Hereditary Poikilocytic Anemia Associated With the Co-Inheritance of Two Alpha Spectrin Abnormalities

By Terri A. Iarocci, Gail M. Wagner, Narla Mohandas, Peter A. Lane, and William C. Mentzer

This report describes a black family in which two distinct structural defects of alpha spectrin were inherited singly and in combination. The propositus, who has a poikilocytic hemolytic anemia that shares many of the features of hereditary pyropoikilocytosis (HPP) or homozygous elliptocytosis, is a compound heterozygote for both the spectrin alpha^165 and spectrin alpha^150e defects as demonstrated by electrophoretic analysis of spectrin tryptic fragments. The spectrin alpha^165 defect alone was found in his mother and sibling, while the spectrin alpha^150e defect was present in the father and another sibling. The red cell spectrin content was normal in all family members. The functional consequences of inheritance of these two spectrin defects were compared with those found in an unrelated patient with classic HPP who had the alpha^150e spectrin defect and was spectrin deficient as well. Prolonged incubation at 37°C resulted in striking budding, fragmentation, and spering of classic HPP red cells but only minimal changes in propositus cells. The percentage of spectrin dimers was increased tenfold in classic HPP, sevenfold in the propositus, and threefold in other family members. Mechanical stability of erythrocyte ghosts, measured by ektacytometry, was reduced severely in both classic HPP and in the propositus, but only moderately in other family members. Thus, co-inheritance of two alpha spectrin defects can result in a poikilocytic hemolytic anemia milder than that usually found in HPP. The greater clinical severity of HPP may be a consequence of the presence of spectrin deficiency, a finding absent in the propositus.

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MATERIALS AND METHODS

Electrophoresis reagents were purchased from Bio-Rad Laboratories, Richmond, CA; diisopropylfluorophosphate (DFP) and phenylmethylsulfonyl fluoride (PMSF) from Sigma Chemical Company, St Louis; Seakem agarose from FMC Corporation, Rockland, ME; Trypsin-TPCK from Worthington/Cooper Biomedical, Inc, Malvern, PA. Horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies were obtained from Pel-Freeze Biologicals, Rogers, AR.

The study protocol was approved by the Committee on Human Research of the University of California, San Francisco.

Phlebotomy and routine hematology. Routine blood counts and reticulocyte determination were performed on EDTA anticoagulated blood. For all other studies, samples were anticoagulated in acid citrate dextrose and stored at 4°C until studied. All studies were performed within 12 to 24 hours of phlebotomy.

Red cell membrane protein analysis. Red cell membrane ghosts were prepared at 0 to 4°C using techniques that we used previously.7 PMSF 0.2 mmol/L, was added to all preparative solutions to minimize proteolysis. Red cell membrane proteins were analyzed by a single dimensional SDS-PAGE3 using either a 4.5% acrylamide stacking gel and a 4% to 15% nonlinear acrylamide separating gel or a nonlinear 3.5% to 17% polyacrylamide gradient SDS slab gel.3 The spectrin to band 3 ratio was determined by excising the Coomassie blue stained bands (spectrin and band 3), eluting the dye in 25% pyridine overnight, and reading the absorbance at 605 nm.10

Spectrin analysis. A crude spectrin extract was obtained from fresh ghosts by low ionic strength extraction for 16 hours at 0 to 4°C.11 The percentage of spectrin dimers (expressed as dimers/dimers plus tetramers) present in this extract was determined by low temperature (0 to 4°C), nondenaturing 2.5% acrylamide, 0.3% agarose composite gel electrophoresis.12 Spectrin dimers and tetramers were quantitated by densitometry at 545 nm.

An aliquot of the crude spectrin extract was subjected to limited tryptic digestion. PMSF was removed by overnight dialysis in 200 vol of 20 mmol/L Tris buffer, pH 8.0. Trypsin concentration in the extracts was determined with the Bio-Rad protein assay (Bio-Rad Laboratories) using bovine serum albumin as the standard. The spectrin extracts were digested for 16 hours at 4°C using an enzyme to substrate ratio of 1:200 (wt:wt).13 To more completely characterize the kinetics of digestion spectrin was also digested for periods from 30 minutes to 4 hours at 4°C using an enzyme to substrate ratio of 1:20 (wt:wt). Digestion was terminated by adding diisopropylfluorophosphate to a 1 mmol/L final concentration. The spectrin tryptic fragments were analyzed by two dimensional electrophoresis. Isoelectric focusing tube gels were prepared with a pH gradient of 3.5-9.5 and a pH 4-6 second-dimension gel. Gels were stained with Coomassie blue to visualize spectrin and the major protein bands.

