

INHERITANCE PATTERN AND CLINICAL RESPONSE TO SPLENECTOMY AS A REFLECTION OF ERYTHROCYTE SPECTRIN DEFICIENCY IN HEREDITARY SPHEROCYTOSIS

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Abstract To determine how various inheritance patterns and responses to splenectomy relate to erythrocyte spectrin deficiencies in hereditary spherocytosis, we measured the spectrin content of erythrocytes by radioimmunoassay in 33 patients with this disease.

Patients with the dominant form of hereditary spherocytosis generally had mild anemia, with spectrin at 63 to 81 percent of normal levels. Patients with the nondominant form of the disease had anemia ranging from severe to mild, with corresponding spectrin levels of 30 to 74 percent; their siblings were affected similarly. Distantly related homozygotes had different clinical severities with correspondingly different spectrin levels. The parents and

offspring of patients with the nondominant form were clinically normal but consistently had subtle erythrocyte abnormalities. Spectrin levels in all patients were inversely related to osmotic fragility ($P < 0.0001$), and they were also correlated with the clinical response to splenectomy: patients with spectrin levels above 70 percent achieved normal blood counts, those with levels of 40 to 70 percent had compensated hemolysis, and those with levels below 40 percent improved but remained anemic ($P < 0.0001$).

We conclude that the inheritance pattern and response to splenectomy in hereditary spherocytosis reflect erythrocyte spectrin deficiencies as determined by radioimmunoassay. (N Engl J Med 1986; 315:1579-83.)

HEREDITARY spherocytosis is the most common inherited anemia affecting persons of northern European ancestry, and it has intrigued clinicians and investigators for decades.¹ The increased sensitivity of the erythrocytes in spherocytosis to hypotonic lysis suggests that a simple but variable defect in the membrane skeleton leads to a reduction in the surface area of the membrane relative to its volume, which is responsible for the clinical problems and abnormal shape and fragility of the erythrocytes. Defects have recently been identified in the red-cell membranes of patients with spherocytosis. Recessively inherited spherocytosis in severely anemic mice is associated with gross deficiencies of spectrin^{2,3}; a similar illness has been described in two humans.⁴ Three other families have been shown^{5,6} to have abnormal associations of spectrin, actin, and protein 4.1, whereas most patients with hereditary spherocytosis have been partially deficient in spectrin.⁷

There is considerable clinical heterogeneity among families with spherocytosis.⁸⁻¹⁰ Approximately 75 percent of cases of spherocytosis occur in families with a dominantly inherited anemia, yet 25 percent of cases have a negative family history. There is also a wide range of clinical severity; we recently reported a correlation between spectrin content and the severity of disease in 14 patients.⁷ The purpose of this study was to determine whether the degree of spectrin deficiency

is related to specific clinical features of the anemia, with the hope that measurement of spectrin by radioimmunoassay may permit the identification of inheritance type and predict the clinical outcome after splenectomy.

METHODS

Blood samples were obtained from patients from throughout the United States and abroad. All the cases of hereditary spherocytosis fulfilled certain criteria: at least occasional spherocytes were seen in peripheral-blood smears with polychromatophilia before splenectomy, Coombs' tests were negative, and osmotic fragility was increased. Fifteen families were white, one was black, one was Hispanic, and one was Korean.

Blood was drawn simultaneously by venipuncture from patients and a control; it was anticoagulated with acid-citrate-dextrose (for radioimmunoassays) or heparin (for measurement of osmotic fragility), packed in ice, shipped by jet to Baltimore, and analyzed within 24 hours. The osmotic fragility of unincubated erythrocytes was compared with that of a normal control by the method of Dacie,⁹ to determine the precise degree of increased sensitivity to osmotic lysis. Sodium dodecyl sulfate-polyacrylamide-gel electrophoresis was performed with use of the buffer system of Fairbanks et al.¹¹ and 3.5 to 17 percent nonlinear gradient slab gels. The amount of spectrin relative to band 3 was measured by the dye-elution method.¹² The spectrin radioimmunoassay measured competition between unlabeled spectrin in detergent-lysed erythrocytes and ¹²⁵I-labeled spectrin, for binding to a limited number of sites on protein A-bearing staphylococci coated with antispectrin IgG, as described elsewhere.⁷ Serum haptoglobin was measured by the serial radial diffusion method (Behring Diagnostics, La Jolla, Calif.). Statistical analyses, including curve-fitting models and the one-sided t-test, were performed with use of the Hewlett-Packard HP-41C Stat Pac.

