Hereditary Spherocytosis Associated with a Variant of Band 3 Protein in the Erythrocyte Membrane

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A 17-year-old woman with hereditary spherocytosis was found to be heterozygous for an unusual variant of the band 3 protein in erythrocyte membranes. The variant had a molecular weight of 95,000 daltons which was larger by about 3,000 daltons than the 92,000 m.w. normal band 3, and was phosphorylated less efficiently when intact cells were incubated with $^{32}$P-inorganic phosphate. It is discussed that this variant may affect the integrity of the membrane skeletons.

Key Words: Hemolytic anemia, Membrane skeleton, Phosphorylation

Hereditary spherocytosis varies its manifestations and clinical severity. It is likely that much of the variability results from genetic heterogeneity. Although this disease is generally acknowledged to be a disorder of the red cell membrane, the underlying biochemical lesion has not been defined. We now report a case of hereditary spherocytosis associated with an unusual red cell membrane abnormality that was detected by sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis. Results of a study on membrane phosphorylation by intrinsic protein kinase system are presented.

CASE REPORT

The patient, a 17-year-old woman, was referred for investigation because of anemia, slight jaundice and splenomegaly which had been present since childhood. Despite the persistent anemia and slight icterus her health status had been otherwise unremarkable. Examination revealed non-tender 5 cm splenomegaly. Hemoglobin was 7.7 g/dl, MCV 94.6 cu, MCH 34.6 pg, MCHC 36.7%, and reticulocytes 18.2 per cent. White blood cell and platelet counts and the white cell differential count were normal. Serum bilirubin was 6 mg/dl, of which 4.7 mg/dl was the indirect form. Aspiration of sternal marrow showed marked normoblastic hyperplasia, myeloid to erythroid ratio being 1:1.7. The half life of the chromium-tagged red cells was 5 days.

Electrophoresis of hemolysates on cellulose acetate revealed no abnormal hemoglobin, nor were there any unstable hemoglobins detectable by isopropanol test. Hemoglobins F and A2 were not increased. A peripheral blood film showed numerous spherocytes typical of hereditary spherocytosis. An X-ray film of the skull showed marked “hair-on-end” appearance (Fig 1). From the clinical and laboratory data, a diagnosis of hereditary spherocytosis was made. In November 1973, both splenectomy and cholecystectomy were performed. An enlarged spleen weighing 600 g and a gallbladder containing numerous sand-like stones were removed. Nine weeks after the operation the patient's hemoglobin was 14 g/dl, reticulocytes 0.25%, and red cell survival 29 days. All the previous findings char-
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Fig 1. Bony abnormality in the skull. Note that widening of the medullary spaces, thinning of the cortex, and a sparseness of the trabecular pattern form the typical hair-on-end appearance.

acteristic of hemolytic anemia disappeared. Although osmotically fragile red cells upon incubation are persistent (Fig 2), she has been asymptomatic for 10 years of follow-up since the operation.

The mother, brother and sisters of the patient were investigated, there being no family history of hereditary spherocytosis. The father was said to have died of icterus and anemia at the age of 45, and therefore might be suspected to be a trait-carrier.

MATERIALS AND METHODS

Heparinized venous blood was obtained from the patient post-splenectomy. Erythrocyte ghosts were prepared by hypotonic lysis, and solubilized as described by Fairbanks et al. All procedures were performed at 0°C to 5°C. Electrophoresis was performed in 0.1% SDS by the method of Porzio and Pearson. Sialoglycoproteins were purified from ghosts with lithium diiodosalicylate-phenol as described by Marchesi and Andrews.

Phosphorylation by intrinsic protein kinase system of the membrane was examined using fresh intact erythrocytes by the method of Shapiro and Marchesi. After 6 hours of incubation with 32P-inorganic phosphate, ghosts were prepared for electrophoresis on SDS-polyacrylamide gels, followed by autoradiography. The gels were sliced into 2 mm sections, solubilized with 50% hydrogen peroxide, and the radioactivity determined by liquid scintillation.

RESULTS

The separation of polypeptides is shown in Fig 3. Proteins are numbered 1 to 7 according to the convention of Steck. The sharp leading edge of band 3 of the patient's red cells is markedly decreased in intensity by about 50%, and a variant consisting of about half as much of the band 3 appears to be present, moving slower than the normal counterpart. An apparent molecular weight of this variant was calculated to be 95,000 daltons, and that of the normal band 3 was 92,000 daltons. The electrophoretic behavior of the band 3 proteins was reproducible highly with different samples, each freshly obtained during the period of 3 years. Three other cases of hereditary spherocytosis were compared but showed membrane patterns similar to the control preparation. All these preparations from hereditary spherocytosis showed, however, striking increases in the amount of residual globin.