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3.5 to 10 by the method of O'Farrell with modifications. Each gel was loaded with 150 μg of protein. For electrophoresis of the focused proteins in the second dimension we used a 10% to 15% polyacrylamide gradient SDS slab gel.

For immunologic analysis of the crude spectrin digests, spectrin tryptic fragments were transferred from SDS slab gels onto nitrocellulose paper (0.45 mm pores) and subsequently reacted with a polyclonal rabbit anti-alpha spectrin antibody (specific for the alpha I tryptic domain) as previously described.

Red cell thermal fragility. Morphologic changes (membrane fragmentation and budding) were assessed after heating washed red cells to various temperatures between 37° and 50°C for ten minutes as previously described.

Incubation of erythrocytes at 37°C. The rapidity and extent to which membrane budding and fragmentation occurred during long-term incubation of RBC at 37°C was evaluated in vitro. Washed erythrocytes (hematocrit 20%) were incubated at 37°C in 10 mmol/L Tris-buffered saline, pH 7.4 containing penicillin and streptomycin to inhibit bacterial growth. During incubation, samples were removed and fixed in 3% glutaraldehyde in normal saline. Cellular morphology was examined by interference phase microscopy (Carl Zeiss Inc, Oberkochen, West Germany).

Deformability measurements. The deformability of intact red cells was measured in the ektacytometer, a couette viscometer, which applies a well defined laminar shear stress to the cell while simultaneously monitoring the extent of cell deformation by laser defractometry. The deformability of the red cells was continuously recorded as the suspending medium osmolality was linearly increased from 50 to 300 mosm/kg in order to obtain information about initial cell surface area, surface area to volume ratio, and cell water content.

Erythrocyte ghost mechanical stability. Red cell ghost fragmentation was assayed in a Technicon digital processing ektacytometer. Resealed ghosts were suspended in a dextran solution of 97 cp viscosity (dextran 40,000, 35 g/dL wt/vol, 290 mosm/kg, pH 7.4). Laser diffraction patterns produced by the ghosts in suspension give a signal proportional to the average cellular ellipticity. This signal, designated as the deformability index (DI), decreases with time as the samples are subjected to a continuous shear stress of 750 dynes/cm². In order to compare different samples of ghosts, the time required for ghost deformability to decrease to 60% of its maximal value (T60) was determined. The ratio of the observed T60 to that of a simultaneously run control sample was then used to calculate relative ghost mechanical stability, expressed as a percentage of normal.

CASE HISTORY

The propositus is a 4½-year-old (BD 06/18/83) black boy from the Yoruba tribe, Kwara State, Nigeria. At birth, he exhibited severe poikilocytosis, hemolytic anemia, and hyperbilirubinemia requiring exchange transfusion and phototherapy. Hemolytic anemia persisted after birth. The propositus is a 4½-year-old black boy from the Yoruba tribe, Kwara State, Nigeria. At birth, he exhibited severe poikilocytosis, hemolytic anemia, and hyperbilirubinemia requiring exchange transfusion and phototherapy. Hemolytic anemia persisted after birth.

There was a two-day history of fever (Tmax = 104°) and a blood count obtained on 10/4/83 showed a hematocrit of 20%, reticulocyte count 3%. By 7 months of age the poikilocytosis had resolved and red cell morphology was elliptocytic, like that of the mother. The pedigree of the family is shown in Fig 1.

An unrelated, 23-year-old, splenectomized black woman (patient K.B.) who has HPP was used for comparative purposes in many of the studies of this family. The classic features of HPP, namely an alpha spectrin mutation, spectrin deficiency, a greatly increased fraction of spectrin dimers, poikilocytic and spherocytic red cell morphology, a lowered threshold for thermal fragmentation of red cells, extreme red cell mechanical fragility, and severe hemolytic anemia were all found in this individual. Her clinical course has been summarized elsewhere.

