Study of a Kindred With Hereditary Spherocytosis and Glyceraldehyde-3-Phosphate Dehydrogenase Deficiency

By Shaun R. McCann, Barbara Finkel, Shirley Cadman, and David W. Allen

A patient with hereditary spherocytosis (HS) was found to have glyceraldehyde-3-phosphate dehydrogenase (G3PD) deficiency by electrophoresis of the isolated red cell membranes on polyacrylamide gels with sodium dodecyl sulfate (PAGE SDS) as demonstrated by a diminished band 6 (G3PD) and confirmed by specific enzyme assay. Thirteen members of his family were studied: four were normal, two had HS alone, three had G3PD deficiency alone, and four had both HS and G3PD deficiency. G3PD deficient kindred members were probably heterozygous, since their red cell enzyme, while qualitatively normal, was present in half normal amounts. The G3PD deficiency alone was asymptomatic, and there was no evidence that the combination of HS with G3PD deficiency increased the clinical severity of the disease. However, G3PD deficiency, when combined with HS, was associated with an increase in protein band 4.5 on PAGE SDS. This band was also increased by incubation of normal red cells without glucose, and appeared to be a protein absorbed to the membrane as a consequence of metabolic stress. Hence, red cells with the combined abnormalities of both HS and G3PD deficiency showed signs of the exceptional metabolic stress to which they were exposed.

HEREDITARY SPHEROCYTOSIS (HS) may result from a defect in red cell (RBC) membrane proteins. Although abnormalities in these proteins have been reported, no consistent pattern of electrophoretic findings has been established. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (PAGE SDS) is an effective and highly reproducible means of examining RBC membrane proteins, and general agreement has been reached on the nomenclature and apparent molecular weight of the peptides separated by this procedure. Of the peptides separated on PAGE SDS, band 6 has been identified as the polypeptide subunit of G3PD. There is a single report of G3PD deficiency in the published literature, but the relationship of this deficiency to the hemo-
lytic anemia present in the patient is unclear. As part of a study of the effects of metabolic stress on membranes derived from incubated RBC, we have observed both increased band 6 with sulfhydryl inhibitors and increased band 4.5 when RBC are incubated in calcium-containing, phosphate-buffered saline without added glucose. Thus, when a patient with HS was found to have RBC membranes with deficient band 6 and increased 4.5 on PAGE SDS, a more intensive study of the patient and his family was undertaken.

MATERIALS AND METHODS

The patient and 13 family members were interviewed and examined. Cell counts, hemoglobins, hematocrits, and red cell indices were determined on a Coulter model S cell counter. Blood smears, reticulocyte counts, incubated and unincubated osmotic fragilities (OF), and autohemolysis tests were performed as described by Dacie and Lewis. Total and direct reacting bilirubin levels were measured in all patients.

RBC membranes were isolated at 0°C from heparinized blood within 5 hr after drawing by the method of Dodge et al., as modified by Fairbanks et al., and stored at -60°C. Prior to electrophoresis, RBC membranes were solubilized in 1% SDS containing 1% mercaptoethanol and heated for 2 min at 100°C to destroy possible proteases. PAGE SDS was performed on 0.5 x 10-cm gels of 5% acrylamide in 0.1% SDS, 0.1 M sodium phosphate buffer pH 7 at 22°C (10 mA/tube), stained with Coomassie blue and scanned on a Canalco gel scanner. The relative amount of each component was calculated from its area of the scan. Hemoglobin, measured by the benzidine method of Dacie and Lewis, comprised 4.2 ± 1.5% of the total membrane protein.

G3PD assays were performed at 25°C by the method of Velick. G3PD in the total hemolysate was determined by adding 1 volume of packed washed RBC to 20 volumes of 0.05% mercaptoethanol, and 0.0027 M ethylenediamine tetraacetic acid (EDTA), pH 7. G3PD in RBC membranes was assayed by dissolving a 50-μl aliquot of the membranes in 100 μl of 0.1% Triton X-100 in 0.015 M sodium pyrophosphate, and 0.03 M sodium arsenate. Duplicate assays were performed and the enzymatic activity expressed in μmoles of reduced nicotinamide adenine dinucleotide (NADH) produced per minute per milligram of membrane protein.

