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Hereditary pyropoikilocytosis and elliptocytosis in a Caucasian family

Transmission of the same molecular defect in spectrin through three generations with different clinical expression

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Summary. Hereditary pyropoikilocytosis (HPP) is a severe hemolytic anemia characterized by a material instability of the red cell membrane leading to cell fragmentation. This fragility may be correlated with functional and structural defects of spectrin. Most HPP patients have been black. We now report three HPP patients from a Caucasian family, the proposita and her two maternal uncles. The proposita's mother and daughter presented mild type I hereditary elliptocytosis (HE), while the proposita's father was clinically and hematologically normal. Our studies revealed a defective ability of spectrin to self-associate, resulting in an excess of spectrin dimer in 4°C extracts in the three HPP patients and to a similar extent in HE relatives. Limited tryptic digestion of spectrin showed a molecular variant in the αI domain as expressed by a decreased amount of 80000-dalton peptide with a concomitant increase in the 74000-dalton peptide. Investigations in the proposita's father revealed no abnormalities of the erythrocyte membrane. The co-transmission of HPP and HE phenotypes in the same lineage might suggest variability in the clinical expression of the same molecular defect and lead us to discuss the hypothesis of a double heterozygosity in HPP patients.

Introduction

Hereditary pyropoikilocytosis (HPP) is a relatively rare, but severe, congenital hemolytic anemia characterized by marked poikilocytosis with microcytes, microspherocytes, and fragmented cells, leading to a very small mean corpuscular volume. As first noted by Zarkowsky et al. (1975), the erythrocytes from these patients were particularly heat sensitive; they fragment between 43°–46°C, while normal cells exhibit a similar fragmentation at 49°C. The membrane protein structure, which maintains erythrocyte shape and consists mainly of spectrin, actin, and band 4.1 (Cohen 1983), is particularly unstable to mechanical stress in HPP patients (Liu et al. 1981). In these patients as in a subset of hereditary elliptocytosis (HE) patients, membrane fragility may be correlated with a molecular defect in spectrin. Spectrin, composed of the two subunits α and β, exists, in the membrane as tetramers or higher polymers resulting from head to head association of heterodimers. In HPP as in some HE patients, called type I HE (Liu et al. 1982), this dimer-dimer association is defective (Liu et al. 1981; Dhermy et al. 1983; Coetzer and Zail 1982; Evans et al. 1983; Mentzer et al. 1984a). Limited tryptic digestion of spectrin has revealed structural abnormalities in the αI domain (mol. wt. 80000 daltons) which contains the dimer-dimer interaction site: three variants were detected and characterized by the appearance of an abnormal peptide with a molecular weight of either 74000 daltons ($SpD\alpha^{I/74}$), 65000 daltons ($SpD\alpha^{I/65}$), or 46000–50000 daltons ($SpD\alpha^{I/46-50}$). The $SpD\alpha^{I/74}$ and the $SpD\alpha^{I/46-50}$ were found in HPP (Knowles et al. 1983; Lawler et al. 1982, 1983) as well as in type I HE (Dhermy et al. 1984a, Lawler et al. 1984; Lecomte et al. 1984, 1985a, b; Marchesi et al. 1986; Morris et al. 1986), the $SpD\alpha^{I/65}$ variant was observed only in type I HE (Garbarz et al. 1986; Lawler et al. 1985; Lecomte et al. 1985b).

HPP differs from HE as it apparently is inherited in a recessive manner. Both parents usually have been found to be clinically and hematologically normal (Knowles et al. 1983; Lawler et al. 1982, 1983), although in some kindreds one parent exhibits typical mild HE (Zarkowsky et al. 1975). When biochemical studies were available in both parents, one parent exhibited the same spectrin defect as his offspring. The severity of HPP as compared with the asymptomatic parent bearing type I HE is not yet understood. As observed in type I HE, most HPP patients are Blacks. We reported studies in a Caucasian family in which three members, the proposita and her two maternal uncles, had typical HPP with defective spectrin dimer self-association correlated with a molecular variant of the αI domain. The proposita's mother and daughter experienced typical mild HE with the same functional and molecular spectrin defect as that found in HPP relatives and to a similar extent. All investigations in the proband's father revealed no abnormalities in the erythrocyte membranes.

