A New Abnormal Variant of Spectrin in Black Patients With Hereditary Elliptocytosis

By Marie-Christine Lecomte, Didier Dhermy, Constance Solis, Anna Ester, Claude Féo, Huguette Gautero, Odile Bournier, and Pierre Boivin

Seven black patients with mild hereditary elliptocytosis (HE) from five unrelated families were studied. The erythrocytes of these patients exhibited an abnormal thermal sensitivity (between 45 °C and 47 °C instead of 49 °C). An important defect of spectrin dimer self-association was detected in two ways: (1) the proportions of spectrin dimer (SpD) extracted from membranes at 4 °C under low ionic strength conditions were increased between 25% and 56% (normal value 15% ± 2%); (2) the spectrin dimer → tetramer conversion in solution were defective with an association constant value between 0.4 and 2.4 × 10^5 M⁻¹ for a normal value of 6 ± 0.4 × 10^5 M⁻¹. Spectrin (Sp) from HE patients and normal volunteers (32 black and 22 white subjects) was submitted to limited trypsic digestion, followed by one- or two-dimensional separation of the peptides. Peptide patterns of crude Sp from all seven HE patients exhibited a marked and reproducible decrease in 80,000-dalton peptide (previously identified as the dimer interaction domain of the α-chain) and a concomitant appearance of a novel 65,000-dalton peptide. A minor fragment at 28,000 daltons was also decreased. Tryptic digestion of HE spectrin dimer and tetramer (SpT), isolated after the SpD self-association procedure in solution, revealed modifications (decrease in the 80,000-dalton peptide and presence of a 65,000-dalton peptide) predominantly in HE SpD when peptide patterns of HE SpT were quite similar to control SpT patterns. Immunoblots with anti-α-chain antibodies revealed that the 65,000-dalton peptide derived from the α-chain. Kinetic studies of Sp digestion showed that the 65,000-dalton peptide did not result from further digestion of a 74,000 intermediate and was not a precursor of 46,000- to 50,000-dalton peptides. These results show a new structural defect of Sp-α-chain, associated with a defective Sp dimer self-association in HE.

The shape of erythrocytes and their viscoelastic properties depend on a submembranous protein network named “membrane skeleton,” which consists of interconnected proteins, mainly spectrin, protein 4.1, and actin. Spectrin, the predominant component, is a heterodimer composed of two polypeptide chains, α and β, of 240,000 and 220,000 daltons, respectively. Spectrin (Sp) is present in the membrane under a tetrameric or possibly higher polymeric form due to head-to-head self-association of heterodimers, and linked by actin polymers and protein 4.1 to form a two-dimensional network. Ankyrin binds the skeletal network to the membrane lipid bilayer through its high-affinity associations with the spectrin β-chain and the integral protein band 3.

A number of defects of the skeletal components have recently been described in certain hereditary hemolytic anemias, such as hereditary spherocytosis (HS), hereditary elliptocytosis, (HE) and hereditary pyropoikilocytosis (HPP), the latter being characterized by an unusual thermal sensitivity of the erythrocytes. In HE, the ghosts, as well as the skeletons, have the same elliptical morphology as the erythrocytes. According to recent studies, HE, which represents a heterogeneous disorder from a clinical point of view, is also a heterogeneous disorder from a biochemical point of view. Different molecular abnormalities of the membrane proteins have been described: HE can be associated with a complete or a partial deficiency of protein 4.1 (HE [4.1]) and HE [4.1'], respectively; a spectrin molecular abnormality has been suggested in some HE cases because of its increased susceptibility to thermal denaturation; defects of spectrin dimer self-association have been found in many HE cases; these cases were defined as type I HE. These functional defects were subsequently characterized by limited trypsic peptide mapping of spectrin. In some HE the molecular abnormality was localized within the α-chain, HE (SpD α -SpD), and characterized by a decrease in an 80,000-dalton peptide with a concomitant increase in a 74,000- 16 or 46,000- to 50,000-dalton peptides. A definitive Sp dimer self-association was related to an abnormal shortened β-chain in one HE family: HE (SpD β -SpD).

In the present work we report the results obtained with seven black patients from five unrelated families...
and suffering from mild HE. Two of the patients presented hemolytic variants. The erythrocytes of these patients exhibited an abnormal thermal sensitivity (between 45°C and 47°C instead of 49°C). All seven patients presented an important Sp self-association defect. Limited tryptic digestion of HE Sp revealed an abnormal peptide pattern, characterized by a decrease in the 80,000-dalton peptide and the presence of an unusual, so far undescribed, 65,000-dalton peptide.

