DEFICIENT RED-CELL SPECTRIN IN SEVERE, RECESSIVELY INHERITED SPHEROCYTOSIS

PETER AGRE, M.D., EUGENE P. ORRINGER, M.D., AND VANN BENNETT, M.D., PH.D.

RED-CELL membrane architecture has been extensively studied in recent years. When the cells are hypotonically lysed and washed free of hemoglobin, the resulting membrane ghosts consist of the lipid bilayer and associated proteins. When ghosts are extracted with non-ionic detergents, lipid and integral membrane proteins are removed, leaving a cytoskeleton that is thought to be responsible for the shape, strength, and reversible deformability of the cell. The cytoskeleton consists of an assembly of polyptides: spectrin (the largest and most abundant protein of the membrane), band 4.1, and actin. The cytoskeleton is attached to the membrane by the association of spectrin with ankyrin, a protein that is attached to the cytoplasmic end of band 3 (the anion channel that spans the lipid bilayer).

Certain disorders of red-cell shape are probably due to abnormalities of the membrane cytoskeleton. Hereditary pyropoikilocytosis is a rare disorder characterized by bizarre forms of red cells that are abnormally sensitive to heat-induced fragmentation because of inherently defective spectrin. Other patients have been described with hemolytic anemias and bizarre red-cell shapes due to a reduced number of high-affinity ankyrin-binding sites on the membrane. Spectrin from the red cells of some patients with elliptocytosis has a temperature-sensitive loss of tertiary structure 0.6°C below that seen in normal spectrin. Other patients with elliptocytosis have spectrin that yields abnormal trypsin-digestion fragments, and patients with homozgyous elliptocytosis due to red cells lacking band 4.1 have been identified. Hereditary spherocytosis has been studied extensively, and the principal molecular defect has remained elusive, although recent preliminary data suggest that defective interactions between band 4.1 and spectrin may occur in some patients. Strains of mice with extreme spherocytic anemias due to recessive traits have been discovered, and the cell membranes are markedly deficient.

From the Department of Cell Biology and Anatomy, The Johns Hopkins University School of Medicine, Baltimore, and the Department of Medicine, North Carolina Memorial Hospital, Chapel Hill. Address reprint requests to Dr. Agre at the Department of Cell Biology and Anatomy, The Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205. Supported by grants from the National Institutes of Health (1-R01-AM29808-1), the Wellcome Foundation, and the Muscular Dystrophy Association. Dr. Agre is the recipient of a Clinical Investigator Award, and Dr. Bennett is the recipient of a Research Career Development Award, each from the National Heart, Lung, and Blood Institute.

in spectrin. The relative deficiencies correlate with the degrees of hemolysis, and incorporation of normal spectrin into these cells has resulted in improved structural integrity.

We present our experience with two patients, the daughters of related but normal parents, who had nearly fatal hemolytic anemia requiring early splenectomy. Both had striking clinical improvement, but spherocytosis persisted. Red-cell-membrane ghosts were at least 50% deficient in spectrin when analyzed by sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) with band 1 reduced more than band 2. The spectrin deficiency was also detected when whole-red-cell lysates were analyzed by radioimmunoassay. Great care was taken to avoid membrane proteolysis, and there was no evidence of spectrin fragmentation. No defect in membrane binding of spectrin or in the membrane-binding sites (ankyrin) was identified. These patients had a severe form of spherocytosis that was probably due to an inadequate amount of spectrin, and the manner of inheritance was probably mendelian recessive.

Case Reports

Patient 1 was a seven-year-old white girl who weighed 2980 g at birth. No neonatal jaundice was noted, but when she was three months old her hemoglobin was 4.5 g per 100 ml. The red cells were spherocytic, but Coombs' tests were negative. Bone-marrow aspiration showed extreme erythroid hyperplasia (myeloid:erythroid ratio, 0.6). She received transfusions on three occasions, with improved growth. After the age of six months her hematocrit remained about 12% per cent, with 15 to 35 per cent reticulocytes, without transfusions. At 17 months she was icteric and had a Grade II/VI systolic ejection murmur at the left sternal border; her liver was palpable 4 cm below the right costal margin, and her spleen extended to the umbilicus. A chest film showed borderline cardiomegaly; the hemoglobin was 4.5 g per 100 ml, the white-cell count 13,300 (16 per cent were nucleated red cells), and the platelet count 190,000. Tests of the osmotic fragility of fresh erythrocytes showed 27 per cent hemolysis at 0.95 per cent sodium chloride, 50 per cent hemolysis at 0.73 per cent sodium chloride (normal, 0.39 to 0.45 per cent sodium chloride), and 90 per cent hemolysis at 0.58 per cent sodium chloride. Tests of incubated erythrocytes' osmotic fragility showed 50 per cent hemolysis at 0.83 per cent sodium chloride (normal, 0.5 per cent sodium chloride) and 90 per cent hemolysis at 0.7 per cent sodium chloride. The total bilirubin was 4.1 mg per 100 ml (74 mg/dl per liter), the direct bilirubin 0.3 mg per 100 ml (5.1 mg/dl per liter), and the dehydrogenase 1303 IU. Red-cell glycolytic enzyme and hemoglobin analyses gave normal results. The spleen was then removed, and weighed 55 g, and showed marked congestion with hemosiderin deposition. The hemoglobin concentration after splenectomy remained at 11 to 13 g per 100 ml, with 5 to 6 per cent reticulocytes, but the red cells were still remarkably spectrinic (Fig. 1).

