Identification of the Hereditary Pyropoikilocytosis Carrier State

By William C. Mentzer, Tikva Turetsky, Narla Mohandas, Stanley Schrier, Chuen-Shang C. Wu, and Harold Koenig

We evaluated the hematologic, rheologic, and biochemical features of erythrocytes obtained from 10 relatives of a 5-yr-old black female with hereditary pyropoikilocytosis (HPP) and severe hemolytic anemia. Erythrocyte morphology was normal in the father and five other relatives, but ghost mechanical fragility and drug-induced red cell endocytosis were increased, as was the percentage of spectrin dimers noted on 3.2% non-denaturing PAGE of spectrin extracts. Identical changes were also noted in the mother and her sister, whose erythrocytes were elliptocytic and exhibited morphological changes upon heating to 45°-48°C (normal 49°C). The two other family members were normal in every respect. SDS-PAGE analysis of membrane proteins demonstrated diminished amounts of spectrin in HPP erythrocytes, but was normal in other family members. A diffuse band (mol wt 575,000-865,000), composed entirely of spectrin, was apparent adjacent to the dimer region on nondenaturing PAGE of spectrin extracts. The nature of such defects has not yet been fully elucidated at the molecular level. Available information regarding the genetics of the HPP syndrome is limited. Usually both parents have been clinically and hematologically normal, although occasionally, one parent has exhibited nonhemolytic elliptocytosis. Liu and Palek found increased mechanical fragility of membrane cytoskeletal preparations and abnormal spectrin dimer–dimer interactions in the nonelliptocytic mothers of two HPP patients. Dhermy and coworkers noted similar in vitro abnormalities in spectrin obtained from the mother of a patient with severe elliptocytic hemolytic anemia and, in addition, found that heated maternal red cells became less deformable than did control cells at temperatures between 45°C and 49°C. No biochemical information has been available on elliptocytic HPP relatives. We have recently obtained such information through study of a single patient with HPP and ten of her relatives. In this family, two different abnormalities of the membrane skeleton, one associated with elliptocytosis and the other not, were detected. The HPP propositus appeared to be a compound heterozygote for these two distinct genetic abnormalities.

MATERIALS AND METHODS

Deformability Measurements

We measured the deformability of intact red cells and resealed erythrocyte ghosts in an ektacytometer. This device imposes a well-defined laminar shear stress field on the cells, while simultaneously monitoring the extent of cell deformation by laser diffractometry. A "deformability index" (DI) is obtained, which is equivalent to the ellipticity of the deforming cells. In the standard mode of operation, DI is recorded continuously as a function of shear rate. For measurement of intact red cell deformability, 10 μl of a 40% red cell suspension was thoroughly mixed with 3 ml of polyvinyl pyrrolidone (PVP, mol wt 360,000, 4 g/dl w/v, 32.6 cp at 20°C, 290 mosmole/kg, pH 7.4). This suspension produced a maximum shear stress of 170 dynes/sq cm at 100 rpm. Numerical values of the maximum deformability index reached, defined as DI_max, were used to compare the deformability of different samples. For measurement of the deformability of resealed membranes, 30 μl of packed resealed ghosts (approximately 250 × 10^6) were suspended in 3 ml Stratran (22 cp viscosity, 290 mosmole/kg, pH 7.4). The ektacytometer was also used to measure whole cell deformability of red cells as a continuous function of the suspending medium osmolality at a constant applied shear stress of 170 dynes/sq cm (osmotic gradient ektacytometry). For these studies, the DI of red cells was continuously recorded as the suspending medium osmolality was linearly increased from 50 to 500 mosmole/kg. As previously shown, the curve showing the variation of DI with suspending medium osmolality can be analyzed to provide information about initial cell surface area, surface area-to-volume ratio, and cell water content.
Membrane Stability Assay

