Molecular Defect of Spectrin in the Family of a Child with Congenital Hemolytic Poikilocytic Anemia

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Summary

We present the study of a black family in which the proband suffered from a severe neonatal hemolytic anemia with poikilocytosis. Both the parents, sister's, and brother's proband were clinically normal. The presence of poikilocytes in proband led to a search for a red cell membrane skeleton defect. Owing to recent improvements in the erythrocyte membrane knowledge, it is now possible to approach the diagnosis by means of biochemical evaluation of both parents, even if they are asymptomatic. So, the first time discovery of a spectrin self-association defect in both parents allowed us to suspect double inheritance of this abnormality in the proband. A complete morphological and biochemical evaluation of the family allowed us to propound the diagnosis of heterozygous type I hereditary elliptocytosis (HE) for both parents and the sister and the diagnosis of homozygous type I HE for the proband owing to the following reasons: slight ovalocytosis was present in both parents and the sister; cell deformability ektacytometric studies gave the same profiles of curve as those observed in patients with HE. Defective spectrin dimer self-association found in both parents was also observed in the sister and proband, associated with the same abnormal spectrin digest pattern, namely a decrease in the amount of a 80,000-dalton peptide and a corresponding increase in a 74,000-dalton peptide. However, clinical presentation of the proband was consistent either with hereditary pyropoikilocytosis or homozygous hereditary elliptocytosis; erythrocyte thermal sensitivity studies in the proband could not be conclusive because of the presence of transfused cells. Both these diagnoses are discussed in detail. Other modifications of spectrin tryptic patterns were detectable and were not related to the functional defect of spectrin since the proband's normal brother appeared homozygous for these modifications.

Abbreviations

HPP, hereditary pyropoikilocytosis
HE, hereditary elliptocytosis
PMSF, phenylmethylsulfonyl fluoride
SDS, sodium dodecyl sulfate
BME, β-mercaptoethanol

PBS, phosphate-buffered saline
EI, ektacytometric index
NDGE, nondenaturing gel electrophoresis
TPCK, L-1-tosylamidophenylethyl chloromethyl ketone

The human red cell contains on the internal face of its lipid bilayer a two-dimensional proteinaceous meshwork named membrane skeleton which is the major determinant of membrane shape and stability (7). The skeleton is mainly composed of spectrin, actin, protein 4.1, and ankyrin. Spectrin is basically a heterodimer composed of an α and β chain (molecular weights, 240,000 and 220,000 daltons, respectively). Each α and β subunit was resolved into several peptide domains by restricted tryptic cleavage. These domains were named αI to αV and βI to βIV (36). The spectrin dimer associates head to head to form a tetramer which represents the putative basic unit of the membrane skeleton. Spectrin tetramers are cross-linked by actin and protein 4.1 to form a meshwork (39). Ankyrin fastens the skeleton to the lipid bilayer by means of its interactions between spectrin β chain and the integral membrane protein band 3 (3). Liu and Palek (24) have shown that stability of the normal membrane skeleton is related to its high content in the tetrameric form of spectrin. If tetramer is experimentally induced to transform into dimer in the membrane, the mechanical stability of the skeleton decreases.

Several cases of hemolytic neonatal anemia with poikilocytosis and bizarre red cell morphology have been related to inherited defects of the membrane skeleton: a complete deficit of protein 4.1 has been reported in three children (11, 37). Their parents were related and showed elliptocytosis and partial deficit of protein 4.1. Agre *et al.* (1) have reported two unrelated families with a form of hemolytic poikilocytic anemia. The four affected patients presented a reduction in the number of high affinity membrane-binding sites for ankyrin. HPP is characterized by an unusual thermal sensitivity of erythrocytes which fragment at 43–46°C instead of 49°C in normal subjects (42). It is now obvious that this disease is related to defective spectrin dimer self-association (8, 26). The same functional defect of spectrin has been also found in several cases of HE, termed type I HE (6, 25). More recently, Evans *et al.* (10) have reported a family in which three children had homozygous HE with severe poikilocytic anemia. A marked defective spectrin dimer self-association was found in all three patients. Their unrelated parents displayed mild HE and a lesser but significant defect in spectrin self-association. Lawler *et al.* (19), Knowles *et al.* (17), and Liu *et al.*

(23) described alterations in the trypsin-resistant domains of spectrin in HPP and in few cases of type I HE. These alterations correlated with the defective self-association of spectrin dimer.

We studied a black family in which the proband suffered from a severe neonatal hemolytic anemia with poikilocytosis. Both parents', the sister's, and brother's proband were clinically normal. Morphology and deformability of the red cells and spectrin self-association studies performed in this family led us to discuss the diagnosis of homozygous type I HE in the proband. In addition, the functional defect of spectrin appeared to be related to an abnormal limited tryptic digest pattern of the spectrin molecule. The observation of this family illustrates the contribution of biochemical studies to the diagnosis of poikilocytic hemolytic anemia in children and to the identification of asymptomatic carriers.

