Abnormal Spectrin in Hereditary Elliptocytosis

By Sally L. Marchesi, William J. Knowles, Jon S. Morrow, Marcia Bologna, and Vincent T. Marchesi

An abnormal α subunit of erythrocyte spectrin has been described in hereditary pyropoikilocytosis (HPP), a rare hemolytic anemia characterized by erythrocyte budding and fragmentation. In HPP spectrin, the N-terminal domain of the α subunit (α T80) shows increased susceptibility to tryptic digestion, resulting in cleavage to a 50,000-d peptide, presumably due to a change in primary structure of the α I domain which alters conformation and generates tide, presumably direct evidence to support this hypothesis. Although inheritance of HE occurs in an autosomal-dominant fashion, its expression is extremely variable, the number of elliptocytes in the peripheral smear ranging from <10% to 100%. Compensated hemolysis is common, but significant anemia occurs in only a small number of patients. Poikilocytic variants of HE with severe hemolysis may occur in early childhood in kindreds with elliptocytosis; the peripheral smears of these children show marked microcytosis, pyknocytosis, and fragmentation of erythrocytes. In some of these cases, both parents have HE, are consanguineous, or both, whereas in others only one parent has HE or both parents are normal hematologically. Frequently, the severity of the hemolysis ameliorates with age, and the patient develops more typical elliptocytosis with mild to moderate hemolysis.

In 1975, Zarkowsky et al7 described three children from two kindreds with severe hemolytic anemia characterized by striking poikilocytosis, pyknocytosis, microspherocytes and budding forms. One parent had mild elliptocytosis, whereas the other three parents were normal. Because the morphologic changes in the peripheral blood of the patients resembled that seen in heat injury, heat-induced fragmentation of the patients' cell was studied; it was found that the patients' cells fragmented at 45°C in contrast to 49°C for normal cells, and the syndrome was thus termed hereditary pyropoikilocytosis (HPP). Calorimetric studies show a major structural transition in HPP ghosts and spectrin at 45°C compared with the normal transition at 49°C. These studies strongly suggested that spectrin was abnormal in HPP.

We and others10-13 have reported the analysis of erythrocyte spectrins from HPP patients and their relatives, some of whom have HE, by two-dimensional isoelectric focusing (IEF) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of intermediate-sized tryptic peptides. These studies have demonstrated an alteration in affected subjects of the N-terminal 80,000-dalton domain of the spectrin α subunit (α T80), which has been shown to bind to dimeric spectrin to promote formation of spectrin oligomers.14 In a previous study from our laboratory, spectrin from HPP patients and from one sibling with HE showed increased susceptibility of α T80 to mild tryptic digestion, with production of 50 kd and 21 kd peptides which are not generated in significant amounts from normal spectrin under the conditions used. Similar observations have been made in HE and HPP spectrin by Lawler et al.12,13 Spectrin from these individuals demonstrates decreased ability to selfassociate to form tetramer and higher oligomers, as shown either by an increased content of dimer relative to higher forms in fresh extracts at 4°C5,11-13 or by impaired concentration-dependent oligomer formation under isonic conditions at 30°C.10 These studies have been interpreted to mean that a molecular alteration (amino acid substitution, deletion) in α T80, as evidenced by the abnormal susceptibility to tryptic digestion, is responsible for decreasing oligomer stability and consequently decreased stability of the membrane skeleton, resulting in red cell shape change, fragmentation, and hemolytic anemia.

A relationship between HPP and HE is suggested by: (1) the clinical resemblance of HPP to poikilocytic infantile variants of HE, some of which have been thought to represent homozygous HE,12,14 (2) the occurrence of HE in parents or other family members of some patients with HPP.10,13,15

From the Departments of Internal Medicine and Pathology, Yale University School of Medicine, New Haven, CT.

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Address reprint requests to Dr Sally L. Marchesi, B124 Brady, Department of Pathology, Yale University School of Medicine, 310 Cedar St, New Haven, CT 06510.

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and (3) a mildly decreased thermal stability of red cells from some kindreds with HE, in which fragmentation occurs at 47 °C to 48 °C.14

In a strict sense, the term HPP should not be applied to patients with poikilocytic variants of HE unless heat stability studies have been performed; however, these variants of HE (which may be called poikilocytic hemolytic elliptocytosis (PHE)) should probably be grouped with HPP until the specific molecular pathologies producing this clinical picture are better defined.

