Double Inheritance of an Alpha I/65 Spectrin Variant in a Child With Homozygous Elliptocytosis

By Michel Garbarz, Marie Christine Lecomte, Didier Dhermy, Claude Feo, Isabelle Chaveroche, Huguette Gautero, Odile Bournier, Christiane Picat, Anne Goeppe, and Pierre Boivin

Hemolytic anemia with red cell fragmentation, poikilocytosis, and elliptocytosis was discovered in a 6-week-old black infant. Both parents and a brother of the proband had compensated mild Hereditary Elliptocytosis (HE). Elliptocytosis was prominent in the proband’s father with the presence of numerous rod-shaped cells whereas, in the proband’s mother, elliptocytosis was less marked and cells were less elongated than in the father. The proband’s red cells fragmented at 45°C instead of 49°C for control cells. The proband’s red cells were less elongated than in the father. The proband’s red cells fragmented at 45°C instead of 49°C for control cells. Both the parents’ and brother’s red cells fragmented at 47°C. The deformability of the proband’s red cells was markedly reduced when measured with the ektacytometer; the red cells of both the proband’s parent and brother exhibited an intermediate decrease in red cell deformability. Spectrin self-association was defective in the proband.

The deformability of the proband’s red cells was markedly reduced when measured with the ektacytometer; the red cells of both the proband’s parent and brother exhibited an intermediate decrease in red cell deformability. Spectrin self-association was defective in the proband as well as in his parents and brother. Limited trypsin digestion of the proband’s spectrin, followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), revealed a complete absence of the normal 80,000 dalton alpha I domain and the presence of an abnormal 65,000 dalton peptide. Two-dimensional isoelectric focusing/SDS-PAGE of limited tryptic digests of spectrin from both the proband’s parents and brother revealed a decrease in the normal 80,000 alpha I domain and the presence of the 65,000 peptide variant. On the basis of biochemical studies performed on the patients’ spectrin, we concluded that the proband had homoyzogus HE, having inherited the structural defect of spectrin present in a heterozygous state in each of his parents. On a clinical and morphologic level, homozygous HE imitates two other forms of congenital hemolytic anemia associated with a spectrin self-association defect: HE with pycnocytosis in infancy and Hereditary Pyropoikilocytosis. This report emphasizes the importance of confronting clinical and rheological as well as biochemical investigations in studying and discussing different entities.

I T IS now known that normal red cell stability and deformability depend upon a submembranous network of proteins called the skeleton, which is attached to the inner surface of the red cell membrane. Spectrin, the predominant protein of the skeleton, forms long fibers that are mainly spectrin tetramers formed by two heterodimers joined “head to head.” Each dimer is composed of two different chains denoted α and β. The distal ends of spectrin are linked together by short polymers of actin. Protein band 4.1 binds to the distal end of the spectrin molecule and regulates the interactions between spectrin and actin. The spectrin-actin-protein 4.1 network is attached to the membrane via ankyrin, a protein that binds spectrin to the anion channel transmembrane protein band 3.

The effect of the skeleton on cell stability has been illustrated by recently discovered hereditary deficiency, or dysfunction, of membrane skeletal proteins in patients with congenital hemolytic anemia2 such as Hereditary Elliptocytosis (HE),3-7 Hereditary Pyropoikilocytosis (HPP),8-10 and Hereditary Spherocytosis.11-14 The patient’s father had typical mild HE with poikilocytosis in infancy, homozygous HE, or HPP, a disorder closely related to HE.2,3,13,23 A few patients with homozygous HE have been reported.24-29 The patients had severe hemolytic anemia with marked red cell fragmentation, poikilocytosis, and elliptocytosis, and both parents of the patients had classical mild HE. In three families, the skeletal protein defect was determined. Féo and Tchernia24 reported three young patients in whom homozygous HE was associated with a complete absence of protein band 4.1. The parents had mild HE and exhibited a partial deficiency in protein band 4.1. Evans and colleagues28 reported three children with homozygous HE and profound defective spectrin dimer-dimer association. The parents had classical mild HE and displayed a lesser but significant defect in spectrin self-association (the cases of homozygous HE associated with defective spectrin self-association have been termed type I HE).3 Dhermy and colleagues29 reported a patient with severe transfusion-dependent poikilocytic hemolytic anemia. The parents had mild HE and exhibited defective spectrin self-association; this defect was associated in both parents with a structural alteration in the spectrin molecule: abnormal patterns in tryptic digestion of spectrin were observed, namely, a decrease in the amount of the 80 Kd peptide of the spectrin α chain involved in dimer-dimer association and a corresponding increase in a 74 Kd peptide derived from the same region.