CASE REPORT

The family we studied is of Malian extraction. The clinical report of the proband Mam. T., a male child, has been extensively described (14). Briefly, Mam. T. was born in October 1981 after 38 weeks of a normal pregnancy. He was the third child alive (a boy was stillborn after 36 weeks of pregnancy without apparent etiology). Delivery was normal, no congenital defects were apparent, but the amniotic fluid was colored. At 6 h, icterus and hepatomegaly were noted. Hemoglobinuria was present and bilirubinemia was 240 mmol/liter. Hemoglobin was 8.3 g/dl, red blood cell count was 3.5×10^{12}/liter, hematocrit was 23%, reticulocyte count was 6×10^{11}/liter, and erythroblast count was 5×10^{10}/liter. On blood smears, anisochromy, anisocytosis, and poikilocytosis were noted. The blood group of both the mother and the child was O Rh(+). The Coombs test was negative. Red cell enzymes (glucose-6-phosphate dehydrogenase and pyruvate kinase) were normal. Bone marrow studies showed erythroblastic hyperplasia without maturation abnormalities. However, marked iron overload was noticed in the reticuloendothelial cells. Serological tests of infectious diseases were negative (rubella, herpes, toxoplasmosis, Epstein-Barr virus). Two exchange transfusions were required at 16 and 26 h because of bilirubin level greater than 550 nmol/liter but he relapsed into anemia and was transfused again at days 6, 14, and 26. During the subsequent months, the child continued to have a severe hemolytic anemia and until today, he requires blood transfusions every month. Nevertheless, growth and development have been within normal limits. Hemoglobin chain synthesis was normal but a heterozygous HbS state was depicted. HLA typing of the child and both parents was not consistent with a false paternity and showed that the parents were not related.

The parents (mother Men. T. and father Abd. T.), sister Rab. T., and brother Han. T. have no signifiant medical history and nothing suggested the possibility of a congenital hemolytic disease. The last sister, Mag. T., was born prematurely and died 3 months later because of respiratory failure and septicemia. A sample of blood, collected on the 3rd day of her life, was available for red blood morphological and deformability studies. She never experienced hemolytic anemia. Red cell enzymes (glucose-6-phosphate dehydrogenase and pyruvate kinase) were normal in both parents and hemoglobin studies revealed heterozygous HbS-α-thalassemia in the father and were normal in the mother and brother; Rab. T. was found to be heterozygous HbS. Serum iron was in the range of normal values in the mother (11 µmol/liter with a total iron-binding capacity of 61 µmol/liter).

MATERIALS AND METHODS

Materials. βME, glutaraldehyde, EDTA, Tris, glycerol, sucrose for density gradient studies, and TPCK-trypsin (3.5 units/mg) were from Merck (Darmstadt, G. F. R.). PMSF and diisopropyl fluorophosphate were from Sigma (St Louis, MO). All materials used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis were from Bio-Rad Laboratories (Richmond, CA). Samples

of venous blood, obtained from the different members of the family were anticoagulated with heparin and used within 24 h. Studies performed in the proband were done 7 weeks after a transfusion.

Methods. Routine hematologic determinations. Results were obtained with a Coulter Counter Model S. Reticulocytes counts were made after new methylene blue staining.

Morphological studies. Erythrocytes were examined on dried smears stained with May-Grünwald-Geimsa and on wet smears; for this latter purpose, fresh blood samples were immediately fixed in 5 mM NaPO₄, 150 mM NaCl, pH 7.4 (PBS) with 1% glutaraldehyde and examined by light phase contrast microscopy.

Red cell thermal sensitivity study. Control and patient erythrocyte thermal sensitivity was examined as previously described (42).

Deformability measurements (osmotic gradient ektacytometry). Whole cell deformability was measured in the ektacytometer (4) as a continuous function of the suspending medium osmolality (12). Cell deformability is expressed as EI which is equivalent to the ellipticity of the deforming cells. The EI was measured during progressive variation of the osmolality from 60 to 450 mosm kg^{-1} at 22°C. Each patient erythrocyte deformability measurement was compared with that of a normal control.

Preparation of the erythrocyte membranes. Blood samples were centrifuged and the buffy coat was removed. Erythrocytes were washed twice in 5 mM NaPO₄, 150mM NaCl, pH 8.0. Ghosts were prepared according to Dodge *et al.* (9) except that the lysis buffer was 5 mM NaPO₄, 0.3 mM PMSF, pH 8.0.

SDS-polyacrylamide gel electrophoresis. Slab gel electrophoresis of ghosts was performed according to Laemmli (18) using a 5–15% polyacrylamide gradient gel.

Spectrin extraction. For low ionic strength extraction, erythrocyte ghosts were washed twice in 0.3 mM NaPO₄, pH 8.0. The packed membranes were then suspended in an equal volume of low ionic strength buffer containing 0.3 mM NaPO₄, 0.1 mM EDTA, 0.1 mM PMSF, 0.1 mM βME, pH 8.0, and either incubated at 37°C for 30 min and immediately cooled on ice (37°C extract) or dialyzed overnight at 4°C against the same buffer (4°C extract). Supernatant extract and ghost residues were separated by centrifugation at 150,000 × g for 45 min.

Study of spectrin dimer-dimer association in solution and determination of equilibrium constant K_a. The supernatant containing crude spectrin (37°C extract) was dialyzed overnight at 4°C against 100 volumes of 5 mM NaPO₄, 0.3 mM PMSF, 150 mM NaCl, 0.1 mM EDTA, 0.2 mM βME, pH 8.0. After estimation of the protein concentration by absorbance at 280 nm, taking an $E_{1 cm}^{1\%}$ (280 nm) of 10.7 (15) and adjustment of required concentrations, each sample of dialyzed proteins was incubated for 240 min at 30°C to induce spectrin dimer to tetramer transformation. After cooling each incubated sample (mean volume, 0.5 ml; concentration range, 0.5–2 mg/ml) was applied to a 10–30% (w/v of dialysis buffer) linear sucrose gradient. Ultracentrifugation was performed in a SW 40 Beckman rotor at 40,000 rpm for 15 h at 4°C. Gradients were eluted at 4°C from the top to the bottom and the absorbance was automatically monitored at 280 nm. Fraction α of spectrin converted into tetramer after 240-min incubation at 30°C was determined from the sedimentation profile by measuring the areas under the peaks corresponding to spectrin dimer (D) and tetramer (T). α is given by the ratio $(T/D + T)$; writing c for the total molar concentration of spectrin (expressed as a dimer of molecular weight 460,000) K_a is given by $\alpha/2c(1-\alpha)^2$