To obtain partially purified G3PD from control and patient RBC, 0.5 ml aliquots of the RBC membranes were thawed and washed at 0° for 1 min, with 0.005 M phosphate buffer pH 8, and centrifuged immediately (1200 g, 3 min). This procedure removed most of the remaining hemoglobin and some spectrin (SDS PAGE bands 1 and 2). The sediment was then extracted with 0.15 M sodium chloride, 0.005 M sodium phosphate, 0.01 M EDTA, and 0.05% dithiothreitol, pH 8, for 1 hr at 25°C. After centrifugation the supernatant was used directly or concentrated by Diaflo UM 10 ultrafiltration. Such material, containing more than 90% band 6 (G3PD) by SDS PAGE and gel scan, was used for determining the specific activity, pH optimum, thermal stability, and Michaelis constants with results within the reported normal range.

Blood for sterile incubation in vitro was obtained aseptically, centrifuged, and the plasma, white cells, and platelets removed. The RBC were washed 3 times with sterile Dulbecco's phosphate-buffered saline, pH 7.4 (containing 1.8 mEq/liter calcium), and incubated in the same solution without added glucose for 24 hr, before RBC membranes were prepared.

Statistical analysis of all results, including derivation of arithmetic mean, standard deviation of the mean, Student's t test of independent variables, and analysis of covariance, employed an electronic calculator (Compucorp 342 Statistician).

RESULTS

The proband was a 43-yr-old veteran who had undergone splenectomy at age 22 for anemia and jaundice and cholecystectomy at 29 for recurrent abdominal pain and cholelithiasis. His family tree (Fig. 1) includes individuals who were studied, as well as family members concerning whom only historical informa-
tion was available. A representative sample of this large kindred of French-Canadian descent was obtained, including family members who were normal, some with G3PD deficiency alone, some with HS alone, and some with both G3PD deficiency and HS. It was apparent that the two abnormalities segregated independently.

Table 1 shows the results of the present study. All patients considered to have HS either had been splenectomized or had splenomegaly (H), except for B, in whom physical examination of the abdomen was unsatisfactory. Jaundice was not clinically evident. G3PD deficiency alone was not accompanied by clinical manifestations. Blood chemistries were normal in all family members except H, who had HS, was not splenectomized, and whose total bilirubin was 2.2 mg/dl (0.1 mg/dl direct reacting). Both H and B, HS patients who still retained their spleens, were the only members with significantly elevated reticulocyte counts. None of the individuals studied was anemic. Spherocytes were present in the peripheral blood smears of all patients with HS. No morphologic difference was noted in family members with isolated G3PD deficiency and normals, nor was there any difference in appearance of red cells between patients with HS alone and those with combined HS and G3PD deficiency. The results of the incubated OF are presented in Table 1, as that concentration of sodium chloride at which 50% of the red cells were hemolyzed. No difference in this value is evident comparing those patients with HS alone and those with HS and G3PD deficiency. The results of autohemolysis tests confirm the OF studies. It is of interest that the degree of autohemolysis detected in the nonsplenectomized HS–G3PD-deficient patient (H) was only partially corrected by the addition of glucose (see Discussion).
Table 1. Comparison of Clinical and Hematologic Data
With Biochemical Features of RBC Membranes