We have now investigated another patient with homozygous type I HE. This patient exhibited defective spectrin self-association, and peptide maps of his spectrin tryptic digests revealed a complete absence of the normal 80 Kd peptide, and the presence of an unusual peptide of 65 Kd. The patient’s father and mother had typical mild HE and displayed defective spectrin self-association, associated with...
a decrease in the normal 80 Kd α I domain and the presence of the 65 Kd variant of spectrin α chain. We discuss this case with the aid of clinical, rheological, and biochemical investigations and in the light of two other kinds of congenital poikilocytic anemia with a spectrin self-association defect, HE with pycnocytes in infancy and HPP.

CASE REPORT

This family is of Malian extraction. The propositus Mar.S., a female baby, was born in August 1984. Delivery was normal, and no congenital defect was apparent. At six day, a moderate icterus was noted. Hemoglobin was 12.5 g/dL, packed cell volume (PCV) 35%, and mean cell volume (MCV) 90 fl. Five weeks later, she was referred to the hospital for anemia. Clinical examination was found to be normal. The spleen was not palpable. Hb was 8.4 g per dL, red blood cell count was 2.9 x 10^{12}/L, PCV was 23.4% and MCV 80 fl. The reticulocyte count was 2.5 x 10^{11}/L. Elliptocytes and poikilocytes were observed on blood smears.

The parents (mother Ka.S, father Dj.S.) were not related. They had no significant medical history. In both parents hematologic data were normal (Table 1). Elliptocytosis was prominent in the father, whereas in the mother, only few elliptocytes were present on blood smears. A brother of the propositus (A S) was 18 months old at the time of this study and had no medical history. Elliptocytosis was discovered fortuitously during a routine blood examination. Hb was 10.3 g/dL, with red blood cell count 4.5 x 10^{12}/L, MCV 65 fl, and reticulocytes 4.5 x 10^{9}/L. Serum iron concentration was 9 mmol/L (normal range 13 to 31 mmol/L). Hemoglobin electrophoresis was normal.

MATERIALS AND METHODS

Materials. Beta mercaptoethanol (BME), glutaraldehyde, ethylene diamine tetraacetic acid (EDTA), TRIS, glyceral sucrose for density gradient studies, and L-1 tosylamido phenylethyl chloromethyl ketone (TPCK) Trypsin (3.5 units per mg) were from Merck (Darmstadt, GFR); phenylmethylsulfonyl fluoride (PMSF) and Disopropyl fluorophosphate (DFP) were from Sigma, St Louis. All materials used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were from Biorad Laboratories (Richmond, Calif). Ammonia were obtained from LKB (LKB-Bromma, Sweden). Patient samples of venous blood obtained were drawn in sterile tubes, anticoagulated with heparin, and used within 24 hours.

Methods. Routine hematologic determinations were obtained with a coulter counter model S. Reticulocyte counts were made after new methylene blue staining. Morphologic studies: erythrocytes were examined on dried smears stained with May Grunwald Giemsa and on wet smears as previously described. Red cell thermal sensitivity was examined as described. Deformability measurements (osmotic gradient ektacytometry): whole-cell deformability was measured in the ektacytometer as a continuous function of the suspending medium osmolality as previously described. Preparation of the erythrocyte membranes: the erythrocytes were washed three times in 5 mmol/L NaPO4, 147 mmol/L NaCl pH 8.0. The ghosts were prepared according to Litman except that 0.3 mmol/L PMSF was added to the lysis buffer. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of isolated membranes was performed using a 5% to 15% polyacrylamide gradient slab gel according to Laemmlli. Gels were stained by Coomassie blue. To estimate spectrin/band 3 ratios, gels were quantified on a DU8 Beckman (Gagny, France) integrating spectrophotometer.