Distribution of spectrin species in the 4°C extract. The distribution of spectrin species in the 4°C extract was studied using two methods: (i) the supernatant containing crude spectrin (4°C extract) was dialyzed at 4°C against 100 volumes of 5 mM NaPO₄, 0.3 mM PMSF, 150 mM NaCl, 0.1 mM EDTA, 0.1 mM βME, pH 8.0. Samples were submitted to sucrose gradient velocity sedimentation and fractions of spectrin dimer and tetramer were estimated from the sedimentation profile as described above; (ii) spectrin species contained in the 4°C extracts were

separated by NDGE. NDGE was performed using 140 × 100 × 1.5 mm slab gel with 3.5% acrylamide in 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, 10% glycerol, pH 7.4 (5). Samples were applied to the gel in a buffer containing 10 mM Tris, 1 mM EDTA, 10% glycerol, 0.005% bromophenol blue, pH 8.0. Gels were loaded and electrophoresed at 4°C for 48 h employing 50 V. Gels were stained by Coomassie blue (5) and scanned at 550 nm in a DU8 Beckman spectrophotometer with a gel scanner system. The proportion of spectrin dimer was estimated by measuring the ratio $D/D + T$, D and T corresponding respectively to the surface of dimer and tetramer peaks.

Limited tryptic digestion. Two preparations of spectrin were submitted to tryptic digestion: the 37°C spectrin extract (adjusted to 0.5 mg/ml) and pure spectrin dimer (adjusted to 0.1 mg/ml); dimer was separated from tetramer by sucrose gradient sedimentation after the spectrin dimer self-association procedure. Limited tryptic digestions were performed with TPCK-trypsin at 0°C for 20 h in PBS, pH 8.0, with an enzyme/substrate ratio of 1:100 (w/w). Digestions were ended by adding diisopropyl fluorophosphate (final concentration, 1 mM). Digests were electrophoresed on a 7–22% SDS-polyacrylamide gradient slab gel (18) with a 3% stacking gel. Dimensions of the gels were 150 × 135 × 1.5 mm. Gels were run at 100 V until the dye reached the bottom line, then stained by silver (29) and scanned either through the white light beam provided by the spectrophotometer or at 633 nm. For each experiment, normal control spectrin was digested and electrophoresed in the same conditions as the patient's spectrin.

RESULTS

Hematological investigations (Table 1). Erythrocyte indices of the five studied family members were obtained at the time of the biochemical investigation.

Morphological studies revealed the presence of ovalocytic cells. On dried blood smears, the mother showed moderate anisocytosis; the father exhibited more prominent anisocytosis with the presence of 2–3% elliptic cells. On a glutaraldehyde-fixed wet smear preparation, both parents (Fig. 1, *B* and *C*) exhibited the presence of 30 to 40% ovalocytic or "roundish" cells as defined by Lipton (22). Sister Rab. T. red cell morphology was similar to that of her father Abd. T. (Fig. 1*E*).

Morphological studies in proband Mam. T. were more difficult to interpret because of the presence of transfused cells (the study was done 7 weeks after a transfusion). Two cell populations were observed on wet preparation (Fig. 1*A*). One population was normocytic and mostly discocytic. The other (estimated at 20%) was microcytic and composed of microspherocytes and microovalocytes. The normocytic population corresponded probably to the transfused red cells but a careful examination revealed that about 10% of the cells were slightly ovalocytic and similar to those observed in both parents.

A blood sample from the sister Mag. T. was collected at the 3rd day of life; wet preparations (Fig. 1*F*) showed anisocytosis, poikilocytosis, and numerous stomatocytes (premature infant). Careful examination revealed the presence of few schizocytes, ovalocytes, and elliptocytes.

Erythrocyte morphology in brother Han. T. was normal on both dried and wet preparation (Fig. 1*D*).

Osmotic gradient ektacytometry revealed abnormal cell deformability. Both parents (Fig. 2), sister Rab. T., and sister Mag. T. (Fig. 3) had red cells less deformable than control red cells. EI was decreased in isotonic conditions. EI improved steadily when cells were in moderate hypotonic conditions but the optimum EI was always lower than that of the control. The minimal EI values (determined in extreme hypotonic and hypertonic conditions) were obtained at the same osmolalities as controls.

In proband Mam. T., EI in isotonic conditions was decreased (Fig. 2); no steady increase of EI was observed in moderate hypotonic conditions. As shown in Fig. 3, brother Han. T. red cell deformability was normal and similar to control.

Table 1. *Hematological investigations**

	Birth date	Hb (g/dl)	Red cell count (×10^{12}/liter)	Mean cell volume (fl)	Reticulocytes (%)
Mother Men. T.	1959	13.3	4.53	87	1
Father Abd. T.	1948	15.9	5.63	82	0.9
Brother Han. T.	1979	12	4.2	82	1.4
Sister Rab. T.	1980	11	3.9	83	1.8
Proband Mam. T.	1981	9	3.3	75	0.3

