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Elliptical erythrocyte membrane skeletons and heat-sensitive spectrin in hereditary elliptocytosis

(thermal denaturation/erythrocyte cytoskeleton/circular dichroism)

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ABSTRACT **Erythrocyte membranes (ghosts) and membrane skeletons (submembranous reticulums of spectrin, actin, and protein 4.1 prepared by extracting ghosts with Triton X-100) from 15 patients with hereditary elliptocytosis (HE) were elliptical, which indicates that the primary defect responsible for the abnormal shape of these cells resides in the skeleton. The protein composition of HE skeletons was normal, but in three kindreds purified spectrin heterodimer from 7/7 HE patients was heat sensitive and denatured at 48.0 ± 0.1°C instead of 49.0 ± 0.3°C ($P < 0.0005$). Heat sensitivity was detected by precipitation and, in the spectrin from one patient, by changes in circular dichroism. In one other kindred spectrin dimer from 3/3 patients denatured at the normal temperature. In two of the three kindreds with heat-sensitive spectrin, intact erythrocytes exhibited budding and fragmentation at the temperature at which spectrin denatured. In the third kindred spectrin was heat sensitive, but erythrocytes were not. The symptoms in the latter kindred were clinically more severe (hemolytic HE with spherocytosis) than in the other three (mild HE). We conclude that defects in the erythrocyte membrane skeleton may be a common feature of HE. As judged by heat denaturation of erythrocytes and purified spectrin dimer, three phenotypically distinct forms of HE exist, two of which are characterized by defective, heat-sensitive spectrin. It remains to be determined whether the molecular defect in spectrin responsible for heat sensitivity is the primary genetic defect responsible for HE.**

In hereditary elliptocytosis (HE) a variable number of the circulating erythrocytes are permanently deformed into elliptical or rodlike shapes. Two groups of investigators (1, 2) have reported that ghosts of elliptocytes are also elliptical, which indicates that a membrane defect is responsible for their abnormal shape. The nature of this defect is unknown, but logically it might involve spectrin, a membrane protein that is believed to be a major determinant of erythrocyte shape, deformability, and integrity (3).

Spectrin is a long, flexible fibrous protein (4) that is located on the cytoplasmic surface of the erythrocyte membrane (5). Recent studies show it normally associates with actin and protein 4.1* to form a submembrane protein meshwork (8–11) that is anchored to band 3, the anion exchange channel, by band 2.1 or ankyrin (12–16). This "membrane skeleton" can be isolated by extracting ghosts or intact erythrocytes with Triton X-100 (12, 17). Membrane skeletons of normal and irreversibly sickled erythrocytes retain the shape of the ghost from which they are prepared (12, 17, 18) and must be an important determinant of the shape of these cells. We wondered if the membrane skeletons of hereditary elliptocytes would also be abnormal and, if so, whether spectrin or one of the other skeletal protein components would be defective.

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MATERIALS AND METHODS

Blood obtained from normal individuals and 15 patients with HE (defined as having 20% or more elliptocytes and no hypochromia or macrocytosis on a peripheral blood smear) was collected in citrate/phosphate/dextrose solution (0.15 ml/ml of blood), stored at 4°C, and used within 72 hr. Ghosts and membrane skeletons were prepared and examined by light microscopy as described (18). Polyacrylamide gel electrophoresis in $NaDodSO_4$ was performed by the procedure of Fairbanks *et al.* (6) as modified by Steck (7).

Spectrin Purification. Spectrin dimer was extracted from ghosts in 0.1 mM EDTA at pH 8 (37°C, 15 min) and purified by chromatography on 4% agarose (Bio-Gel A-15m, 200–400 mesh, Bio-Rad) in 100 mM NaCl/10 mM Tris·HCl/0.1 mM EDTA, pH 8. Under these conditions 40–60% of the spectrin eluted in a fraction with a partition coefficient, K_{av}, of 0.5 and was homogeneous (>98% pure) by $NaDodSO_4$ gel electrophoresis. Normal and HE spectrin were equally extractable at 37°C and had indistinguishable chromatographic patterns in the 10 patients tested.

