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Research Article

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We conclude that membrane ("structure") proteins of HS red cells are genetically altered in such a way as to interfere with their proper conformation, perhaps into fibrils. Potentially many mutations in membrane proteins might preclude this alignment, with the result that normal erythrocyte biconcavity and plasticity is prevented [...]

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Abnormal Membrane Protein of Red Blood Cells in Hereditary Spherocytosis

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ABSTRACT We present evidence that the hereditary hemolytic disease, hereditary spherocytosis (HS), involves an abnormality in protein of the red cell membrane. Unlike that from normal red cells, lipid-free proteins extracted from HS red cell membranes fail to increase in sedimentation rate when treated with cations; such treatment of normal membrane proteins has been shown by others to cause the formation of microfilaments. That microfilament formation might be defective in HS red cell membranes is supported by observations with vinblastine. This compound, a potent precipitant of filamentous, structure proteins throughout phylogeny, precipitates significantly less HS membrane protein than normal. The resistance of HS membrane protein to changes in conformation by cations is observable at the cellular level as well. That is, both normal and HS red cells agglutinate after repeated washing and suspension in electrolyte-free media. Tiny concentrations of Ca^{++} (5×10^{-6} M) changes the surfaces of normal cells in such a way as to cause disagglutination; HS red cells resist this change and remain agglutinated unless Ca^{++} concentrations are increased many-fold.

We conclude that membrane ("structure") proteins of HS red cells are genetically altered in such a way as to interfere with their proper conformation, perhaps into fibrils. Potentially many mutations in membrane proteins might preclude this alignment, with the result that normal erythrocyte biconcavity and plasticity is prevented and the clinical syndrome of hereditary spherocytosis is manifest.

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INTRODUCTION

Recently summarized evidence (1) suggests that an abnormality in the red cell membrane underlies hereditary spherocytosis (HS).¹ This evidence includes: (a) red cell membranes in this disease leak sodium at excessive rates (2, 3); (b) "buds" of membrane form prematurely and fragment off the HS red cell during incubation, thereby enhancing spherocytosis (4, 5); (c) HS red cell ghosts are intrinsically stiffer than normal, resisting passage into glass microcapillary tubes (6); and (d) these membranes have a unique blistered appearance when view with surface scanning electron microscopy (7). The autosomal dominant inheritance of HS makes it unlikely that a deficiency of a metabolic enzyme, which is generally a recessively inherited phenomenon, is causative, although claims (8) and refutations (9) of such a mechanism have been made. Instead, search for a mutation in the structural lipid or protein elements of the red cell membrane would seem worthwhile.

Numerous studies of membrane lipids of HS red cells have documented minor abnormalities, most possibly related to the increased glycolytic rates and cation fluxes of hereditary spherocytes (10). Until recently, investigations of red cell membrane protein have been hampered, since methods for extracting this protein have generally been highly denaturative. With such procedures, multiple protein bands are found by gel electrophoresis, and conflicting reports as to differences (11) and identity (12) of HS and normal membrane preparations have appeared. Recently Marchesi and coworkers, using a gentle dialysis technique, have extracted a homogeneous protein component from red cell membranes (13). This material, termed "spectrin," aligns

¹ *Abbreviations used in this paper:* HS, hereditary spherocytosis; PMB, paramercuribenzoate.

into long microfilaments when treated with divalent cations and ATP, behavior reminiscent of actin after its analogous extraction from skeletal muscle.

Although further work (14) indicates that actin and spectrin are not identical, the possibility that a fibrillar protein occurs in red cell membranes is of interest in suggesting a mechanism for the spheroidicity and rigidity of hereditary spherocytes. Strong evidence has been accumulating that the shape and motility (or "contractility") of cells generally derive from structural elements such as microtubules and microfilaments (15). The molecular characteristics of these morphological elements have been examined and allow a useful hypothesis that structure proteins are a class of similar, though not identical, molecules (16). There are indications that the microfilamentous proteins that may underlie cell shape share important properties—such as the binding of myosin and the precipitation by vinblastine—with molecules responsible for motility, such as actin filaments of muscle and the microfilaments involved in cytoplasmic streaming (17, 18). The "actin-like" properties of membrane protein of red cells ("spectrin") and the vinblastine precipitability of red cell membrane protein to be described in the present studies, suggest that the proteins of membranes are part of this class of structure protein. If so, the inherited shape abnormality of the HS erythrocyte might result from an abnormality of the structure proteins of its membrane. The present studies, by utilizing guidelines worked out in the investigation of structure proteins from other tissues, substantiate this view. The results have been reported in preliminary form elsewhere (19, 20).