The mean corpuscular volume (MCV) was 74 fl, and the mean corpuscular hemoglobin concentration (MCHC) was 39 g per 100 ml. Her physical examination is now normal, and her weight has increased from the 20th to the 30th percentile. She has had hemoglobinuria during viral illnesses and has received daily folic acid supplements and penicillin prophylaxis. Red-cell fragmentation has been measured at several temperatures from 44°C to 49.5°C, and no abnormal instability has been detected.

Patient 2 was the five-year-old sister of Patient 1. She weighed 2530 g and was noted to have spherocytosis when she was born two weeks prematurely. Her serum bilirubin rose to 17 mg per 100 ml (292 μmol per liter) when she was a newborn, and she required ultraviolet light and exchange transfusion. Her hematocrit steadily declined over the next 10 weeks to 7 per cent; thereafter, she received transfusions every four to six weeks. Her growth rate slowed whenever her hematocrit declined below 14 per cent. When evaluated for splenectomy at the age of seven months, she was pale but not icteric. She had a Grade I/VI systolic ejection murmur at the left sternal border. Her liver was palpable 1 cm below the right costal margin, and her spleen was palpable 2.5 cm below the left costal margin and weighed 82 g when removed. She also recovered well; her hemoglobin has been 11 to 12 g per 100 ml, her MCV 74 fl, and her MCHC 38 g per 100 ml, with 6 to 7 per cent reticulocytes. Her red-cell morphology has remained identical to her sister's, and their subsequent clinical courses have been similar.

The parents were found to be fourth cousins, and parentage was confirmed by HLA typing. There were no other pregnancies. Neither parent had had any medical problem (no symptoms of cholelithiasis), and both had had normal physical examinations without palpable spleens. Both had normal red-cell morphology (Fig. 1). Red-cell indexes for the mother included a hemoglobin level of 13.9 to 14.5 g per 100 ml, an MCV of 91 to 99 fl, and an MCHC of 31 to 34 g per 100 ml, with 1.8 per cent reticulocytes. Serum haptoglobin was slightly reduced at 90 mg per 100 ml (normal, 100 to 300). Indexes in the father included a hemoglobin level of 15 to 16.4 g per 100 ml, an MCV of 92 to 95 fl, and an MCHC of 33 to 34 g per 100 ml, with 1.6 per cent reticulocytes. Serum haptoglobin was low normal at 120 mg per 100 ml. The osmotic fragility of fresh and incubated erythrocytes, serum bilirubin, and hemopexin were normal in both parents. All grandparents, all uncles, and the aunt also had normal red cells, and no ancestors were known to have blood disorders.

Methods

Samples of venous blood were obtained from the patients on four different occasions, anticoagulated with acid citrate dextrose, and stored on ice for four to 18 hours. Whole-blood samples were sedi-

membrane-glucosidase saline with 0.75 per cent dextan T500 at 1xg 2°C, and the red cells were then washed in physiologic saline. Membrane ghosts were prepared by hypotonic lysis as described elsewhere, and no residual white cells remained. We avoided proteolysis by including a 1:1000 concentration of diisopropylfluorophosphate, 20 μg of phenyl methyl sulfonyl fluoride per milliliter, 1 mM EDTA, and 5 μg of pepstatin A per milliliter in ice-cold lysis buffers. SDS-PAGE was performed with 4 to 17 per cent gradient slab gels with diisothiureitol, as described elsewhere. Samples from each of the four blood donors were subjected to electrophoresis five to 20 times each. Gels were stained with Coomassie blue and scanned with a Gilford 260 spectrophotometer. Protein peaks were cut from the tracings and weighed (a process known as SDS-PAGE scanning). Unstained SDS-PAGE slabs of membrane-ghost samples and whole-red-cell lysates were electrophoretically transferred to nitrocellulose paper, incubated with antispectrin IgG, washed, and incubated with 125I-protein A (a technique known as immunoblot). Autoradiographs were prepared with Kodak XO-mat AR film.

