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

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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. 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, as well as in his parents and brother. Limited tryptic 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 homzygous HE, having inherited the structural defect of spectrin present in a heterozygous state in each of his parents. On a clinical and morphologic level, homzygous HE mimics other forms of congenital hemolytic anemia associated with a spectrin self-association defect: HE with pycnodysostosis 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.

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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 pycnocytosis 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 day six, a moderate icterus was discovered fortuitously during a routine blood examination. Hb 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 10^9/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, ethylenediamine tetraacetic acid (EDTA), TRIS, glycerol sucrose for density gradient studies, and L-L 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). Ampholines 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. Normal values for reticulocyte counts were 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 Laemmli. Gels were stained by Coomassie blue. To estimate spectrin/band 3 ratios, gels were quantified on a DU8 Beckman (Gagny, France) integrating spectrophotometer.

RESULTS

Hematologic indices of the four members of the family were obtained at the time of the biochemical 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 roundish cells; 3% to 5% of the cells were rod-shaped. The proband’s brother’s red cells were mainly heterogeneous.

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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 (EI). When EI is plotted versus the osmolality of the suspending medium, the curve obtained shows three remarkable points (Fig 3): the EI 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 hemolysis, and the index in hypertonicity (HYPER), which corresponds to the osmolality at which EI 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 EI ISO of 0.28 and 0.34, respectively (controls: 0.55 ± 0.04); EI improved steadily when the cells were under moderate hypotonic conditions but the optimum EI 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 EI of 0.15 and the ektacytometric profile was reminiscent of that of both parents with the same trapezoid shape.

In these three patients, EI 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 (EI ISO at 0.28) but the EI HYPO was shifted to the left (120 mOsm kg⁻¹) (not shown). It is known that in iron-deficient cells, the EI HYPO value is obtained at a lower osmolality than that in normal cells.

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
association constant $K_a$, with a value of $3 \times 10^9\text{ mol/L}^{-1}$, respectively. (Control value: $6 \pm 0.4 \times 10^9\text{ mol/L}^{-1}$). 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 was 50% 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 αII 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 E1 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 α1 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 I HE with the spectrin α1/65 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 alpha I 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 alpha 1/74 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 alpha 1/65 parents exhibited lower amounts of spectrin dimer and the homozygous proband had almost twice the spectrin dimer than did either of his parents. For

![Figure 4. SDS-PAGE of limited tryptic digests of spectrin from proband (MS), proband's father (DS), proband's mother (KS), proband's brother (AS). Spectrin crude extracts were treated with TPCK trypsin (enzyme/substrate ratio: 1/100) at 0 °C for 20 hours. Gels were stained with Coomassie blue. The position of variable bands is indicated by arrows.](image-url)
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 pycnocytosis 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 biochemical 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 ektacytometric 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 ektacytometric profile observed in HPP is different from that of homozygous HE: the ektacytometric index in isotonicity is extremely reduced and the minimal ektacytometric 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.
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REFERENCES


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