* Erythrocyte indices of the proband Mam. T. were obtained 7 weeks after a transfusion.
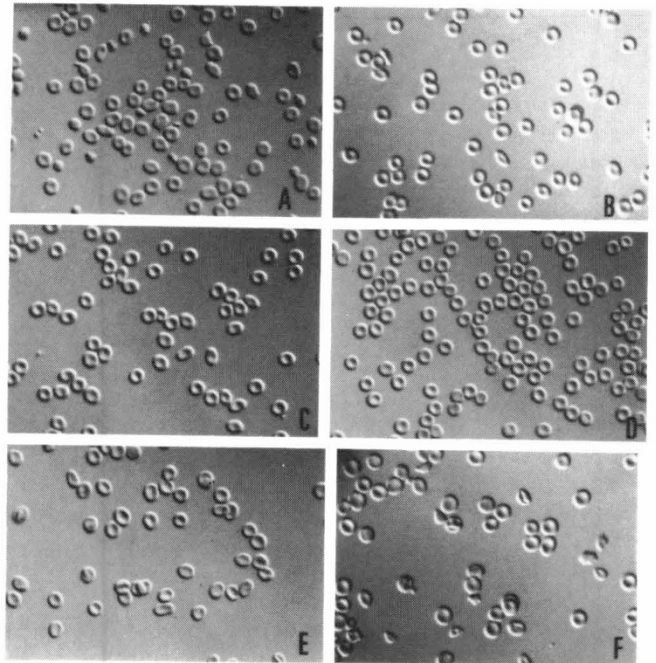

Fig. 1. Study of erythrocyte morphology. Erythrocytes were examined on glutaraldehyde-fixed wet smear preparations by light phase contrast microscopy (× 500). *A*, proband Man. T.; *B*, father Abd. T.; *C*, mother Men. T.; *D*, brother Han. T.; *E*, sister Rab. T.; *F*, sister Mag. T.

Erythrocyte thermal sensitivity. Thermal sensitivity was found normal in both parents, brother Han. T., and sister Rab. T. In the proband, no fragmentation was observed until 49°C but this study could not be conclusive because of the small percentage of abnormal erythrocytes present in the red cell population.

SDS-polyacrylamide gel electrophoresis patterns (Fig. 4). Erythrocyte membrane electrophoresis of the five family members exhibited normal patterns.

Spectrin dimer-dimer association was decreased both in membrane and in solution. In the 4°C extract, the spectrin dimer-tetramer equilibrium is kinetically trapped in its native state in the membrane. In normal subjects, the tetrameric state is largely predominant. The normal percentage of spectrin dimer, determined by means of sucrose gradient velocity centrifugation was found to be 15 ± 2% (n = 24). Similar values were obtained using NDGE: 18 ± 4% (n = 15).

Table 2 shows the percentage of spectrin dimer in the 4°C extract found in the five studied family members using sucrose gradient velocity sedimentation. Moderate but significant increase of spectrin dimer was found in proband. Both parents and sister Rab. T. exhibited greater amounts of spectrin dimer (values between 45 and 48%). The percentage of spectrin dimer was also determined twice in mother Men. T. using NDGE. The mean value was 45% which is similar to that obtained using sucrose gradient separation.

As reported (8, 26), conversion of spectrin dimer into tetramer can be directly studied in the 37°C extract. After incubation at
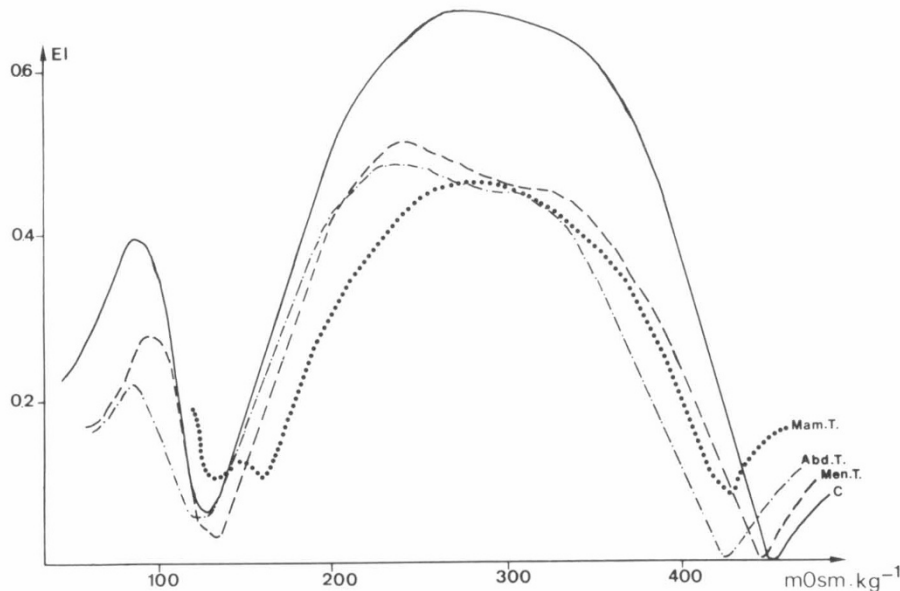
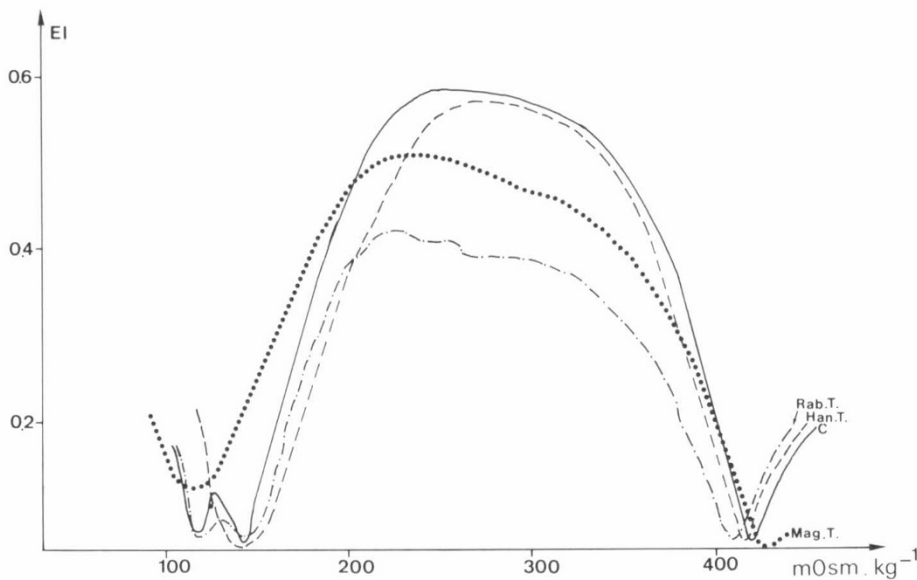Fig. 2. Osmotic gradient ektacytometry. Curves of both parents (Abd. T. and Men.T.) are compared to control *C*.