Effect of Temperature on Spectrin Solubility and Erythrocyte Shape. Purified spectrin heterodimer (1 ml, 0.15–0.30 mg/ml) was heated to various temperatures between 46°C and 54°C in quartz cuvettes inserted in Peltier thermoelectric cuvette holders attached to a digital controller (Perkin–Elmer Instruments, Norwalk, CT). This device rapidly adjusted the cuvette holders to the desired temperature and maintained them within ±0.05°C. One to 10 samples could be heated simultaneously. The empty cuvettes were equilibrated at each new temperature for exactly 5 min before the spectrin samples were added. Each sample was heated for exactly 10 min. Approximately 2.5 min was required for the samples to reach the desired temperatures. The heated samples were then cooled to 0°C and centrifuged at 100,000 × *g* for 60 min. Soluble spectrin was defined as the protein (measured by absorbance at 280 nm) remaining in the supernatant after centrifugation. With this technique repeated thermal denaturation studies on different spectrin preparations from the same individual differed by less than 15%.

Intact erythrocytes were washed in 10 vol of phosphate-buffered saline (143 mM NaCl/7 mM sodium phosphate, pH 7.4), diluted to a hematocrit of 2% in the same buffer and heated in parallel with their extracted spectrin. [The composition of the buffer seemed to be of some importance, because addition of albumin (0.5%) altered the effect of heat on erythrocyte shape.]

Abbreviation: HE, hereditary elliptocytosis.

* Nomenclature: We use the system proposed by Fairbanks *et al.* (6) and modified by Steck (7) for numbering the polypeptide bands on $NaDodSO_4$ gels of erythrocyte membranes.

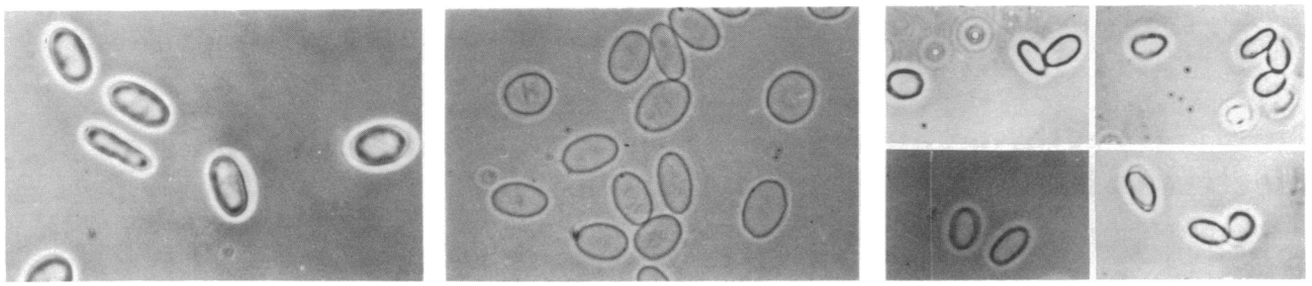

FIG. 1. Phase-contrast photomicrographs of HE erythrocytes (*Left*), membranes or "ghosts" (*Center*), and Triton X-100-extracted ghosts or "membrane skeletons" (*Right*). (×850.)

The heated erythrocytes were added to an equal volume of 2% (vol/vol) glutaraldehyde in phosphate-buffered saline and fixed at room temperature for 60 min, and the proportion (500 cells counted) of normal-appearing cells was determined by phase microscopy.

The mean denaturation temperature ($t_{1/2}$) was defined as the temperature at which half of the total observed change (between 46°C and 54°C) in spectrin solubility or erythrocyte shape had occurred.

Circular Dichroism. Purified normal and HE spectrin dimer in 100 mM NaCl/10 mM Tris·HCl/0.1 mM EDTA, pH 8, were adjusted to an absorbance of 1.035 at 220 nm and placed in 1-cm path length water-jacketed cuvettes. Temperature was adjusted by circulating heated water through the cuvettes and was measured by a digital thermometer equipped with a thermistor probe. The sample was exposed sequentially, for exactly 5 min, to a series of 14 temperatures between 42°C and 54°C and the change in ellipticity was monitored at 221 nm in a Cary model 61 circular dichroism instrument.