<table>
<thead>
<tr>
<th>Kindred Subgroups</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Spleen*</th>
<th>Hemoglobin (g/dl)</th>
<th>Reticulocytes</th>
<th>Incubated Of 50% Hemolysis (NaCl g/dl)</th>
<th>Autohemolysis 48 hr</th>
<th>G3PD (µm/min/mg)</th>
<th>RBC Membrane PAGE SDS (%)</th>
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<tr>
<td>Normal</td>
<td>C</td>
<td>F</td>
<td>0</td>
<td>13.5</td>
<td>0.6</td>
<td>0.48</td>
<td>1.8</td>
<td>1.3</td>
<td>0.73</td>
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<tr>
<td></td>
<td>J</td>
<td>F</td>
<td>0</td>
<td>12.5</td>
<td>0.6</td>
<td>0.47</td>
<td>0.6</td>
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<td></td>
<td>L</td>
<td>F</td>
<td>0</td>
<td>13.5</td>
<td>1.0</td>
<td>0.53</td>
<td>0.4</td>
<td>0.2</td>
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<td></td>
<td>M</td>
<td>M</td>
<td>0</td>
<td>15.1</td>
<td>0.4</td>
<td>0.53</td>
<td>0.5</td>
<td>0.2</td>
<td>0.90</td>
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<td></td>
<td>Control</td>
<td>48</td>
<td>M</td>
<td>15.4</td>
<td>1.6</td>
<td>0.52</td>
<td>1.3</td>
<td>0.8</td>
<td>0.98</td>
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<td></td>
<td>14.0 ± 1.2</td>
<td>0.8 ± 0.5</td>
<td>0.51 ± 0.03</td>
<td>0.9 ± 0.6</td>
<td>0.6 ± 0.5</td>
<td>0.84 ± 0.12</td>
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<td>Hereditary</td>
<td>spherocytosis</td>
<td>B</td>
<td>11</td>
<td>F</td>
<td>13.8</td>
<td>10.7</td>
<td>0.58</td>
<td>24.7</td>
<td>1.6</td>
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<td></td>
<td>14.9 ± 1.5</td>
<td>6.3 ± 0.2</td>
<td>6.02 ± 0.05</td>
<td>25.4 ± 0.9</td>
<td>2.9 ± 1.8</td>
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<td>G3PD deficiency</td>
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<td>11.7</td>
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<td>0.45</td>
<td>1.3</td>
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<td>G</td>
<td>M</td>
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<td>16.5</td>
<td>2.1</td>
<td>0.50</td>
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<td>0.7</td>
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<td></td>
<td>K</td>
<td>F</td>
<td>0</td>
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<td>0.53</td>
<td>0.8</td>
<td>0.6</td>
<td>0.41</td>
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<td>14.2 ± 2.4</td>
<td>1.2 ± 0.8</td>
<td>0.49 ± 0.04</td>
<td>1.2 ± 0.3</td>
<td>0.7 ± 0.1</td>
<td>0.42 ± 0.03</td>
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<td>Hereditary</td>
<td>spherocytosis and</td>
<td>Proband</td>
<td>43</td>
<td>M</td>
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<td>3.7</td>
<td>0.64</td>
<td>28.0</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>G3PD deficiency</td>
<td>D</td>
<td>40</td>
<td>F</td>
<td>14.3</td>
<td>1.9</td>
<td>0.60</td>
<td>7.4</td>
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<td></td>
<td>15.3 ± 1.1</td>
<td>4.3 ± 0.4</td>
<td>0.63 ± 0.03</td>
<td>16.1 ± 7.8</td>
<td>4.1 ± 3.6</td>
<td>0.44 ± 0.06</td>
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</table>

*0, spleen not palpable; +, spleen enlarged; R, splenectomy.
Figure 2 is a photograph of the PAGE SDS gels of the entire kindred identified as in Table 1. Note the decrease in band 6 in members A, G, and K with G3PD deficiency alone, and in D, E, H, and I with hereditary spherocytosis and G3PD deficiency combined.