Thus, in this Caucasian kindred, we observed the transmission through three generations of a spectrin molecular defect to a similar extent, but with a different clinical expression. Because of these observations we will discuss here the hypothesis of a double heterozygosity in HPP patients.

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Materials and methods

Clinical materials

We studied a Caucasian family in which three members presented with HPP and two others, with mild HE. The predigree is shown in Fig. 1. The proposita BEP, a young female adult, and her two maternal uncles (JCB and DAB) had a history of neonatal hemolytic anemia followed by splenectomy. Several blood transfusions were required before splenectomy, which was done at 9 months for BEP, at 7 years for patient JCB, and at 11 years for patient DAB. The major clinical and laboratory features are summarized in Table 1. The proposita's mother (CB) and daughter (CP), who was 1 year old, experienced typical mild HE at the time of our studies. At age of 2 months, the child (CP) presented with hemolytic anemia (Hb 7.8 g/dl, reticulocyte count 200000/mm^3) with anisocytosis and poikilocytosis. At 7 months she still showed poikilocytosis and a high reticulocyte count, but her Hb was increased to 11 g/dl. At 9 months, her poikilocytosis disappeared, and the child presented with a compensated hemolytic anemia: Hb 13 g/dl, reticulocyte count 100000–200000 per mm^3. The proposita's father (JB) and her husband (JP) were clinically and hematologically normal. The proposita's maternal grandmother (GB) was known to have anemia, but was not available for study.

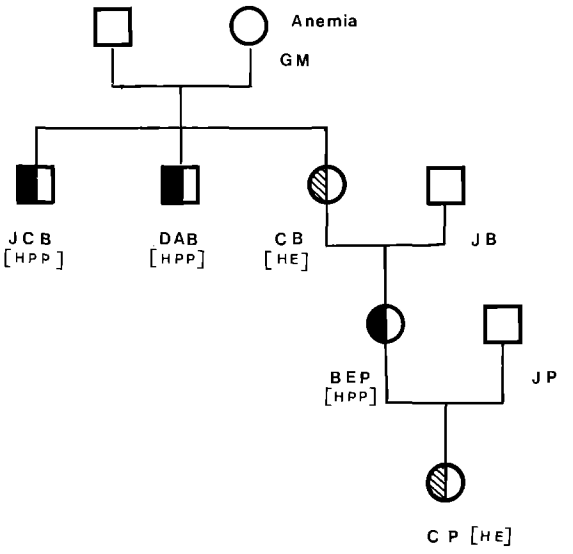

Fig. 1. Pedigree. Distribution of HPP (◧) and HE (▨)

Table 1. Hematological investigations

Subject		Clinical presentation	Splenectomy	Hemoglobin (g/dl)	Mean corpuscular volume (fl)	Reticulocytes (%)
Proposita	(BEP)	HPP	+ At 9 mo	12.8	73	20
Daughter	(CP)	HE		13	80	6
Mother	(CB)	HE		14.1	94	8.8
Father	(JB)	Normal		16.8	95	1.5
Maternal uncle	(DAB)	HPP	At 11 y	13.8	70	3.6
Maternal uncle	(JCB)	HPP	At 7 y	14.4	73	7.2
Husband	(JP)	Normal		16.3	101	1.8

Blood samples were collected in sterile vacutainer tubes containing heparin and were analyzed within 24 h.

Thermal sensitivity and morphology of erythrocytes

The thermal sensitivity of patient and control erythrocytes was studied as previously described (Zarkowsky et al. 1975). Normal and heated cells were examined by light phase-constrast microscopy after fixation in 1% glutaraldehyde, 5 m*M* phosphate buffer, and 150 m*M* NaCl at pH 7.4.

Membrane stability assay

The measurement of membrane resistance to shear-induced fragmentation was performed using the ektacytometer as described by Mohandas et al. (1982).

Erythrocyte deformability measurements

Whole cell deformability was measured in the ektacytometer (Groner et al. 1980) as a function of the suspending medium osmolality as previously described (Féo et al. 1982).

Preparation and analysis of red cell ghosts

Red cell ghosts were prepared by hypotonic lysis of washed red cells in 0.1 m*M* PMSF, 5 m*M* sodium phosphate buffer, pH 8.0, followed by three or four washes in the same buffer.