CASE REPORT

Patient DD

The hematologic data of this young woman were hemoglobin (Hb), 12.4 g/dL; packed cell volume (PCV), 37%; mean cell volume (MCV), 83 fl; mean cell hemoglobin concentration (MCHC), 33.3%; reticulocyte count, 45,000/µL. Studies of hemoglobin were normal. Elliptocytes were the predominant cells (95%) with some fragmented cells (2%).

Patient MD

A chronic hemolytic anemia with moderate splenomegaly was discovered in 1975 in this 45-year-old black male, a native of Mali. The hematologic data were found to be relatively stable during the time of our study: Hb, 9.8 to 11 g/dL; PCV, 32% to 35%; MCV, 82 to 83 fl; MCHC, 29% to 31%; reticulocyte count, 148,000 to 360,000/µL; serum bilirubin, 43 µmol/L; serum iron, 14.5 µmol/L. No abnormal hemoglobin was detected. A glucose-6-phosphate dehydrogenase (G6PD) deficiency was observed. The blood smears showed a predominant elliptocytosis (about 95%) with rod-shaped cells and fragmented cells (about 5%). The survival of autologous red cells, labeled with 51Cr, was markedly decreased (half-life, 13.5 days; normal value, 30 ± 4 days).

Patient ML

This patient, a 29-year-old black woman, was a native of Antilles. The red cell indices were Hb, 12.4 g/dL; MCV, 90 fl; MCHC, 32%; reticulocyte count, 43,000/µL. Studies of hemoglobin were normal. Elliptocytes were the predominant cells (about 98%) observed in blood smears.

Family M

This black family originated from Antilles. CM, a male baby, presented a hemolytic anemia a few days after birth. At the age of 20 days, the blood findings were Hb, 7.8 g/dL; MCV, 94.3 fl; reticulocyte count, 75,000/µL. Smears of the peripheral blood showed many elliptocytes (about 30%) and fragmented cells. The Hb, the G6PD, and the pyruvate kinase (PK) were found to be normal. The patient received two blood transfusions. At the age of 6 months (when our study began), the elliptocytes were predominant (about 80%) and the schizocyte number decreased: Hb, 11.2 g/dL; MCV, 73 fl; MCHC, 32.2%; reticulocyte count, 18,480/µL.

The father (JM) was found to be clinically normal; the hematologic data of the latter subject were Hb, 15 g/dL; MCV, 95 fl; MCHC, 33%; reticulocyte count, 38,240/µL. Studies of hemoglobin were normal. A large number of elliptocytes (about 90%) was observed in blood smears. The mother was clinically and hematologically normal.

Family Y

This black family originated from Mali. AY, a 3-year-old female child, presented a mild anemia with sporadic hemolysis. The hematologic data were Hb, 10.2 to 13 g/dL; PCV, 35.8%; MCV, 74 fl; MCHC, 36.6%; reticulocyte count, 9,600 to 57,500/µL. Studies of hemoglobin were normal. Elliptocytes were the predominant cells (85%) with some rod-shaped cells. Her mother (DY), born in 1960, was pregnant when our study began; the hematologic data were Hb, 9.2 g/dL; PCV, 27%; MCV, 92 fl; MCHC, 34.4%; reticulocyte count, 29,000/µL; most of the cells (90%) were slightly elliptical. The hematologic data of the father (BY) were Hb, 12.6 g/dL; PCV; 35.5%; MCV, 75 fl, MCHC, 35.4%; reticulocyte count, 92,600/µL. The blood smears showed mostly discocytes, although some elliptocytes (10%) were also present. MY, a 6-month-old male child, AY’s brother, presented at the time of our first study an anemia due to iron deficiency; Hb, 7.2 g/dL; MCV, 54 fl. The red blood cells were mainly discocytes with some microcytes. After iron therapy, the hemogram was normalized, and the blood smears showed normal erythrocytes.

Control Subjects

Black volunteers used as controls were from Antilles and from different African ethnic groups.