RESULTS

Morphological Studies: Elliptical Ghosts and Membrane Skeletons. In all 15 HE patients examined, many of the ghosts and membrane skeletons were elliptical (Fig. 1). Quantitation in five HE patients from four different kindreds showed that 91% ± 14% (SD) of hereditary elliptocytes formed elliptical ghosts and 97% ± 9% of the elliptical ghosts formed elliptical membrane skeletons. Because elliptical ghosts and membrane skeletons were rarely found in control preparations (<2%), and because we have previously shown that the Triton X-100 extraction procedure does not alter the intrinsic shape of membrane skeletons (18), we concluded that a defect in one or more of the membrane skeletal proteins must be responsible for the shape of hereditary elliptocytes.

Protein Composition of Ghosts and Membrane Skeletons. As judged by $NaDodSO_4$ gel electrophoresis, there was no difference between the protein compositions of normal and HE ghosts or membrane skeletons, except for a small increase in the amount of membrane-associated globin in HE ghosts (data not shown). The protein composition of the membrane skeletons was quite limited. The predominant components were spectrin, actin, and protein 4.1. Small amounts of protein 3 and, often, proteins 4.2 and 7 were also typically present. The remaining proteins were found in the Triton X-100 extracts.

Because the quantitative composition of HE membranes was normal, we searched for a qualitative defect in one of the membrane skeletal components, focusing first on the thermal sta-

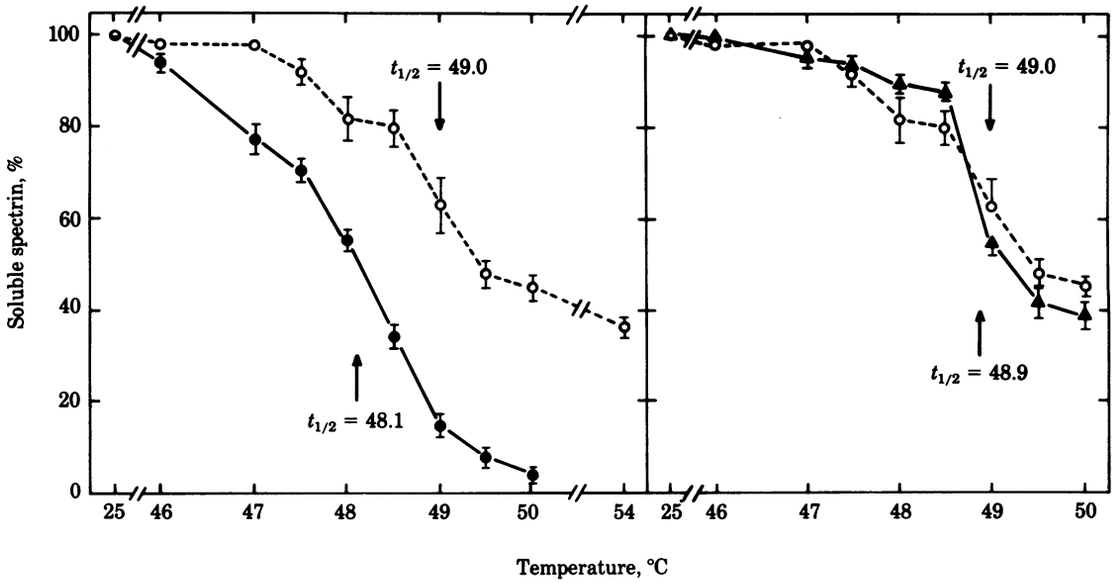

FIG. 2. Heat denaturation of HE spectrin heterodimer. (*Left*) Heat-sensitive spectrin: composite curves (mean ± SEM) of spectrin dimer from seven HE patients (●) (kindreds 1–3, Table 1) and six normal individuals (○). (*Right*) Heat-stable spectrin: composite curve of spectrin dimer from three HE patients (▲) (kindred 4, Table 1) compared to normal (○). In all experiments purified spectrin dimer was heated at various temperatures between 46°C and 54°C for exactly 10 min, cooled to 0°C, and centrifuged at 100,000 × *g* for 60 min. Soluble spectrin was defined as the percent of the original A_{280} remaining in solution. The numbers shown represent the $t_{1/2}$ for the change in spectrin solubility.