Spectrin interaction studies. Spectrin was extracted by incubating the membranes in low ionic strength buffer either at 37 °C or at 4 °C. Spectrin dimer-dimer association in solution was studied directly in the 37 °C extracts. The samples were incubated for 240 minutes at 30 °C to induce spectrin dimer-to-tetramer transformation. Dimer and tetramer species were separated by sucrose gradient velocity centrifugation and equilibrium constant Ka was determined as described. The distribution of spectrin species in the 4 °C extracts was studied using nondenaturant gel electrophoresis (NDGE) and the percentage of spectrin dimer was determined as described.

Limited tryptic digestion of spectrin. Crude spectrin extracts (1 mg/ml) were digested at 0 °C for 20 h in 20 mmol/L NaPO4, 100 mmol/L NaCl pH 8.0. The enzyme/substrate ratio was 1/100 (w/w). The digestion was ended by adding of DFP (1 mmol/L final concentration). Each assay was done in duplicate. Spectrin peptides from the tryptic digestion were separated on a 7% to 15% polycrylamide gradient gel as described. Gels were stained with Coomassie brilliant blue and the bands were quantified on a DU8 Beckman (Gagny, France) integrating spectrophotometer.

Isoelectric focusing. Isoelectric focusing followed by SDS-PAGE was performed according to O'Farrell's method with the modifications reported by Speicher. Samples of tryptic digest were lyophilized and solubilized in 9.5 mol/L urea, 2% Triton X 100, 5% BME, and 2.4% ampholines, pH 3.5-10, 4-6, 5-7, respectively. Samples were electrofocused for 16 hours at 340 volts in 4% polycrylamide tube gels (0.5 x 13 cm) containing 2.4% ampholines. The second dimension was performed on 7% to 15% linear gradient slab gels (140 x 100 x 1.5 mm) as described. Gels were stained with Coomassie blue.

RESULTS

Hematologic indices of the four members of the family were obtained at the time of the biochemic investigations. The results are summarized in Table 1.

Morphologic studies. On wet preparations, the morphology of the proband's red cell was characterized by the presence of numerous elliptocytes of different sizes and of various degrees of ellipticity, triangle shaped cells, and microcytes (Fig 1). The calculated MCV was 77.5 fl. But the distribution curve was bimodal, showing a proportion of about 20% of very microcytic red cells. This percentage corresponded with the number of microcytes observed on the wet preparation. The dispersion of the volumes is very broad since the proband's curve is superimposed on that of the father in the area where the volumes exceed the normal mode (>90 fl; Fig 2). In the father, elliptocytosis was prominent (about 100%) with cells moderately to extremely elongated; no fragmented cells were observed. In the mother, elliptocytosis was less important (about 30%) with a majority of microcytes. The spleen was not palpable. Hb was 8.4 g per dL, red blood cell count was 2.9 x 10^{12}/L, MCV 80 fl. The reticulocyte count was 2.5 x 10^{11}/L. Elliptocytes and poikilocytes were observed on blood smears.