RESULTS

Quantitation of Spectrin

The extent of protein deficiencies in cell membranes in certain inherited anemias was first estimated by densitometric analysis of sodium dodecyl sulfate-polyacrylamide electrophoresis gels after staining.^{4,13,14} The anion transporter (also referred to as band 3) is an index of the surface area of the membrane, and

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reductions in the amount of spectrin as compared with band 3 can usually be detected by this method. In this study, 33 patients with hereditary spherocytosis from 18 families with diverse backgrounds were studied with both gel analyses and spectrin radioimmunoassays. It is clear (Fig. 1) that the gel method overestimated the spectrin content, which was determined by radioimmunoassay to range from 30 to 81 percent of normal. Furthermore, unlike the gel method, which yields lower ratios of spectrin to band 3 after splenectomy (apparently due to increased⁷ band 3), radioimmunoassay determined that the spectrin content was nearly identical in two patients with hereditary spherocytosis who were studied before and several months after splenectomy, as well as in members of the four families containing both patients who had undergone splenectomy and those who had not. Spectrin deficiency may be a unique feature of hereditary spherocytosis and pyropoikilocytosis (a related disorder¹⁴), since patients with spherocytosis that was not inherited (antibody-mediated disease) and those with other kinds of anemia were not deficient in spectrin (Table 1).

Inheritance Patterns

Patients and unaffected members of the families with spherocytosis were studied with the spectrin radioimmunoassay; selected genealogies are shown in Figure 2. Most patients with spherocytosis have a mild anemia of dominant inheritance, and affected members of these families were always found to have comparable spectrin deficiencies, with the amount of spectrin ranging from 63 to 81 percent of normal (Families E and F). Although their parents and offspring were clinically normal, patients with the nondominant form of the disorder had anemia ranging from severe to mild, with corresponding spectrin levels ranging from 30 to 74 percent of normal. Parental consanguinity was established in two kindreds, indicating that some families have a recessively inherited anemia (Families