Table 1. Effects of heating on spectrin solubility and shape of normal and HE erythrocytes

Kindred	Patient	Spectrin $t_{1/2}$, °C	Heat-labile spectrin, %	Heat-stable spectrin, %	Erythrocytes $t_{1/2}$, °C
1*	A.S.	48.09	91	9	48.02
	R.S.	48.08	97	3	48.00
	D.S.	48.05	100	0	47.68
	Mean ± SEM	48.07 ± 0.01	96 ± 3	4 ± 3	47.90 ± 0.11
2*	N.H.	48.22	98	2	48.05
3†	M.Ja.	47.95	99	1	49.05
	L.Ja.	47.98	97	3	48.75
	R.Ja.	48.00	100	0	49.42
	Mean ± SEM	47.98 ± 0.02	99 ± 1	1 ± 1	49.07 ± 0.19
4*	A.Jo.	48.89	66	34	48.75
	E.Jo.	48.85	64	36	49.13
	J.Jo.	48.83	60	40	49.15
	Mean ± SEM	48.86 ± 0.02	63 ± 2	37 ± 2	49.01 ± 0.13
Normals	B.P.	48.60	65	35	48.85
	M.L.	48.65	66	34	48.95
	S.W.	48.88	66	34	49.07
	M.E.	49.12	70	30	49.22
	M.T.	49.15	65	35	48.70
	B.H.	49.33	60	40	48.83
	Mean ± SEM	48.96 ± 0.12	65 ± 1	35 ± 1	48.86 ± 0.08

* These kindreds have mild HE (3).
† This kindred has hemolytic HE with spherocytosis (3).

bility of spectrin, the major skeletal protein.

Heat Denaturation of Normal Spectrin Dimer. Pure normal spectrin heterodimer contained two apparent subcomponents (Fig. 2, left). About two-thirds precipitated (100,000 × *g*, 60 min) when spectrin was heated for 10 min to temperatures of 50–54°C. In six normal samples, the mean denaturation temperature ($t_{1/2}$) was 48.96 ± 0.12°C (SEM), and 65.3% ± 1.3% (SEM) of the spectrin was heat labile (Fig. 2, Table 1). The composite curve (Fig. 2) suggested that the normal spectrin samples might actually contain two different heat-labile components with apparent denaturation temperatures of 47.6°C (≈20% of the total sample) and 49.2°C (≈45%). However, this relatively subtle division was evident in only four of the six normal samples tested, and we are currently uncertain whether it is valid.

The molecular basis of the heterogeneous thermal denaturation of spectrin is unknown. $NaDodSO_4$ gels of the precipitable (heat-labile) and nonprecipitable (heat-stable) spectrin components showed only the usual spectrin pattern (data not shown).

Heat Denaturation of HE Spectrin Dimer. Thermal denaturation of purified spectrin dimer was abnormal in patients of some kindreds with hereditary elliptocytosis (Fig. 2 *Left* and Table 1). Three such kindreds were observed: two were black families with typical mild HE (3) (kindreds 1 and 2 in Table 1: <3% reticulocytes, no anemia, little poikilocytosis or spherocytosis) and one was a white family with typical hemolytic HE with spherocytosis (3) (kindred 3 in Table 1: 6–12% reticulocytes, mild anemia and splenomegaly, mild to moderate microspherocytosis and microelliptocytosis, and increased osmotic fragility). There was no discernible difference among the seven patients in these families in the heat stability of spectrin dimer. The mean denaturation temperature of 48.05 ± 0.03°C (SEM) (Fig. 2 *Left*) was significantly different from normal ($P < 0.0005$, *t* test). In addition, unlike normal, patients with heat-sensitive spectrin did not show a nonprecipitable subcomponent.

In one set of three experiments, thermal denaturation of spectrin dimer from patient A.S. (kindred 1, Table 1) was followed by circular dichroism instead of solubility (Fig. 3). A similar but somewhat smaller difference in heat stability was observed by this technique.

In contrast, patients from a third black kindred with mild HE had normal thermal denaturation of spectrin dimer (Fig. 2 *Right*; kindred 4 in Table 1).