Fig. 3. Osmotic gradient ektacytometry. Curves of sister Rab. T., brother Han. T., and sister Mag. T. are compared to control *C*.

30°C for 240 min in order to reach the equilibrium state, we found the association constant (K_a) for normal spectrin to be 6 (+ 0.4) $\times$ 10^5 M^{-1} (n = 42). K_a values, determined in each member of the family are presented in Table 2. Spectrin dimer self-association was defective in both parents, sister Rab. T., and proband Mam. T.

Limited tryptic digestion of spectrin from normal subjects. As proposed by Lawler *et al.* (19), an enzyme/substrate ratio of 1:100 was chosen to investigate both control and patient spectrin. The crude 37°C extracts were extensively used as substrate because peptide patterns obtained either from 37°C extracts or gel filtration purified spectrin were quite similar. Peptide patterns produced by limited tryptic digestion of spectrin in 23 white and 31 black normal subjects (native of Africa or Antilles) were reproducible and no variations were observed in the 70,000- to 110,000-dalton peptide range. However, some variations were noticed in 35,000-, 37,000-, 40,000-, and 48,000-dalton peptides as previously described (19).

Limited tryptic digestion of spectrin from the five family members. Results are summarized in Table 2. Two different preparations of spectrin were subjected to tryptic digestion: crude

spectrin extracted at 37°C and spectrin dimer separated from tetramer after the spectrin dimer self-association procedure.

Tryptic digest patterns of the 37°C extracts. As shown in Fig. 5, densitometric tracings of both parental tryptic digest patterns revealed a decrease in the 80,000-dalton peptide, an increase in a 78,500-dalton peptide, and a broadening of a 74,000-dalton peptide. Densitometric tracing of the proband's pattern (Fig. 6) displayed the same findings but to a lesser extent. Furthermore, a 28,000-dalton peptide was markedly reduced in both parents (Fig. 7A). The other modifications in the 35,000- to 48,000-dalton peptides range are shown in Table 2.

Tryptic digest patterns of isolated spectrin dimer. As shown in Fig. 7, *B* and *C*, the spectrum of spectrin dimer tryptic digests was different from that obtained with the 37°C extract: a larger quantity of material associated with the high molecular weight peptides was present. The discrepancy could be explained by the five times lower concentration of protein used in the digestion procedure and the presence of sucrose in the dimer sample (about 20%, w/v). In both parents (Fig. 7, *B* and *C*), the decrease in the 80,000-dalton peptide and increase in the 74,000-dalton peptide were more manifest than in the 37°C extract tryptic patterns.
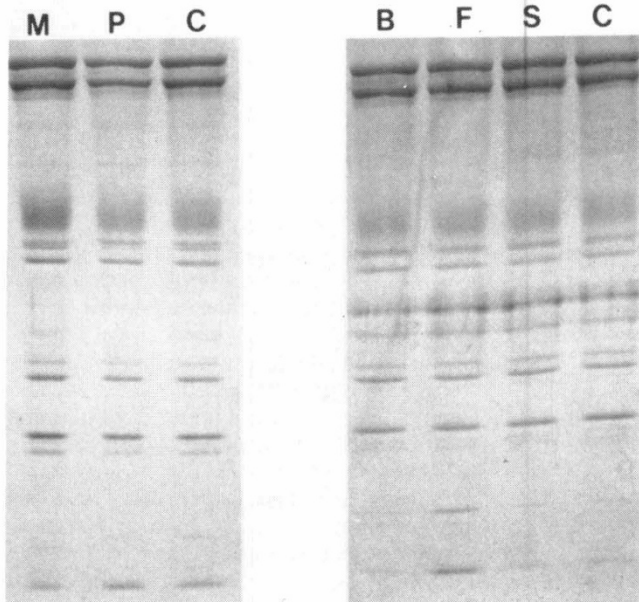
Fig. 4. SDS-polyacrylamide gel electrophoresis. *M*, mother Men. T.; *P*, proband Mam. T.; *B*, brother Han. T.; *F*, father Abd. T.; *S*, sister Rab. T.; *C*, control.

The decrease in the 28,000-dalton peptide was more apparent in the mother (Fig. 7*C*). The tryptic pattern of sister Rab. T. (Fig. 7*B*) was indistinguishable from that of her father and the tryptic pattern of normal brother Han. T. (Fig. 7, *B* and *C*) did not show any modifications in the 80,000-, 74,000-, and 28,000-dalton peptides. The other modifications in the 35,000- to 50,000-dalton peptides range are shown in Table 2.