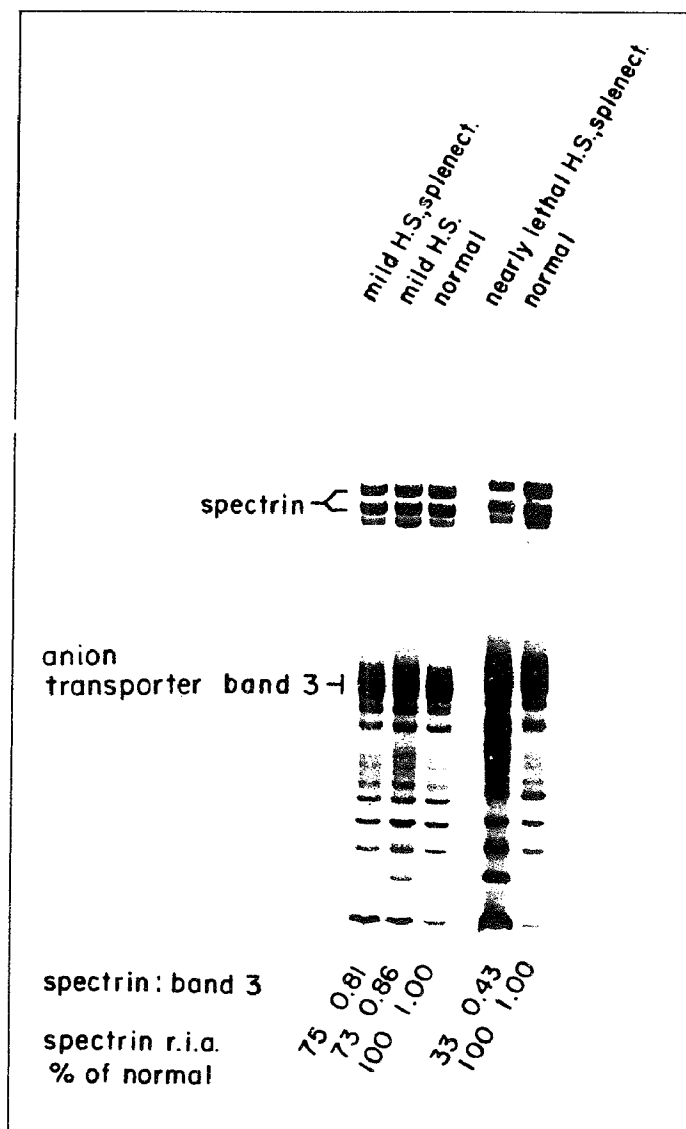


Figure 1. Comparison of Two Methods of Determining the Spectrin Content of Erythrocyte Membranes: Gel Electrophoresis and Spectrin Radioimmunoassay.

Membranes from washed erythrocytes were subjected to electrophoresis through sodium dodecyl sulfate-polyacrylamide gels that were stained with Coomassie blue (upper panel). The relative concentrations of spectrin and the anion transporter (band 3) were estimated after elution of the dye and normalized as spectrin:band 3 ratios. Erythrocytes from the same patients were evaluated with the spectrin radioimmunoassay, and the correlation between these determinations was plotted (lower panel). All points are below the "line of equivalence," indicating that the gel method uniformly overestimated the actual spectrin content.

Patients with the nondominant form of spherocytosis are indicated by triangles, those with the dominant form by circles, and those who had previously undergone splenectomy by open triangles or circles. The cross-hatched areas represent the range for 10 normal controls and 4 controls who had undergone splenectomy. H.S. denotes hereditary spherocytosis, and r.i.a. radioimmunoassay.

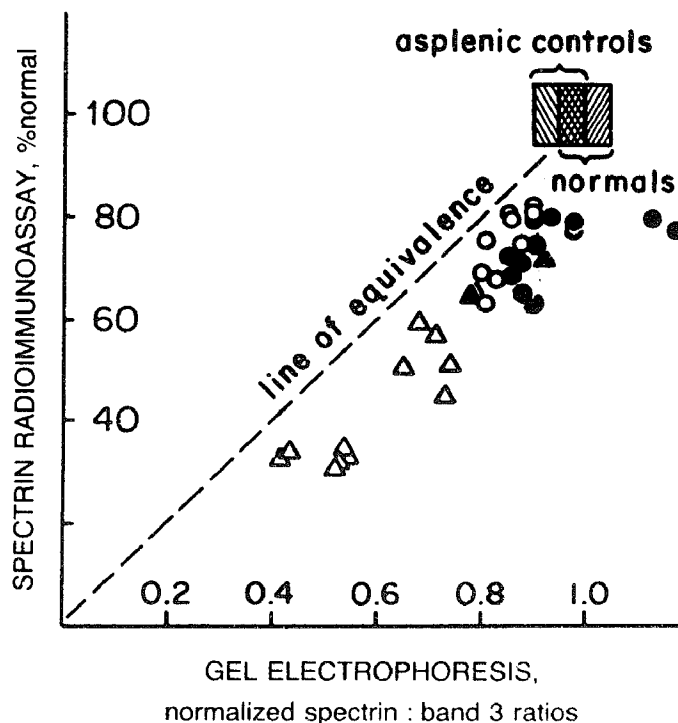


Table 1. Spectrin Radioimmunoassay of Erythrocytes from Patients with Conditions Other Than Hereditary Spherocytosis.