METHODS

Patients. Red cells were extracted from 12 patients representing seven families with typical HS. Four had undergone splenectomy in the remote past and four were studied before and after splenectomy. The results were unaffected by the splenectomy status of the patients. Normal volunteer blood was extracted and analyzed in parallel. In addition, the following other abnormally shaped red cells were also extracted: (a) spherocytes from a patient with severe Coombs' positive hemolytic anemia; (b) burr cells from a uremic patient; and (c) macroreticulocytes from a patient in early remission of folic acid deficiency.

Membrane protein extraction. Lipid-free protein in yield of 70–80% was extracted from red cell membranes by our previously described technique (21). As previously demonstrated no lipid (measured as phospholipid or cholesterol) is present in the water-soluble protein extract using this technique. No difference in yields from normal and HS red cells were detected. Briefly, red cells from 60–75 ml of heparinized blood were washed three to four times with isotonic (0.3 M) sucrose, previously deionized with a mixed cation-anion exchange resin.^a After three washes in which buffy coats were removed, ghosts were prepared by rapidly lysing

the washed red cells with 0.2% Triton X-100^a in the presence of a large excess of the ion-exchange resin. White ghosts were obtained by washing three times in 0.25 M deionized sucrose, adjusted first to pH 6.8 and finally to pH 7.5 with micromolar amounts of ammonium hydroxide. As previously demonstrated, this technique, which depends upon ghosts being prepared in extremely low ionic strength media, permits most of the membrane protein to be solubilized in alkaline media. The material solubilized from these ghosts with dialysis against water adjusted to pH 9.5 with ammonia vapor was ultracentrifuged at 160,000 *g*. Lipid-free protein is found in the supernatant solution; the precipitate comprises lipid-rich tiny vesicles. The amino acid composition, sedimentation characteristics, and other properties of this extracted protein have been described (21). Of interest is the relatively slow sedimentation of the protein obtained in these low ionic-strength preparations (approx. 2.5S) as compared with values reported (22) using other extraction techniques (approx. 5–10S). The protein solutions, at the same pH (8.5–9.0) and concentration (2.0 mg/ml), were ultracentrifuged,⁴ their schlieren patterns analyzed every 16 min and approximate *S*₂₀ values calculated as previously described (21). When appropriate, cations such as K⁺ and Ca⁺⁺, as the chloride salts, were added in 1/10 final-volume portions.

Agglutination-disagglutination studies. Red cells reversibly agglutinate in low ionic strength media. Thus, after two to three washes in resin-treated isotonic sucrose, both normal and HS red cells agglutinate and fall to the bottom of their centrifuge tubes. To 1 volume of a roughly 10% suspension of agglutinated red cells was added 1/10 volume of varying concentrations of CaCl₂ and disagglutination evaluated after 3 min. Observations of disagglutination was simplified by placing the calcium-treated suspensions in glass microhematocrit capillary tubes, wherein agglutinated cells rapidly settled out. Magnesium at similar concentration could replace calcium, whereas with potassium and sodium an approximately five-fold increase in concentration was required to disagglutinate red cells.

Vinblastine precipitation of protein extracts. 1 volume of protein extract, adjusted to 1 mg/ml, was added to 1 volume of 5 mM TES buffer, pH 9.0. 1/5 volume 5 mM CaCl₂ and 1/5 volume 5 mM ATP were added⁵ followed after 5 min at 37°C by 1/5 volume of vinblastine,⁶ 10 mg/ml. The resulting white precipitate was centrifuged at 2000 *g*, washed once with H₂O, dried, and analyzed for nitrogen by the method of Schiffman, Kabat, and Thompson (23). The precipitate completely disappeared at 4°C and reappeared at 37°C, behavior reminiscent of microtubules viewed in various intact cells (24).

^a Calbiochem, Los Angeles, Calif.

⁴ Spinco model E.

⁵ Ancillary studies revealed 75–85% less vinblastine precipitation in the absence of Ca⁺⁺. Little if any additional effect of ATP, even in doses three times higher, could be demonstrated. The use of vinblastine and Ca⁺⁺ is predicated on their ability to facilitate filament formation of structure proteins. They have been shown to do so either individually or additively (27). Since the dose-response curve of precipitation of structure protein by vinblastine or calcium is sigmoidal in shape, doses of these compounds were chosen which were well below maximal, so that differences between normal and HS extracts could be detected.

⁶ Available as crystalline Velban, Eli Lilly and Co., Indianapolis, Ind.