DISCUSSION

The case reported herein illustrates the difficulty of neonatal hemolysis diagnosis in a transfusion-dependent infant. The presence of poikilocytes in blood leads one to search for a red cell membrane skeleton defect. Owing to recent additions to knowledge of the erythrocyte membrane, it is now possible to approach the diagnosis by means of biochemical evaluations of both parents, even if they are asymptomatic. So the first time discovery of a spectrin self-association defect in both parents allowed us to suspect double inheritance of this abnormality in the proband. A complete evaluation of the family confirmed our hypothesis. The moderate increase of spectrin dimer in the proband 4°C extract (26%) compared to the values found in other family members could be explained by the presence in the blood of transfused cells. Until today, three congenital hemolytic disorders with poikilocytosis have been found to be related to a defective self-association of spectrin: HPP (8, 17, 20, 26), homozygous HE

Table 2. *Biochemical investigations**

	Spectrin association constant in solution† (×10⁵ M⁻¹)	Spectrin dimer in the 4°C extract (%)‡	Modifications of limited tryptic digest patterns§													
			80-kd		74-kd		50-kd	48-kd	47-kd	45-kd	40-kd	37-kd		35-kd		28-kd	
			CS	SD	CS	SD	SD	CS	SD	CS	CS	CS	SD	CS	SD	CS	SD
Mother Men. T.	1.9 ± 0.4 (4)	48.5 (2)	↓¶	↓	↑	↑	↑	−	↓	↑	−	+	+	↓	↓	↓	↓
Father Abd. T.	2.3 (2)	45	↓¶	↓	↑	↑	↑	−	↓			+	+	↓	↓	↓	
Brother Han. T.	5.7 (2)	16	ND		ND		↑↑	ND	−	ND	ND	ND	+	ND	−	ND	
Sister Rab. T.	2.45 (2)	47	ND	↓	ND	↑	↑	ND	↓	ND	ND	ND	+	ND	↓	ND	↓
Proband Mam. T.	1.9 (2)	26 (2)	↓¶		↑						↑						

* Only unequivocal modifications of proband's spectrin tryptic pattern noticed in the 37°C extract digest are presented. Numbers in parentheses, number of samples.

† Normal values are $6 \pm 0.4 \times 10^5$ M⁻¹.

‡ Normal values are 15 ± 2%.

§ CS, crude spectrin extract performed at 37°C. SD, spectrin dimer isolated from spectrin tetramer after self-association process. ND, not done. + and − correspond respectively to the appearance and disappearance of the peptide. ↑ and ↓ correspond respectively to the increase and decrease in the peptide amount.

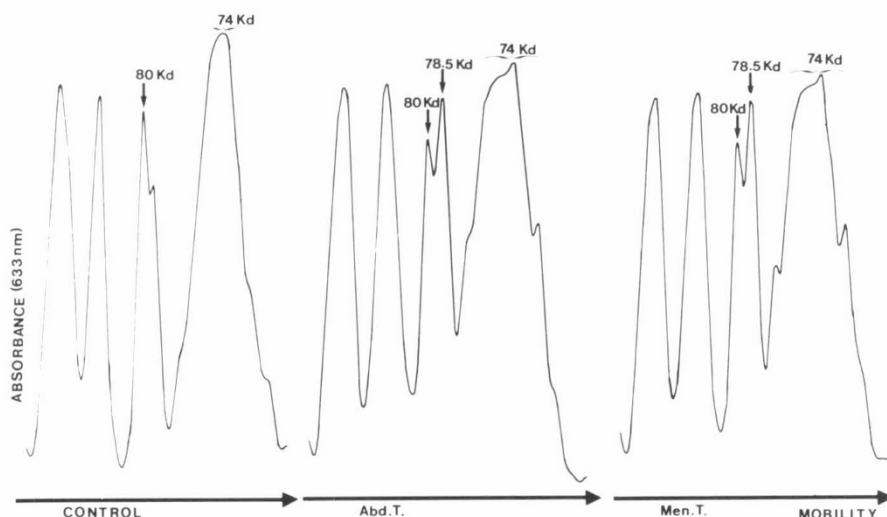¶ The decrease in the 80,000-dalton peptide was concomitant with increase in a 78,500-dalton peptide.

Fig. 5. Tryptic digest pattern densitometric tracings restricted to the 70,000–110,000-dalton region, of both parents Abd. T. and Men. T. and control.

(10), and HE with pyknocytosis in infants (33; personal observation); the latter disease is not consistent with the clinical features of our case because severe hemolysis and poikilocytosis persisted after 18 months (34). Blood smears in HPP (8, 26, 34, 42) and homozygous HE (10, 13, 22, 32, 35, 41) are quite similar, characterized by extreme poikilocytosis, with fragments, budding cells, microspherocytes, microelliptocytes, and other bizarre poikilocytes. Notwithstanding the presence of transfused cells in the proband we studied, it was possible to recognize a poikilocytic population.

Degree of elliptocytosis may be artifactually diminished in dried smears (27) and, as illustrated in our studies, it is useful to examine glutaraldehyde-fixed wet smear preparations. By this technique, we depicted ovalocytic cells in both parents, two sisters, and the proband. The few elliptocytes shown in the father's smear could be related to the thalassemia trait. Many authors have previously noticed that, in some HE, the cells may be only slightly eccentric and not readily appreciated as abnormal (2, 40, 41). Ektacytometric studies were determinant in this field. Osmotic gradient ektacytometry curves obtained in both parents and two sisters were similar and the general aspect of the curves was consistent with hereditary elliptocytosis. In our experience, this characteristic profile, with a steady increase of the EI in moderate hypotonic conditions, was only found in HE. It was never observed in hematological disorders such as iron deficiency, thalassemia trait, or megaloblastic anemia where anisocytosis with few ovalocytes or elliptocytes could be present.

