Molecular Determinants of Clinical Expression of Hereditary Elliptocytosis and Pyropoikilocytosis

By Theresa Coetzter, Jack Lawler, Josef T. Prchal, and Jiri Palek

The clinical severity of common hereditary elliptocytosis (HE) is highly variable, ranging from an asymptomatic carrier state to a severe hemolytic anemia. To elucidate the molecular basis of this variable clinical expression, we evaluated 56 subjects from 24 HE kindred, who carry α spectrin mutants characterized by a spectrin dimer (SpD) self-association defect related to a structural abnormality of the α I domain of spectrin. Twenty-nine subjects had common HE, 13 subjects have a closely related disorder, hereditary pyropoikilocytosis (HPP), and 14 are asymptomatic carriers. We compared the severity of hemolysis with the following biochemical parameters: (a) spectrin heterodimer self-association, as manifested by the percentage of SpD in the 4°C low ionic strength spectrin extract; (b) spectrin structure, as examined by limited tryptic digestion of spectrin; and (c) spectrin content of the RBC membrane.

Our analysis indicates that the severity of hemolysis may be correlated with quantitative differences in the percentage of SpD in the 4°C spectrin extract, as well as the total spectrin content of the membrane. Thus, HPP subjects, who have the most severe hemolytic anemia, have the highest percentage of SpD as well as a decreased spectrin content. HE subjects and asymptomatic carriers, respectively, have a lower percentage of SpD and a normal spectrin content. Factors influencing these two determinants include functional differences between the individual spectrin mutants, the relative amounts of mutant spectrin present in the cells, the stability of mutant spectrin, and the possibility of a superimposed genetic defect involving spectrin synthesis.

HEREDITARY ELLIPTOCYTOSIS (HE) is a group of disorders that is heterogeneous in terms of inheritance, severity of hemolysis, and RBC morphology as well as the underlying molecular defect. 12 Nine clinical variants of common HE have been delineated. 3 These include an asymptomatic carrier state; nonhemolytic HE; HE with either minimal, sporadic, chronic, or severe hemolysis; and HE with neonatal poikilocytosis and hereditary pyropoikilocytosis (HPP). This clinical heterogeneity of the HE/HPP syndrome is paralleled by a biochemical heterogeneity, and several different molecular defects have been described in HE and HPP. 3 The most common defect found in a subpopulation of HE patients and in all HPP patients is the spectrin dimer (SpD) self-association defect, 4,5 in which the abnormality is localized in the α I domain of spectrin, which represents the SpD-SpD contact site. 6,7 On a functional level, this defect is manifested by an impaired SpD self-association both in solution and in the membrane, resulting in an increased amount of SpD extracted from the membrane under conditions that reflect the SpD-SpT equilibrium present in the RBC membrane in situ. 8-10 On a structural level, the defect is detected by limited tryptic digestion of spectrin, which cleaves the α and β chains into distinct domains: α I through α V and β I through β IV. 11 To date, three major variants of the α I domain of spectrin have been detected in HE and HPP: Sp α 1/74, Sp α 1/46, and Sp α 1/65 (nomenclature according to references 2-5).

The clinical severity of these spectrin α I domain defects varies from that of an asymptomatic carrier state to a severe hemolytic disease. In this study, we attempted to elucidate the molecular basis of these heterogeneous clinical phenotypes. We compared the clinical expression of 56 patients from 24 kindred with HE and/or HPP who have defective SpD self-association to the biochemical expression on (a) a functional level as manifested by the percentage of SpD present in the membrane, (b) a structural level by limited tryptic digestion of spectrin, and (c) a quantitative level by estimating the amounts of normal and abnormal spectrin present in the membrane.

