Mechanical Properties of Sickle Cell Membranes

By Richard Messmann, Susan Gannon, Sharada Sarnaik, and Robert M. Johnson

The mechanical properties of sickle erythrocyte membranes were evaluated in the ektacytometer. When ghosts from the total red blood cell population were examined, the rigidity of the resealed ghosts and their rate of fragmentation by shear stress (t₁/₂) were normal. However, fractionation on Starch density gradients revealed that sickle cells were heterogeneous in their membrane mechanical properties. The ghosts from dense cell fractions exhibited both increased rigidity and decreased stability. Presumably, these altered mechanical properties are a reflection of the well-documented biochemical damage found in irreversibly sickle cell membranes. Nevertheless, neither of the alterations in mechanical properties is likely to be a significant element in the hemolysis of sickle cell anemia.

There are two distinct clinical features of sickle cell (SS) disease. It is unique among the anemias in exhibiting a high incidence of vaso-occlusive events, which are attributed to intravascular sickling or to red blood cell (RBC) adherence to the endothelium. In addition, hemolysis in SS disease can be extremely rapid, leading to a relatively severe anemia. There are numerous reports of biochemical changes in sickle cell membrane proteins and lipids that may be related to oxidative events. It is a plausible hypothesis that the secondary biochemical alterations to membrane structural proteins would increase the hemolytic rate. For example, it is well known that membranes with genetically altered structural proteins are often mechanically fragile, and this is associated with hemolytic anemia. In order to evaluate the possible role of membrane instability in the hemolytic process in SS disease, we have directly examined the mechanical fragility of the sickle cell membrane.

Materials and Methods

Assays of membrane mechanical properties were done in the ektacytometer (Technicon Corp, Tarrytown, NY), an instrument for the measurement of the response of a population of RBCs or RBC membranes to shear. The basic configuration developed by Bessis and Mohandas is composed of a Couette viscometer with two concentric cylinders constructed of an optically clear material. The cells or membranes are suspended in a viscous medium and introduced into a 0.5 mm gap between the cylinders. After cylinder rotation begins, the cells or ghosts elongate under stress and orient themselves with their long axis normal to the rotational axis. Elongation is monitored by the diffraction of a laser beam directed normally to the cylinder axis. Information about the stress response is derived from the laser diffraction pattern, which is circular when the cells or ghosts are undeformed but becomes elliptical as they elongate under stress. An automated signal analyzer measures the length (L) and width (W) of the first diffraction ring and calculates the ellipticity index (EI): EI = (L - W)/(L + W).

Resealed ghost preparation. Careful attention to the conditions of lysis and resealing were necessary to obtain reproducible results. In addition, the viscosity of all solutions used in the ektacytometer was measured before use and carefully adjusted to the values given in the procedures.

Erythrocytes were washed twice in 0.9% NaCl to remove of the buffy coat. After each wash, the cells were collected by centrifugation (3,000 rpm for 2 minutes). The cells were cooled to 4°C, and lysed in 30 volumes of ice cold 5 mmol/L NaPi, pH 8.0, 1 mmol/L EDTA, 20 μg/mL phenylmethylsulfonyl fluoride. After 5 minutes at 0°C, the ghosts were collected by centrifugation at 15,000 rpm for 10 minutes in the Sorvall SS34 rotor (Dupont Co, Wilmington, DE). The supernatant and the tightly packed pink button at the bottom of the centrifuge tube were removed by aspiration. The pink membranes were then resealed by the addition of 5 volumes of cold PBS followed by incubation for 1 hour at 37°C. The resealed ghosts were pelleted at 18,000 rpm for 10 minutes, and the supernatant was aspirated.