We report here studies of spectrin from two kindreds with hereditary elliptocytosis; one family of five (a mother and four children) which includes two individuals with compensated hemolysis, and a second family (grandmother, mother, and child) with elliptocytosis that is expressed in the child as a severe poikilocytic hemolytic anemia. The spectrin of all affected family members shows an alteration in the N-terminal 80-kd domain of the α subunit (α I T80), which is identical qualitatively to that previously reported for hereditary pyropoikilocytosis, but which involves a smaller fraction of the total α subunit.

MATERIALS AND METHODS

Blood collection, preparation of red cell ghosts and extraction of spectrin. Blood was collected by venipuncture into ACD anticoagulant; informed consent was obtained from all donors and the quantity of blood drawn was appropriate to the estimated blood volume and hematocrit of the subject. Red cell ghosts were prepared by lysis of washed red cells for 30 minutes at 0 °C in 10 to 15 vol of 5 mmol/L of sodium phosphate, 1 mmol/L of EDTA pH 8.0 containing 130 μmol/L of phenylmethylsulfonyl fluoride (PMSF) and 0.1 mmol/L of diisopropyl fluorophosphate (DFP), followed by three washes with the same buffer. Spectrin was extracted by 48-hour dialysis of ghosts at 4 °C into 0.1 mmol/L of EDTA, pH 9.4, containing 130 μmol/L of PMSF and 0.1 mmol/L of DFP. The vesiculated ghosts were removed from the spectrin extract by 2× centrifugation at 48,000 g for 60 minutes at 4 °C. The spectrin extract was then dialyzed into buffer containing 10 mmol/L of EDTA, 1 mmol/L of NaCl, 130 mmol/L of KC1, 1 mmol/L of EDTA, 0.5 mmol/L of EDTA, 0.5 mmol/L of Na2HPO4, pH 7.4. Serial dilutions of spectrin in the same buffer were incubated for three hours at 30 °C prior to electrophoresis. In some cases, non-denaturing gels were run in the second dimension on 7% to 12% acrylamide gradients in SDS according to Laemmli.15

Quantitation of spectrin oligomers and peptides from Coomassie blue-stained acrylamide gels was performed by elution of excised gel slices with 1.0 mL of 25% pyridine for three to four days until the gel slice was colorless. Optical density at 700 nm of the eluates was measured in a Varian DMS 90 spectrophotometer.

125I peptide maps. Two-dimensional maps of 125I-labeled limit chymotryptic peptides were prepared from intermediate-sized tryptic peptides cut from two-dimensional polyacrylamide gels. Na125I (carrier free, 350 to 600 mCi/mL) was obtained from Amersham (Arlington Heights, Ill). Labeling of excised gel bands was performed by the method of Elder26 with modifications.27 Digestion with chymotrypsin and two-dimensional cellulose peptide maps were formed as previously described.27

NTCB cleavage. Spectrin was solubilized in 7 mol/L of guanidine, 200 mmol/L of Tris HCl, and 1 mmol/L of EDTA at pH 8.0, and was cleaved at cysteine residues by 2-nitro-5-thiocyanobenzoic acid (NTCB) at a concentration 10 times the cysteine content. After one-hour incubation at RT, the pH was raised to 9.0, and the solution was incubated at 37 °C overnight. The reaction was terminated by addition of 50 mmol/L of β-mercaptoethanol. The spectrin fragments produced were analyzed by two-dimensional IEF/SDS-PAGE and the peptide mapping procedure described above.

Photomicrographs. Wright-Giemsa stained blood smears were photographed at 200 x magnification on a Olympus BH-2 photomicroscope.