RESULTS

Representative blood counts obtained on each family member and on patient K.B., who has classic HPP, are presented in Table 1. Only K.B. and the propositus, IIw, were anemic and had reticulocytosis. Both exhibited microcytosis, which was particularly striking in K.B. Although red cell morphology was distinctly abnormal in both individuals (elliptocytes, microelliptocytes, cells with projections, and irregularly shaped microfragments), spherocytes were seen far more frequently in the blood of K.B. (Fig 2). Mild poikilocytosis was seen at birth in the younger sister, IIw, who subsequently developed the morphology of common hereditary elliptocytosis (Fig 2)

Red cell membrane protein composition, assessed by SDS-PAGE, was normal in all family members except the propositus. His pattern and that of patient KB revealed increased amounts of protein 4.5, protein 8, and globin, findings previously reported in other patients with HPP. The spectrin to band 3 ratio, assessed by Fairbanks non-linear acrylamide gel electrophoresis, was normal in the propositus and his family, indicating that spectrin was present in normal amounts (Table 1). In contrast, the spectrin to band 3 ratio was decreased in patient KB. The percentage of spectrin dimers present in cold low ionic strength spectrin extracts

![Fig 1. Family pedigree. 1. Carrier of spectrin alpha^A defect (normal red cell morphology); 2. carrier of spectrin alpha^M defect (HE); 3. compound heterozygote for both spectrin alpha^A and alpha^M defects.](https://www.bloodjournal.org)
was increased approximately tenfold in K.B., sevenfold in the propositus, and threefold in other family members (Table 2).

Electrophoretic analysis of limited tryptic digests of spectrin from family members revealed three distinct patterns, as shown in Fig 3. The maternal spectrin tryptic digest (Fig 3C) and that of the younger sister (not shown) were characterized by a modest decrease in the 80 kd alpha I fragment and an increase in the 65 kd fragment. Spectrin digests from the father (Fig 3B) and the older sister (not shown) both showed a decrease in the 80 kd fragment accompanied by the appearance of new fragments with molecular weights of 50 and 21 kd. The propositus showed a marked decrease in the 80 kd alpha I tryptic fragment with a concomitant increase in the 65 kd fragment and the appearance of new fragments with molecular weights of 50 and 21 kd (Fig 3D). Immunoblot analysis of the tryptic fragments using a polyclonal anti-alpha I spectrin antibody confirmed that the 65 and 50 kd fragments were derived from the 80 kd alpha I spectrin domain (Fig 4). To evaluate whether the residual 80 kd alpha I tryptic fragment noted in the propositus (Fig 3D) represented residual undigested mutant alpha spectrin or normal spectrin, digestion was carried out using more trypsin (1:20 enzyme to substrate ratio) for periods from 30 to 240 minutes. Under these conditions, the 80 kd alpha I fragment could be identified in normal digests until 180 minutes, while it was absent at all time points in digestes prepared from propositus spectrin. In contrast, the abnormal 65 kd, 50 kd, and 21 kd tryptic fragments were evident in propositus digests (data not shown). Thus, the residual 80 kd alpha I tryptic fragment noted in 1:200 trypsin propositus digests (Fig 3D) was undigested mutant spectrin, not normal spectrin. Limited tryptic digestion of spectrin from patient K.B. was not performed by us but previously was reported by others to reveal the presence of the spectrin alpha1/50a defect.

In order to characterize red cell membrane function in this family, we performed several studies of red cell stability in vitro. Heated erythrocytes from the propositus first exhibited morphologic changes (budding and fragmentation) at 44 to 46°C, a temperature well below that required to produce similar changes in normal erythrocytes or in erythrocytes from other family members (Table 2). Prolonged incubation at 37°C of HPP red cells (patient K.B.) in glucose free buffer produced extensive budding, fragmentation, and spherocyte formation (Fig 5). These changes as well as visible evidence of hemolysis were first noted within six hours of incubation. Normal red cells and red cells from the parents developed echinocytic changes during incubation, but even after 28 hours no budding, fragmentation or hemolysis was noted. Propositus red cells became echinocytic more quickly than did normal cells, but by 28 hours had developed only minimal budding, fragmentation, spherocytosis, and hemolysis.

The mechanical stability of erythrocyte ghosts from the propositus (II1), measured at 3% years of age, was reduced to a level only 18% of normal (Fig 6). Classic HPP ghosts (patient K.B.) displayed even greater fragility (data not shown). Ghost mechanical stability was 17% to 29% and 36% in the two family members (I5, II1) who had the alpha1/50a elliptocytosis defect and 39% and 43% in the two individuals (I1, II1) who had the alpha1/50a silent carrier defect.