To obtain a measure of membrane resistance to shear-induced fragmentation, we performed a membrane fragmentation assay using the ektacytometer. Resealed ghosts were suspended in a dextran solution of 97 cp viscosity (dextran mol wt 40,000, 35 g/dl w/v, 290 mosmole/kg, pH 7.4). Rotation of the ektacytometer chamber at a speed of 110 rpm generated a shear stress of 575 dynes/sq cm. Continuous application of this stress resulted in progressive fragmentation of the intact membranes into small undeformable spherical fragments. This process was detected as a decrease in the Dl, which was monitored as a function of time. The time required for the Dl to fall to 10% of its maximum value was taken as a measure of the susceptibility of ghosts to shear-induced fragmentation, and hence, of membrane stability.

Red Cell Thermal Fragility

Blood was collected in sodium heparin or acid citrate dextrose (formula A), and stored at 4°C for not more than 48 hr before processing. Blood was filtered through cotton and washed once with phosphate-buffered saline, pH 7.4, and then twice with 132 mM sodium chloride, 5 mM potassium chloride, 10 mM sodium phosphate, and 20 g/dl glucose, pH 7.4 (BSKG). A 50% erythrocyte suspension was added to prewarmed BSKG solution and heated in a water bath for 10 min at various temperatures between 37°C and 50°C and then immediately transferred to ice. Red cells in the heated suspensions were fixed in 1% glutaraldehyde in isotonic phosphate-buffered saline, and then examined by phase-contrast microscopy for membrane distortions and budding. Fragmentation of the heated red cells was also evaluated by measuring the accumulation of small particles in the suspensions using a Coulter Counter Model ZBI, (Coulter Electronics, Inc., Hialeah, FL). A suspension of 10,000–20,000 cells/ml in isotonic buffered saline was counted and then sized utilizing a Channelizer II (Coulter Electronics, Inc.). Fragmentation was defined as $F = F_{0.04}/F_{0.96}$, where $F_{0.04}$ and $F_{0.96}$ are the fractions of particles integrated when the lowest channel size limit is selected to 0.04 and the largest to 0.96, respectively.

Ghost Preparation

Blood was filtered through cotton to remove white cells, then washed 3 times in phosphate-buffered saline, pH 7.4. Membranes were prepared by hypotonic lysis at 4°C essentially as described by Dodge et al. using 5 mM sodium phosphate, pH 8.0, with added 0.2 mM phenyl-methyl-sulfonyl-fluoride (PMSF) to minimize proteolysis. Ghosts were stored at −70°C if not analyzed immediately.

Spectrin Extraction

Fresh ghosts were washed twice and then dialyzed for 20–24 hr at 0–4°C in low ionic strength buffer containing 0.1 mM sodium phosphate, 0.1 mM ethylene diamine tetraacetic acid (EDTA), and 0.2 mM PMSF at pH 8.0. The extracted ghosts were then centrifuged at 39,100 g for 40 min at 4°C and the supernatant harvested. Changes in the extractability of spectrin induced by prior heating of erythrocytes to various temperatures between 37°C and 50°C were assessed as described by Mohandas and Greenquist. The effect of heat on the circular dichroism of spectrin was determined in 4°C low ionic strength extracts, as described by Chang and coworkers. Spectrin was obtained for studies of dimer to tetramer transformation in solution by extraction of ghosts for 20 min at 37°C in low ionic strength buffer containing 0.1 mM sodium phosphate, 0.1 mM EDTA, 0.1 mM PMSF, 0.1 mM N-acetyl-L-lysine chloromethyl ketone (TLCK), and 0.1 mM 2-mercaptoethanol, pH 8.0.