RESULTS

Erythrocyte spectrin obtained from 22 members of seven kindreds with hereditary elliptocytosis has been analyzed by mild tryptic digestion at 0 °C followed by two-dimensional (IEF/SDS) PAGE of the intermediate-sized tryptic peptides produced. When applied to normal spectrin, this technique generates a map of >50 well-resolved peptides with mol wts ranging from 80 kd to 12 kd.24 Limit chymotryptic mapping of these peptides combined with generation of overlap peptides obtained by enzymatic and chemical cleavage has led to construction of a model of the spectrin heterodimer that includes five major trypsin-resistant domains within the α subunit and four major domains within the β subunit. These are identified in Fig 1. The α I domain is an 80 kd peptide which is relatively resistant to further proteolysis under the mild conditions used (see Fig 1). There is, however, some cleavage of α I T80 to produce a 74 kd peptide just below and at the same IEP as α I T80. The derivation of the 74 kd peptide from α I T80 has been shown by limit peptide mapping and by monoclonal antibody blot.27 α I T74 is frequently seen in digests of normal spectrin, but is variable in quantity.

Two-dimensional peptide maps of spectrin obtained from three of seven HE kindreds studied were normal except for polymorphism in the α II 46 kd domain (α II T46) previously described in blacks.28 Spectrin from affected members of two
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Fig 1. Two-dimensional IEF/SDS polyacrylamide gel electrophoresis of intermediate sized tryptic peptides of spectrin extracted from normal red cells. The major domains of α and β subunits, as defined by mild tryptic digestion, are shown; in some cases, peptides derived from further tryptic cleavage of a parent domain are also shown.

other kindreds with HE showed variably decreased quantities of the 80 kd domain of the α subunit (α1T80) and appearance of a new 50 kd peptide at approximately the same isoelectric point which was previously shown by limit peptide mapping to be derived from the α1T80 domain.10 Concentrated spectrin of affected family members also showed a reduction in oligomer formation of varying degrees when analyzed over a concentration range of ~1 mg/mL to 14 mg/mL. These changes are qualitatively similar to those previously reported for patients with hereditary pyropoikilocytosis (HPP), although in no case was all of the α subunit affected as has been seen in HPP.10 These families are reported in detail below. The remaining two families with HE showed a different structural alteration in the α1 domain, which will be reported separately.

Family R. Family R (Fig 2A) is Puerto Rican and consists of a mother and her four children. M.R. Sr is a 46-year-old woman with hematocrits of 36% to 40%, reticulocyte count of 1%, and peripheral smears showing slight anisocytosis and polychromasia but no elliptocytes. A.R., her 19-year-old son, had hyperbilirubinemia at birth; when he was 6 months of age, his hemoglobin was 9 g/dL, and the smear showed marked elliptocytosis and poikilocytosis. Between the ages of 5 and 17, his hematocrit rose to normal (>40%), and the smears show typical elliptocytosis. His bilirubin is normal, but haptoglobin is 0 to 10 mg/dL (normal 20 to 200 mg/dL). W.R., 16 years old, has a hematocrit of 42%, reticulocyte count of <1%, some ovalocytic erythrocytes, and a haptoglobin of 31 mg/dL. M.R. Jr, 14 years old, has hematocrits ranging from 33% to 39%, with reticulocyte count of 1.5% to 3%, haptoglobin of 0 and 6 mg/dL, and smears showing marked elliptocytosis. A fourth child, S.R., 12, has a hematocrit of 39%, a reticulocyte count of 0.2%, and a normal smear. The father was not available for study. Peripheral smears from this family are shown in Fig 3A through C.

Family S. Family S (Fig 2B) is black and consists of a child, his mother, and maternal grandmother. I.S. is a 57-year-old woman with ovalocytes and occasional fragments on smear (Fig 3D). Her hematocrits range from 35% to 40% with reticulocyte count of 1.5%, and a haptoglobin of 77 mg/dL. Her daughter, E.S., 30 years old, had a hematocrit of 24% at one year of age with a reticulocyte count of 5%; the smear showed marked elliptocytosis, anisocytosis, and poikilocytosis. Her hematocrit gradually rose; at 28 years of age, she had a hematocrit of 34% with a reticulocyte count of 2.4% and a haptoglobin of 0. The smear continues to show marked elliptocytosis (Fig 3E) but without the poikilocytosis seen earlier. T.S., the 12-year-old son of E.S., developed hyperbilirubinemia at birth requiring exchange transfusion. His peripheral smears showed marked poikilocytosis, microcytosis, and fragmentation. At 2 weeks of age, his hematocrit was 22% and his reticulocyte count was 6%. Hemoglobin was AA, G6PD was normal, and Coombs test was negative. At 4 months of age, he developed hepatosplenomegaly. He required five transfusions during the first 6 months of life for hematocrits of 15%; at 2½ years of age, he was transfused for a hematocrit of 12% during a viral infection. Between the ages of 4 and 9 years, his hematocrits were stable at 27% with a reticulocyte count of 14% to 19%. He underwent splenectomy at age 10. Since then, his hematocrits have been 33% to 40% with reticulocyte counts of 1% to 3%. HgbA2 is normal but hgbF is 23% and Heinz body prep is negative. At 4 months of age, he developed hyperbilirubinemia; PHE/HPP. poikilocytic hemolytic anemia clinically resembling HPP.