MATERIALS AND METHODS

Subjects. Fifty-six patients from 24 unrelated kindred were studied. Twenty-nine subjects have common HE, including one patient with severe homozygous HE; 13 subjects have HPP; and 14 are asymptomatic carriers. Five of the kindred are white (two with HE/HPP occurring in the same family), one subject has a white mother and a Black father, and the remainder are black. The clinical diagnosis of HE was based on the presence of elliptocytes (±10% to 100%) since infancy, inherited in most cases as an autosomal dominant trait. For HPP patients, the clinical diagnosis was based on...
the characteristic and persistent RBC morphology of striking poikilocytosis and microspherocytosis, a thermal instability of the RBCs, an autosomal recessive mode of inheritance, and the absence of identifiable abnormalities in glycolytic enzymes and hemoglobin (with the exception of one subject who is homozygous for HbC). Five of the 13 HPP patients and 7 of the 29 subjects with HE were of identifiable abnormalities in glycolytic enzymes and hemoglobin.

Erythrocyte ghosts were prepared by hypotonic lysis at 4°C of washed RBCs in 3 mmol/L NaPO₄, pH 8, 0.1 mmol/L EDTA, and 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF), a protease inhibitor included to minimize proteolysis. Membrane protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 5.6% polyacrylamide gels and stained with Coomassie blue. Protein concentrations were measured by the BioRad protein assay using bovine serum albumin (BSA) as standard. The amount of spectrin on the membrane was expressed as a ratio of spectrin to band 3 and was quantitated by densitometry of the stained gels at 540 nm and integration of the area underneath the peaks, or by eluting the Coomassie blue stain from the gels with 25% pyridine (vol/vol). In the case of Sp α 1/74, the amount of abnormal spectrin cannot be accurately quantitated by measuring the amount of 74,000-daHn, spectrin produced since normal control spectrin produces a certain, although much smaller, amount of the 74,000-daHn peptide. For HE Sp α 1/65 variants, neither is precise quantitation of the mutant spectrin possible, since the abnormal 65,000-daHn peptide partly overlaps with the α II domain. However, quantitation of the ratio of these two peptides to the 80,000-daHn peptide is used for comparative purposes in Table 3.

Stability of membrane skeletons. Erythrocyte membrane skeletons were prepared in Triton X-100 and subjected to shear stress in a concentric cylinder-rod shearing apparatus as described by Liu and Palek.

RESULTS

Clinical characterization. Clinical data on 56 patients from 24 unrelated kindred are shown in Table 1. All subjects have an increased amount of SpD in the membrane and a structural abnormality of the α I domain of spectrin, except in the case of two patients with mild HE and two asymptomatic carriers in whom the structural abnormality of spectrin has not yet been identified (referred to as SpD-SpD?). Classification of the clinical presentation is based on that of Palek and includes the following clinical variants: asymptomatic carriers, mild HE with no or minimal hemolysis, HE

<table>
<thead>
<tr>
<th>Clinical Phenotype</th>
<th>RBC Morphology</th>
<th>Subjects (n)</th>
<th>Kindred (n)</th>
<th>Hb (g/dL)</th>
<th>Hct (%)</th>
<th>MCV (fL)</th>
<th>Reticulocytes (%)</th>
<th>Molecular Defect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymptomatic carrier</td>
<td>Normal (&lt;2% elliptocytes)</td>
<td>14</td>
<td>11</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>6 Spα 1/74</td>
</tr>
<tr>
<td>Mild HE, no/minimal hemolysis</td>
<td>± 10% to 100% elliptocytes</td>
<td>20</td>
<td>12</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>0.2-4</td>
<td>12 Spα 1/74</td>
</tr>
<tr>
<td>Mild HE, chronic hemolysis</td>
<td>15% to 60% elliptocytes</td>
<td>3</td>
<td>1</td>
<td>12-13</td>
<td>35-36</td>
<td>56-72</td>
<td>18-23</td>
<td>3 Spα 1/74</td>
</tr>
<tr>
<td>Severe Homozygous HE</td>
<td>Elliptocytes, poikilocytes, microspherocytes, fragments</td>
<td>1</td>
<td>1</td>
<td>7</td>
<td>20</td>
<td>81</td>
<td>30</td>
<td>1 Spα 1/74</td>
</tr>
<tr>
<td>HE with neonatal poikilocytosis</td>
<td>Elliptocytes, poikilocytes, fragments → 50% to 100% elliptocytes</td>
<td>5</td>
<td>4</td>
<td>5-11</td>
<td>18-33</td>
<td>50-84</td>
<td>0.3-7</td>
<td>2 Spα 1/74</td>
</tr>
<tr>
<td>HPP</td>
<td>Poikilocytes, microspherocytes, fragments, few, if any, elliptocytes</td>
<td>13</td>
<td>12</td>
<td>4-8</td>
<td>13-26</td>
<td>25-72</td>
<td>9-40</td>
<td>5 Spα 1/74</td>
</tr>
</tbody>
</table>