Pure spectrin and the cytoplasmic 43,000-dalton fragment of band 3 were prepared from normal red cells and labeled with 125I. High-affinity rabbit IgG antibody against human spectrin was purified by elution from spectrin-Sepharose affinity gels. Affinity-purified rabbit IgG antibody against the 43,000-dalton fragment of band 3 was made by a similar method. Spectrin in whole red cells lysed in 2 M urea, 0.1 M glycine, 1 per cent Triton X-100, and 1:1000 diisopropylfluorophosphate was measured by radioimmunoaassay with antispectrin IgG, 125I-spectrin, and protein A-bearing staphylococci, as described elsewhere. Band 3 was measured similarly. Reassociation of spectrin with spectrin-depleted inside-out vesicles and competitive inhibition of binding were performed as described by Bennett and Branton. Measurements of the phospholipid content of membrane ghosts were also performed. Blood samples were immediately fixed in physiologic.
saline with 0.25 per cent glutaraldehyde for scanning electron micrographs. Statistical evaluations were calculated from a normal distribution.

RESULTS

Membrane-ghost proteins were separated by SDS-PAGE and stained with Coomassie blue (Fig. 2). Preparations from Patients 1 and 2 had notable reductions in the amount of spectrin and very small reductions in the amounts of bands 4.1 and 4.2 and actin, but the amounts of ankyrin and bands 3, 6, and 7 appeared normal. The amounts of band 8 and globin appeared to be somewhat increased, as is commonly seen in several sorts of hemolytic anemia. Sialoglycoproteins stained with periodic acid–Schiff were not different from those in controls (data not shown).

The quantity of each membrane protein was measured by scanning the SDS-PAGE slabs of ghosts. Spectrin content was normalized to the amount of band 3, an index of membrane surface area. The spectrin:band 3 ratios were found to be highly reproducible in several nonanemic adults (Table 1). The ratio in the second daughter, who was more severely affected clinically, was even lower than her sister’s. Their parents, however, had normal ratios. Several other controls were checked, and all had ratios similar to those of the normal adults. Nonanemic children and a nonanemic patient who had undergone splenectomy had slightly higher ratios, and the ratios in three patients with elliptocytosis were slightly lower. A patient with non-spherocytic poikilocytosis, microcytosis, and reticulocytosis had a slightly lower ratio. An unrelated patient with severe, nondominantly inherited spherocytosis was identified, and his cells had a significantly lower ratio. Two other unrelated patients with typical hereditary spherocytosis (moderate hemolysis of dominant inheritance) had slightly lower ratios. It was concluded that the observed reductions of spectrin in the membranes in Patients 1 and 2 were reproducible and were probably not due to age, splenectomy, microcytosis, or the reticulocyte count.

Spectrin comprises two polypeptide chains, and more detailed SDS-PAGE scanning of the daughters’ membrane ghosts showed that band 1 was reduced more than band 2 (to 34 and 50 per cent of the control values, respectively). Measurements of lesser bands showed that band 4.1 and actin were slightly reduced to 80 to 90 per cent of the control values and that band 4.2 was reduced by amounts varying from 65 to 90 per cent of the control values.

Proteolysis Not Detected

Spectrin and other membrane proteins can be proteolytically degraded during the preparation of ghosts. This is especially troublesome when one is studying anemias with increased reticulocytes, which are known to be rich in proteases. Steps taken to prevent this included the prompt processing of blood, the removal of all white cells by dextran sedimentation, and the use of four protease inhibitors in ice-cold lysis buffers.

Immunoblot analysis revealed no fragmentation of spectrin. Autoradiographs of whole-red-cell lysates and ghosts from Patient 1 and a control are compared in Figure 2. Bands 1 and 2 of spectrin stained intensely, whereas no other peptides reacted. Even after prolonged exposure of the film, no increase in reactivity associated with smaller peptides was found (data not shown).
Patient I and 0.26 in Patient 2; normal, 1.0). Radioimmunoassays of both parents' cells failed to identify a significant spectrin deficiency. Membrane phospholipid content is also an index of membrane surface area, and the daughters had significantly subnormal phospholipid content (mean ± S.D., normalized to control values): 0.91 ± 0.04 μmol per milligram of protein in Patient 1 (P < 0.01) and 1.04 ± 0.01 μmol per milligram of protein in Patient 2 (P < 0.005) (normal, 1.38 ± 0.02). The membrane ghosts from the parents α-δ had slightly subnormal phospholipid content: 1.22 μmol per milligram of protein in the mother and 1.16 μmol per milligram of protein in the father.