Fig 2. Kindreds of the R family (A) and the S family (B). HE, marked elliptocytosis on peripheral smear and evidence of compensated hemolysis; (HE), mild elliptocytosis, no evidence of hemolysis; PHE/HPP, poikilocytic hemolytic anemia clinically resembling HPP.
Fig 3. Peripheral blood smears stained with Wright's-Giemsa. R family (A through C): (A) S.R., normal; (B) W.R., mild HE; (C) A.R., hemolytic HE. S family (D through F): (D) I.S., mild HE; (E) E.S., hemolytic HE, (F) T.S., poikilocytic HE (HPP).

Fig 4. Tryptic peptides of spectrin from members of family R electrophoresed in two dimensions IEF/SDS polyacrylamide gel electrophoresis: (A) M.R., Sr. normal; (B) A.R., and (D) M.R., Jr. with marked elliptocytosis and compensated hemolysis; (C) W.R., with ovalocytosis, no hemolysis. Arrows indicate the α 1 T80 and 50 kd peptides (multiple isoelectric forms). α 1 T21 (>) is shown in Fig 4D.
and in the unaffected mother (Fig 4A). W.R., with mildly elliptocytic cells and no evidence of hemolysis, also shows the abnormal α I 50 kd peptide (Fig 4C). Anti-α I T80 monoclonal antibody (23) binds both α I T80 and α I T50 on Western blot of a tryptic digest of AR spectrin (data not shown) consistent with the limit peptide mapping results of Knowles et al., establishing α I T50 as a degradation product of α I T80.

Figure 5 shows the intermediate tryptic peptides of spectrin from family S. I.S. spectrin (Fig 5B) shows a moderate reduction in α I T80, whereas E.S. and T.S. spectrin (Fig 5C and D) show distinct reductions in the quantity of α I T80: in Fig 5D, α I T74 is relatively increased. All three family members show the prominent α I 50 kd peptide observed in affected members of family R.

A second (21 kd) cleavage product of α I T80, identified in limited tryptic digests of spectrin from patients with HPP reported earlier, has been identified by peptide mapping from spectrin digests of members of the R and S families and is demonstrated in Fig 4D and 5B; it can be identified in variable amounts in digests of all affected family members.

The increased susceptibility of the α I 80 kd domain to proteolytic cleavage and the production of the 50 kd and 21 kd peptides observed in these families closely resembles our earlier findings and those of others for HPP spectrin, but appears to involve variable percentages of the α subunit. In at least three of the HPP patients reported intermediate tryptic digests showed no residual α I T80, whereas in the R and S families, and in some other patients with HPP, residual α I T80 is seen in varying degrees. Spectrin peptides α I T80, α I T74, and α I T50 were cut from two-dimensional gels of tryptic digests from the R and S families, and the protein content was quantified by measuring pyridine-eluted Coomassie blue stain (see Methods section). The results are shown in Fig 6, in which α I T50 and α I T74 are expressed as a mole fraction of the total α I