Nondenaturing Polyacrylamide Gel Electrophoresis

Nondenaturing polyacrylamide gel electrophoresis (PAGE) of low ionic strength membrane skeleton extracts, which consisted predominantly of spectrin, was performed as described by Liu and Palek with modifications. Acrylamide tube gels (3.2%) (with bisacrylamide content equal to 4% of total acrylamide) were prepared in a 40-mM Tris buffer, pH 7.4, which also contained 20 mM sodium acetate and 2 mM EDTA. Crude spectrin extracts (protein concentration 500 µg/ml) were mixed with 1/4 volume of concentrated buffer to give a final solution containing 40 mM Tris–HCl, 20 mM sodium acetate, 2 mM dithiothreitol (DTT), 200 mM sucrose, and 2 mM EDTA, pH 7.4. Electrophoresis was performed in the same Tris/sodium acetate/EDTA buffer described above, at 50 V for 17 hr at 0–4°C. Proteins were stained with Coomassie Brilliant Blue R-250 (Bio-Rad, Richmond, CA) for 2 hr at 60°C or overnight at room temperature. The gels were destained in 25% isopropanol alcohol and 10% acetic acid, and the staining intensity of the protein bands was determined by gel scanning at 545 nm. The relative protein content of each peak was estimated on an Apple II Computer Graphics tablet. These results correlated well with those obtained by cutting the tracings of the protein peaks and weighing on an analytical balance.

SDS-Polyacrylamide Gel Electrophoresis

One-dimensional SDS-PAGE was performed as described by Laemmli with a 4.5% acrylamide stacking gel and a 5%–15% linear acrylamide gradient separating gel. Four percent SDS-polyacrylamide tube gels (Bio-Rad) were used to evaluate the ratio between spectrin and band 3 in extracted and unextracted ghosts. Membrane aliquots were mixed with dissolving buffer to a final concentration of 1 g/dl SDS, 10 mM sodium phosphate, pH 7.0, 0.1 M DTT, 10 g/dl glycerol, and boiled for 3 min. Bromo-phenol blue was used as a tracking dye. Approximately 35 µg protein (determined by absorbance at 280 nm) was loaded on each gel. Two-dimensional electrophoresis combined one-dimensional—3.2% nondenaturing PAGE and SDS electrophoresis in the second dimension. First-dimension nondenaturing gels were stored at −70°C in SDS buffer (3 g/dl SDS, 0.38 M DTT, 8 g/dl glycerol, and 0.19 M Tris–HCl, pH 6.8) until used. For electrophoresis in the second dimension, a 10% acrylamide gel was used. One-dimension gels were secured to the slab gel as described by O’Farrell.

Transformation of Spectrin Dimer to Tetramer in Solution

The extent of dimer association to form tetramers was evaluated as described by Liu and Palek. Spectrin extracted at 37°C was then incubated at 30°C in an isotonic solution containing 5 mM sodium phosphate, pH 7.4, 150 mM sodium chloride, 1 mM mercaptoethanol, 0.1 mM PMSF, 0.1 mM TLCK, and 0.1 mM EDTA. Finally, the incubated extract was electrophoresed on 3.2% nondenaturing gels (as above).

Endocytosis

Drug-induced red cell endocytosis was measured as previously described. All values obtained in family B were compared to control samples that had been processed and run simultaneously.

CASE HISTORY

CB, a 5-year-old black female, was anemic (cord blood hematocrit 42%) and jaundiced during the neonatal period. Erythroblastosis
and striking spherocytosis, as well as many bizarre and fragmented red blood cells, were noted on the peripheral blood smear. Hemolysis persisted throughout infancy and early childhood. The hemoglobin ranged from 4.7 to 10.9 g/dl, and the mean reticulocyte count was 14% (mean absolute reticulocyte count 54,500/cu mm). Transfusions were required on 6 occasions prior to the age of 21 mo for episodes of unusually severe anemia which, in general, were not associated with fever or reticulocytopenia. From 7 to 11 mo of age, she was treated with 2 mg/kg of prednisone daily for a suspected autoimmune hemolytic process, although the direct antiglobulin test was consistently negative. Mean hemoglobin levels rose several grams per deciliter during treatment with prednisone, but there was no decrease in the reticulocyte count. Splenomegaly gradually developed after the first year of life. Splenectomy was performed at the age of 4 yr 10 mo. Following splenectomy, the hemoglobin rose to normal, but evidence of hemolysis persisted. Representative blood counts prior to splenectomy were: Hb 8.4 g/dl, Hct 22%, RBC count 4,280,000/cu mm, mean corpuscular volume (MCV) 51.4 fl, mean corpuscular hemoglobin concentration (MCHC) 38.2%, and reticulocyte count 14%. Four months postsplenectomy, the Hb was 13 g/dl, Hct 32%, RBC count 5,650,000/cu mm, MCV 56.6 fl, MCHC 40.6%, and reticulocyte count 3.1%. The serum bilirubin ranged from 1.6 to 2.5 g/dl, but other serum chemistry values, including iron, folate, and B12 levels, were normal, as were the antiglobulin test and hemoglobin electrophoresis (including quantitation of A2 and F hemoglobins). All family members evaluated by us were in good health and nothing in the history suggested the presence of a congenital hemolytic anemia in other, unevaulated family members.