Mechanical fragmentation of ghosts. The general procedures of Mohandas et al and Mentzer et al were followed with minor modifications. The ghost fragmentation buffer was made by dissolving dextran T40 (Pharmacia, Uppsala, Sweden) in a buffer containing 6.34 mmol/L Na₃H₂PO₄, 2.0 mmol/L NaH₂PO₄, 0.04% NaN₃, 1 mmol/L EDTA, adjusted to 290 mOsM with NaCl and to pH 7.4 ± 0.05 with dilute NaOH. The dextran has two functions in this buffer: it increases the viscosity and, hence, the shear stress to a level sufficient to fragment the ghosts, and it raises the refractive index of the medium. The ghosts are resealed in PBS, and the resulting refractive index difference between the ghost interior and the medium generates the diffraction pattern. Since the viscosity is an exponential function of dextran concentration at these high levels, slight variations in solution preparation can cause significant variations in viscosity and, correspondingly, in the fragmentation time. Solution viscosities were measured in a Cannon-Fenske capillary viscometer, size 300 (Fisher Scientific, Pittsburgh, PA). In early work, dextran was used at a concentration of 36%. In later removal of the dextran.

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experiments, the dextran concentration was reduced so as to increase the sensitivity of the assay. The lower viscosity increases the fragmentation times. To a lesser degree, temperature also affects solution viscosity. The instrument is not thermostated, but room temperature did not vary by more than a few degrees. For the run, 150 \( \mu \text{L} \) of packed resealed membranes were resuspended in 7 mL of fragmentation buffer and loaded into the ektacytometer with the rotational speed at zero. The cylinder rotational speed was brought to 150 rpm and a time sweep was begun simultaneously. Under these conditions, the applied shear stress causes mechanical disruption of the ghosts into smaller vesicles with the same biochemical composition as the original ghost. The index rises initially and then declines as the RBC ghosts fragment (Fig 1A). The \( E_1 \) was recorded until it declined to a constant final value. The value of the initial index is termed \( E_{10} \), and the half time, \( t_{1/2} \), is defined as the time in seconds required for the index to decline to the midway point between the initial and final values.

**Determination of ghost rigidity.** Resealed ghosts (150 \( \mu \text{L} \)) were suspended in 7.0 mL of fragmentation buffer and loaded into the nonrotating viscometer. The index was then recorded as the rotational speed and, the shear stress gradually increased (ramp-up assay, Fig 1B). The index reaches a plateau value, \( E_{1\text{max}} \), which reflects both ghost deformability and surface area.\(^{8,10}\) Ghost deformability independent of area is determined from a plot of \( E_1 \) against the log of the shear stress.\(^{8,10}\)

**Relationship between \( E_1 \) and \( E_{1\text{max}} \).** We also found that the initial value of the index in a fragmentation assay, \( E_{10} \), has the same numerical value as the \( E_{1\text{max}} \), the plateau value of a ramp-up assay. This was shown by determining both values for a number of ghost preparations (Table 1). This finding is reasonable, given the magnitude of the shear stress applied in the fragmentation assay. \( E_{1\text{max}} \) is sensitive to both ghost surface area and deformability.\(^{11,12}\) Therefore, the numerical identity of \( E_{10} \) and \( E_{1\text{max}} \) allowed us to use the results of the ghost fragmentation assay to determine if either of these factors was altered in the ghost preparation. In those instances where \( E_{10} \) was low, the ghosts were further analyzed in the ramp-up assay, which is not sensitive to surface area,\(^9\) to distinguish the two possibilities.

**Fractionation of dense RBCs.** Washed, cellulose-filtered\(^{11} \) erythrocytes were separated on discontinuous Stractan gradients as described by Corash et al\(^{12} \) and Clark et al\(^{13} \). A concentrated solution of Stractan (St. Regis Paper Co, Tacoma, WA) was extensively
dialyzed against distilled water to remove electrolytes and small molecules, and lyophilized. The resulting powder was dissolved in 5 mmol/L sodium phosphate, 1 mmol/L EDTA buffer, pH 7.4, at concentrations (and densities) of 24% (1.096), 25% (1.100), 26% (1.102), and 35% (1.139). The osmolality of each solution was adjusted to 290 mOsm with NaCl. A discontinuous gradient with 2 mL layers of each solution was then pipetted into clear SW41 centrifuge tubes. Control and sickle erythrocytes were washed twice and brought to a hematocrit of approximately 30 in PBS with 0.2% glucose. The washed cells were then carefully layered on top of the gradients and centrifuged for 120 minutes at 25,000 rpm in a Beckman SW41 rotor (Beckman, Spinco Division, Palo Alto, CA). An attempt was made to collect four distinct layers of sickle erythrocytes, but occasionally layers had to be pooled. Fractionated cells were washed three times in PBS to remove Stractan. Packed cell volume was measured after centrifugation to a constant volume in a microhematocrit tube. Mean corpuscular hemoglobin concentration (MCHC) was determined for each layer from the hematocrit and a hemoglobin determination was made with Drabkin’s reagent. An aliquot was preserved in 1% glutaraldehyde for Coulter and irreversible sickle cell (ISC) counts.