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\textbf{Fig 5.} Spectrin tryptic peptides from members of family S electrophoresed in two dimensions IEF sodium dodecyl sulfate polyacrylamide gel electrophoresis: (A) normal spectrin; (B) I.S. with mild elliptocytosis, no hemolysis; (C) E.S. with marked elliptocytosis and compensated hemolysis; (D) T.S. with marked poikilocytosis and hemolytic anemia. Arrows indicate α I T80 and 50 kd peptides in B, C, and D. α I T21 is shown in Fig 5B. Filled triangles (▲), α II 46 kd domain variants; double pointer (▼), α III 52K domain variant. α I T21 is shown in Fig 5B.
domain represented on the gel. The initials of the subjects are arranged in order of decreasing severity of hematologic disease within each of the two families. The data shown are taken from one to three two-dimensional gels of spectrin tryptic peptides from each family member and controls. Subsequent quantitation of α I peptides of multiple (three to seven) tryptic digests of spectrin from T.S., E.S., A.R., and M.R. gave similar results. Initials of family members are placed in order of decreasing severity of hematologic abnormality. Although α I T74 is increased in some of the tryptic digests of members of these families (as illustrated in SD [T.S.]), the cleavage of α I T80 to α I T74 is variable in degree in our experience both in normal and HE families. This is shown in Fig 6 (bottom), which expresses the mole ratio of α I T74 to total α I domain peptides from tryptic digests of the S and R families.

Additional α spectrin variants in S family. In addition to the α I T80 abnormality described above, the S family also shows polymorphism in the α II T46 domain of the type previously reported to be common in blacks.39 In Fig 5A, the α II domain type I migrates as 46 kd peptides at several isoelectric points with cleavage products of 35 kd and 30 kd, and is the type seen in spectrin of all (>100) white subjects studied to date (see also Fig 1). Spectrin of E.S. and I.S. (Figs 5 B and C) shows double heterozygosity for α II T46 types 1 and 2; in type 2, the α II domain migrates as 50 kd, 39 kd, and 35 kd peptides with isoelectric points more basic than α II T46, 35 and 30 (type 1). T.S. spectrin (Fig 5D) is heterozygous for α II T46 types 2 and 3; in the latter, apparent mol wt of α II increases without a change in isoelectric point.

All of the members of the S kindred also show variant α III domain peptides not previously described. A new peptide is seen above and at the same isoelectric point as the normal α III T52 as well as a new α III cleavage product (Fig 5 B through D; see Fig 1). 125I-labeled limit chymotryptic maps of the larger peptide (α III T56) are identical to normal α III T52 shown in Fig 7. This α III variant has been seen to date in four other individuals (three kindreds) all of whom are black. Three of these subjects have HE or poikilocytic hemolytic anemia consistent with HPP, and their spectrin shows the α I T80—α T50 cleavage described above, as well as variant α II domains. One of these patients, K.B., was previously reported.16 Thus, the spectrin of these individuals has alterations in the three of the five domains of the spectrin α subunit.

A number of other differences are apparent on two-dimensional gels of spectrin tryptic digests from normal individuals. These include variability in degree of digestion of the α IV domain and variable intensity of many of the peptides of <30 kd. These differences, which occur randomly, in part reflect differences in protein load, as well as other variables inherent in the experimental technique.

Localization of α I T50 within the α I domain. Cleavage of spectrin at cysteine residues with NTCB followed by two-dimensional IEF/SDS-PAGE yields a complex map of peptides with mol wts between 90 kd and 6 kd. Peptide mapping of some of the NTCB peptides has been performed to identify their location within the domain structure of the spectrin molecule.21 A 67 kd NTCB peptide has been found by peptide mapping of 125I-labeled limit chymotryptic peptides to contain most of the iodinated spots from the α II domain as well as some of the map of α I T50 from HPP spectrin, suggesting that α I T50 is located adjacent to the α II domain. Peptide maps of NTCB 67, α II T46, and α I T50 are shown in Fig 8. The peptide map tracing shows those peptides from α I T50 and α II T46 which are also found in
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Fig 8. Peptide maps of $^{125}$I-labeled limit chymotryptic peptides derived from α I T50, α II T46, and NTCB 67 kd peptides. Horizontal axis, electrophoresis; vertical axis, chromatography. The map tracing is a composite of the α I T50 limit peptides (a) and α II T46 limit peptides (0) which appear in NTCB 67. Line drawing at top illustrates the relative location of the α I and α II domains, α I T50, and the NTCB 67 peptide.