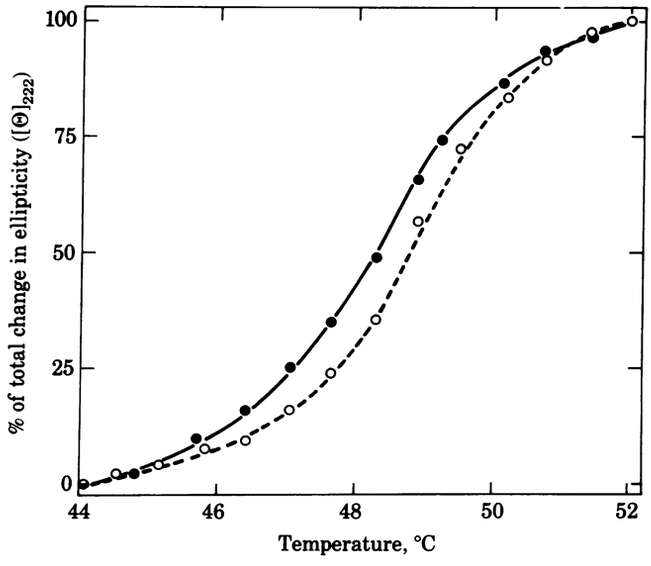

FIG. 3. Change in ellipticity at 221 nm ($[\Theta]_{221}$) of normal (○) and HE (●) (A.S., Table 1) spectrin dimer during heating. The total observed decrease in ellipticity on heating from 42°C to 54°C was 50.5% (normal) and 50.9% (HE). The results shown are representative of three experiments on spectrin from these two individuals.

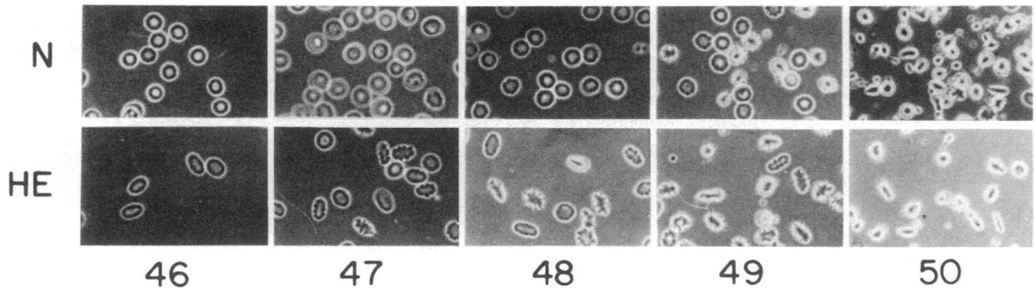

FIG. 4. Phase-contrast photomicrographs of normal (N) and HE (patient A.S., Table 1) erythrocytes after incubation at various temperatures for 10 min. Note that crenation and membrane budding are first evident in HE erythrocytes at 47–48°C but do not appear in normal erythrocytes until 49°C. (×300.)

Morphology of Heated Normal and HE Erythrocytes. It has been known for more than 100 years that erythrocytes heated to temperatures approaching 50°C become spiculated and fragment, leaving spherical remnants of various sizes (19). Similar changes were observed in the present study (Fig. 4). In general the change in erythrocyte shape correlated closely with the heat sensitivity of spectrin dimer (Table 1). Thus in most HE patients with heat-sensitive spectrin, erythrocytes began to crenate at 47°C and membrane budding was first evident at 47–48°C, whereas in normal cells such changes were not evident until 49°C (Fig. 4). In the family with hemolytic HE and spherocytosis, however, the changes in erythrocyte shape induced by heating fell within the normal range, even though isolated spectrin was heat sensitive (kindred 3, Table 1).

DISCUSSION

Our studies strongly suggest that HE is caused by a defect in the membrane skeleton. In all 15 patients examined, ghosts and membrane skeletons retained the elliptocytic shape. Because the composition of HE skeletons was normal, the abnormal shape could not be attributed to the lack of a normal component or the influence of an adsorbed one, and presumably it was due to malfunction of one of the usual skeletal proteins.