RESULTS

Hematologic Studies

With the exception of the anemic HPP propositus, peripheral blood counts and red blood cell indices were normal in all family members we evaluated (Fig. 1). Red blood cell morphology was characteristic of HPP in the propositus, in whom extreme microcytosis, dense spherocytes, microelliptocytes, budding, and irregular red cell fragments were evident. In both the mother and the maternal aunt, the majority of erythrocytes were elliptocytic, but in all other family members, red cell morphology was normal. Complete blood grouping ruled out any linkage between elliptocytosis and Rh genotype in affected family members. Blood grouping studies were not consistent with false paternity.

Membrane Proteins

One-dimension SDS-PAGE analysis revealed more than the usual amount of band 4.5, band 8, and globin in red cell membrane preparations from the propositus and, to a lesser extent, in those from the maternal aunt. The spectrin/band 3 ratio, used to estimate the amount of spectrin present in the membrane, was reduced to 0.63 ± 0.09 (n = 5) in red cell membranes from the propositus (normal 0.92 ± 0.14), but was normal in all other family members. No qualitative abnormalities in membrane proteins were detected in the propositus or other family members (gels not shown).

At 37°C, more than 95% of the spectrin was extractable from red cells of the propositus, other family members, or normal controls. Heating to 47°C reduced the amount of spectrin extractable from HPP erythrocytes to less than 70%, and at 49°C, spectrin became virtually inextractable. Equivalent changes in spectrin extractability from normal red cells required exposure to temperatures 2°C higher than was the case in HPP. When crude spectrin extracts (obtained at 4°C) were heated to temperatures between 32°C and 58°C, the thermal denaturation of spectrin, monitored by circular dichroism measurements, occurred at 49°C in controls and at 47.5°C in an extract prepared from HPP propositus red cells.

To evaluate spectrin dimer–dimer associations we prepared low ionic strength extracts of red cell membranes at 0°C and then subjected the extracts to nondenaturing gel electrophoresis (Fig. 2). A high molecular weight fraction and spectrin tetramers were the predominant species found in normal extracts (Fig. 2, Table 1). Extracts obtained from erythrocytes of the

![Fig. 1. Pedigree of family B.](image-url)

![Fig. 2. Spectrin species in low salt extracts (prepared at 0°C) of red cell membranes from members of family B. Denatometric tracings of nondenaturing 3.2% polyacrylamide electrophoreograms are shown. HMW, high molecular weight complexes of spectrin and other membrane proteins; Sp-T, spectrin tetramers; Sp-D, spectrin dimers.](image-url)
Table 1. Hematologic, Rheologic, and Cytoskeletal Features of the Erythrocytes From Members of Pedigree B