NTCB 67K. These results establish the proximity of α I T50 to the α II domain, as shown in the molecular model at the top of Fig 8. Placement of α I T50 from HPP spectrin in the C terminal half of the α I domain agrees with sequencing data performed on α I T50 derived from normal spectrin (discussed later).

Oligomer formation from HE spectrin. The ability of spectrin heterodimers from family R and family S to form higher oligomers was analyzed by incubation of spectrin at concentrations between 0.8 and 14 mg/mL in isotonic buffer at pH 7.4 for three hours at 30 °C prior to nondenaturing gel electrophoresis. Results are seen in Figs 9 through 11.

Figures 9 and 10 show oligomer formation by spectrin from the R family at concentrations of 0.86 and 8.6 mg/mL. It can be seen that spectrin from a normal individual (C) and from the unaffected mother, M.R. Sr, when incubated at the 8.6 mg/mL (left lanes) forms higher oligomers, whereas the predominant forms of spectrin at 0.86 mg/mL (right lanes) are dimer and tetramer. Spectrin from A.R. and M.R., Jr, who have compensated hemolytic HE, forms less tetramers than normal at low concentrations and forms less oligomers at high concentration. Spectrin of W.R. (mild nonhemolytic HE) forms oligomers normally at the higher concentration; however, at low concentration, it has a moderately increased dimer-tetramer ratio.

Figures 10 and 11 show oligomer formation by spectrin from the S family. Both T.S. and his mother, E.S., have markedly impaired oligomer formation in vitro over a wide

Fig 9. Self-association of spectrin from members of family R. Crude spectrin extracts from family members designated by initials and from a normal unrelated control donor were concentrated and incubated at 30 °C for three hours at 8.6 mg/mL (left lanes) and 0.86 mg/mL (right lanes); 75 μg of each sample was loaded on 2% to 4% acrylamide gels and was run for 48 hours in Na acetate buffer pH 7.4. D, dimer; T, tetramer; H, hexamer; O, octamer. Arrow (right) indicates nonspectrin protein which migrates near hexamer in this gel system. Other nonspectrin proteins are seen migrating ahead of spectrin dimer.
range of concentrations. Crude spectrin extracts from T.S. contain greater than normal amounts of nonspectrin protein, characteristic of spectrin extracts from patients with red cell fragmentation and hemolysis, and include a protein band migrating near spectrin hexamer (arrows). T.S. spectrin also shows smearing of protein between dimer and tetramer; the smear consists largely of spectrin as shown by electrophoresis of the nondenaturing gel into SDS/PAGE in the second dimension (Fig 12). Figure 12 also demonstrates nonspectrin proteins in the crude extract, including a 22 kd peptide (arrow), identified as band 8 (glutathione S transferase) which migrates with the spectrin smear between dimer and tetramer on nondenaturing gels but which separates from spectrin in SDS. No specific interaction between band 8 and the patient’s spectrin could be detected by sucrose density gradients of crude spectrin extracts (data not shown).

Quantitative assessment of spectrin oligomer formation in the R and S families is shown in Figs 13 and 14. In Fig 13, the mole fraction of spectrin dimer and of tetramer and higher oligomers is shown for spectrin from family R incubated as described for Fig 9. The initials of family members are again arranged in order of decreasing expression of hematologic disease. In Fig 14, the mole fractions of dimer and tetramer are shown for E.S. and T.S. over the range of spectrin concentrations described in Fig 11; these are compared with a normal control curve derived from a large number of subjects.

It is evident from Figs 13 and 14 that the mole fraction of dimer is elevated and oligomers are decreased in spectrin from all HE/HPP subjects at low spectrin concentrations, and that these differences are not only maintained but are amplified at high concentrations of spectrin; this is important because the spectrin concentration at the membrane surface probably exceeds 100 to 200 mg/mL. These data also show that the degree of impairment of spectrin self-association parallels the hematologic expression of HE and HPP.