The low reticulocyte count found in the proband (Table 1) cannot be totally explained by transfusion frequency. Bone marrow studies did not show any maturation defect or red cell aplasia. Anomalously low reticulocyte counts with erythroblastic hyperplasia were previously reported by Torlontano *et al.* (38) in some cases of hereditary hemolytic ovalocytosis. On the basis of the studies reported by Prchal *et al.* (34) the youngest erythrocyte population could display increased fragility providing an explanation for the observed low reticulocyte count.

Among tryptic digestion pattern modifications observed in this family, only decrease in the 80,000-dalton peptide and increase in the 74,000-dalton peptide were related to the defective self-association of spectrin. The decrease in the 28,000-dalton peptide, more clearly observed in the 37°C extract digests, also correlated with the spectrin defect. These tryptic digest abnormalities were more readily visualized in the spectrin dimer fraction remaining after dimer to tetramer conversion. This emphasizes the relationship between tryptic pattern modifications and functional spectrin abnormality, since the defective
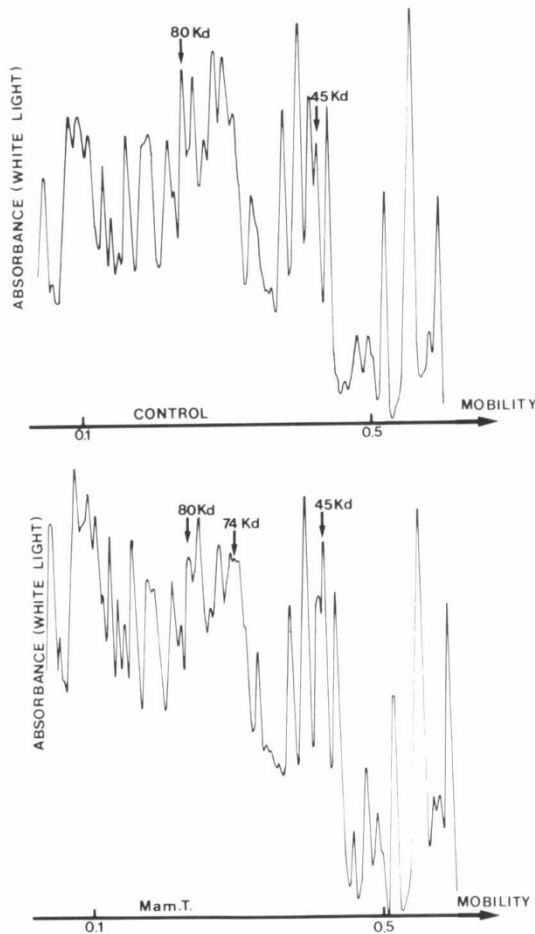

Fig. 6. Tryptic digest pattern densitometric tracings of proband Men. T. and control.
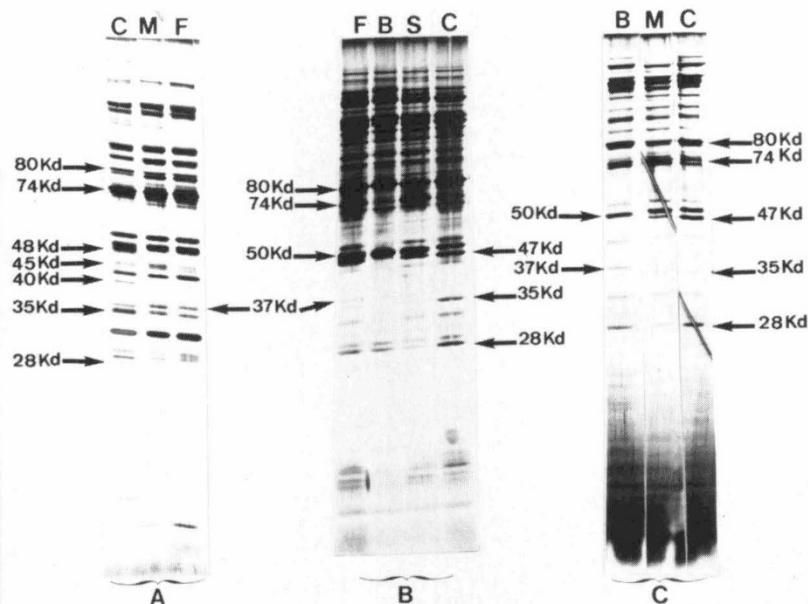

Fig. 7. SDS-polyacrylamide gel electrophoresis patterns of limited tryptic digests. The separating gel was a 7–22% gradient; 10 μg of protein were loaded per lane. Peptides bands were stained by silver *A*, crude spectrin (37°C extract) tryptic digests of mother Men. T. (*M*), father Abd. T. (*F*), and control (*C*). *B* and *C*, isolated spectrin dimer tryptic digests of both parents, brother Han. T. (*B*) and sister Rab. T. (*S*).

spectrin molecule remained mostly in dimeric form. Morrow *et al.* (31) showed that the 80,000-dalton peptide fragment, called αI domain resulting from limited tryptic digestion of spectrin, corresponds to the terminal fragment of the α chain involved in the self-association process. Furthermore, Morrow *et al.* have also demonstrated (30) that trypsin is capable of removing a 6,000-dalton peptide from the 80,000-dalton peptide and the resulting 74,000-dalton peptide has lost the ability to bind the native spectrin dimer. So, the defective spectrin self-association is consistent with the modification of the 80,000- and 74,000-dalton peptides. Identical results have been previously reported by Lawler *et al.* in the two HPP kindred (19) and by Liu *et al.* in some type I HE cases (23). As proposed by Lawler, we could infer that the conformational change of the spectrin molecule, affecting the self-association site of the αI domain renders it more susceptible to tryptic digestion.