Ektacytometry of membranes from fractionated sickle cells. Resealed ghosts were prepared from these dense RBC’s as described previously, except that the heavy red pellet found at the bottom of the sedimented lysed ghosts was not discarded. This pellet is here, white blood cells were essentially completely removed by the sedimented lysed ghosts of high MCHC erythrocytes contains a significant number of ghosts that retain more hemoglobin than membranes from normally hydrated cells. Since these are likely to be the most abnormal cell membranes, it was considered important not to remove these ghosts from the analysis.

The ektacytometric analysis was done as described above for unfractionated erythrocytes. ISC are known to contribute a net negative component to the ektacytometric index because of their elongated shape and failure to deform in the conditions of the ektacytometric experiment. Unlike normal erythrocytes, ISC do not tank tread and deform, but rather rotate around their long axes, oriented 90° to the shear field. Ghosts of ISC are also sickle-shaped, because the morphology of the ISC is dictated by a permanent change in the morphology of the cell membrane. Nevertheless, ghosts of ISC will not generate a negative EI since the second major characteristic of ISC erythrocytes, the elevated MCHC and the resulting increased cytoplasmic viscosity, does not hold for ghosts. Their internal viscosity is less than the medium, and they will deform under shear.

Quantitation of resealing of fractionated erythrocytes. The percentage of input cells recovered as ghosts was determined by fixing an aliquot of ghosts in 0.1% glutaraldehyde in PBS, followed by Coulter counting. Resealing was verified by determining the percentage of the membrane glyceraldehyde-3-phosphate dehydrogenase that was accessible. This enzyme is exclusively associated with the cytoplasmic surface and is sequestered from exogenous substrates if the membrane is resealed.

Other methods. Counts of ISC were done by a single investigator using oxygenated cells fixed in 0.5% glutaraldehyde in PBS. Any cell with an L-W ratio greater than two was scored as an ISC. Blood samples were obtained from the Sickle Cell Clinics at the Children’s Hospital of Detroit, Detroit, MI, and at Harper Hospital, Detroit, MI. All individuals were homozygous HbS by hemoglobin solubility, cellulose acetate, and citrate agar electrophoresis. Blood samples were obtained in heparin during routine clinical visits when the subjects were asymptomatic. A review of clinical history and blood bank records indicated that patients had not received blood transfusions for at least 120 days before the sampling. Samples were obtained from 23 patients, of whom 14 were male. Their ages ranged from 2 to 21 years (median, 11). The ektacytometric measurements were usually done the same day, but always within 28 hours.

RESULTS

Mechanical properties of total SS ghosts. Ghost fragmentation curves in the ektacytometer are characterized by a sharp initial rise in EI as the viscometer begins its rotation, followed by a decline extending over a 3- to 6-minute period. The use of t1/2 to quantitate membrane stability has been well established by Mohandas et al,6 Mentzer et al,6 and Chasis and Mohandas.10 Typical fragmentation curves for ghosts from unfractionated sickle cells are shown in Fig 1A, and data from 23 individuals are summarized in Table 2. The fragility (t1/2) and the initial index (EIo) of the membranes from the total sickle cell population were not significantly different from normal (AA) values. As noted earlier, EIo is numerically equal to EImax, and is lower in rigid or spherocytic membranes. The observed EIo values, therefore, suggest that membrane deformability and surface area are normal in sickle cells. Unfractionated SS membranes were indistinguishable from control ghosts in a ramp-up assay (Fig 1B), verifying their normal deformability.