Abbreviations: Hb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; N, normal value; SpD-SpD?, subjects with increased percentage SpD but an unidentified structural defect.

*Subject is transfusion dependent, and clinical values were obtained 20 days after transfusion.
with chronic hemolysis, HE with neonatal poikilocytosis, and severe homozygous HE and HPP.

Table 1 indicates that each clinical phenotype does not correspond to a specific structural defect of α spectrin, but that each defect occurs in more than one phenotype. The α 1/74 and α 1/46 spectrin variants are found in both HE as well as HPP subjects and asymptomatic carriers. The most recently described spectrin variant, Sp α 1/65, has thus far only been detected in HE patients and in an asymptomatic carrier, possibly indicating that this structural variant is not as severe as the other two types.

Spectrin dimer content of the membrane and clinical severity. When spectrin is extracted from the RBC membrane at 4°C, the equilibrium between spectrin dimers (SpD), tetramers (pT), and oligomers is stabilized, and the relative amounts of the spectrin species obtained reflect the composition of the membrane in situ.28,29 Figure 1 depicts a correlation among disease severity, as reflected by percentage of reticulocytes and hematocrit and the amount of SpD present in RBC membranes of control, HPP and HE subjects, and asymptomatic carriers. The SpD content is expressed as the percentage of SpD of the total SpD and SpT pool in a crude 4°C spectrin extract. In control subjects, spectrin exists mainly in the tetrameric form and the percentage of SpD is very low (large rectangles in Fig 1, 5.0 ± 3.6%, n = 35). In contrast, HPP and HE subjects included in this study, as well as asymptomatic carriers, have an increased amount of SpD in the membrane, reflecting a SpD-SpD self-association defect. This defect is most pronounced in all HPP patients (circles in Fig 1) who have a severe hemolytic anemia (reticulocytes >10%, hematocrit <28), followed by HE patients (rectangles in Fig 1) most of whom have a milder disorder (reticulocytes >5%, hematocrit ≥28), and then by asymptomatic carriers (triangles in Fig 1). The differences in the percentage of dimers among patients with low, intermediate, and high reticulocyte and hematocrit values are highly statistically significant (Fig 1). These results indicate that the percentage of SpD in the membrane correlates with the severity of the hemolytic disease and that patients with the highest proportion of SpD in the membrane are the most severely affected. This variation in the levels of SpD is due both to differences in the functional properties of the various mutant spectrins28 (manuscript in preparation) and to differences in the quantity of abnormal spectrin present (see below).

The patient with homozygous HE is not included in the statistical analysis since she is transfusion dependent. She had been transfused 3 weeks prior to our studies, and the low result of 25% SpD in the membrane reflects the SpD content of a mixed population of transfused normal cells and patient cells. However, as an analogy to a similar case report of homozygous HE28 and also due to the high levels of SpD found in the membranes of both her parents and sister who are HE heterozygotes (range 38% to 46% SpD), we speculate that she would have a very severe functional and structural defect of spectrin reminiscent of HPP, and that the percentage SpD in her membranes would be markedly increased in accordance with the life-threatening clinical presentation.