<table>
<thead>
<tr>
<th></th>
<th>Ghost Mechanical Fraility (T%, sec)</th>
<th>Spectrin Dimers (%)</th>
<th>Drug-Induced RBC Endocytosis†</th>
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<tr>
<td><strong>HPP</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III-8</td>
<td>20</td>
<td>65.5 (24.5)</td>
<td>3.07</td>
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<tr>
<td>Elliptocytic HPP carriers</td>
<td></td>
<td></td>
<td>4.17</td>
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<tr>
<td>II-6</td>
<td>48</td>
<td>38.9 (20.8)</td>
<td>2.80</td>
</tr>
<tr>
<td>II-7</td>
<td>37</td>
<td>44.6 (8.9)</td>
<td>9.46</td>
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<tr>
<td>Nonelliptocytic HPP carriers</td>
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<td></td>
<td>1.66</td>
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<td>I-2</td>
<td>61</td>
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<td>I-3</td>
<td>64</td>
<td>25.6</td>
<td>2.26</td>
</tr>
<tr>
<td>Normal family members</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>II-3</td>
<td>130</td>
<td>4.2</td>
<td>1.12</td>
</tr>
<tr>
<td>II-8</td>
<td>135</td>
<td>5.2</td>
<td>1.25</td>
</tr>
<tr>
<td>Normal controls</td>
<td>133 ± 14*</td>
<td>6.3 ± 4.2*</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*Mean ± standard deviation.
†The percentage of dimers in samples from III-8, II-6, and II-7 could not be measured accurately due to the presence of an adjacent, higher molecular weight species (see Fig. 2). The value given is for both dimer and the adjacent species. An estimate of the percentage of dimer alone is indicated in parentheses.
‡Expressed as fold increase when compared to simultaneously measured normal control. To estimate the significance of the fold increase, regression analysis of control values versus affected family member (HPP and carriers) was done. For primaquine, the estimate of the mean fold increase (slope of regression line) was 2.47, which was significantly greater than 1 (p < 0.005). The estimated mean fold increase for vinblastine was 2.11 (p < 0.025).

father and five other nonelliptocytic family members contained an increased proportion of spectrin dimers but were otherwise normal. Dimers were also increased in extracts obtained from the two elliptocytic family members and an additional peak, designated x, was evident between the dimer and tetramer peaks. Extracts from red cells of the propositus had an even greater proportion of dimers and an accentuation of the x component noted in the elliptocytic relatives. The nature of the x component was evaluated by subjecting extracts to two-dimensional gel electrophoresis (non-denaturing PAGE:SDS-PAGE). These studies indicated that the x component consisted solely of spectrin (Fig. 3). Only normal-sized α and β-spectrin subunits were evident on SDS-gel electrophoresis. Other properties of the x component are listed in Table 2. The apparent molecular weight ranged between 575,000 and 665,000. In extracts obtained at low temperature (0°C) from propositus red cells, the x component comprised nearly half of the total amount of lower molecular weight spectrin (tetramer or smaller). Lesser amounts of the x component were present in the red cells of elliptocytic relatives. Appreciable amounts of the x component were also obtained by extraction of spectrin at higher temperature (37°C). Prolonged electrophoresis (48 hr) at low voltage (25 V) or electrophoresis in 0.3% agarose plus 2.5% acrylamide gels had no effect on the amount of the x component. Following nondenaturing electrophoresis of crude spectrin extracts obtained from maternal (Table 1, II-6) red cells, the x component was isolated by excision of the appropriate gel segment. The x component was then electroeluted and evaluated by SDS-PAGE. The ratio of band 1 to band 2 in the x component was 1.30, similar to the whole crude spectrin extract (1.19).

The ability of spectrin dimers to reassociate in vitro was evaluated by first obtaining dimer-rich preparations of spectrin by extraction at 37°C and then promoting subsequent transformation of dimers to tetramers by incubation at 30°C as described by Liu and Palek (Fig. 4). In normal individuals, more than 95% of spectrin was in the form of dimers following extraction. In the propositus and the two elliptocytic relatives, there seemed to be relatively more tetramers than seen in normal individuals, but, because the x component and tetramer were not separated from one another by the electrophoretic technique employed, the amount of tetramer could not be quantitated. As shown in Fig. 4, there seemed to be less reassociation of spectrin dimers into tetramers in the two elliptocytic relatives than in a normal control. Almost no reassociation of dimers to tetramers was noted in spectrin from the HPP propositus. In vitro dimer–tetramer transformation had little or no influence on the amount of the x component (Fig. 4).