MATERIALS AND METHODS

Chemical and Reagents

Acrylamide, NN'-methylene bisacrylamide, and TEMED were purchased from Eastman-Kodak (Rochester, NY). Sodium dodecyl sulfate (SDS), diisopropylfluorophosphate (DFP), and Coomassie brilliant blue were from Sigma Chemical Co (St Louis). Standards for molecular weight determination and Sepharose CL-4B were from Pharmacia Fine Chemicals (Uppsala, Sweden). Peroxidase and rabbit IgG were from Nordic Laboratories (Tilburg, Netherlands). TPCK trypsin and other reagents were obtained from Merck (Darmstadt, RDA).
Erythrocyte Morphology

Blood samples were collected on heparin and analyzed within 24 hours. The cells were examined by light phase contrast microscopy, after fixation of 1% glutaraldehyde in 5 mmol/L phosphate buffer, 150 mmol/L NaCl (phosphate-buffered saline [PBS]), pH 7.4.

Thermal Sensitivity of Erythrocytes

The thermal sensitivity of patient and control erythrocytes was studied as previously described.21

Preparation of Erythrocyte Membranes

The erythrocytes were washed three times in PBS, pH 8.0. The ghosts were prepared according to the method of Litman et al, except that 0.1 mmol/L phenylmethyl sulfonyl fluoride (PMSF) ghosts were prepared according to the method of Litman et al.22

Polyacrylamide Slab Gel Electrophoresis

Nondenaturing gel electrophoresis (NDGE) was carried out with 140 x 100 x 1.5-mm slab gels and 2% to 5% acrylamide gradients in 40 mmol/L Tris, 20 mmol/L sodium acetate, 2 mmol/L EDTA, pH 7.4.23 The sample buffer consisted in 10 mmol/L Tris, 1 mmol/L EDTA, 10% glycerol, 0.005% bromphenol blue, pH 8.0. The gels were electrophoresed at 50 V, for 48 hours, at 4°C, and stained with Coomassie brilliant blue.24 The electrophoresis of the membrane proteins was carried out with a discontinuous system described by Laemmli.25 The gels were electrophoresed at 50 V, for 48 hours, at 4°C, and stained with Coomassie brilliant blue.26 The spectrin dimers were separated on 140 x 100 x 1.5-mm gels with a 7% to 22% or 10% to 20% polyacrylamide gradient. Gels were stained with the silver staining procedure described by Morrissey27 or with Coomassie brilliant blue and scanned at 630 nm and 550 nm, respectively, in a DU8 Beckman (Gagny, France) spectrophotometer.

Isoelectric Focusing

Isoelectric focusing followed by SDS-polyacrylamide gel electrophoresis were performed by the method of O’Farrell28 with modifications reported by Speicher.29 Samples of tryptic digest were lyophilized and solubilized in 9.5 mol/L urea, 2% Triton X-100 (Sigma, St Louis), 5% β-mercaptoethanol, and 2.4% amphoties (pH 3.5 to 10, 4 to 6, 5 to 7). Samples were electrofocused for 16 hours at 340 V in 4% polyacrylamide tube gels (0.15 x 13 cm) containing 2.4% mercaptoethanol, 0.3 mmol/L phosphate buffer, pH 8.0. Samples were applied to a 1% to 30% linear sucrose gradient. The percentages of Sp-D and Sp-T were evaluated from the sedimentation profiles, as described above. (2) The Sp-species of the 4°C extracts were separated by NDGE. 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 obtained by densiometric tracings.

Limited Tryptic Digestion of Spectrin

Tryptic digestion was done at 0°C for 20 hours in PBS, pH 8.0. The substrate concentrations were always adjusted to 0.5 mg/mL for spectrin extracts and 0.1 mg/mL for SpD and SpT, assuming an absorbance at 280 nm, E 1% 1 cm = 10.1.27 The enzyme-substrate ratios were either 1:20 or 1:100 (w/t). The digestion was ended by addition of DFP (final concentration, 1 mmol/L) and by heating the samples at 100°C for three minutes in presence of 2% SDS and 5% β-mercaptoethanol. The assays were done in duplicate.