Further analysis of the data in Fig 1 shows that there is no significant difference in the percentage of SpD between the Sp α 1/74 and Sp α 1/46 subjects in each clinical category. However, the structural α 1/65 defect differs in functional severity from the α 1/74 and α 1/46 spectrin variants. HE Sp α 1/65 subjects show a significantly smaller increase in the amount of SpD in the membrane (18.4% ± 4.8%, n = 7) as compared with the HE Sp α 1/74 and HE Sp α 1/46 variants (39.4% ± 6.3%, n = 19, *P* < .0005). Furthermore, the asymptomatic carrier of the α 1/65 defect has a normal level of SpD in the membrane. Of interest also is one of the asymptomatic carriers of the α 1/46 defect who has a reproducibly normal SpD content.

We previously showed that the SpD self-association defect is reflected in a decreased stability of the RBC membrane skeleton when exposed to shear stress.23,29 Although we find this test useful in screening for membrane skeletal defects, it is inaccurate and poorly reproducible to permit quantitative comparisons.

The relationship between the percentage of SpD in the membrane of the HE patients (n = 28) and the percentage of elliptocytes on their peripheral blood smears was also examined. There appears to be no significant correlation between
the elliptocytic morphology of the RBCs and the self-association state of spectrin in the membrane as indicated by linear regression analysis, which yielded a coefficient of correlation of 0.29 (data not shown).

Quantitation of abnormal spectrin in the cells. The clinical presentation of asymptomatic carrier, HE, and HPP was compared with the approximate percentage of abnormal α 1/46 spectrin present, as indicated in Table 2. Control individuals do not produce any of the abnormal 46,000-dalton or 17,000-dalton peptides, and quantitation of these peptides therefore reflects the amount of mutant α spectrin present. Asymptomatic carriers contain ±25% of the α 1/46 spectrin, HE patients ~50%, and HPP subjects 100% of the abnormal spectrin under the digestion conditions used. A similar approach, however, cannot be used for Sp α 1/74 variants since the cleavage site producing the 74,000-dalton peptide is present in the normal α I domain of control spectrin. Accessibility to this site, however, is limited in normal individuals, and only a small amount (27% ± 6%) of the 74,000-dalton peptide, relative to the 80,000-dalton peptide, is produced. In contrast, the α 1/74 mutant spectrin is characterized by markedly increased susceptibility of this site to cleavage.2,10

Total spectrin content of the RBC membrane. The spectrin content of erythrocyte membranes, expressed as a Sp/band 3 ratio, was correlated with clinical expression as illustrated in Fig 2. In control membranes, there are approximately equal amounts of spectrin and band 3 as indicated by a Sp/band 3 ratio of 1.0 ± 0.1 (n = 60). In contrast, all 13 HPP subjects show a marked decrease in the Sp/band 3 ratio to a mean value of 0.74 ± 0.05, reflecting a decreased spectrin content of the membrane. This partial spectrin deficiency is not affected by the type of mutant spectrin present in the cells, since there is no significant difference between the Sp/band 3 ratio of HPP Sp α 1/74 (0.75 ± 0.05, n = 7) and HPP Sp α 1/46 (0.72 ± 0.05, n = 5) and a value of 0.71 for the double heterozygote HPP Sp α 1/74, 46.

HE patients (n = 23) have a normal Sp/band 3 ratio of 0.99 ± 0.07, and asymptomatic carriers also fall into the normal range with a mean Sp/band 3 ratio of 0.98 ± 0.06 (n = 11). Thus, even though the functional and structural defects of spectrin in HE and in asymptomatic carriers are qualitatively the same as in HPP subjects, the spectrin content in HE and carriers is not affected.