| CONDITION (NO. OF PATIENTS IF MORE THAN 1) | SPECTRIN % of normal |
|---|-------------------------|
| Warm-reacting autoimmune hemolytic anemia, circulating spherocytes, increased osmotic fragility | 97 |
| Neonatal spherocytosis, presumed isoimmune | 98 |
| Normal neonatal erythrocytes (2) | 103, 106 |
| Hereditary ovalocytosis, Rh-linked | 94 |
| Hereditary elliptocytosis, alpha-spectrin variant | 93 |
| Splenectomy (controls) (2): trauma, thrombocytopenia | 98, 106 |
| Uncharacterized hemolytic anemia, increased reticulocytes | 101 |
| Beta-thalassemia trait, MCV 67 fl* | 96 |
| Macrocytic anemia (4), MCV 100–122 fl* | 112–116 |
| Acanthocytosis, preleukemia | 103 |

*MCV denotes mean corpuscular volume (in femtoliters).

A and B). Affected siblings within these families had anemias of comparable severity and similar spectrin deficiencies, whereas distantly related homozygotes from a large kindred centered in Bug Hill, North Carolina (Family A, generations VII and VIII) had anemias of different severities and correspondingly different spectrin levels, indicating that other factors may affect expression.

Presumed Carriers of Nondominant Spherocytosis

Patients without a positive family history tend to be seen frequently in referral settings. Both parents and all the offspring of 14 patients with spherocytosis were found to be clinically normal; all had normal hematocrits and peripheral-blood morphology, none had splenomegaly, and none reported a history of anemia or jaundice. Since it was considered likely that many of the patients with nondominant disease had parents who were silent carriers for a trait expressed only in homozygotes or double heterozygotes, careful analysis was undertaken to seek subtle manifestations of possible heterozygosity in the parents. Although no striking abnormalities were found, minor ones were usually noted (Fig. 3). Most of the parents had erythrocyte spectrin levels near the lower end of the normal range, indicating a minimal spectrin deficiency. Likewise, the median osmotic fragility of unincubated erythrocytes in both parents was consistently a small degree above that in the controls, indicating that there was a minimal reduction of membrane surface area relative to volume. Similarly, their reticulocyte counts were slightly above normal. Not one of these indexes is sufficiently convincing to permit clear identification of any suspected heterozygote. Nevertheless, the patterns of inheritance (Fig. 2) and these subtle abnormalities (Fig. 3) suggest that most of the parents carried mutations that affected heterozygotes only minimally.

Osmotic Fragility

Testing for osmotic fragility is a standard method for diagnosing spherocytosis; a rough correlation between increased sensitivity to hypotonic lysis and red-cell survival has been reported in several patients.¹⁵ In this study we have employed a tightly controlled, quantitative osmotic-fragility test to determine the precise degree of increased sensitivity to osmotic lysis.⁹ The increased median osmotic fragility was plotted against the erythrocyte spectrin content as determined by radioimmunoassay (Fig. 4, Panel A). The data obtained in all the patients with hereditary spherocytosis were clearly different from those in the controls and fit a computer-generated equation indicating a correlation between reduced membrane surface area and spectrin deficiency. The curve projects above the patient data to the normal median fragility ($X = 0$) at 97 percent of the normal erythrocyte level of spectrin, suggesting that persons with spectrin levels only slightly below normal will