Mechanical fragility of resealed ghosts from dense cells. Although we found that membrane mechanical properties were apparently normal when the total sickle erythrocyte population was examined in the ektacytometer, only a subset of sickle erythrocytes in an individual at a given time will have acquired membrane abnormalities. The severely dehydrated dense fraction of sickle cells is known to have undergone various biochemical alterations, and this fraction contains most of the ISC. We fractionated the RBCs from six patients on Stractan gradients to determine the membrane properties of the dense cells. Figure 2 shows four sets of fragmentation assays with fractionated sickle cells, and

<table>
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<tr>
<th>Table 1. Correlation Between Initial Value of EI and EImax</th>
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<tr>
<td>AA Patients</td>
</tr>
<tr>
<td>CC</td>
</tr>
<tr>
<td>EIo</td>
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<tr>
<td>EImax</td>
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Abbreviations: EIo, the initial value of EI in a ghost fragmentation experiment (Fig 1A); EImax, the plateau value of EI for ghosts as shear force is increased in a ramp-up assay (Fig 1B).

Table 2. EI0 and t1/2 in Resealed Ghosts From the Total RBC Population of a Group of Sickle Cell Patients and Controls

<table>
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<tr>
<th>Group (N)</th>
<th>EI0</th>
<th>t1/2 (s)</th>
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<tbody>
<tr>
<td>Controls (7)</td>
<td>0.784 ± 0.021</td>
<td>125 ± 15</td>
</tr>
<tr>
<td>Patients (23)</td>
<td>0.760 ± 0.067</td>
<td>135 ± 28</td>
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</table>

The controls are normal AA erythrocytes. Many individuals were tested more than once, and in such cases, all the values were averaged. Membrane values for controls and SS patients did not differ statistically (Students’ t test).
Fig 2. Ghost fragmentation curves from density-separated sickle cells. Four experiments are shown. The dextran concentration used for fragmentation was reduced over the experimental period in an attempt to increase the sensitivity of the assay for short $t_{1/2}$ values. For this reason, $t_{1/2}$ values generally increase from experiments A to D. The solid line on each graph represents the control (AA) ghosts. ISC counts (%) of the fractions were as follows, for experiments A, B, C, and D, respectively: (- - - -): 2, 7, and 0; (- - - -): 28, 30, 46, and 9; (- - - -): 0, 36, 45, and 18; and (- - - -): 37, 39, 57, and 24.

Table 3 summarizes the data from six experiments, showing the percentages of the input RBCs found in the Stractan layers, together with the MCHC and %ISC. The $t_{1/2}$ and $E_{10}$ of density fractions revealed a striking heterogeneity in the mechanical properties of the resealed ghosts. The lightest cell fraction, SS1, had increased membrane stability, while the dense cells, SS2 through SS4, comprising about 25% of the erythrocytes, had diminished stability (shorter $t_{1/2}$). The normal $t_{1/2}$ of total sickle membrane masked this heterogeneity in the ghosts' ability to resist shear.

**Ghost rigidity in dense fractions.** The $E_{10}$ was also lower in membranes from dense cells. Since $E_{10}$ ($E_{10_{mw}}$) is influenced by both membrane rigidity and surface area, the lower $E_{10}$ in ISC-rich fractions could be a consequence of membrane rigidity or reduced membrane area. However, it is known that ISC have normal amounts of phospholipid per cell, showing that they have not lost surface area. Moreover, we directly demonstrated by means of ramp assays (Fig 3), which are not affected by ghost morphology, that ghosts were less deformable. The membranes of the densest cells were clearly rigid, since at any shear stress they deform less than membranes from control cells or normally hydrated sickle cells. The deformability of resealed ghosts derived from the majority top fraction lacking ISC was normal. Similar results on membrane rigidity in dense sickle cells have been obtained by Fortier et al.