Red Cell Fragmentation and Deformability

Red cell thermal fragility was evaluated by heating cells suspended in buffered saline and glucose to various temperatures between 44° and 50°C.
Fig. 2. Two-dimensional gel electrophoretograms of low salt extracts of red cell membranes, prepared at 0°C. First dimension: nondenaturing 3.2% polyacrylamide gel electrophoresis. Second dimension: SDS-PAGE (10% polyacrylamide). (Top gel) Normal control; (Bottom gel) mother of HPP propositus. A diffuse band (x) is evident between the spectrin dimer (D) and tetramer (T) regions in the extract prepared from the HPP family member, but not in the control extract. In both extracts, the high molecular weight component (HMW) is composed of spectrin plus other smaller proteins, notably band 5. In contrast, no proteins smaller than spectrin are evident below the x component, spectrin dimers (D), or spectrin tetramers (T). The small amounts of band 5 and band 7 present in low salt extracts, but not complexed with spectrin, are visible in the lower left quadrant of both gels.

In these conditions, control red cells first exhibited budding and other membrane distortions at 49°C and released small fragments between 49° and 50°C. In contrast, in the propositus, both budding (44°) and fragmentation (46°) were noted at considerably lower temperatures. Membrane distortions and budding were first noted at 45°C in the maternal aunt and 48°C in the mother, and in both women fragmentation occurred between 49° and 50°C. Red cell thermal stability was normal in other family members.

The osmotic fragility of both fresh and incubated red cells from the propositus was markedly increased. The effect of osmolarity on red cell deformability was also explored, using the technique of osmotic gradient ektacytometry (Fig. 5). In hypotonic media, the nadir of the deformability index, which coincides with the mean osmotic fragility, was shifted to higher osmolalities in red cells from the propositus, which is consistent with their increased osmotic fragility. Propositus red cells were less deformable than normal at all osmolalities between 150 and 380 mosmole. The maximum deformability of erythrocytes from elliptocytic family members was also subnormal. The red cells of two nonelliptocytic relatives were completely normal, and those of remaining family members were slightly less deformable than normal, but not to the extent noted in

Table 2. Properties of Abnormal Spectrin Species (x Component)
(see Fig. 2)

<table>
<thead>
<tr>
<th></th>
<th>Apparent Mol. Wt.</th>
<th>Amount Present*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>4°C 37°C 30°Ct</td>
<td></td>
</tr>
<tr>
<td>Elliptocytosis (II-6)</td>
<td>575,000</td>
<td>24.6 13.7 14.2</td>
</tr>
<tr>
<td>Elliptocytosis (II-7)</td>
<td>665,000</td>
<td>35.7 24.9 27.8</td>
</tr>
<tr>
<td>HPP propositus (III-1)</td>
<td>585,000</td>
<td>42.8 20.7 25.4</td>
</tr>
</tbody>
</table>

*Abnormal species/dimer + tetramer + abnormal species × 100.
†Following 4-hr incubation in high salt buffer to promote dimer to tetramer transformation.

Fig. 3. Spectrin dimer to tetramer transformation in solution. (—) Spectrin species in low salt extracts prepared at 37°C. (-----) Spectrin species following incubation of 37°C extracts for 4 hr at 30°C (see Materials and Methods). Denaturation tracings of nondenaturing 3.2% polyacrylamide electrophoretograms are shown. HMW, high molecular weight complexes of spectrin and other membrane proteins; SpT, spectrin tetramers; SpD, spectrin dimers.