**Functional Analysis of Spectrin by Reassociation**

Qualitative abnormalities of spectrin and membrane-attachment sites (ankyrin) were sought. Membrane-attachment sites were measured directly with 125I-labeled spectrin (prepared from a normal control) and spectrin-depleted inside-out membrane vesicles prepared from the daughters, their mother, and a control (Fig. 4A). The patients' and controls' vesicles bound 125I-spectrin similarly, indicating that there was no detectable abnormality of the membrane-attachment site. The spectrin itself was analyzed by competitive binding experiments (Fig. 4B). The unlabeled spectrin from the daughters, the mother, and the control all displaced 125I-labeled spectrin equivalently. Spectrin tetramer-dimer equilibrium was also normal 15 (data not shown). This suggests that although the daughters' spectrin was diminished in quantity,

**Table 1. Spectrin:Band 3 Ratios in Patients and Controls.**

<table>
<thead>
<tr>
<th>SUBJECTS (NO.)</th>
<th>SPECTRIN: BAND 3 RATIO</th>
<th>S.D.</th>
<th>P VALUE ♦</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daughter 1</td>
<td>0.46</td>
<td>0.07</td>
<td>&lt;0.0002</td>
</tr>
<tr>
<td>Daughter 2</td>
<td>0.40</td>
<td>0.12</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Mother</td>
<td>1.04</td>
<td>0.10</td>
<td>NS</td>
</tr>
<tr>
<td>Father</td>
<td>1.00</td>
<td>0.13</td>
<td>—</td>
</tr>
<tr>
<td>Normal adults (9)</td>
<td>1.09</td>
<td>0.09</td>
<td>NS</td>
</tr>
<tr>
<td>Normal children (2)</td>
<td>1.09</td>
<td>0.15</td>
<td>NS</td>
</tr>
<tr>
<td>Splenectomized control</td>
<td>1.09</td>
<td>0.15</td>
<td>NS</td>
</tr>
<tr>
<td>Patients with nonspheroctic hemolytic anemia ‡</td>
<td>0.89</td>
<td>0.12</td>
<td>NS</td>
</tr>
<tr>
<td>Patients with elliptocytosis (3)</td>
<td>0.89</td>
<td>0.08</td>
<td>NS</td>
</tr>
<tr>
<td>Patients with atypical spherocticity §</td>
<td>0.60</td>
<td>0.06</td>
<td>&lt;0.004</td>
</tr>
<tr>
<td>Patients with typical spherocticity (2) ¶</td>
<td>0.85</td>
<td>0.09</td>
<td>NS</td>
</tr>
</tbody>
</table>

*The ratio of spectrin to band 3 in membrane ghosts was determined from sodium dodecyl sulfate polyacrylamide-gel electrophoresis slabs stained with Coomassie blue. Values are derived from multiple tracings of preparations from several blood samples from each subject.

♦ These patients were unrelated 25-year-old and 26-year-old women with family histories typical of those in hereditary spherocticosis. Both underwent splenectomies as young adults because of spherocticosis and moderate hemolysis.
the spectrin bound to the membrane and formed tetramers normally.

**Discussion**

Both these patients, the daughters of apparently normal, but distantly related parents, had extreme spherocytic anemia. The patients' red-cell membranes had less than 50 per cent of the normal amount of spectrin when measured in both ghosts and whole-red-cell lysates. Great care was taken to prevent proteolysis, and no spectrin fragments were detected. No defects in the membrane-attachment sites (ankyrin) or in the ability of spectrin to attach to membranes or to form tetramers were identified. Although our patients' cells were significantly deficient in spectrin, they were only slightly deficient in bands 4.1 and 4.2 and actin, and only total absence of band 4.1 has been associated with substantial hemolysis. The principal structural defect in the daughters' cells was probably a deficiency of spectrin.