^a AG 501-X8 Bio-Rad Labs, Richmond, Calif.

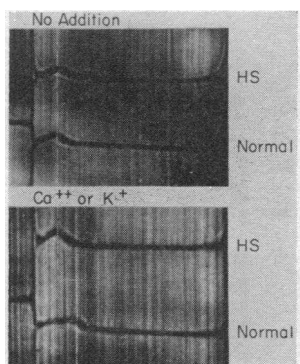


FIGURE 1 Failure of cations to aggregate HS membrane protein. At virtually zero ionic strength (top) both HS and normal membrane protein have sedimentation rates of roughly 2.5S. Increasing ionic strength with Ca^{++} , or Mg^{++} (1 mM) or by Na^+ or K^+ (20 mM) (bottom) increases sedimentation rate and heterogeneity of normal, but not HS protein. Ultracentrifuge patterns: speed 59,780 rpm; 32 min at speed; temperature 19.8°C ; protein concentrations 2 mg/ml; pH = 9.0.

RESULTS

In low ionic strength media, soluble membrane proteins from both HS and normal red cells sediment virtually identically in the analytical ultracentrifuge (upper, Fig. 1). S_{20} values of roughly 2.5 are usual. As previously reported (21), increasing ionic strength by adding

cations such as potassium or calcium, increases the sedimentation rate of protein extracted from normal red cells (bottom-most tracing, Fig. 1). That is, the protein peak becomes more diffuse and travels more rapidly toward the bottom of the centrifuge tube, toward the right in Fig. 1. In contrast, membrane protein from hereditary spherocytes resists this change, the protein peak maintaining its shape and migration velocity (next to the bottom tracing, Fig. 1).

The sedimentation characteristics of proteins from red cells of 12 HS patients and a similar number of controls is summarized in Fig. 2. The sedimentation coefficient of normal membrane protein (left, Fig. 2) increases from roughly 2.5S in cation-free media to approximately 6S when potassium or other cations (not shown) are added. This conformational alteration is markedly deficient in membrane protein extracted from all HS patients (right portion, Fig. 2). The difference in response of proteins from the two cell types is highly significant ($P < 0.001$). No significant difference from normal was observed in the sedimentation characteristics or extractability of membrane protein from patients with (a) macrocytic reticulocytosis, (b) spherocytosis secondary to Coombs' positive hemolytic anemia, or (c) burr cells of severe uremia.

Further evidence of aberrant behavior of HS membrane protein was gathered using vinblastine. This

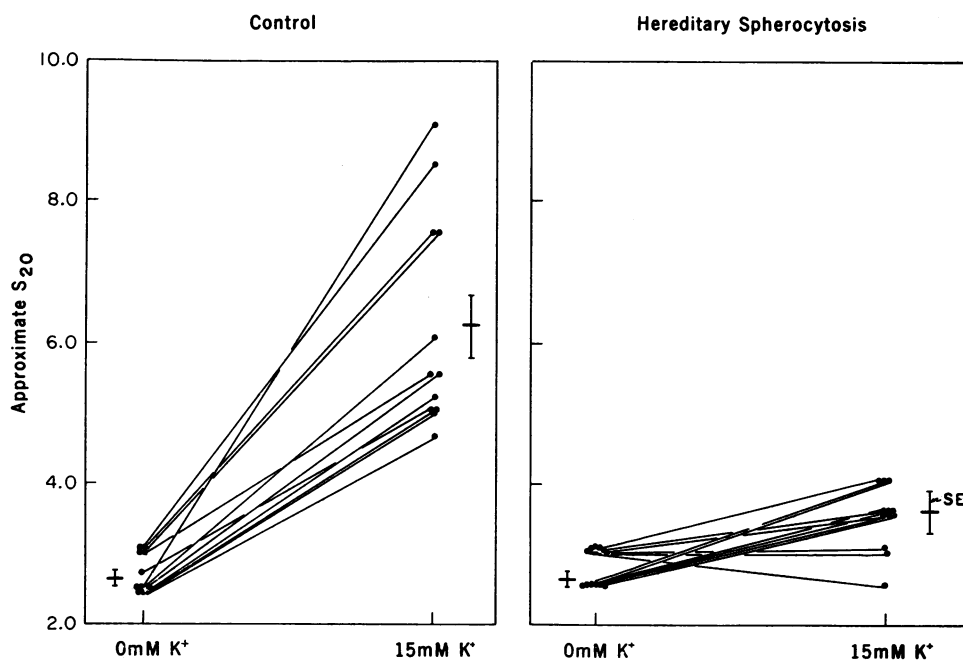


FIGURE 2 Summary of sedimentation changes induced by K^+ . Sedimentation rates of protein extracted from normal (left) but not from HS (right) red cell membranes increased strikingly with addition of 15 mM K^+ . The difference between red cell types is highly significant ($P < 0.001$).