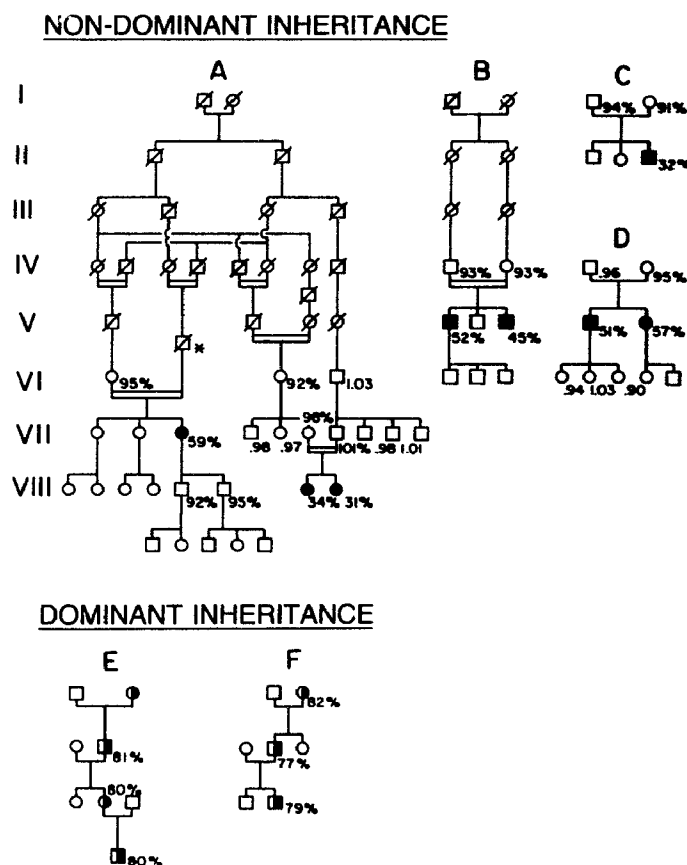


Figure 2. Selected Genealogies of Three Families with Spherocytosis.

Symbols indicate that family members were asymptomatic with normal clinical laboratory data (\square , \circ); were deceased, with no clinical laboratory data available (\square , \circ); were deceased, with normal clinical laboratory data and no history of anemia (\square , \circ); had the nondominant form of spherocytosis (\blacksquare , \bullet); or had the dominant form of spherocytosis (\blacksquare , \bullet). The symbols also indicate the percentage of normal spectrin content as determined by radioimmunoassay (e.g., \circ 92%), the normalized spectrin:band 3 ratio (e.g., \square .98), or the presence of a consanguineous marriage (\square — \circ).

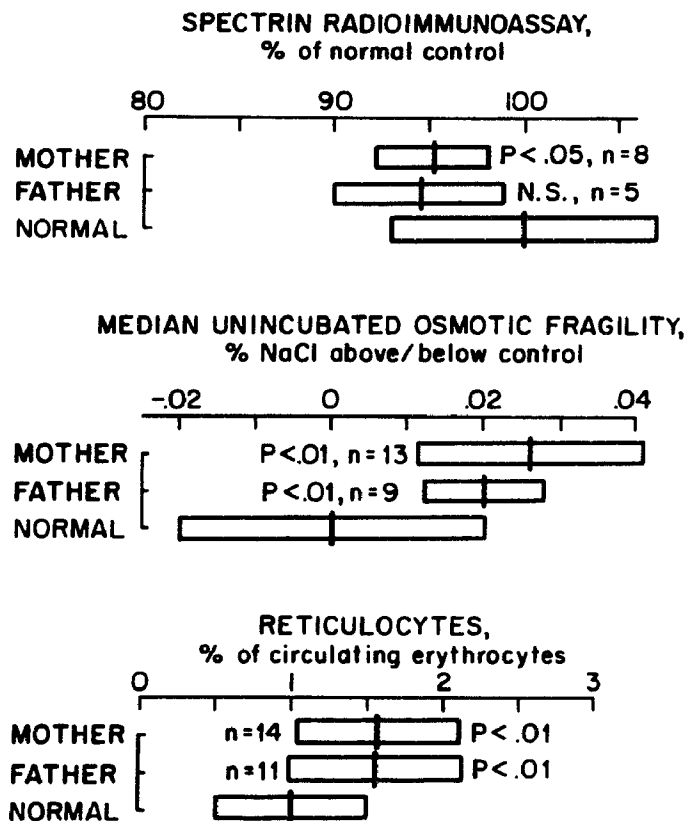


Figure 3. Subtle Erythrocyte Abnormalities in the Parents of Patients with Nondominant Spherocytosis. Erythrocyte spectrin levels, osmotic fragility, and reticulocyte counts were determined and compared with the normal range. The vertical bar indicates the mean, and the horizontal column ± 1 SD. N.S. denotes not significant.