Membrane proteins were analyzed by electrophoresis in SDS-polyacrylamide slab gel (SDS-PAGE) with a 5%–15% polyacrylamide gradient as described by Laemmli (1970). Gels were stained with Coomassie blue. To estimate spectrin/band-3 ratios, SDS-polyacrylamide slab gel was performed as described by Agre et al. (1985) and scanned on a DU8 Beckman spectrophotometer at 550 n*m* after Coomassie blue staining.

Study of spectrin dimer-tetramer equilibrium

As previously reported by Liu et al. (1981), spectrin was extracted by incubating white ghosts in low ionic strength buffer pH 8 (0.3 m*M* sodium phosphate, 0.1 m*M* PMSF, 0.1 m*M* EDTA) either for 30 min at 37°C or overnight at 4°C. Spectrin extracts, separated from membrane residues by centrifugation, were dialyzed at 4°C against saline phosphate buffer, pH 8, containing 0.1 m*M* EDTA, 0.1 m*M* β-mercaptothanol, and 0.3 m*M* PMSF.

Spectrin dimer-dimer association in solution was performed as described by Dhermy et al. (1984a). Spectrin dimers (Sp-D) from 37°C extracts were incubated at 30°C under isotonic conditions for 240 min to induce tetramer formation. The distribution of spectrin species in the 4°C extracts and in the 37°C extracts after dimer-tetramer conversion was determined by ultracentrifugation on a linear 10%–30% sucrose gradient or by nondenaturing gel electrophoresis (Dhermy et al. 1984a).

Limited tryptic digestion of spectrin

Tryptic digestion was done at 0°C for 20 h in PBS, pH 8, with an enzyme-substrate ratio of 1:100. Substrate concentrations were always adjusted to 1 mg/ml for spectrin extracts, an absorbance at 280 n*m*, $E_{1cm}^{1\%} = 10.1$ (Clarke 1971) being

assumed. Digestion was ended by addition of diisopropylfluorophosphate (DFP) (final concentration 1 m*M*). All assays were done in duplicate. Spectrin peptides were separated on slab gels with a 7%–15% polyacrylamide gradient. Gels were stained with Coomassie blue and scanned at 550 n*m* in a DU8 Beckman spectrophotometer.

Tryptic peptides were also separated by two-dimensional gel electrophoresis (isoelectrofocusing followed by SDS-PAGE) using O'Farrell's method (1975) with modifications described by Speicher et al. (1982). Samples were electrofocused for 16 h at 340 V in 4% polyacrylamide tube gels (0.15 × 13 cm) containing 2.4% ampholytes. The second dimension analysis was performed on 7%–15% linear gradient slab gels (140 × 160 × 1.5 mm) as described by Laemmli (1970).

Chemicals and reagents

Acrylamide, NN'-methylene bisacrylamide, TEMED, sodium, dodecyl sulfate (SDS), DFP, and Coomassie brilliant blue were from Sigma Chemical Co. Standards for molecular weight determination were from Pharmacia fine chemicals. TPCK trypsin and other reagents were obtained from Merck.

Results

Red cell morphology and stability

The erythrocyte morphologies in the proposita BEP and her two maternal uncles DAB and JCB were similar and characterized by marked poikilocytosis with many budding and fragmented cells, microspherocytes, and some elliptocytes (2%). In both HE relatives (the proposita's mother and daughter, CB and CP respectively) the blood smears showed predominent elliptocytosis. In other healthy family members (in the proband's father (JB), and in her husband (JP)), red cell morphology was normal. Erythrocytes from all patients were abnormally sensitive to heat. They began to fragment at 45°C for patients BEP, DAB, and JCB and at 47°C for HE relatives CP and CB. Budding and fragmentation occurred at 49°C for erythrocytes from subjects JB and JP and controls. The mechanical stabilities of whole erythrocytes and their ghosts were studied in the three patients (BEP, DAB, JCB) and in the subjects JB and JP. Erythrocytes or ghosts were submitted to a constant shear force (1400 dynes/cm^2), and fragmentation was monitored with an ektacytometer. Erythrocytes from the proposita and her maternal uncles were extremely fragile. Conversely, mechanical erythrocyte stabilities were normal in the proposita's father and husband (Fig. 2).