Deficient spectrin can occur because of any of several different defects. Increased intracellular proteolysis, inherently unstable spectrin, defective membrane binding, and reduced spectrin synthesis are all possible explanations. It is unlikely that the deficiency was a proteolytic artifact of ghost preparation, because of the preventive measures taken, the lack of spectrin fragments, and the reduction of spectrin in whole cells. Furthermore, the magnitude of the reduction was nearly identical on each of several occasions. Nevertheless, in vivo proteolysis can occur, and subtle deficiencies in bands 4.1 and 4.2 and actin were also detected. Hereditary pyropoikilocytosis is an illness characterized by inherently unstable spectrin, and red cells in this disorder are slightly deficient in spectrin relative to band 3 (85 per cent of normal). Our patients had a much more severe anemia than that of patients with hereditary pyropoikilocytosis and had neither increased heat-induced red-cell fragmentation nor defective spectrin tetramer-dimer equilibrium. However, an inherent instability of our patients' spectrin cannot be ruled out. Interactions of spectrin with band 4.1 were not measured, but reassociation studies suggested that spectrin-membrane associations were normal and were not a likely explanation for the spectrin deficiency. Reduced synthesis of spectrin, however, is a likely explanation. Furthermore, band 1 was reduced more than band 2, and that may be the principal molecular defect, with secondary reductions in the other peptides resulting from the lack of their binding sites on band 1. However, until direct methods of measuring spectrin synthesis in the patients' red-cell progenitors or methods of restriction analysis of spectrin genes are available, no definite conclusions can be made.
Although the red cells of the two daughters appeared very spherocytic, their illness showed some interesting differences from the illness typically described as hereditary spherocytosis. Unlike typical hereditary spherocytosis, in which some cells are usually spherocytes and some are normal, all our patients' red cells were microcytic, most were very spherocytic, and none appeared normal. Although the clinical courses in typical hereditary spherocytosis vary, these patients rarely require transfusion, and many of them are discovered only by chance observation. In one study of 76 patients with this disease before splenectomy, the lowest hemoglobin level was 7.4 g per 100 ml. Our patients had life-threatening anemia and remained mildly anemic even after splenectomy. Typical hereditary spherocytosis is characterized by increased fresh osmotic fragility, yet in two large studies the most fragile sample underwent 50 percent hemolysis in 0.53 percent sodium chloride. Our patients' cells were extraordinarily fragile, and accurate fragility testing was difficult because of considerable hemolysis, even in physiologic saline. Indeed, these patients were atypical of those with spherocytosis in that they had the hallmarks of typical hereditary spherocytosis, but to an extreme degree.

Four strains of hemolytic spherocytic anemia have been discovered in mice. All are inherited as autosomal recessive traits, and all are characterized by the presence of less than 50 percent of the normal amount of spectrin. Heterozygotes of each mouse strain appear normal, as do double heterozygotes of different strains. It appears that hemolytic spherocytosis in mice results from several different specific but non-overlapping genetic defects, each producing a deficiency in spectrin. It is likely that the specific genetic breakdown resulting in one of the mouse strains of the disease is fundamentally similar to that responsible for our patients' illness.

Hereditary spherocytosis in human beings is thought to be an autosomal dominant disorder, with about 20 to 25 percent of the cases appearing sporadically; no examples of homozygous hereditary spherocytosis are known. Although our patients' parents were distantly related, there was no known anemia in preceding generations. The parents were clinically normal and had a normal amount of spectrin; however, both had a slight decrease in membrane phospholipid and borderline-low serum haptoglobins. This suggests that the daughters were homozygous for a recessive trait. We have recently learned of another
group of distant relatives, with no known parental consanguinity, who have a very mild form of spherocytosis of dominant inheritance. They may be heterozygotes with the same allele who for unknown reasons express the trait, albeit subtly. Still another distant relative, also the offspring of normal but related parents, has been identified; her illness is clinically and biochemically similar to that of the two patients described here. (Agrè P: unpublished data).

These observations, together with the identification of an unrelated person with a similar illness, suggest that spectrin deficiency may be found in other patients who appear to have sporadic hereditary spherocytosis. It is interesting to speculate that even smaller deficiencies in spectrin could result in some degree of spherocytosis. Just as thalassemia has a clinical spectrum from nearly undetectable states to intraterine death due to unbalanced synthesis of globin chains, some spherocytic anemias with a variety of clinical states may result from different genetic defects that lead to a reduction in synthesis or to some post-transcriptional modification of spectrin.

We are indebted to the patients, their parents, and their grandmothers; to Dr. M. M. Billah for phospholipid analysis; to Ms. Celine Henderson for electron microscopy; and to Drs. Campbell McMillan, Peter Zuck, and John C. Parker of the University of North Carolina for referring patients.

REFERENCES