Whole cell deformability and cell surface to volume relationships were evaluated by osmotic gradient ektacytometry (Fig 7). Deformability was severely reduced in red cells from the propositus, although not to the extent seen in classic HPP. The point of minimal deformability (on the hypotonic arm of the curve) was normal in the propositus, indicating that mean osmotic fragility was normal,16 but was shifted toward greater tonicity in patient K.B., reflecting the large number of spherocytes present in her blood. Red cells from the mother with elliptocytosis (I5) showed a modest reduction in the maximum deformability index accompanied by the usual flattening of the curve characteristic of HE. The younger sister’s (II1) red cells, studied in the first 2 weeks of life showed a shift to the left in the hypotonic arm, a normal

Table 1. Hematologic Data

<table>
<thead>
<tr>
<th>Subject</th>
<th>Hb (g/dL)</th>
<th>MCV (fl)</th>
<th>MCHC (g/dL)</th>
<th>RDW (%)</th>
<th>Reticulocytes (%)</th>
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<td>Father (I1)</td>
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<td>35</td>
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<tr>
<td>Sister (II1)</td>
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<td>34</td>
<td>13.2</td>
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<tr>
<td>Mother (I1)</td>
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<td>83</td>
<td>35</td>
<td>12.7</td>
<td>2.3</td>
</tr>
<tr>
<td>Sister (II1)</td>
<td>12.0</td>
<td>73</td>
<td>35.7</td>
<td>14.6</td>
<td>1.0</td>
</tr>
<tr>
<td>Propositus (II1)</td>
<td>10.7</td>
<td>61</td>
<td>35.7</td>
<td>38</td>
<td>10.3</td>
</tr>
<tr>
<td>HPP (patient K.B.)</td>
<td>9.9</td>
<td>52</td>
<td>38.7</td>
<td>34</td>
<td>12</td>
</tr>
</tbody>
</table>

Fig 2. Wright-stained peripheral blood smears (original magnification ×500). Occasional microspherocytes and elliptocytes were noted in the younger sister, II1, at birth (A). By 7 months of age (B), only elliptocytes and discocytes were seen. Bizarre red cell shapes, elliptocytes, fragments, and budding forms were seen in the propositus, II1, (C) and in patient K.B. with classic HPP (D) who also exhibited profound spherocytosis.
finding in the newborn period. By 7 months of age her pattern had changed to that of her mother with elliptocytosis. Similar reductions in red cell deformability were found in the red cells of the father and the older sister (II,), neither of whom had elliptocytosis.

**DISCUSSION**

Seven distinct structural mutations of alpha spectrin, designated alpha1/74, alpha1/65, alpha1/6', alpha1/5β, alpha1/5α, alpha1/43, and truncated alpha spectrin have been identified in individuals with HE or HPP by SDS-PAGE evaluation of red cell membrane proteins or by analysis of tryptic peptides following limited digestion. The family reported here is of particular interest because two of these mutations were present, both singly and in combination. The father and the older sister, whose red cell morphology was normal, were carriers of the spectrin alpha1/5α variant and resembled other carriers of this defect. The mother and younger sister, who had elliptocytosis, were found to be carriers of the spectrin alpha1/65 tryptic variant. The younger sister exhibited mild neonatal poikilocytosis which subsequently (by 7 months of age) evolved to elliptocytosis. The propositus inherited both the alpha1/65 and the alpha1/5α spectrin defects and exhibited many characteristics of HPP including autosomal recessive inheritance, poikilocytic red cell morphology, susceptibility of red cells to thermal fragmentation at 45 to 46°C, striking red cell membrane mechanical fragility, and the presence of a greatly increased proportion of spectrin dimers. The failure of these abnormalities to resolve by 4 years of age precluded the possibility that the propositus actually suffered from transient neonatal poikilocytosis.