Purification of Sp Dimers and Sp α-Chains

The Sp dimers were purified from 37°C membrane extracts by chromatography on a Sepharose CL-4B column. The column buffer was 5 mmol/L EDTA, 0.2 mmol/L PMSF, 1 mmol/L β-mercaptoethanol, 150 mmol/L NaCl, 10 mmol/L phosphate buffer, pH 8.0. The Sp α-chains were separated from the Sp dimers by chromatography on hydroxypatite, as described by Calvert et al.29

Preparation of Anti-Sp α-Chain Immune Sera

An Sp a-chain preparation (200 μg) was mixed with complete Freund’s adjuvant (50% vol/vol) and injected into an albino rabbit. Two booster injections were administered at one-month intervals, using incomplete Freund’s adjuvant.

Immunoblotting Technique

Red cell membrane proteins (20 μg) and frozen tryptic digests (30 μg) were separated by SDS-PAGE (as described earlier) and transferred electrophoretically onto nitrocellulose sheets.28 The saturation of the binding sites and the reaction with anti-Sp chain immune serum (1/2,000 vol/vol) were carried out essentially as described previously.11 Bovine serum albumin-coated papers were incubated for five hours at room temperature with the anti-Sp chain immune serum (1/2,000, vol/vol). After three washes, the sheets were incubated for two hours at room temperature in peroxidase-conjugated goat anti-rabbit IgG (1:1,000, vol/vol). The antibody-labeled proteins were visualized by immersing the sheets for one to five minutes in the substrate solution (0.0025% 0-dianisidine, 0.01% H₂O₂, 10 mmol/L Tris-HCl, pH 7.4).
A NEW VARIANT OF SPECTRIN IN ELLIPTOCYTOSIS

Table 1. Study of Erythrocyte Thermal Sensitivity

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Incubation Time (min)</th>
<th>Percentage of Fragmented Cells</th>
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<tr>
<td></td>
<td>10</td>
<td>20</td>
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<tr>
<td>Controls</td>
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<td>0</td>
</tr>
<tr>
<td>I DD (HE)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>II MD (HE)</td>
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<td>5</td>
</tr>
<tr>
<td>III ML (HE)</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>IV CM (HE)</td>
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<td>0</td>
</tr>
<tr>
<td>V JM (HE)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>0</td>
</tr>
<tr>
<td>VY AY (HE)</td>
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</tr>
<tr>
<td>V MY (HE)</td>
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</table>

RESULTS

Thermal Sensitivity of Erythrocytes

The first morphological change due to heat treatment is the crenation of the cells. In normal erythrocytes, crenation is observed only after heating at 47 °C for 20 to 40 minutes. Fragmentation with budding occurs at 49 °C and increases with the time of incubation, as previously reported. The thermal sensitivity of erythrocytes was found to be abnormally high in all seven patients studied, but varied greatly from patient to patient and within the same family. The results are summarized in Table 1. The most fragmentable cells were found in patient MD, an adult with a marked elliptocytosis: crenated cells appeared after ten minutes of incubation at 45 °C and fragmentation was total after 20 minutes of incubation. The erythrocytes of patient AY also began to fragment at 45 °C, but only 10% were affected after 40 minutes of incubation; at 47 °C, the fragmentation was quite clear and increased with the time of incubation (50% after ten minutes). In her mother, DY, and in four other patients (DD, ML, CM, JM), the critical temperature was 47 °C, with a fragmentation between 10% and 60% of the cells. No significant differences were observed between the controls and the other members of the Y family (BY and MY).

Electrophoresis of Membrane Proteins

Neither qualitative nor quantitative abnormalities of membrane proteins were observed by SDS-polyacrylamide gel electrophoresis (data not shown).

Study of Spectrin Self-Association

The native state of spectrin in the membrane is reflected in the 4 °C extracts. The predominant species in normal subjects: the mean percentage of remaining SpD, estimated from sucrose gradient ultracentrifugation, was 15% ± 2% (n = 24). Similar values were obtained using nondenaturing gel electrophoresis: 18% ± 4% (n = 15). The percentage of SpD in 4 °C extracts from the seven patients was significantly increased, between 25% and 56% (Table 2). As previously reported, the transformation of Sp dimers into tetramers can be directly studied in 37 °C extracts. After incubation of the 37 °C extracts at 30 °C for 240 minutes, Sp dimers are converted into tetramers, with an association constant (Ka) for normal spectrin of $6 \pm 0.4 \times 10^3 \text{M}^{-1}$ (n = 42). The Ka values determined for the patients are shown in Table 2. The Sp dimer self-association was defective in all seven HE patients, with Ka values ranging from 0.4 to $2.4 \times 10^3 \text{M}^{-1}$ (Table 2).