A densitometric scan of the PAGE SDS of the RBC membranes from a normal control is shown in Fig. 3A. Figure 3B demonstrates that the proband’s RBC membranes, when compared with the control membranes (Fig. 3A), showed decreased band 6. This change has not been observed previously in HS patients. As Table 1 demonstrates, the proband and seven other members of his family have approximately half the normal amount of band 6. Since band 6 is known to correspond to G3PD, G3PD assays of RBC membranes were also performed (Table 1). Figure 4 relates membrane G3PD activities to the percentage of band 6 in membrane polypeptides. The line derived by analysis of covariance had an $r$ value of 0.808, showing positive correlation at the 0.0005 level of significance. The clustering of the family members into two groups with the involved individuals having half normal band 6 and half normal enzymatic activity is evident. Examination of Fig. 4 shows that the statistically derived line intercepts the per cent band 6 axis close to the origin, a finding which suggests that most of the band 6 is enzymatically active. No significant difference was found between band 6 levels or in enzymatic activities between membranes with G3PD deficiency alone and with G3PD deficiency and HS.

In the family study reported in Table 1, the G3PD assay was performed on isolated RBC membranes. Under the conditions of hemolysis, almost all the G3PD was membrane-bound, and membrane preparation and assay of G3PD allowed both direct comparison with band 6 in PAGE SDS and permitted the simultaneous measurement of band 4.5. Total RBC G3PD on other occasions was 26 $\mu$moles/min/g Hb for the proband compared to 40 $\mu$moles/min/g Hb
Fig. 3. (A) Normal red cell membrane polypeptides on SDS PAGE. Scan of Coomassie blue stained 5% gel. Bands identified as in Steck.6 (B) Membrane polypeptides on PAGE SDS of proband. Note increase in band 4.5 and decrease in band 6. (C) Membrane polypeptides on PAGE SDS from normal RBC incubated for 24 hr in Dulbecco’s phosphate-buffered saline without added glucose. Note increase in band 4.5.
for the control. In addition determination of whole RBC G3PD at 37°C by Dr. Kouichi R. Tanaka, UCLA School of Medicine gave values of 16.7, 18.6, and 17.8 μmoles/min/10¹⁰ RBC for the proband and 34.7 and 38.1 μmoles/min/10¹⁰ RBC for the control. Thus, total RBC G3PD of the proband was also half normal. Dr. Tanaka also measured 19 other RBC enzymes all of which were normal, except for the acetylcholinesterase, which was increased in the proband consistent with the diagnosis of hereditary spherocytosis.

In those individuals in whom RBC levels of G3PD were decreased, the isolated enzyme appeared to be qualitatively normal. Thus, G3PD that was 90% pure by PAGE SDS had the same enzymatic activity per milligram of protein in deficient individuals as in controls. The thermal stability (Fig. 5) and pH optimum (Fig. 6) of G3PD from deficient individuals resembled control values.

Kinetic studies in which the Michaelis constant, $K_m$, was derived by double reciprocal plots showed no differences in the affinities of the G3PD of the proband and normal G3PD for both G3P (Fig. 7) and NAD (Fig. 8).

A second abnormality detected in the RBC membrane proteins of the proband was an increase in the first component of polypeptide 4.5 (compare
Fig. 6. pH optimum curve: comparison of activity of G3PD preparations from proband with the average of control values at the pH shown in the pyrophosphate arsenate assay buffer.

Fig. 7. Double reciprocal plot of activity of G3PD from proband (14 µg protein) compared with two concentrations of control G3PD preparations (32 µg lower curve, and 8 µg upper curve). Here concentrations of G3P were varied. For both control and proband, $K_{mG3P} = 60$ µmoles/liter.
HEREDITARY SPHEROCYTOSIS

Fig. 2. Double reciprocal plot of activity of G3PD from proband, compared with G3PD from control (both 32 μg). Here concentrations of NAD were varied. For both control and proband, \( K_{\text{m}} \text{NAD} = 11 \) μmoles/liter.

