RBC. We predicted an increased heat sensitivity of the B₁₂-deficient RBC. We observed just such an increase in heat-induced cell fragmentation that was not observed in RBC from cobalamin-deficient controls or cells from patients with other forms of anemia. It is unlikely that heat stress caused RBC fragmentation while the patient was B₁₂ deficient. Rather, heat sensitivity is a marker of membrane instability, and we sought the biochemical basis for this by examining the quality and quantity of structural membrane proteins.

Spectrin in HPP cells exhibits defective self-association of dimers into the tetrameric form present in the membrane lattice. Heat-induced injury to HPP cells results in the formation of high molecular weight (HMW) complexes comprised of spectrin with small contributions of other membrane proteins. The presence of these HMW complexes, the loss of structural proteins from the membrane when these complexes are formed, and/or membrane injury induced by splenic removal of the complexes may destabilize the entire RBC membrane, leading to RBC fragmentation. Splenic pitting of HMW complexes leads to a relative loss of spectrin, measured by comparing the amount of bands 1 + 2 protein with band 3, which is not a component of these precipitates.

The small number of cells available for study precluded a study of the spectrin dimer—tetramer equilibrium or a direct search for HMW complexes. However, we found a normal (1 + 2)/3 ratio in the highly heat-sensitive, cobalamin-deficient cells. We interpret this as evidence that the microcytes do not arise as lipid-rich, structural protein-depleted "buds" of larger unstable cells.

For technical reasons, storage of the RBC as glycerolized frozen cells was required before our studies could be performed. That this step did not alter the thermal sensitivity of the RBC or the character and quantity of membrane proteins is indicated by several lines of evidence. First, the heat curves obtained in this study on the deglycerolized RBC are identical to those observed by others. Second, we observed that heat curves on fresh cells from two normal controls and two cobalamin-deficient patients did not differ from those obtained on the same sample after freezing and deglycerolization. The curves on fresh RBC from two non-hemolytic HE patients were shifted only 0.5°C toward less heat sensitivity (data not shown). Finally, the bands (1 + 2)/3 ratios observed for frozen deglycerolized RBC are very similar to those reported by others for normal nonfrozen RBC.

We conclude that the paradoxical production of microcytes and fragmented RBC by B₁₂ deficiency in HE is reflected in a heat-sensitive RBC membrane. This reversible lesion is not associated with major changes in the quantity of membrane proteins or identifiable electrophoretic abnormalities. Furthermore, cobalamin deficiency alone does not result in this abnormal heat sensitivity of the membrane or perturbations of the PAGE protein electrophorogram. This latter observation is in contrast with one published report in which proteolysis of the RBC membranes may have altered the PAGE proteins.

The central role of cobalamin in methylation reactions usually results in problems of nuclear maturation without abnormalities of protein synthesis. It is conceivable that subtle changes in fatty acid constituents of the membrane brought about by cobalamin deficiency could compromise membrane integrity in the presence of a mutant spectrin, as in HE. Increased quantities of odd-chain and branched-chain fatty acids have been observed in myelin from the central nervous system.
system of cobalamin-deficient monkeys\textsuperscript{22} and from peripheral nerves of cobalamin-deficient humans.\textsuperscript{23} Whatever the underlying mechanism, our observations provide further evidence for the heterogeneity of the HE syndromes and the tenuous grasp that some of the nonhemolytic forms have upon maintenance of erythrocyte integrity.

ACKNOWLEDGMENT

We are indebted to Dr. Harvey Klein at the National Institutes of Health, Clinical Center Blood Bank for blood typing of the family; to Drs. Cory Spencer and Thomas Cosgriff for their referral of cobalamin-deficient patients to us; and Dr. Patrick Lorenz for his assistance in developing the PAGE technique. Finally, we are indebted to Dr. Daniel G. Wright and Dr. William H. Crosby for their interest and support of this project.

REFERENCES

Increased heat sensitivity of red blood cells in hereditary elliptocytosis with acquired cobalamin (vitamin B12) deficiency

EB Schoomaker, WM Butler and LF Diehl