Limited Tryptic Digestion of Spectrin From Normal Subjects

The peptide patterns obtained from a control extract were similar and reproducible, although some minor variations were observed between experiments. In order to control these variations due to experimental conditions, the spectrin from normal subjects was always prepared, digested, and electrophoresed at the same time as the patient samples. A certain variability was detected among normal control individuals, essen-

Table 2. Association Constant of Sp Dimer from 37 °C Extracts and Percentage of Sp-D in 4 °C Extracts

<table>
<thead>
<tr>
<th></th>
<th>Association Constant (Ka) in Solution 10^3 M (^{-1})</th>
<th>Percentage of Sp-D in 4 °C Extracts</th>
</tr>
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<tr>
<td></td>
<td>n = 42</td>
<td>15 ± 2</td>
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<tr>
<td>Controls</td>
<td>6.0 ± 0.4</td>
<td>n = 24</td>
</tr>
<tr>
<td>DD</td>
<td>1.65</td>
<td>25</td>
</tr>
<tr>
<td>MD</td>
<td>0.4</td>
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<tr>
<td>ML</td>
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<tr>
<td>CM</td>
<td>1.6</td>
<td>32</td>
</tr>
<tr>
<td>JM</td>
<td>1.0</td>
<td>32</td>
</tr>
<tr>
<td>DY</td>
<td>2.4</td>
<td>32</td>
</tr>
<tr>
<td>BY</td>
<td>ND</td>
<td>32</td>
</tr>
<tr>
<td>AY</td>
<td>2.1 ± 0.17</td>
<td>40</td>
</tr>
<tr>
<td>LMY</td>
<td>6.0</td>
<td>17</td>
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Spectrin extracts from 22 normal white subjects and 32 normal black subjects were digested with an enzyme-substrate ratio of 1:100. A 40,000-dalton peptide was absent in a few cases, in white as well as in black subjects; a 37,000-dalton peptide appeared in the peptide patterns of 14 out of 32 black individuals; the presence of a 37,000-dalton peptide was concomitant with the decrease or the disappearance of a 35,000-dalton peptide. The 37,000-dalton peptide was not observed in any of the 22 white normal subjects. The increase of a 50,000-dalton peptide and the decrease of a 48,000-dalton peptide was frequently observed in individuals who exhibited the 37,000-dalton peptide. The study of a black family permitted us to demonstrate the genetic transmission of the latter variations, the variant being found in heterozygous form (both of 35,000- and 37,000-dalton peptides) in both parents and in heterozygous or homozygous form in the children.

Limited tryptic digestion of crude spectrin extracts (enzyme-substrate ratio 1:100) from the seven HE patients revealed similar abnormal patterns characterized by a decrease in the staining intensity of the 80,000- and 28,000-dalton peptides and the appearance of a 65,000-dalton peptide (Figs 1 and 2). These decreases were especially important in patient MD. Densitometer scans of stained gels indicated that the 80,000-dalton peptide was decreased about 75% in patient MD and between 40% and 60% in the six other patients, as compared with controls. A slight decrease of a 74,000-dalton peptide was observed in all patients. In the M family, the child (CM), who presented elliptocytosis with pyknocytosis at birth, displayed the same abnormal pattern observed in his HE parent (JM). Two patients (AY and DY) were again examined after a four-month period; identical results were obtained. The 65,000-dalton peptide was never found.

**Fig 1.** SDS-PAGE of limited tryptic digests of spectrin from controls (C). HE patients (ML, MD, CM, MJ, DY, AY), AY’s brother (MY), and AY’s father (BY); Sp 37 °C extracts were treated with TPCK trypsin (enzyme-substrate ratio: 1:100) at 0 °C for 20 hours. (a) Gels stained with silver; (b) gels stained with Coomasie brilliant blue. The arrows indicate the position of variable bands with the molecular weight.
in the Sp peptide patterns of 54 white and black control subjects and in the studied normal members of the Y family (father BY and child MY). In father BY, the 50,000- and 37,000-dalton peptides were observed concomitantly with a diminution in the 48,000- and 35,000-dalton peptides. The 37,000-dalton peptide was not observed in any of the seven patients. Two-dimensional electrophoresis (isoelectric focusing followed by SDS-PAGE) of tryptic digests from normal spectrin showed a separation of the major fragments similar to that previously reported (Fig 3). The 65,000-dalton fragment and the decreased amount of 80,000-dalton peptide were predominantly observed in HE SpD as compared with HE SpT or normal Sp (Fig 2).