Most of the clinical and biochemical features of HPP may also be found in homozygous HE. For example, Garbarz et al described a child with homozygous HE who had inherited an alpha1/65 spectrin mutation from each parent. Like individuals with HPP, this child had poikilocytic red cell morphology, altered erythrocyte thermal sensitivity, reduced red cell deformability, and an increase in the proportion of spectrin dimers. Unlike individuals with HPP, the red cell spectrin content was normal and spherocytes were not a prominent feature of the blood smear. The propositus we studied does not fully meet the criteria for classification as homozygous or doubly heterozygous HE, since elliptocytes were found in only one parent. However, the clinical and biochemical features of the case are otherwise completely consistent with this diagnosis. In particular, the paucity of spherocytes and the normal spectrin content in the red cells of the propositus are features that more closely resemble homozygous HE than HPP. The limitations of the traditional system of classification of the poikilocytic anemias, which is based largely on clinical features, inheritance pattern and tests of red cell thermal sensitivity have been pointed out previously and were encountered, as discussed above, in the propositus reported here.

The genetic basis of HPP is not fully understood. In most instances, one parent has an identifiable defect in alpha spectrin while the other is normal. It is speculated that the apparently normal parent may actually have a clinically undetectable abnormality resulting in diminished synthesis of alpha spectrin. Such an abnormality would be silent when present in isolation because alpha spectrin is normally produced in excess and even reduced synthesis will fully meet the requirements of the cell. When co-inherited with another...
gene coding for an alpha spectrin structural defect, however, defective synthesis of normal alpha spectrin would increase the proportion of the structural mutant and thus result in a clinical syndrome of greater severity. A relationship between the amount of alpha spectrin mutant(s) and clinical severity is also evident in the rare individual found to be a compound heterozygote for two separate spectrin defects. Here the total amount of abnormal spectrin is increased by the presence of a second spectrin structural variant, rather than by decreased synthesis of normal spectrin. The previously unreported combined inheritance of a spectrin alpha<sup>165</sup> and a spectrin alpha<sup>150α</sup> mutation that we found in the propositus is an example of this type of compound heterozygote.

Of particular interest was the mild clinical course noted in the propositus. Although transfusions were needed at birth for hyperbilirubinemia and at 2 years following an acute, infection-associated anemic crisis, anemia was usually minimal, particularly after the first year of life. Similarly, the rheologic changes observed on osmotic gradient ektacytometry and the extent of increase in the proportion of spectrin dimers, while abnormal, were not of the magnitude observed in classic HPP as exemplified by patient K.B. and by other reported cases. Although poikilocytes were abundant, there were fewer microspherocytes. Prolonged incubation of propositus red cells at 37°C did not lead to the extensive budding, fragmentation, and spherocyte formation that we observed in red cells from patient K.B. and that was previously described by Zarkowsky et al in another individual with classic HPP.

The two factors most likely to have modified clinical severity are the degree to which mutant spectrin participated in tetramer formation and the lack of spectrin deficiency. The abnormal spectrins present in the red cells of the propositus are known to be capable of participating in tetramer formation to a limited extent. As a result, the percentage of spectrin dimers was increased to only approximately 35% instead of the >50% observed in classic HPP. In contrast, in another individual with HPP who was doubly heterozygous for the Sp alpha<sup>150</sup> and the Sp alpha<sup>174</sup> defects, spectrin dimers were increased to >50%, reflecting the severely impaired ability of the alpha<sup>174</sup> mutation to form tetramers. There is a direct correlation between the percentage of spectrin dimers present in the membrane and diminished red cell membrane stability. Thus, it is not surprising to find in the propositus that the degree of red cell fragmentation in vivo and diminution of ghost mechanical stability in vitro fell somewhere between classic HPP on the one hand and HE or asymptomatic carriers of spectrin abnormalities on the other. The second factor, normal membrane spectrin content, is a finding quite unlike the spectrin.
deficiency usually encountered in classic HPP. In hereditary spherocytosis, another hemolytic anemia characterized by spectrin deficiency, the extent of the deficiency correlates well with clinical severity. The least deficient subjects have the mildest clinical course and by analogy one might expect the same to be true in poikilocytic anemias like HPP or homozygous HE.

The abundance of microspherocytes found in classic HPP patients such as K.B. may also be related to the extent of spectrin deficiency. Similarly, the relative paucity of spherocytes in the propositus is consistent with the normal spectrin content of his red cells. That spherocytes are seen at all implies that his dysfunctional spectrin may have effects on cell processes leading to spherocyte formation (eg, vesiculation, endocytosis, and/or fragmentation) similar to those of spectrin deficiency. However, spectrin deficiency seems to be a more important factor in spherocyte formation since in the two individuals we studied (K.B. and the propositus), both of whom had dysfunctional spectrin, only K.B., whose red cells were also spectrin deficient, exhibited extensive spherocytosis.

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