<table>
<thead>
<tr>
<th></th>
<th>Hb (g/dL)</th>
<th>Red Cell Count (x 10^{12}/L)</th>
<th>Mean Cell Volume (fl)</th>
<th>Reticulocytes (x 10^{9}/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Father</td>
<td>14.4</td>
<td>4.76</td>
<td>93</td>
<td>47.600</td>
</tr>
<tr>
<td>Mother</td>
<td>12.5</td>
<td>4.42</td>
<td>83</td>
<td>75.000</td>
</tr>
<tr>
<td>Proband</td>
<td>8.4</td>
<td>2.90</td>
<td>80</td>
<td>252.000</td>
</tr>
<tr>
<td>Brother</td>
<td>10.3</td>
<td>4.50</td>
<td>65</td>
<td>45.000</td>
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hemolysis, and the index in hypertonicity (HYPER), which corresponds to the osmolality at which El equals half the normal maximum on the hypertonic arm of the curve. Red cell deformability was found to be abnormal in all four patients (Fig 3): the proband’s father and mother displayed a decrease in red cell deformability with an El ISO of 0.28 and 0.34, respectively (controls: 0.55 ± 0.04); El improved steadily when the cells were under moderate hypotonic conditions but the optimum El was shifted markedly toward hypotonic values (200 mOsm) and was always lower than that of the control. The two curves had an asymmetric trapezoid shape. In the proband cell deformability was markedly reduced with an El of 0.15 and the ektacytometric profile was reminiscent of that of both parents with the same trapezoid shape. In these three patients, El HYPO values were obtained at the same osmolalities as in controls (141 ± 5 mOsm kg⁻¹). The proband’s microcytic brother exhibited an osmotic deformability profile similar to that of his parents (El ISO at 0.28) but the El HYPO was shifted to the left (120 mOsm kg⁻¹) (not shown). It is known that in iron-deficient cells, the El HYPO value is obtained at a lower osmolality than that in normal cells.³⁹

SDS-PAGE of the red cell membranes gave normal patterns in both the proband’s parents and brother. The electrophoretic pattern of the proband showed a decrease in subcomponent 4.1a and an increase in subcomponent 4.1b related to the hypereticulocytosis. No reduction in the amount of spectrin relative to band 3 was observed in the proband or in either of her parents. Spectrin to band 3 ratio was 1.29 in the proband, 1.31 in the proband’s father, and 1.37 in the proband’s mother (spectrin to band 3 ratio in controls: 1.25 ± 0.22; n = 12). Spectrin dimer-dimer association was defective in the four members of the family; the proportion of spectrin dimer was increased in the 4 °C extracts of the proband (41%) and in a lesser extent in both parents and brother (respectively, 28%, 28%, and 24% for a control value of 13% ± 3). The study of spectrin dimer self-association in solution performed only in the proband’s mother and brother revealed a decreased

Fig 3. Changes in erythrocyte deformability as measured by the ektacytometric index (El) during continuous variation of the suspending medium osmolality. Curves of father (DS), mother (KS), proband (MS), are compared to control (C).

Fig 1. Morphology of erythrocytes from the four family members. Father (A) Mother (B) Proband (C) Proband’s brother (D).

elliptocytes (about 70%) which were moderately elongated; few microcytes and schizocytes were present.

Red cell thermal sensitivity. Red cell thermal sensitivity was abnormal in the four patients. The most fragmentable cells were found in the proband: her red cells began to fragment at 45 °C (instead of 49 °C for control red cells). In both the proband’s parents and brother, the critical temperature of red cell fragmentation was 47 °C.

Red cell deformability studies. The deformability of the red cells is expressed as an ektacytometric index (El). When El is plotted versus the osmolality of the suspending medium, the curve obtained shows three remarkable points (Fig 3): the El in isotonicity (ISO), the minimum index in hypotonicity (HYPO) around 140 mOsm kg⁻¹, which corresponds to the maximum volume of the near-spherical cells just prior to

Fig 2. Red cell volume distribution measured by a Coulter C 1000 model E (Margency, France). The mean value was determined by integrating the area of the curve. We estimated the percentage of microcytes by comparing the area of the curve from the origin to channel 22 with the total area. Channel 22 (45 fl) corresponds to the channel under which less than 3% of normal cells are found. (C) curve of control. (M) Proband’s mother’s curve; (P) Proband’s curve.
association constant $K_a$, with a value of 3 and $1.8 \times 10^3$ mol/L$^{-1}$, respectively. (Control value: $6 \pm 0.4 \times 10^3$ mol/L$^{-1}$). All results are summarized in Table 2.