Other tryptic pattern modifications, nonrelated to the functional spectrin abnormality, were also noticed (Table 2). Study of family T. allowed us to consider that the presence of both the 37,000- and 35,000-dalton peptides corresponds to a heterozygous state of a genetic polymorphism, previously depicted using Cleveland digest of α chain (21). The 37,000-dalton peptide, found in brother Han. T. corresponds to the homozygous state. The variations of the 47,000- and the 50,000-dalton peptides paralleled those of the 35,000- and 37,000-dalton peptides. Recently, Knowles *et al.* (16) showed that the digestion of the spectrin αII domain generates 46,000- and 35,000-dalton peptides which probably correspond to our 45,000- and 35,000-dalton peptides. The authors also described αII domain variations in a black population identical to those described in family T.

We think that it is reasonable to propound the diagnosis of heterozygous type I HE for both parents and two sisters and the diagnosis of homozygous type I HE for the proband (Fig. 8) owing to the following reasons: slight ovalocytosis was present in both parents and two sisters; cell deformability ektacytometric studies gave the same profiles of curve as those observed in patients with HE; defective spectrin dimer self-association was found in both parents, sister Rab. T., and proband associated with the same abnormal spectrin digest pattern. Nevertheless, each of the above data is not conclusive and has to be discussed. Elliptocytes were slightly eccentric and only appreciated on wet smear preparations. Ektacytometric studies confirmed the presence in these patients of a red cell population exhibiting the abnormal deformability characteristic of that observed in HE, but this syllogism might be found to be erroneous in the future. Clinical presentation of the proband is consistent either with HPP or with homozygous HE. Erythrocyte thermal sensitivity studies in the proband cannot be conclusive because of the presence of transfused cells. In HPP kindred reported by Lawler *et al.* (19), spectrin abnormalities identical to those we described were found only in one of the two parents (HPP carriers). However, Mentzer *et al.* (28) reported recently HPP kindred in which a spectrin dimer excess in the 4°C extracts was found in both parents, one of whom also exhibited elliptocytosis. In addition, the structural alterations of the spectrin molecule peptide mapping found in the family T. has been described in HPP as well as in some cases of type I HE.

Clinical, rheological, and biochemical investigations have to be carried on in an attempt to understand better the relationship between HE and HPP. It remains that the erythrocyte membrane biochemistry has already established itself as a permanent companion in diagnosis of many congenital hemolytic disorders.

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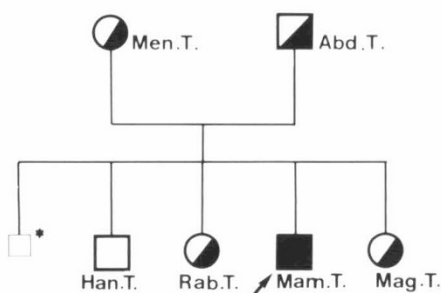27. Lux SA, Glader BE 1981 Disorder of the red cell membrane. In: Nathan DG,

Fig. 8. Pedigree of T. family. ◐, ◩, heterozygous elliptocytosis; ■, homozygous elliptocytosis; ★, stillborn; ↗, proband.

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Iron Is Sequestered as Ferritin in Macrophages in Skeletal Muscle of Vitamin E-deficient Rabbits

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Summary

Weanling rabbits were fed a purified diet with or without vitamin E supplementation to evaluate the abnormal sequestration of iron in skeletal muscle associated with vitamin E deficiency. A severe myopathy developed in unsupplemented rabbits within 3 to 4 weeks. At this time, the concentration of soluble nonheme iron in biceps femoris muscles had increased from 2.1 ± 0.4 µg/g wet weight (mean ± SD) for six control rabbits to 4.3 ± 1.4 for 10 vitamin E-deficient rabbits, and total nonheme iron had increased from 5.0 ± 1.2 to 8.4 ± 3.3. Soleus muscles had even greater increases in total and soluble nonheme iron concentrations. Intramuscular injection of iron-dextran caused large increases in total and soluble nonheme iron in noninjected muscle of vitamin E-deficient rabbits, which further exaggerated the difference between the two groups. By radioimmunoassay using an antibody to rabbit liver ferritin, the concentration of ferritin in biceps femoris muscles increased from 0.47 ± 0.18 µg/g wet weight for seven control rabbits to 6.34 ± 1.70 for 14 vitamin E-

deficient rabbits. Uptake of intravenously injected transferrin-bound iron into muscle of vitamin E-deficient rabbits was not increased in a short term experiment (6 h), but radioiron did accumulate in muscle in a long term experiment (6 days). There was no trapping of heat-damaged erythrocytes, no phagocytosis of intravenously injected carbon particles, and no erythrophagocytosis in muscle. An immunohistological staining method designed to detect ferritin in tissue sections stained muscle from normal rabbits very scantily but intensely stained macrophages in the muscle of vitamin E-deficient rabbits. We conclude that macrophages in skeletal muscle of vitamin E-deficient rabbits take up iron from transferrin and incorporate it into ferritin, in which form it is relatively unavailable for erythropoiesis because of slow release.

Young vitamin E-deficient rabbits have high serum iron-binding capacity, low serum iron, and high erythrocyte free protoporphyrin concentrations, and they recover slowly from anemia induced by phlebotomy (2). These abnormalities are secondary to iron sequestration in muscle rather than to a decrease in the total amount of iron in the body (2). Since vitamin E-deficiency is known to cause an extensive necrotizing myopathy accompanied by infiltration of macrophages (1), the present study evaluated the involvement of macrophages in the sequestration of iron.

MATERIALS AND METHODS

Young New Zealand White rabbits initially weighing approximately 650 g were used in all except one experiment. Rabbits