**DISCUSSION**

HE appears to be a heterogeneous disease caused by deficiencies or abnormalities in several components of the membrane skeleton including, to date, the α and β subunits of spectrin and protein 4.1. All HE kindreds reported with abnormal spectrin have also evidenced abnormal spectrin self-association, which is thought to be responsible, at least in part, for the shape change, decreased mechanical fragility, and shortened survival observed. We have described here two families with HE whose spectrin demonstrates an abnormal α I domain (α I T80) detected by its increased susceptibility to tryptic cleavage. Normal α I T80 is highly resistant to further tryptic cleavage under the mild digestion conditions used, yielding small (and variable) amounts of a 74 kd peptide and a 50 kd peptide, as well as lower mol wt fragments (S. Marchesi and J. Letsinger, unpublished observations). The α I 74 kd and 50 kd peptides produced from normal spectrin have been shown by Speicher...
et al.\textsuperscript{28} to result from cleavage of peptide from the N terminus of the \(\alpha\) subunit. The spectrin of HPP patients is characterized by an \(\alpha\) I T80 domain which is readily cleaved under the same conditions to a 50 kd peptide\textsuperscript{10} (or 46 kd peptide\textsuperscript{11-13}) presumably due to altered conformation of the 50 kd cleavage site leaving little, if any, residual intact \(\alpha\) I T80 peptide. By contrast, a smaller fraction of the \(\alpha\) subunit is abnormal in patients with HE; whereas two-dimensional peptide maps of all affected individuals demonstrate cleavage of \(\alpha\) I T80 to \(\alpha\) I T80 peptide. A variable amount of unaltered \(\alpha\) I T80 remains as shown above and by others.\textsuperscript{13}

The cleavage of \(\alpha\) I T80 to \(\alpha\) I T74, although frequently increased in this population, does not correlate quantitatively with hemolytic expression of HE (Fig 6). It should be noted, however, that other investigators have determined the \(\alpha\) I 80 \(\rightarrow\) 74 cleavage to be typical of a subpopulation of patients with HPP and HE.\textsuperscript{11-13} The differences between their results and ours may be due to methodology, in particular due to characteristics of the digestion conditions including pH and ionic strength, which are known to affect specific cleavage sites (W. Knowles and J. Morrow, unpublished observations).

The most likely interpretation of our observations is that an altered amino acid sequence within the \(\alpha\) I domain of HE and HPP spectrin produces conformational changes in neighboring portions of the peptide, resulting in increased exposure of the (normal) tryptic cleavage site at lysine 252,\textsuperscript{28} producing an increased quantity of the 50 kd \(\alpha\) I peptide, and possibly increasing cleavage at arginine 39 to produce \(\alpha\) I T74. The sequence data suggest that both (normal) 50 kd and 74 kd peptides extend to the same COOH terminal site as the intact \(\alpha\) I 80 kd.\textsuperscript{28} The 50 kd peptide that appears in digests of HE and HPP spectrin is thought to be identical to the normal 50 kd cleavage product, based on its isoelectric point, monoclonal antibody studies, and \textsuperscript{125}I limit peptide mapping which shows only normal peptides of \(\alpha\) I 80 kd.

The presumptive alteration in \(\alpha\) I primary structure and resultant conformational change is thought to be responsible for the abnormal spectrin–spectrin interactions observed in patients with HE (and HPP) because the \(\alpha\) I domain is known to associate specifically with spectrin dimers to promote oligomer formation.\textsuperscript{14} Impairment of spectrin self-association has frequently been reported in HPP and HE, expressed either as increased percentage of dimer in crude extracts at 4 °C,\textsuperscript{11-13,15,29,30} or as inability to form dimers and oligomers after incubation at 30 °C over a range of concentrations.\textsuperscript{10} Oligomer formation by spectrin from HE subjects in the two families reported here was reduced in all cases; however, the degree of impairment varied from slight in the patients with mild nonhemolytic HE to severe in the case of patient T.S. with poikilocytic hemolytic anemia, approaching our early findings in HPP.\textsuperscript{10} As shown in Figs 6, 13, and 14, the severity of clinical expression of HE in members of the kindreds described is proportional to the fractional quantity of \(\alpha\) I T80 with altered conformation as well as to the degree that oligomer formation is reduced.

**Figure 13.** Quantitation of dimer, tetramer, and higher oligomers of spectrin from family R by pyridine elution of Coomassie blue dye stain from native gels shown in Fig 9. See legend for Fig 7 for experimental detail. \(\bigstar\) dimer; \(\bigcirc\) tetramer; \(\bigcirc\) hexamer; \(\bigcirc\) octamer.