spectrin levels. Fourteen of the 15 patients with spectrin levels more than 70 percent of normal had the dominant form of the disease; yet of 8 who had undergone splenectomy, only 2 had continuing hemolysis. Thirteen of 18 patients with spectrin levels less than 70 percent of normal had the nondominant form of the disease, and although most had evidence of hemolysis persisting after splenectomy, all were completely compensated except for those with spectrin levels under 40 percent. The clinical response to splenectomy was also related to the clinical course before the operation, and all 9 patients with spectrin levels less than 55 percent of normal required multiple transfusions before splenectomy. All 9 underwent splenectomy before the age of five, and 7 before the age of two. Thirteen of 15 patients with spectrin levels above 70 percent of normal either never received a transfusion before splenectomy or received only one, and only 3 underwent splenectomy before the age of 12.

have slight reductions in membrane surface area, as was noted for the presumed carriers of nondominant spherocytosis (Fig. 3).

Relation of Spectrin Levels and Responses to Splenectomy

Although all the patients in this study were described as being clinically improved after splenectomy, all did not achieve normal clinical laboratory values. The percentage of circulating reticulocytes in 23 patients was determined twice or more after splenectomy (Fig. 4, Panel B), and the erythrocyte spectrin level was inversely correlated with the average reticulocyte counts. Serum haptoglobin levels also reflect ongoing hemolysis, and spectrin levels were correlated with haptoglobin levels after splenectomy (Fig. 4, Panel C). However, the hematocrits of all the patients reached normal levels after splenectomy, except those of the six patients with the lowest spectrin levels, whose hematocrits rose only to 27 to 31 percent.

Erythrocyte spectrin content appears to be closely related to inheritance pattern and clinical outcome after splenectomy. Furthermore, there may be important thresholds of disease associated with certain

DISCUSSION

Although the rate of hemolysis has been related to the spectrin content of erythrocytes, the relations

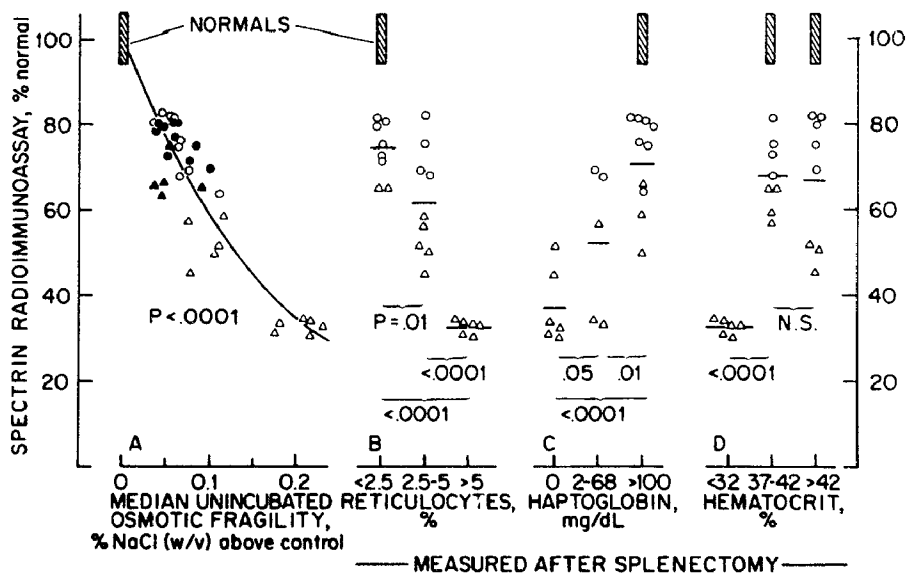


Figure 4. Correlation of Spectrin Level with Osmotic Fragility and Clinical Indexes after Splenectomy.