*Limited tryptic digestion of patient’s spectrin.* Limited tryptic digestion of crude spectrin extract from the proband revealed a complete absence of the 80 Kd peptide and the presence of an abnormal peptide of 65 Kd (Fig 4, right panel). In the three other members of the family (father, mother, and brother AS) we observed a decrease in the 80 Kd peptide and the presence of the 65 Kd peptide (Fig 4, left and right panels). Densitometric tracings (not shown) enabled us to estimate that the ratio 65/80 = 65% in the father, 40% ($n = 4$) in the mother, and 48% ($n = 2$) in the brother. Variations at the level of 35 to 37 Kd (decrease in the 35 and presence of a 37 Kd peptide) peptides were observed in the mother.

Two-dimensional electrophoresis (isoelectric focusing followed by SDS-PAGE) of tryptic digest of spectrin could only be performed in the parents and proband’s brother. They confirmed the decrease in the 80 Kd peptide and the presence of the 65 Kd peptide which focused between pH 5.15 and 5.2 (Fig 5).

In the proband’s mother we also observed variations in peptides related to the αI domain: a decrease of the two spots at 48 Kd level and the spot at 35 Kd, with the concomitant presence of two spots at 50 Kd and a spot at 37 Kd. The two spots at 50 Kd were also modified in their isoelectric point which was less acidic (Fig 5). These modifications corresponded to the “1/2” variant described by Knowles.40

**DISCUSSION**

We report the case of a child suffering from a congenital hemolytic anemia with poikilocytosis and elliptocytosis. The mean cell volume was slightly decreased and this corresponded with the presence of moderate fragmentation (Fig 2). This was associated with a normal EI HYPO. (Fig 3). The proband’s erythrocytes were particularly sensitive to heat treatment (45 °C instead of 49 °C for normal erythrocytes). Both the proband’s parents and brother exhibited mild HE and their red cells fragmented at 47 °C.

The diagnosis of homozygous HE was established in the proband with the aid of the biochemical studies performed in each member of the family. Both the proband’s parents had defective spectrin self-association with an increased amount of spectrin dimer in the spectrin 4 °C extract. Peptide mapping of the tryptic digests of their spectrin showed a reduction of about 50% in the normal 80 Kd αI spectrin domain, and the presence of an abnormal 65 Kd variant. The proband exhibited defective spectrin self-association and had greater amounts of spectrin dimer in the spectrin 4 °C extracts than his heterozygous parents. The proband had inherited the structural alteration of spectrin from each of his parents, and the 80 Kd peptide was absent on the peptide maps of tryptic digests of his spectrin, only the 65 Kd variant being present. The proband’s brother had mild HE and was heterozygous for the structural alteration of spectrin like each of his parents.

This is the first reported case of homozygous type 1 HE with the spectrin αI/65 variant. This structural defect has been previously reported in seven cases of heterozygous HE,41 and reports on three other HE patients with the same 65 Kd variant have been recently published.42 The abnormal 65 Kd peptide was shown to derive from the 80 Kd αI domain.41,42

In the present case of homozygous HE, it was possible to follow the transmission of the spectrin structural defect from each heterozygous parent to the homozygous proband. In the cases of homozygous HE reported by Evans,28 the probands were found to have twice the amount of spectrin dimer as their heterozygous parents but the type of the variant was not known. In the case of homozygous αI/74 reported by Dhermy,29 the transmission of the defect could not be traced in the proband because the severity of the anemia required monthly transfusions. Interestingly, in this latter case and in Evans’ cases (in which the type of the variant was not known), the proportion of spectrin dimer in the heterozygous parent was about 40%. In our present case, the proband’s heterozygous αI/65 parents exhibited lower amounts of spectrin dimer and the homozygous proband had almost twice the spectrin dimer than did either of his parents. For

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**Table 2. Functional Studies of Spectrin**