Study of erythrocyte deformability by the ektacytometer

Deformability of erythrocytes is expressed as an ektacytometric index EI. When EI is plotted versus the osmolality of the suspending medium, the resulting curve shows three remarkable points: (1) the EI in isotonicity; (2) the minimum index in hypotonicity around 140 mosmol/kg which corresponds to the maximum volume of near-spherical cells just prior to hemolysis; and (3) the index in hypertonicity which corresponds to the osmolality at which EI equals half the normal maximum on the hypertonic arm of the curve and reflects the effects of the internal viscosity of the cell. Deformability of erythrocytes from the proposita, BEP, and her maternal uncles DAB and

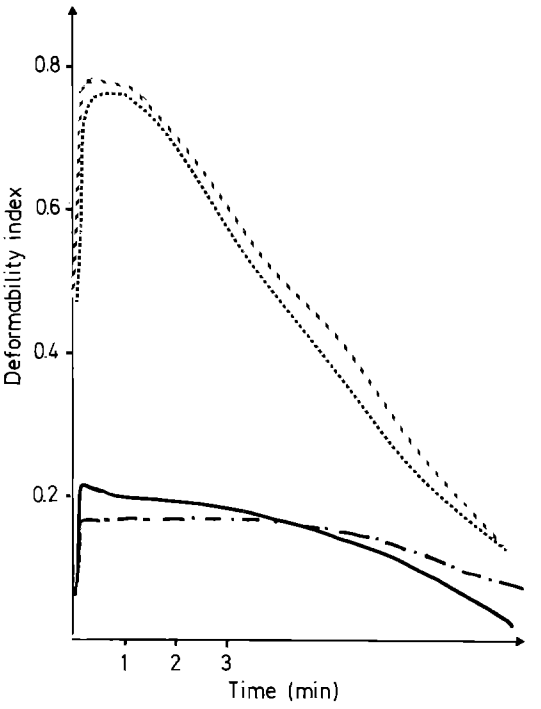

Fig. 2. Fragmentation curves of red cells from the proposita, her father (JB), and a maternal uncle (DAB). ·····, Control; - - - - - - -, father JB; ———, proposita; —·—·—, uncle DAB

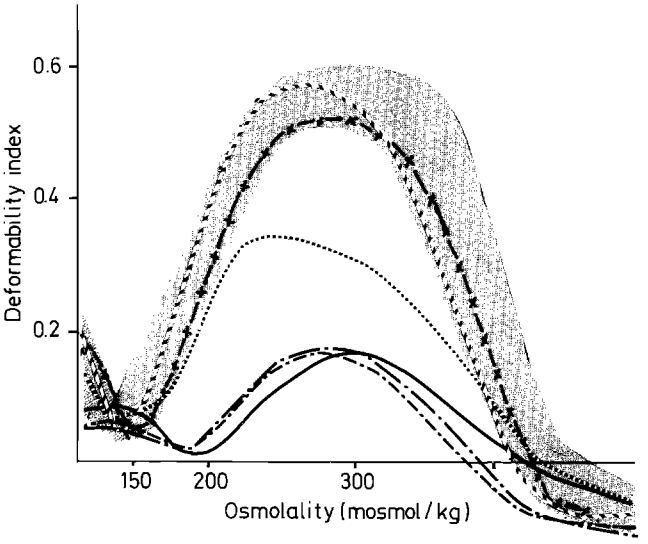

Fig. 3. Osmotic deformability profiles of red cells. ▨, Controls; - - - - - - -, father JB; ·····, daughter CP; –x–x–, husband JP; ———, proposita; -———-, uncle DAB; -----, uncle JCB

JCB was dramatically reduced as indicated by an EI in isotonicity between 0.13 and 0.16 (normal value 0.55 ± 0.04) (Fig. 3). The EI in hypotonicity was shifted to higher osmolality, which is characteristic of the presence of native spherocytic cells. Erythrocytes from the daughter CP generated trapezoidal shaped curves with a decreased EI in isotonicity (Fig. 3). As we have previously reported (Dhermy et al. 1984a, 1986; Garbarz et al. 1986), this trapezoidal curve is typical of HE with spectrin defects. Red cell deformability of the two subjects JB and JP was normal.