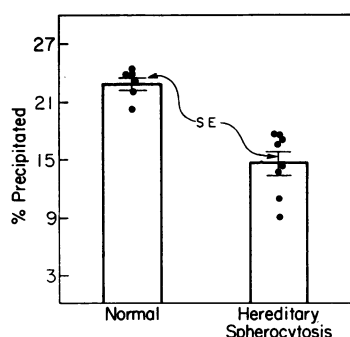


FIGURE 3 Vinblastine precipitability of RBC membrane protein. A significantly less ($P < 0.01$) amount of membrane protein derived from HS red cells (right) was precipitated by vinblastine than from normal cells (left). Conditions were identical in all experiments and are described in the text.

compound is a potent precipitant of microfilamentous proteins and as such has been used to separate such proteins from various tissues (25, 26). Its ability to precipitate the microtubular protein of mitotic spindles underlies its use as an antimetabolic, chemotherapeutic agent; pertinent to the present studies it precipitates actin (27) and, as shown in Fig. 3, red cell membrane protein. A significantly lower proportion of HS membrane protein (right, Fig. 3) is rendered insoluble by vinblastine than normal protein (left, Fig. 3).

The resistance of membrane protein from HS red cells to undergo alteration (sedimentation acceleration) in the presence of cations, is reflected by a similar resistance of intact HS cells to undergo changes in surface characteristics with added cations. Both normal and spherocytic red cells agglutinate and fall to the bottom of glass capillary tubes when repeatedly washed in cation-free isotonic sucrose (left, Fig. 4). Tiny amounts of calcium disagglutinate normal cells (right tube, middle portion, Fig. 4), whereas no disagglutination of spherocytes occur even with a 40-fold increase in calcium concentration (right, Fig. 4). With a 100-fold increment, or 5 mM concentration, calcium did disagglutinate these cells (not shown). Magnesium, potassium, or sodium, could replace calcium in these studies with similar results. The differences in cation-induced disagglutination of HS and normal red cells persisted even when the cells were washed free of plasma protein by use of buffered saline (pH 7.4) before the sucrose washings. Evidence that agglutination of red cells in low ionic strength may at times reflect adsorbed gamma globulin (34) would, thus, not seem germane to the differences portrayed in Fig. 4. The marked resistance of HS cells to disagglutinate was not shared by heat-induced (50°C) spherocytes, spur cells from a pa-

tient with terminal hepatic cirrhosis, uremic burr cells, or hereditary acanthocytes.

DISCUSSION

The present studies demonstrate an abnormality in a component of the membrane proteins extracted from hereditary spherocytes. The rate of sedimentation of solubilized membrane proteins from normal red cells is markedly accelerated when ionic strength is increased. Proteins from HS red cell membranes resist this change in sedimentation velocity. Although these studies allow no firm conclusion as to the relation of increased sedimentability of membrane proteins to normal biconcavity and plasticity of red cells, it is tempting to speculate that a relationship does exist. Aggregation into microfilaments has been shown to occur when normal red cell membrane protein is exposed to divalent cations (13). The geometry of filaments would seem well suited to provide plasticity to the red cell. Thus, individual fibers, by undergoing hinge-like shortening (or pleating), might produce one type of shape flexibility, while individual fibrils by sliding, one over the other, might provide yet another. If so, defective microfilament formation in HS red cell membranes might underlie their abnormal shape and increased rigidity. It is acknowledged that our studies show only a diminished propensity of HS membrane proteins to increase in sedimentation rate with added cations and not necessarily defective microfilament formation. However, the decreased precipitation of HS membrane proteins by added vinblastine (Fig. 3), a widely used, and possibly specific (25-27), precipitant of filamentous structure proteins, supports, but in no way proves, that defective microfilament formation is involved. That is, we suggest that the alignment of small subunits into fibrils probably underlies the cation-induced increment in sedimentation of normal membrane protein; this alignment