The hatched panels indicate the normal ranges for each index. Symbols are defined in the legend to Figure 1.

The osmotic fragility of unincubated erythrocytes was measured in patient and control samples (Panel A). The concentrations of sodium chloride producing 50 percent hemolysis (patient minus control) are plotted on the X axis. The curve represents a computer-generated fitting of the patient data to an exponential function ($y = ae^{bx}$, where $a = 96.7$ percent of normal spectrin content, $b = -5.10$, $n = 35$, and $r = -0.917$).

Panels B, C, and D show the percentage of circulating reticulocytes (on two occasions), the serum haptoglobin level, and the hematocrit as determined for each patient at least four months after splenectomy.

between hereditary spherocytosis and other clinical problems remain puzzling. For example, only a fraction of the patients whose erythrocytes had less than 50 percent of the normal amount of spectrin reported having had neonatal jaundice. Likewise, associations between the spectrin content of erythrocytes and the occurrence of gallstones or leg ulcers remain to be established.

This study provides little explanation for the mechanisms leading to the deficiency of spectrin. Immunoblots (not shown) failed to demonstrate the presence of proteolytic-degradation products of spectrin in any patients with spherocytosis. It must be considered likely that a variety of primary defects in coding, intervening, or promoter regions of either alpha- or beta-spectrin genes could produce variable degrees of spectrin deficiency, since the subunits depend on dimerization for stability. The development of probes for alpha- and beta-spectrin genes, as well as those for other skeletal proteins, may permit identification of the mutant gene in the larger kindreds through analysis of restriction-fragment polymorphism. The similarity between nondominant spherocytosis in humans and that in mice is striking. It is likely that some families, like the mice, may have anemias resulting from reduced synthesis or stability of alpha- or beta-spectrin or ankyrin.¹⁶ Interestingly, the beta subunit is limiting in avian biosynthesis of spectrin¹⁷; beta defects may result in a dominantly inherited anemia in humans, whereas heterozygotes for a defect in alpha synthesis may compensate almost entirely. Expression in the homozygotes, nonetheless, may also be influenced by other factors, since there was a great range of anemias and spectrin deficiencies in this group. Siblings with nondominant spherocytosis had comparable degrees of spectrin deficiency, but distantly related homozygotes had anemias of different severities and different degrees of spectrin deficiency (Fig. 2, Family A). Some families may be deficient in spectrin because of defective spectrin-protein 4.1 interactions (as described in one patient¹) or primary defects in peptides other than spectrin. As described previously,^{4,7} relatively smaller reductions in ankyrin and other peptides were frequently observed in blood with gross spectrin deficiencies, but these were more likely the result than the cause of spectrin deficiency.

Establishing the diagnosis of hereditary spherocytosis is not a simple matter when other family members are clinically normal or unavailable. The standard osmotic-fragility test as performed by clinical laboratories can usually identify patients with spherocytosis qualitatively. However, reduced erythrocyte volume, as in iron deficiency, will obscure the increased fragility,¹⁸ and osmotic-fragility profiles for autoimmune hemolytic anemias resemble those of hereditary spherocytosis (although the Coombs' test will readily distinguish the two). A more convenient form of the spectrin radioimmunoassay could be used in

clinical laboratories, and the advantages of such evaluations may be important. Not only could a firm diagnosis of hereditary spherocytosis be established, but important prognostic information concerning the eventual clinical outcome could be obtained, along with a clearer elucidation of the most likely mode of inheritance. Furthermore, since the indications for transfusion and the ideal age for splenectomy are not clearly defined, the spectrin radioimmunoassay may provide a useful guide to the identification of patients who may not respond completely to splenectomy, thus remaining at risk for folate deficiency or for life-threatening anemia after certain parvovirus injections.

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