Table 2. Summary of biochemical data

Subject		Temperature stability[a]	Mechanical stability[b]	Spectrin/band 3[c]	Sp-D in 4°C extracts (%)	Association constant of Sp-D in solution ($\times 10/M$)	Tryptic fragments 74 kd/80 kd + 74 kd (%)
Proposita	(BEP)	45°C	↓↓	0.67	59 ($n = 3$)	0.9 ($n = 2$)	54 ($n = 4$)
Daughter	(CP)	47°C	ND	0.86	53 ($n = 1$)	ND	53 ($n = 2$)
Mother	(CB)	47°C	ND	0.89	49 ($n = 2$)	2.2 ($n = 2$)	42 ($n = 4$)
Father	(JB)	49°C	N	0.95	13 ($n = 1$)	5.2 ($n = 2$)	ND
Maternal uncle	(DAB)	45°C	↓↓	↓	46 ($n = 1$)	ND	62 ($n = 2$)
Maternal uncle	(JCB)	45°C	↓↓	↓	45 ($n = 1$)	ND	65 ($n = 2$)
Husband	(JP)	49°C	N	ND	3.5 ($n = 1$)	ND	ND
Control		49°C		0.98 ± 0.06	13.0 ± 3.6 ($n = 27$)	6.0 ± 0.4 ($n = 42$)	

[a] Temperature at which erythrocytes fragment and form membrane projections
[b] The mechanical stability of erythrocytes was classified as normal (N), considerably decreased (↓↓), or not done (ND)
[c] Mean of two evaluations. N, normal; ↓, decrease

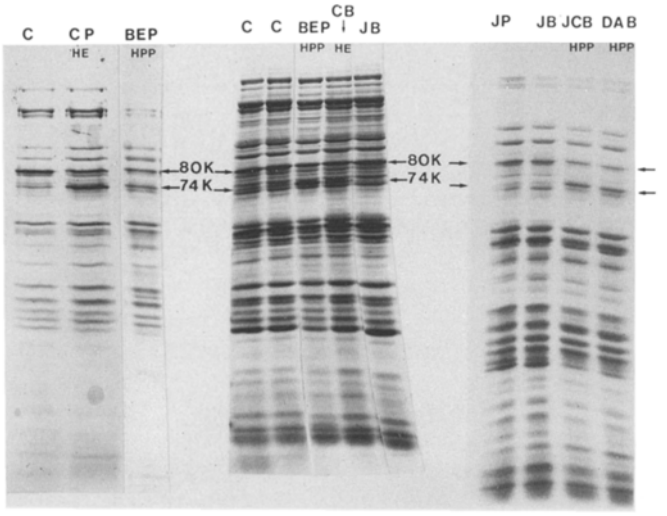

Fig. 4. SDS-polyacrylamide gel of tryptic digests of spectrin from the proposita (BEP), her daughter (CP), mother (CB), father (JB), maternal uncles (JCB, DAB), 2nd husband (JP)