![Double Reciprocal Plot](image)

Fig. 3B with Fig. 3A). Polypeptide 4.5 with an apparent molecular weight of 60,000 also increased after sterile incubation of normal RBC in vitro at 37°C in calcium-containing phosphate-buffered saline without added glucose (Fig. 3C). Evidently band 4.5 is one of the cytoplasmic proteins absorbed on the membrane during glucose deprivation. A highly significant increase in band 4.5 \( (p < 0.005) \) was present in the membranes of patients with the combined abnormality (Table 1), while there was no statistically significant increase in band 4.5 of patients with HS alone. While band 4.2 was decreased in the proband (Fig. 3B), there was no significant overall decrease in this band in his HS kindred \(^3\) (Fig. 2). Likewise, there was no significant difference in the per cent of other membrane polypeptides or in membrane-bound hemoglobin between the various kindred subgroups shown in Table 1. Other abnormalities in PAGE SDS of the 1-day incubated RBC (Fig. 3C), such as the relative decrease of band 3, are being investigated. An RBC membrane PAGE SDS pattern very similar to that seen in Fig. 3C results from membranes obtained with fresh RBC and the addition of 0.01% calcium chloride to the hemolyzing solution, \(^6\) confirming the role of increased RBC calcium uptake in the changes produced by sterile incubation. \(^15\)

DISCUSSION

This kindred offers a unique opportunity to analyze the interaction of a partial deficiency of a glycolytic enzyme with the increased demands for glycolysis in HS. In HS cells, glucose catabolism is increased 35%, apparently to compensate for a leaky RBC membrane; \(^17\) it is likely that the deficiency of a
glycolytic enzyme so closely linked to cation transport presents a special hazard for such cells.18 Thus, RBC from H, the patient with HS and G3PD deficiency, who had not been splenectomized, are not well protected against autohemolysis by addition of glucose (see Table 1).

The increase in polypeptide 4.5 provides insight into the mechanisms of red cell aging and destruction. The fall in intracellular ATP in red cells incubated in vitro without added glucose results in a gain in intracellular calcium, and increased hemoglobin and nonhemoglobin protein in the membrane fraction, which are associated with decreased cellular deformability and filterability.15 It has been suggested that these changes result from increased absorption of cytoplasmic proteins to the inner surface of the membrane resulting from a calcium-induced sol to gel transformation.15 We have recently investigated these cytoplasmic proteins by PAGE SDS of the membrane and have identified band 4.5 as one of the primary cytoplasmic polypeptides binding to the red cell membrane in glucose-deprived cells. Thus, it seems quite likely that the increased band 4.5 observed here in cell membranes from patients with both HS and G3PD deficiency is provided by a similar mechanism. Such doubly affected cells having an increased requirement for glycolysis with a diminished glycolytic capacity may be especially likely to have increased band 4.5. A similar increase in 4.5 should be sought in other hemolytic anemias associated with metabolic stress, and indeed, in larger sample populations of patients with HS alone.

Study of this kindred demonstrates that HS and G3PD deficiency traits segregate independently and occur in both males and females, and thus are probably present on separate autosomal genes. G3PD-deficient family members have approximately half the normal RBC enzyme content. The enzyme present, however, is normal in all properties tested. Thus, it is likely that the abnormal gene in this kindred results in production of no G3PD recognizable either by PAGE SDS of the membranes or by specific assay. In none of the patients with G3PD deficiency alone has hemolytic anemia been present, in distinction to the previous case report.6

The results reported here emphasize the value of family studies for identifying a particular chemical abnormality with a clinical finding. Although it was initially assumed from studies of the proband alone that band 6 (G3PD) deficiency was associated with HS,19 it was clear from examination of other family members that this enzyme deficiency was not etiologically related to this disease.

REFERENCES


7. Allen DW, Cadman S: Increased binding of cytoplasmic proteins to the red cell membrane produced by metabolic stress or sulfhydryl inhibition. Fed Proc 34:552, 1975


Study of a kindred with hereditary spherocytosis and glyceraldehyde-3-phosphate dehydrogenase deficiency

SR McCann, B Finkel, S Cadman and DW Allen