In three of the five kindreds examined, purified spectrin dimer was heat sensitive. This abnormality was present in all of the patients tested in these three kindreds and was not detected in normal individuals or in patients from one other HE family. HE patients with heat-sensitive spectrin must have a molecular abnormality of spectrin, but it is not clear whether this is is a primary sequence defect or an abnormality in a posttranslational process. The only known posttranslation modifications of spectrin are proteolysis (20), phosphorylation (21), and oxidation (22). No proteolysis was detected on $NaDodSO_4$ gels of unheated HE spectrin; however, the loss of a relatively small proteolytic fragment (<10,000 daltons) might go undetected in a protein this size. Phosphorylation of heat-sensitive spectrin, measured by the incorporation of $^{32}PO_4$ into intact erythrocytes (23), was also normal in one experiment (L. C. Wolfe, personal communication). The possibility that HE spectrin was abnormally oxidized was not tested. If spectrin heat sensitivity is due to a primary sequence defect, it presumably reflects the genetic defect responsible for the disease. Unfortunately, such a defect may be difficult to detect because spectrin is such an enormous molecule (≈4070 amino acids in the heterodimer) that the effects of a single amino acid substitution will be muted.

It might be argued that the observed change in spectrin heat denaturation cannot be directly linked to the primary genetic defect because it affects the entire population of spectrin molecules (Fig. 2 *Left*). HE is a dominant disorder (3) and, thus, only about 50% of the spectrin molecules should be genetically defective. However, because heat denaturation results in precipitation, it must be preceded by or associated with aggregation of spectrin molecules. If the aggregation involves normal spectrin dimers as well as those derived from the HE gene, a hybrid curve, lying between the heat denaturation curves of the spectrins produced by the normal and HE genes, would result. In one experiment such a curve was observed when a 1:1 mixture of HE and normal spectrin was heated (data not shown). However, further studies will be needed to prove this hypothesis.

It is important to note that the presence or absence of heat-sensitive spectrin does not appear to correlate with the degree of elliptocytosis or hemolysis. This is not surprising, because instability of spectrin at 48°C (but not at 37°C) obviously does not contribute directly to the pathogenesis of HE; it simply indicates diminished conformational stability of the molecule. Presumably, in kindreds with hemolysis and heat sensitivity, the conformational change also affects sensitive functional regions of spectrin such as the actin (8), protein 4.1 (10), ankyrin (10), or spectrin (i.e., dimer–dimer) (24) binding sites, which are not affected (or are affected to a lesser degree) in nonhemolytic variants. The recent development of assays for each of these functional interactions (8, 10, 13) should allow this hypothesis to be tested directly in the near future.

The observation that heat-sensitive spectrin can be detected by heating intact erythrocytes as well as purified spectrin dimer is interesting for two reasons. First, it offers a simple test for detecting the spectrin defect and differentiating it from similar, but more severe, defects, such as those observed in hereditary pyropoikilocytosis (25) or in some neonates with hereditary elliptocytosis (26). Second, it provides additional proof that spectrin function is important for membrane structural stability (3, 27). However, as shown by kindred 3 (Table 1), the correspondence between the heat sensitivity of erythrocytes and their isolated spectrin is not exact. In this kindred, either association of spectrin with its membrane protein neighbors compensates for heat lability, or the thermal change leading to heat precipitation does not involve the regions of the molecule that function to prevent membrane fragmentation. In either case, one can infer that the spectrin defect in this kindred differs from that observed in kindreds 1 and 2, a conclusion that is also evident clinically.

Thus three phenotypes can be identified on the basis of the heat denaturation of both spectrin and erythrocytes: heat-sensitive spectrin and heat-sensitive erythrocytes (kindreds 1 and 2), heat-sensitive spectrin and heat-stable erythrocytes (kindred 3), and heat-stable spectrin and erythrocytes (kindred 4). Previous genetic studies have shown that at least two forms of HE exist: a form in which the elliptocytosis gene (El_1) is linked to

the Rh locus on chromosome 1 (28, 29) and a form(s) in which it is not. Unfortunately Rh blood groups were not determined in the present study, so it is not known which, if any, of the phenotypes described above correspond to the El_1 genotype.

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