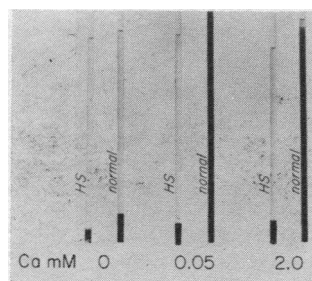


FIGURE 4 Failure of Ca^{++} to disagglutinate hereditary spherocytes. Red cells agglutinate in electrolyte-free (sucrose) media and fall to the bottom of capillary tubes (left pair). Addition of tiny concentrations of Ca^{++} disagglutinate normal, but not HS red cells (middle pair). Even with 40-fold increases in calcium concentration this dichotomy persists (right pair).

would seem to be retarded in HS.⁷ Regardless of geometry, we should like to speculate that an abnormality in membrane protein structure might also underlie the excessive agglutinability of HS red cells in low ionic strength media (Fig. 4). The propensity of normal membrane proteins to undergo physical change (increased sedimentation rate) with added cations correlates with the ease of disagglutination of normal cells with added low concentrations of cations; conversely HS membrane proteins resist change with added cations, and concomitantly HS red cell disagglutinate only with difficulty, requiring much higher cation concentrations.

It is unclear whether one, or a mixture of, protein molecules are responsible for the differences demonstrated in the present studies. Our protein solutions are heterogeneous when examined by ultracentrifugation and acrylamide gel electrophoresis. In addition, at least two immunodiffusion bands are detected with antisera prepared in rabbits against whole protein extracts.⁸ The filamentous protein, spectrin, is present in our preparations (22); however, whether the aberrant protein in the present studies is spectrin, alone or in combination, or not at all, is not presently known. The possibility also exists that our extraction procedure fails to extract the protein moiety from HS cells which is responsible for the cation-alterable sedimentation characteristics of normal extracts. However, since the over-all yield of proteins from normal and HS red cell membranes was not consistently different, this explanation seems unlikely.

The mechanism by which membrane proteins increase in sedimentability is currently under investigation. The possibility that disulfide bonds form between thiols of protein subunits is attractive for many reasons. Relevant to structure proteins generally, this mechanism has been suggested for the analogous, cation-induced aggregation of myosin subunits (29). In addition, we and others have previously shown that red cells in which membrane thiol groups are blocked by paramercuribenzoate (PMB) mimic hereditary spherocytes in nu-

merous ways; for instance, in hyperpermeability to sodium (30), increased rigidity (31), proclivity to fragmentation (5), increased osmotic fragility (30), microspherocytic shape (5), and specific entrapment by the spleen when reinjected into the human circulation (32). In studies to be presented subsequently, protein extracted from membranes of such PMB-treated red cells has been found to resist cation-induced increases in sedimentation.

A different, but possibly related, type of cation-induced conformational change in red cell membranes has been recently described (33). A "sol-gel" transformation in red cell membranes produces ghost rigidity when intracellular calcium is accumulated. This phenomenon differs from the alteration of membrane protein induced by calcium (and other ions) described in the present studies. That is, numerous cations including Mg^{++} increase sedimentation rates of our membrane proteins, whereas calcium alone has been reported specifically related to the membrane rigidification process; in fact, Mg^{++} inhibits this change (33). It is of interest that calcium concentrations higher than those used in our aggregation studies (above 10 mM) will cause gross precipitation of our membrane protein preparations (27). Perhaps an earlier stage of this "denaturation" is the analogue of the Ca^{++} -induced rigidification noted with whole membranes.

We conclude that some component of extracted HS membrane proteins is genetically altered in such a way as to inhibit its attaining a proper conformation. We speculate, but cannot as yet prove, that such a conformation might be fibrillar and that it presumably underlies normal erythrocyte biconcavity and plasticity. It seems likely that potentially many mutations might preclude whatever this appropriate alignment might be. In fact in ultracentrifuge studies preliminarily reported (20), at least two different genotypes have been found, perhaps explaining the variable severity of this disease in different families.

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⁷ Although increased sedimentation rates of proteins usually signify aggregation of subunits into higher molecular weight structures, recent work with synthetic biopolymers suggests other interpretations as well (28). Thus, especially at the low ionic strength used in the present studies, addition of cations may increase sedimentability by dampening the repulsive charges of molecules which tend to inhibit sedimentation of molecules at near-zero ionic strength. Regardless of interpretation, a fundamental abnormality in protein structure in HS red cell membranes must underlie its aberrant behavior in the ultracentrifuge. That aggregation of red cell membrane protein does indeed occur, at least at higher ionic strength, is documented by the frank precipitation of such protein induced by calcium concentrations in excess of 10 mM (27).

⁸ Kaplan, M. E., and H. S. Jacob. Unpublished results.

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