Fig 2. Osmotic fragility of red cells from the patient compared with that of cells from a normal subject, before and after incubation of erythrocytes at 37°C for 24 hours.
Fig 3. Sodium dodecylsulfate-polyacrylamide gel electrophoresis of membrane proteins. Stained for carbohydrates by the PAS procedure (first and second gels), and for proteins with Coomassie blue (third and fourth gels) according to Fairbanks et al.\textsuperscript{3}. Membrane preparations were from a normal control (A) and from patient (B). Arrow indicates the band 3 variant with substantial difference in the electrophoretic mobility. No difference was observed either in the electrophoretic pattern or in the amount of sialoglycoproteins of patient's red cells as compared to those of normal subjects.

As shown in Fig 4, the pattern of phosphorylation in the patient's red cell appears to be similar to those of the normal control. About 90\% of the total radioactivity could be ascribed to the phospholipid fraction. Of the proteins, band 2 was the most prominent for labelling, and a small fraction of radioactivity was associated with band 3. The extent of phosphorylation of the membrane substrates of the kinase was increased in this patient when whole ghosts were analysed. The slower moving variant of band 3 was phosphorylated less intensely than the normal band 3 fraction, having one-fifth as high an activity incorporated (Table 1).

Table 1. Phosphate Incorporation into Intact Erythrocyte Membranes*

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<tr>
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<th>Band 2</th>
<th>Band 3</th>
<th>Whole ghosts</th>
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<tbody>
<tr>
<td>Normal control</td>
<td>12,600</td>
<td>2,390</td>
<td>186,000</td>
</tr>
<tr>
<td>Patient</td>
<td>8,150</td>
<td>1) 2,330</td>
<td>455,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2) 580 (variant)</td>
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*Average of triplicate determinations for cpm of $^{32}$P incorporated per mg membrane proteins.

**DISCUSSION**

The structural alteration of the major protein that is associated with hereditary spherocytosis seems to be an important finding, since it may suggest that a deficient function, if any, is related to the disease. The band 3 protein is an integral membrane component that spans the lipid bilayer\textsuperscript{9} and has been implicated in a variety of functions, such as anion transport, glucose uptake and water permeability\textsuperscript{1,4}. This protein may be composed of at least two kinds of protein which migrate to form the leading edge and the trailing rim, respectively. If this is the case, the faster moving band is separated into two variants.
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distinct bands in this patient. Another, more likely, explanation may be that band 3 protein is composed of single homogeneous protein which had no or variable amount of carbohydrate chains, representing the sharp leading front and the trailing portions of the band\(^{10}\). If one assumes that spherocytosis has as its molecular basis the structural alteration of red cell membrane protein, we should see at least theoretically a normal in addition to the variant protein, since most patients are likely to be heterozygous for the gene. Examination of the gel electrophoresis seems to support this explanation.

Despite decades of intensive research, the precise molecular defect for spherocytosis has not been identified\(^{1}\). At the cellular level the membrane lesion is expressed as a progressive loss of surface area, which is enhanced by metabolic deprivation\(^{1,9}\). Recent evidence indicates that the structural stability of red cell membrane is almost entirely determined by the membrane skeleton, a filamentous meshwork of proteins lining the inner membrane surface\(^{1}\). The skeleton is composed predominantly of 4 proteins: spectrin, actin, protein 4.1, and ankyrin. Spectrin fastens to ankyrin, which tethers the skeleton to the membrane by means of its connection to the band 3 protein\(^{10}\). Six to 7% of American population is heterozygous for a slightly larger, but apparently normal variant of band 3 protein\(^{12}\). In this regard, it will be important to compare the function of the structurally altered band 3 protein, which may affect the stability of membrane skeltons of red cells in this patient.

The data indicate that although glycolysis is more active in the patient's red cells than in the normal, and thereby more APTs are generated during incubation, the phosphorylation reactions in band 2 and 3 proteins are less efficient in the patient's red cells than in the normal. The results might be comparable with those of Beutler et al.\(^{9}\). As discussed by the previous authors, the difference in phosphorylation between spherocytosis and normal membranes may be secondary to the disturbed membrane relationship, not fundamental to the kinase system itself. An altered phosphorylation in the variant of band 3 protein is associated with hereditary spherocytosis in this patient. However, its specificity and its relationship to the disease still remain to be explored.

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REFERENCES