HE with neonatal poikilocytosis. Five patients in this study exhibited HE with neonatal poikilocytosis. This condition was noted in association with all three structural variants of spectrin as indicated in Table 1. Table 3 depicts an α 1/74 and the α 1/65 variant in which the change in the RBC morphology is compared with the aboveoutlined biochemical parameters. This type of analysis was not performed on the α 1/46 variants. Table 3 indicates that the percentage of SpD in the membrane, as well as the relative amounts of the abnormal α I peptide, remains constant during the transformation from HPP to HE phenotype. The Sp/band 3 ratio also remains within the control range during this transition. These data indicate that the functional and structural defects of spectrin remain unaltered during the morphologic transi-

Table 3. Correlation of RBC Morphology with Biochemical Features of Spectrin in HE With Neonatal Poikilocytosis

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>Percentage of Abnormal Spectrin</th>
<th>74 kd (%)</th>
<th>65 kd (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DD (HE Spα 1/74)</td>
<td>2 wk</td>
<td>55</td>
<td>47</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>15 mo</td>
<td>55</td>
<td>47</td>
<td>0.12</td>
</tr>
<tr>
<td>TE (HE Spα 1/65)</td>
<td>3 wk</td>
<td>55</td>
<td>47</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>3½ mo</td>
<td>55</td>
<td>47</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>10 mo</td>
<td>55</td>
<td>47</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>16 mo</td>
<td>55</td>
<td>47</td>
<td>0.12</td>
</tr>
</tbody>
</table>

*Densitometric scanning of tryptic digest maps of spectrin was used to estimate 74 kd (%) and is expressed as a percentage of the total 74,000 ± 80,000-dalton peptides.
†Percentage 65,000 dalton + βII peptides in the total 65,000 + βII + 80,000-dalton peptides is given as 65 kd (%).
tion, implying that intracellular factors rather than structural changes in the spectrin molecule are responsible for the change in clinical presentation.

**DISCUSSION**

In recent years, we and other researchers identified four structural variants of α spectrin associated with HE. These mutant spectrins also exhibit a functional SpD self-association defect. During these studies, a marked variability became apparent in the clinical expression of the HE disorder, ranging from an asymptomatic carrier state to mild HE with minimal or moderate hemolysis and ultimately to HPP, which presents as a severe hemolytic disease. The purpose of the present study was to elucidate the molecular basis of these heterogeneous clinical phenotypes. To accomplish this objective, we compared the biochemical expression of the underlying molecular defects in 56 HE/HPP subjects from 24 kindreds who exhibit a SpD self-association defect with the clinical severity and morphologic expression of the disorder. Our analysis of the functional and structural abnormalities of spectrin in these HE/HPP patients indicates that the heterogeneity of clinical expression may be correlated with two major factors: the SpD content and the total spectrin content of the membrane.

**SpD content of the membrane.** The role of the SpD self-association defect as a determinant of the clinical severity is illustrated by the highly statistical differences in the percentage of SpD between the low, intermediate, and high reticulocyte and hematocrit groups. In addition, all HPP patients, who have a very severe hemolytic anemia, have the highest amount of SpD in the membrane, whereas HE subjects have a considerably lower percentage of SpD and are clinically much less affected (Fig 1). Furthermore, asymptomatic carriers have an even lower percentage of SpD in the membrane.

The amount of SpD in turn appears to be related to at least two factors. The first factor involves differences between mutant α spectrins in terms of their functional capacity to self-associate into SpT. This possibility is raised by previous studies in our laboratory[10,28] that demonstrated that the self-association of α 1/74 spectrin is severely impaired whereas the α 1/46 spectrin variant retains some capacity to form SpT.

The second factor is the amount of dysfunctional α spectrin present. For Sp α 1/46 variants, the amount of abnormal α I peptides (46,000 and 17,000 daltons) may be quantitated and corresponds to the amount of abnormal spectrin present, since control spectrin does not produce any of these peptides. The clinical severity correlates with the mutant spectrin content, since HPP Sp α 1/46 individuals have no normal α I domain (ie, 100% abnormal spectrin), followed by HE Sp α 1/46 subjects and carriers who contain ~50% and 25% of abnormal spectrin, respectively (Table 2). This type of correlation is not possible for the α 1/74 and α 1/65 spectrin variants, since the amount of abnormal α I peptides cannot be accurately quantitated, as previously discussed.