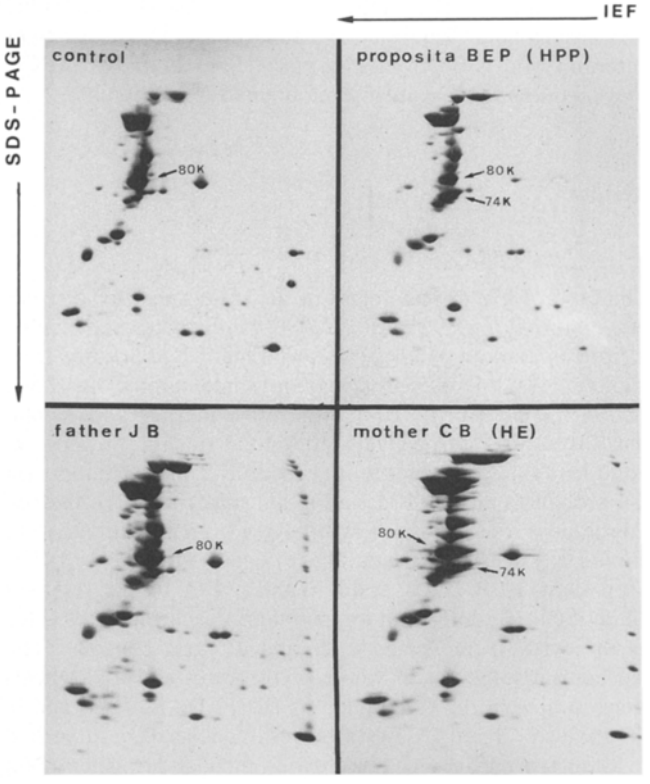

Fig. 5. Two-dimensional separation of tryptic digests of spectrin from the HPP proposita BEP, her HE mother (CB), her HE daughter (CP), and her father (JB)

Membrane protein composition

In the proposita and her two uncles, SDS-PAGE of membrane proteins exhibited an obvious decrease in the spectrin quantities in comparison with controls. This spectrin decrease was more accurately estimated by the spectrin/band-3 ratio, which was found equal to 0.67 in the proposita, the normal value being 0.98 ± 0.06. Neither quantitative nor qualitative abnormalities were detected in other family members.

Dimer-tetramer equilibrium

Spectrin exists in the membrane as a tetramer and higher polymers. We investigated the capacity of spectrin to self-associate by measuring the percentage of spectrin dimer in the 4°C extracts, which reflects its native state in the membrane. The 4°C extracts from erythrocytes of the three patients BEP, DAB, and JCB and the HE relatives contained an increased proportion of spectrin dimer, between 45% and 59% (normal value 13% ± 3.6%) (Table 2). The association constant of spectrin dimer in solution was decreased in the proposita BEP and her mother (Table 2).

Analysis of spectrin tryptic peptides by one- or two-dimensional gel electrophoresis

As we have previously reported a control sample was studied concurrently with the patient samples. The peptide maps showed a decrease in the 80000-dalton and 28000-dalton peptides with a concomitant increase in the 74000-dalton peptide (Figs. 4–5). Densitometer tracings indicated that the decrease in the 80000-dalton peptide was quite similar in the proposita, her uncles, her mother, and her daughter (Table 2). The peptide patterns from the proposita's father and husband were identical to control patterns.

Discussion

The three Caucasian patients, the proposita BEP and her two maternal uncles DAB and JCB exhibited a number of the clinical and hematological features previously associated with HPP by Zarkowsky et al. (1975): (1) severe hemolytic anemia with hyperreticulocytosis requiring blood transfusion and then splenectomy, which was successful; (2) significant poikylocytosis and microcytosis with microspherocytes on wet blood smears; (3) increased erythrocyte thermal sensitivity at 45°C. Microspherocytosis is more obvious on osmotic gradient ektacytometry curves where the minimum index in hypotonicity is shifted to higher osmolality values, reflecting the presence of osmotically fragile cells. As in some previously reported cases of HPP (Coetzer and Palek 1986; Mentzer et al. 1984a), studies of whole membrane proteins by means of SDS-polyacrylamide gel electrophoresis revealed a partial spectrin deficiency in the three HPP patients (BEP, DAB, and JCB). Although not well elucidated, this spectrin deficiency did not result from degradation of spectrin during the life span of HPP cells in blood circulation as demonstrated by Coetzer and Palek (1986). A proteolytic degradation might occur in bone marrow prior to the release of cells into the circulation. Hereditary pyropoikilocytosis seems to be the only congenital hemolytic anemia with a spectrin self-association defect where a partial deficiency of the protein was observed; however such a partial deficiency of Sp was found in a subset of HS (Agre et al. 1985; Dhermy et al. 1984b).

As revealed by all spectrin studies performed in HPP (Dhermy et al. 1983; Knowles et al. 1983; Lawler et al. 1982, 1983; Liu et al. 1981, 1982; Mentzer et al. 1984a), the three HPP patients BEP, DAB, and JCB exhibited a defective self-association of spectrin dimer, expressed by an excess of dimer species in the membrane and a decreased value of the association constant in solution. This spectrin functional defect was correlated with a structural abnormality revealed by limited tryptic digestion and consisting in a decrease in the 80000-dalton peptide (αI domain) and a concomitant increase in the 74000-dalton peptide. This pathological spectrin variant, written $SpD\alpha^{I/74}$ (Palek and Lux 1983), has been reported in black HPP patients (Lawler et al. 1982) and in black and white HE patients (Dhermy et al. 1984a; Lawler et al. 1984; Lecomte et al. 1984).