<table>
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<tr>
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<th>Equilibrium Constants of Spectrin Dimer-Dimer Association in Solution ($K_a$ $10^4$ mol/L)</th>
<th>Percentage Spectrin Dimer in 4 °C Crude Spectrin Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>$6.00 \pm 0.4$ ($n = 42$)</td>
<td>$13% \pm 3%$ ($n = 27$)</td>
</tr>
<tr>
<td>Father (DS)</td>
<td>ND</td>
<td>28%</td>
</tr>
<tr>
<td>Mother (KS)</td>
<td>2.3</td>
<td>28% ($n = 2$)</td>
</tr>
<tr>
<td>Proband (MS)</td>
<td>ND</td>
<td>41%</td>
</tr>
<tr>
<td>Brother (AS)</td>
<td>1.8</td>
<td>24%</td>
</tr>
</tbody>
</table>

*ND, not determined.
technical reasons, we could not measure the spectrin dimer association constant in the homozygous proband, which would have been more meaningful in evaluating the functional defect in the alpha 1/65 spectrin variant.

Two other forms of congenital hemolytic anemia associated with a spectrin self-association defect imitate the hematologic picture observed in homozygous HE: HE with poikilocytosis in infancy and HPP. In these two diseases, red cell fragmentation occurs at 43 °C to 45 °C. In HE with poikilocytosis in infancy, one parent has mild HE and exhibits the same increase in the amount of spectrin dimer and the same spectrin tryptic digest pattern as his poikilocytic offspring: after 12 to 14 months the morphologic poikilocytic picture evolves into a morphologic picture similar to that observed in the mild HE parent. Structural studies on spectrin in HPP have shown that one parent contains a structural defect in spectrin (74, 46, or 50 Kd variant) that is similar to a lesser extent to the HPP proband’s defect. The other parent does not carry any identifiable defect but probably transmits to his HPP offspring an anomaly that acts in concert with the inherited membrane skeletal defect, resulting in the HPP phenotype. In the cases of HPP reported by Palek, spectrin self-association is more defective and the normal 80 Kd peptide is more reduced in the HPP proband than in the parent who carries the spectrin defect; however, as we observed recently (unpublished data), the proportion of normal 80 Kd present may be the same in the HPP patient as in his parent or, as reported by Marchesi, the 80 Kd peptide can be totally absent in the HPP proband. In our case of homozygous HE, both the presence of 50% of the normal amount of 80 Kd peptide in each parent and the total absence of 80 Kd peptide in the proband are necessary to make the diagnosis of homozygous HE certain. Also, the amount of spectrin present in the proband’s red cell membranes was normal, whereas this amount was found to be decreased (about 30%) in the HPP patients’ red cell membranes. In addition to the biochemic studies performed on each member of the family, we think that red cell deformability studies performed with the ektacytometer are very useful to confirm the diagnosis of homozygous HE in the proband: red cell deformability was more reduced in the proband than in her heterozygous parents (Fig 3); the trapezoid shape of the proband’s red cell ektacymetric profile was reminiscent of the curve observed in each heterozygous HE parent. In our experience, this kind of asymmetric profile has so far been observed only in HE patients. In our studies concerning HPP patients, as well as in the recent case reported by Mentzer, the ektacymetric profile observed in HPP is different from that of homozygous HE: the ektacymetric index in isotonicity is extremely reduced and the minimal ektacymetric index in hypotonicity is shifted to higher osmolality values (this being consistent with the increased osmotic fragility of red cells in HPP).

In the present case of homozygous HE, biochemical studies performed on patients’ spectrin have shown that the homozygous proband had inherited the structural defect of spectrin present in a heterozygous state in each of his parents.

One interesting question to be asked would be whether the kind of spectrin molecular variant plays a role in the clinical severity of the disease. It has to be pointed out that in the case reported by Dhermy, the proband had severe transfusion-dependent hemolytic anemia and was homozygous for a 74 Kd spectrin variant, while in the present case which is clinically less severe, the proband is homozygous for a 65 Kd spectrin variant.
ACKNOWLEDGMENT

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REFERENCES


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