Kinetic Studies of Sp Tryptic Digestion

To examine a relationship between the defect in the SpD self-association and the presence of the 65,000-dalton peptide in tryptic digests, we isolated SpD from SpT after the SpD → SpT conversion in solution, and we subjected them to tryptic digestion. The 65,000-dalton fragment and the decreased amount of 80,000-dalton peptide were predominately observed in HE SpD as compared with HE SpT or normal Sp (Fig 2).

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Fig 4. Kinetic studies of tryptic digestion of Sp extracts from HE patient (DD) and control. Sp extracts (1 mg/mL) were treated with TPCK trypsin (enzyme-substrate ratio: 1:100) at 0 °C for 30 minutes and 1, 3, and 6 hours. Gels were stained with Coomassie brilliant blue.

dalton peptide. After 20 hours of digestion, the amount of the 80,000-dalton peptide in HE Sp was strongly 50% of control and was equal to the quantity of the 65,000-dalton peptide that had appeared in the same time. At each time of digestion, the amount of the 74,000-dalton peptide was lower in HE Sp than in control. The amount of the 70,000-dalton peptide in the HE patient became normal after six hours of digestion. Between 20 and 30 hours of digestion, no further modifications were observed in the digest patterns of HE Sp.

Immunoblotting With the Anti–Sp α-Chain Immune Serum

The anti–Sp α-chain immune serum was assayed against erythrocyte membrane proteins. The immunoblot pattern showed that the antibodies reacted specifically with the Sp α-chain. The immunoblot of Sp-tryptic peptides with anti–Sp α-chain serum showed that in controls, the anti–α-chain antibodies reacted mostly with the 80,000-dalton peptide (Fig 6). In HE patient immunoblots, the anti–α-chain antibodies
bound to the 65,000-dalton peptide as well as to the remaining 80,000-dalton peptide. In patient MD, who displayed a marked decrease in the 80,000-dalton peptide on silver-stained pattern, no binding of the antibodies of the 80,000-dalton peptide was observed. The anti-Sp α-chain antibodies also reacted with other peptides in both HE patients and controls. These results indicate that the 65,000-dalton peptide comes from the Sp α-chain.

**DISCUSSION**

The seven HE patients reported in this study presented a marked elliptocytosis. A considerable variability in the ellipticity of the red cells was observed. On the basis of clinical evaluations and red cell morphology, these patients corresponded to the common form of mild HE. Two patients (CM and MD) presented hemolytic variants of mild HE. Patient CM had a neonatal hemolytic anemia: elliptocytes and many fragmented spiculated cells were observed on smears; at the age of 6 months, his conditions gradually evolved into a typical mild HE similar to that of his father. This patient represented a case of mild HE with infantile poikilocytosis. In six patients, the erythrocytes were abnormally heat sensitive, exhibiting cell fragmentation at 47 °C instead of the usual 49 °C; the marked fragmentation of MD’s erythrocytes at 45 °C suggested a relationship with an HPP syndrome, although the normal MCV and the erythrocyte population composed mainly of elliptocytes and rod-shaped cells, did not correspond to the hematologic picture observed in HPP (in which the micropoikilocytes and the microspherocytes are predominant). Yet an abnormal thermal sensitivity of the erythrocytes was observed in subpopulations of HE: Tomaselli et al reported that two of the four HE families they studied presented a 50% erythrocyte fragmentation after a ten-minute incubation at 48 °C. Zarkowsky reported a heat-induced erythrocyte fragmentation in neonatal elliptocytosis during the first year of life. These patients, mostly black, had a red cell morphology and a thermal fragmentation at 45 °C similar to that observed in HPP. When the cell fragmentation and the pyknocytosis disappeared, the elliptocytosis became prominent and the red cell heat sensitivity became characteristic of mild HE (47 °C to 48 °C). This form of mild HE is illustrated by the M family, in which the child and his father display the same erythrocyte thermal sensitivity. Unfortunately, heat-induced erythrocyte fragmentation was not studied in the child during his pyknocytosis period. One of the two patients described by Coetzer and Zail was a white adult (CG) with prominent elliptocytosis and normal MCV: as in the adult DM patient, the thermal instability of the erythrocytes was observed at 45 °C, as in the HPP syndrome. Nevertheless, we believe that the diagnosis of the two patients, CG and DM, must remain that of an unclassified HE.