The clinical and red cell morphological features as well as the erythrocyte fragmentation at 45°C described in HPP are also observed in homozygous HE (Garbarz et al. 1986) and in some heterozygous HE children before the age of 2. However HPP and homozygous HE can be distinguished by transmission study of the spectrin molecular defect. We think that HPP must be characterized by the presence of a spectrin defect in only one parent, whereas the membrane skeleton is normal in the other one. So in our family, the $Sp\alpha^{I/74}$ variant was observed in the proposita's mother, but no alterations were detected in erythrocytes from the proposita's father: red cell resistance to heat and shear-stress were identical to that of controls; the membrane had a normal protein content; and no functional and structural defects were observed within the spectrin molecule. In other HPP kindreds where molecular studies of spectrin were done, one parent was also free from any spectrin abnormality. Moreover, the quantity of defective spectrin (roughly 50%) found in the proposita and her maternal uncles argued against the diagnosis of homozygous HE, where the patients have a double inheritance of their parents' molecular defect. Furthermore, the partial spectrin deficiency revealed by SDS-PAGE in HPP has not been observed so far in homozygous HE (Garbarz et al. 1986).

Although HPP is considered as a separate disease, there is an increasing body of evidence for a relationship with type I HE. In all cases where familial and biochemical studies were available, one parent presented the same defective spectrin molecule as his offspring, either with typical mild HE or without morphological modifications of red cells. In the family studied here, the HPP proposita had not only an HE mother, but also an HE child. So in all affected members from three generations, in HPP patients as well as in HE patients, we observed the transmission of the same molecular Sp defect to a similar extent but with a different clinical expression. The severity of HPP versus mild clinical feature of HE in a single family has not yet been elucidated. In most reported families, the proportion of abnormal spectrin reflected either by the amount of spectrin dimer in the 4°C extract or by the quantity of remaining 80000-dalton peptides in spectrin tryptic digests appeared to be somewhat higher in HPP patients than in HE relatives. But the amount of defective spectrin varies, from one kindred to the other, between 30% and almost 100%, with being apparently identical clinical severity (Knowles et al. 1983; Lawler et al. 1982, 1983). In our family the amount of defective spectrin is not significantly different between HPP and HE patients. The type of spectrin variant could not be involved in the severity of the disease since our proposita experienced severe anemia requiring early splenectomy as reported in HPP patients having the $Sp\alpha^{I/46}$ variant (Lawler et al. 1983).

To explain the severity of HPP in comparison with HE, some authors have suggested as a hypothesis that HPP may result from two independent genes, (1) a spectrin defect in alpha I domain transmitted by one parent and (2) an unknown silent mutation by the other one. With regard to the nature of this second factor, several hypotheses have been proposed: either oxidative damage of skeletal proteins (Morris et al. 1986) or a high level of 2,3-diphosphoglycerate which is known to destabilize the cytoskeleton in vitro (Mentzer et al. 1984b; Sheetz and Casaly 1980). The proposita's father has normal skeletal proteins and, as the proposita, he did not present an increased level of 2,3-diphosphoglycerate (data not shown).

The hypothesis of a double heterozygosity in HPP would imply that in our family the second genetic factor must be transmitted twice by two unrelated subjects; so this factor must be frequent, whereas HPP remains a rare disorder in comparison with HE. The alternative transmission of HPP and HE phenotypes from one generation to the next in the same lineage could also suggest an autosomal dominant transmission of one genetic defect having a variable clinical expression. However, as nothing could explain this clinical variability, we have to keep in mind that many young heterozygous HE patients display, during their first to second years of life, clinical and hematological features similar to HPP as observed in the proposita's child CP. But, in contrast to HPP, this HE with pycnocytosis in infancy evolves towards a typical mild HE. An understanding of this "maturation" towards a mild HE phenotype could be very helpful in elucidating the HPP phenotype.

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