Fig. 4. Osmotic deformability profiles for red cells from members of family B.
elliptocytic red cells. Red cell ghost deformability, measured in the ektacytometer, was normal in the two family members in whom whole cell deformability studies were normal, but was decreased in all other relatives studied, in rough proportion to the extent of the deformability decrease noted in whole cells.

To measure the mechanical stability of the red cell membrane, we subjected red cell ghosts to a constant shear force of 575 dynes/sq cm and monitored their fragmentation in the ektacytometer (Fig. 6). The half-time for fragmentation in normal ghosts was 120–145 sec (Table 1). Ghosts from the propositus were extremely fragile, with a half-time of only 20 sec. The two relatives with elliptocytosis, as well as six otherwise apparently normal relatives, including the father, exhibited intermediate abnormalities in ghost mechanical stability. The mechanical stability of ghosts was completely normal in two other family members.

Endocytosis

Red cells from the propositus exhibited increased drug-induced endocytosis using either primaquine or vinblastine as an endocytogenic agent (Table 1). All relatives in whom ghost mechanical stability was abnormal also showed greater than normal primaquine-induced endocytosis, and most, but not all, of these relatives exhibited increased vinblastine-induced endocytosis as well. One relative in whom ghost mechanical stability was normal was studied and did not show increased endocytosis with either primaquine or vinblastine.

DISCUSSION

These studies provide supportive evidence for involvement of spectrin in the cytoskeletal abnormalities of HPP. First, as previously reported in other HPP patients, the relative amount of spectrin in the membrane of the propositus was less than normal as estimated by determinations of the ratio of spectrin to band 3. Whether the apparent lack of spectrin in HPP erythrocytes is due to diminished synthesis or to enhanced degradation of a structurally abnormal molecule cannot be determined from the studies we performed. Second, assembly of the cytoskeleton also appeared to be defective, as reflected by the increased fraction of spectrin dimers present in low salt extracts and by the greatly reduced tetramer formation from dimers noted in vitro. Similar findings have previously been reported by Liu and Palek in two other HPP patients. However, it is important to note that such studies, carried out in dilute solution, may not fully reflect the actual state of spectrin in the intact membrane, where locally high spectrin concentrations may favor the formation of oligomers larger than tetramers. The electrophoretic technique we employed did not allow assessment of such spectrin oligomers. Third, the thermal denaturation of spectrin occurred at a lower temperature in our HPP patient than in controls, which is consistent with earlier studies in HPP by Chang et al. In addition, when red cells from the HPP propositus were heated, spectrin became relatively inextractable at a temperature several degrees below that required to achieve an equivalent effect in normal cells. As was previously noted by Mohandas et al., who studied normal cells, such changes in spectrin extractability closely parallel changes in erythrocyte morphology and fragmentation induced by heating, suggesting a causal relationship between the phase transition in spectrin and the shape changes. Other family members exhibited abnormal spectrin dimer–dimer associations, but did not have a relative decrease in the amount of membrane associated spectrin.

The rheologic studies clearly demonstrated the presence of an intrinsic membrane abnormality in HPP red cells, as ghost as well as whole red cell deformability was decreased and ghost mechanical stability was also impaired. The low deformability index of red cells from the propositus indicated a reduction in the surface area-to-volume ratio, presumably due to the selective loss of membrane components that occurs in HPP. There was, in addition, an increase in the intrinsic rigidity of the cell membrane, since ghost deformability was reduced. The rigidity and density of HPP

Fig. 6. Fragmentation curves for ressealed ghosts prepared from red cells obtained from members of family B. The range for ghosts prepared from normal control red cells is indicated by the shaded area. Symbols for the individual results are the same as in Fig. 5.
cells is likely to contribute to their early demise, as is the case for metabolically depleted cells, cells with primary abnormalities of cation and water balance, and certain hemoglobinopathies. The rheologic abnormalities noted in other family members were milder in character and had no appreciable effect on erythrocyte lifespan.