The seven patients presented a defect of Sp self-association, as previously observed in some HE. This defect showed up as an excess of dimer species in 4 °C extracts, together with a decreased Sp dimer.
association constant in solution. As proposed by Liu et al., these HE should be classified as type I HE. Limited tryptic digestion of normal Sp at 0 °C has allowed the identification of structural domains, a-I to a-V and b-I to b-IV. Morrow et al. have shown that an 80,000-dalton fragment, called a-I domain, derives from a terminal part of the a-chain and is involved in the Sp self-association process. The decrease in the 80,000-dalton peptide, observed in the peptide patterns of the seven patients, suggests a structural alteration in the a-I domain. Such an alteration is consistent with a defect in the dimer-tetramer equilibrium. The decrease in the 80,000-dalton peptide has previously been described in some cases of HE and in HPP; it has always been found associated with a defect of the Sp self-association process. In our seven patients, the decrease in both 80,000- and 28,000-dalton peptides was associated with the appearance of a 65,000-dalton peptide in the Sp tryptic patterns. These Sp digestion abnormalities were essentially found in the Sp dimer remaining after the conversion of dimer to tetramer. Thus, the abnormal tryptic pattern correlates with the defective self-association. The 65,000-dalton peptide comes from the Sp a-chain and is correlated with the decrease in the 80,000-dalton peptide. This peptide has not yet been observed in Sp digests from normal subjects. The Sp molecule is probably functionally defective because of a conformational change affecting the a-I domain, rendering it more susceptible to degradation into a 65,000-dalton peptide. Two structural variations associated with defective Sp self-association have been yet detected within the Sp a-I domain; Lawler et al. have described in two families with type I HE and in two HPP families a decrease in 80,000- and 22,000-dalton peptides concomitant with an increase in the 74,000-dalton peptide. We have also observed such an Sp tryptic pattern in type I HE (one white patient and a black family). Lawler et al. have also reported a decrease in the 80,000-dalton peptide associated with an increase in 46,000- and 17,000-dalton peptides in one type I HE family and one HPP family. The latter tryptic pattern was similar to that described by Knowles et al in HPP and in type I HE, with a decrease in the 80,000-dalton peptide and an increase in 50,000- and 21,000-dalton peptides. We have observed such a peptide pattern in two black families with type I HE (manuscript in preparation).

The kinetic studies of tryptic digestion showed that the 65,000-dalton peptide is not an intermediate of the abnormal peptides previously observed: The 65,000-dalton peptide appeared early during trypsin digestion. The appearance of this fragment corresponded roughly with the loss of the 80,000-dalton peptide. At each studied time of digestion, the amount of the 74,000-dalton peptide was less important in HE spectrin than in control. So these data indicate that the 65,000-dalton peptide results directly from the digestion of the 80,000-dalton peptide. The 65,000-dalton peptide is not a precursor of a 50,000- or 46,000-dalton peptide: Like the 80,000-dalton peptide, the 65,000-dalton peptide was stable up to 30 hours of digestion time. When tryptic digestions were performed with an enzyme-substrate ratio of 1:20, the 65,000-dalton peptide disappeared without modifications of smaller peptides in the 50,000- to 26,000-dalton range. Kinetic studies of HE spectrin with the (SpDaI/46) variant showed that the loss of the 80,000-dalton peptide was directly correlated with the production of a 46,000-dalton peptide and did not go by way of an intermediate peptide of 65,000 daltons (data not shown).

The studies on the Sp tryptic patterns in normal members of the Y family revealed that the father, BY, exhibited the presence of a 37,000-dalton peptide together with the decrease in a 35,000-dalton peptide. In our experience, this Sp variant is commonly observed in normal black populations (50%) and has previously been described. Knowles et al. have demonstrated that this genetic polymorphism is beared to the a-II domain. It should be noted that none of our seven patients exhibited such a 37,000-dalton peptide.

The thermal instability of erythrocytes correspond closely to the heat denaturation of Sp. Indeed, membrane budding and fragmentation occur at the same temperature as Sp denaturation, as demonstrated in normal subjects as well as in HE and HPP. The erythrocytes thermal instability of our seven HE patients may be related to the molecular alteration observed on Sp.

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