**Figure 14.** Quantitation of spectrin dimer and tetramer from T.S. (■), and E.S. (○) by pyridine elution of Coomassie blue dye from the native gels shown in Fig 11. See legend for Fig 9 for experimental details. The control curve (●) is a composite of studies on normal individuals as previously reported.\textsuperscript{125}
Another possible interpretation of our observations is that the abnormal segment of spectrin is located in the \( \alpha I \) domain, and, because of its interaction with the \( \alpha I \) domain, changes in conformation of \( \alpha I \) occur which result in an altered trypsin cleavage pattern. However, we have previously demonstrated\(^{16} \) that trypsin digestion of HPP spectrin which has been denatured and refolded for 60 seconds produces only \( \alpha I \) T50 and \( \alpha I \) T21 peptides, whereas normal spectrin generates only \( \alpha I \) T80. Because the denaturing conditions used results in dissociation of \( \alpha \) and \( \beta \) subunits, and because the refolding period was very short, it seems unlikely that the \( \beta \) subunit is responsible for the altered digestion of the \( \alpha I \) domain.

Dhery et al\(^{13} \) have reported a spectrin \( \beta \) chain variant in a family with elliptocytosis associated with defective self-association of spectrin dimer. No abnormality of the \( \beta I \) domain of spectrin from families R and S was seen on SDS-PAGE of intact spectrin nor on two-dimensional maps of trypic and NTCB digests (data not shown); however, this does not rule out the possibility of an abnormal \( \beta I \).

The increases in apparent mol wt of the variant \( \alpha II \) and \( \alpha III \) domains may be due to insertion of 4 kd or to an amino acid substitution which affects the sites of trypic cleavage for both domains. Available data on the \( \alpha II \) variants do not support these mechanisms for the \( \alpha II \) polymorphism (W. Knowles and D. Speicher, unpublished observations). Alternatively, single amino acid substitutions may affect conformation, changing SDS-binding properties and migration on SDS/PAGE, in addition to altering isoelectric point. No information yet shows how expression of the abnormal \( \alpha I \) T80 may be modulated by the variant \( \alpha II \) and \( \alpha III \) domain peptides observed in family S. The \( \alpha II \) variants, which are seen frequently in blacks in our studies, confer no observable effect on erythrocyte membrane stability when present alone, but their effect in the setting of an abnormal \( \alpha I \) domain is unknown. The \( \alpha III \) variant has been seen in three families in the setting of HE and HPP and with \( \alpha II \) variants; nothing is known about its effect on the expression of the abnormal \( \alpha I \) T80. The only other subject with the \( \alpha III \) variant observed to date had HS but an otherwise normal spectrin trypic digest.

Although HPP appears to be closely related to HE based on family studies, its occurrence within a particular kindred is not predictable. Mentzer et al\(^{11} \) conclude from a study of two families that HPP results from compound heterozygosity for HE and a nonelliptocytic carrier state characterized by red cell ghosts with increased mechanical fragility. Lawler et al\(^{13} \) suggest that HPP red cells contain more of a particular abnormal spectrin than HE cells. There may be multiple alleles coding for the spectrin \( \alpha \) subunit, as in the \( \alpha \) thalassemia syndromes, and with similar consequences. Thus, if members of a family with HE were to have 1, 2, 3, or 4 abnormal alleles coding for the \( \alpha \) subunit, a corresponding proportion of spectrin would contain the abnormal \( \alpha I \) 80K.

As the number of affected alleles increased, the hematologic expression would range from mild HE to severe poikilocytic hemolytic anemia. This concept is particularly attractive because of the multiple \( \alpha \) subunit mutations observed in spectrin from the same individual. Thus, variants in \( \alpha I \), \( \alpha II \), and \( \alpha III \) seen in the spectrin of one individual need not all occur on the same molecule in the four-allele model. It may be that the \( \alpha II \) or \( \alpha III \) variants have no pathophysiologic effect if present alone on an \( \alpha \) subunit, but if present on the same \( \alpha \) subunit as the \( \alpha I \) T80 \( \rightarrow \) 50 mutation, they may interact with and modulate expression of hemolytic disease.

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