The endocytosis studies suggest that the red cell membrane lesion in HPP is different than that present in hereditary spherocytosis and several other hemolytic diseases where drug-induced endocytosis is either moderately or severely reduced. In fact, HPP is the only red cell disorder we have discovered to date where drug-induced endocytosis is regularly increased. Primaquine endocytosis has an absolute requirement for ATP, whereas some vinblastine endocytoses can occur even in the absence of measurable red cell ATP. The fact that both primaquine and vinblastine endocytosis are increased in HPP suggests that the metabolic status of HPP red cells plays a less important role than their unusual membrane features in the as yet undefined abnormality that leads to enhanced endocytosis.

It seems reasonable to assume that the gene responsible for elliptocytosis in this family also contributes to the HPP syndrome noted in the propositus. A variety of membrane protein abnormalities may result in elliptocytosis. Whether each of these genetically distinct forms of elliptocytosis can result in an HPP syndrome if inherited in a homozygous or compound heterozygous state is unknown.

A distinctive feature of cytoskeletal preparations from elliptocytic family members and from the propositus was the presence on nondenaturing PAGE of a diffuse collection of spectrin, designated x, approximately midway between the dimer and tetramer peaks. The possibility that this might represent proteolysis or oligomers formed from abnormally small subunits was eliminated by examination of the composition of this region on SDS-gel electrophoresis. The possibility of spontaneous dissociation of tetramers to dimers during the electrophoretic run was also considered. However, meticulous care was taken to avoid any rise in temperature, which might induce dissociation, and altering the conditions of electrophoresis to reduce heat output had no influence on the abnormality. Furthermore, the x component was also found in spectrin extracts prepared at 37°C, a condition that ensures that the majority of spectrin will be dimeric prior to nondenaturing electrophoresis. Finally, incubation for 4 hr at 30°C, under conditions that favor formation of tetramers, also had no influence on the x component.

The other explanation considered was that the x component was a trimer, composed of a spectrin heterodimer plus a monomeric spectrin subunit. However, the diffuse nature of the abnormal band argues against the presence of a single spectrin species and the apparent molecular weight is well below that predicted for a trimer (690,000). Differences in molecular conformation as well as molecular weight affect electrophoretic migration and might explain the apparent discrepancy in molecular weight. However, the band 1/band 2 ratio near unity observed in the x component obtained from maternal spectrin extracts is not the 2:1 or 1:2 value predicted for a trimer. Although the precise nature of the x component was not revealed by our studies, they do further document the abnormality of spectrin present in this pedigree.

In our hands, changes in ghost mechanical stability, measured with the ektacytometer, proved to be an accurate, relatively simple way to identify nonelliptocytic HPP carriers. The changes in mechanical stability were, in every instance, accompanied by an increase in spectrin dimers, a decrease in red cell deformability, and by an increase in endocytosis. The internal consistency of these various measurements suggests that they are identifying a genuine abnormality in the erythrocyte membrane skeleton, and their resemblance to the more severe defects characteristic of HPP suggest a close relationship to this syndrome. It is of interest, however, that these various abnormalities, so clearly evidenced in vitro, had little or no influence on erythrocyte lifespan in vivo.

The study of pedigree B has provided a clearer view of the genetics of HPP. As proposed in Fig. 1, the propositus appeared to be a compound heterozygote for two different genetic defects, transmitted by elliptocytic and nonelliptocytic carriers. Elliptocytosis is usually inherited as a dominant trait, and it is presumed that the deceased maternal grandfather, who was not tested, was an elliptocytic carrier. Because the maternal grandmother was also found to be an HPP carrier, more complex inheritance patterns remain possible. More direct analysis of the spectrin mutations presumed to be present in this family will be required to distinguish between these alternatives. Until such techniques are perfected, studies such as those described here may be useful to families desiring genetic counseling. Such counseling should be undertaken only with caution, since it seems likely, as is the case for so many other inherited hematologic disorders, that HPP may result from the interaction of a number of different mutations involving spectrin, not all of which can be detected by the laboratory tools available at present.

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