**Total spectrin content of the membrane.** The clinical expression also appears to be related to the net spectrin content of the membrane. HPP RBCs contain ~30% less spectrin than do those of control individuals, whereas all HE patients, most of whom have a much milder form of the disease, as well as asymptomatic carriers, have a normal spectrin content (Fig 2). However, the estimation of spectrin content from the intensity of staining of SDS-PAGE slabs does not accurately quantitate the actual degree of spectrin deficiency, which is likely to be even larger. Because in addition to the partial spectrin deficiency, HPP RBCs also contain the highest fraction of dimeric spectrin, it remains uncertain whether this partial spectrin deficiency in HPP aggravates the other molecular abnormality of spectrin (defective spectrin self-association). However, spectrin deficiency in HPP might be responsible for microspherocytosis in this condition, which is absent in patients with HE. Partial deficiency of spectrin is also a common feature of hereditary spherocytosis[39] and (although it remains to be proven) may underlie the decrease in RBC surface area in this condition.

Several factors could influence the spectrin content of the membrane and hence the clinical expression. These include the following: The instability of mutant spectrin leads to degradation of this protein prior to its assembly on the membrane (as may be the case in HPP Sp α 1/46). Second, a double heterozygous state for an α spectrin mutant and a defect involving synthesis of this protein is suggested by a characteristic pattern of HPP inheritance as previously discussed. Typically, one of the parents of the HPP offspring carries the α spectrin mutant, whereas the other parent is fully asymptomatic. It is likely that this other parent transmitted to the HPP offspring another genetic defect (presumably involving synthesis of spectrin) that increases the quantitative expression of the α spectrin mutant and causes a concomitant partial spectrin deficiency. A third possible factor accounting for the clinical and molecular heterogeneity of HE could be a duplication of the α spectrin gene. This hypothesis is tentatively supported by the correlation between the amount of abnormal spectrin present in Sp α 1/46 subjects and the clinical expression. The Sp α 1/46 asymptomatic carriers have ±25%, HE subjects ±50% and HPP patients 100% of the abnormal spectrin (Table 2). These results could be compatible with a duplicated α spectrin gene, and consequently, inheritance of one, two, or more abnormal alleles of four spectrin alleles could result in an asymptomatic carrier state, mild HE, and homozygous HE or HPP, respectively. The human α spectrin gene has been localized to chromosome 13 and, with the aid of genetic cloning and hybridization techniques as well as studies on kindred carrying several nonlinked variants of α spectrin, the validity of the α spectrin gene duplication hypothesis could be established.

**HE with neonatal poikilocytosis.** HE with neonatal poikilocytosis is an interesting and heterogeneous phenotypic variant of HE illustrating the influence of additional factors on the variability in clinical expression. During the clinical transition of HE, the severity of the underlying molecular abnormalities does not change (Table 3), and the poikilocytosis and hemolysis in the neonate and the subsequent morphologic transition has been considered as possibly related to differences between the RBC microenvironment of the neonate and the adult. The presence of fetal hemoglo-
bin, which has a decreased affinity for 2,3 DPG, would lead to increased levels of 2,3 DPG in the cytoplasm. This could destabilize the spectrin/actin/band 4.1 complex, which could in turn augment the expression of the SpD self-association defect and thus weaken the skeleton even further, leading to poikilocytosis and fragmentation. Whether the neonatal poikilocytosis occurs in all neonates with HE, or in a subset of such patients remains to be established. One neonate with presumed HE, in whom the molecular defect has not been defined, did not have neonatal poikilocytosis. On the other hand, all HE neonates carrying α spectrin defect who are reported in this study did have neonatal poikilocytosis.

In conclusion, this comparative study indicates that HE subjects, who exhibit a SpD self-association defect, have the same defect of α spectrin as do HPP individuals. The variation in the severity of hemolysis, ranging from asymptomatic carrier to HE and HPP, is related to differences in the SpD content and presumably the total spectrin content of the membrane as well.

ACKNOWLEDGMENT

We wish to thank Carol Comerio, Joe Connolly, and Paula Ferro for expert technical assistance; Mia Thurlow and Joan Joos for the artwork; and Loretta Wencis for typing the manuscript. We also thank the patients and their relatives for their cooperation.

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