**Recovery and resealing of ghosts from fractionated cells.** Because of the documented biochemical abnormalities of dense sickle cells, there was concern that they might not reseal completely, leading to nonrepresentative fragmentation data. We therefore assayed the ghost recovery and the accessibility of glyceraldehyde-3-phosphate dehydrogenase, a measure of ghost resealing introduced by Steck and Kant. This is a stringent criterion, since it tests impermeability to...
MECHANICAL PROPERTIES OF SS MEMBRANES

Washed cellulose-filtered RBCs from sickle cell patients were fractionated on Stractan gradients. SS1 through SS4, fractions of increasing density; cell %, percent of the total RBCs that were found in the layer. Resealed ghosts were made from the fractionated cells, and the ghost mechanical properties were determined in a fragmentation assay. Controls were AA erythrocytes from the top layer of parallel Stractan gradients. This layer included at least 95% of all the normal erythrocytes applied to the gradient.

The substrates of glyceraldehyde-3-phosphate dehydrogenase, which are much smaller than the dextran T40 that establishes the refractive index difference across the ghost membrane. Table 4 shows that ghost recovery is better than 90%, and percent resealing is 85% to 90%, which is comparable with published values for unfractionated normal erythrocyte membranes. In fact, contrary to expectation, it was the light fraction of sickle cells that showed reduced recovery and increased accessibility of glyceraldehyde-3-phosphate dehydrogenase. This appeared to be related to the number of reticulocytes in the fraction, and suggests that reticulocytes reseal less effectively than mature RBCs.

**DISCUSSION**

Two membrane properties of sickle cell ghosts, the deformability and the stability under shear stress, were found to be normal when the entire sickle cell population was studied. When ghosts from dense cells were examined, however, diminished resistance to shear and increased membrane rigidity was found. The membrane mechanical properties of the total sickle cell population is dominated by the majority of sickle cells with normal MCHC and density. Our ektacytometric results on membrane rigidity and the earlier findings of Fortier et al are consistent with micropipette measurements, which found non-ISC sickle cells to have essentially normal rheological properties, while ISC have an increased membrane shear modulus and viscosity. The underlying cause of the deformability loss in sickle membranes is not known at the present time, although precipitated HbS or a complex between hemoglobin and skeletal proteins has been implicated.

The loss of membrane mechanical stability in dense erythrocytes reported here is a new aspect of sickle cell disease. The ISC-rich fractions had t1/2 values that were 75% to 80% of normal AA controls. Despite an increase in average ISC count from 28% to 45%, t1/2 did not increase between SS3 and SS4. This finding indicates that the uncharacterized biochemical changes underlying the ISC shape in the SS membrane are not the same as those that cause the observed reduction in membrane stability. The t1/2 of ghosts from the lightest sickle fraction was slightly longer than the control value, which may be a reflection of the high reticulocyte level in these fractions.

The reduction in membrane strength may be related to oxidative damage. Since the membrane skeleton is the structural basis of the erythrocyte's mechanical strength, the oxidation of spectrin, ankyrin, and protein 4.1 reported by Rank et al is especially relevant. Oxidation of sulfhydryls in spectrin is reported to reduce its affinity for the ternary complex with actin and protein 4.1. Since this is one of the major lateral interactions that support shear stress in the erythrocyte, its weakening would be expected to lower membrane stability. Protein 4.1 also has modified thiols, although their effect on binding interactions is not known. Ankyrin in sickle cells is also oxidized and some of the ankyrin molecules are no longer functional spectrin ligands.

### Table 3. Membrane Properties of Density Fractionated Sickles Cells

<table>
<thead>
<tr>
<th>Erythrocytes</th>
<th>Ghosts</th>
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<tbody>
<tr>
<td>Cell %</td>
<td>MCHC %</td>
</tr>
<tr>
<td>SS1 76 ± 12</td>
<td>30.2 ± 2.0</td>
</tr>
<tr>
<td>SS2 8 ± 6</td>
<td>34.5 ± 3.2</td>
</tr>
<tr>
<td>SS3 10 ± 7</td>
<td>37.3 ± 3.8</td>
</tr>
<tr>
<td>SS4 9 ± 5</td>
<td>42.7 ± 4.0</td>
</tr>
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Washed cellulose-filtered RBCs from sickle cell patients were fractionated on Stractan gradients. SS1 through SS4, fractions of increasing density; cell %, percent of the total RBCs that were found in the layer. Resealed ghosts were made from the fractionated cells, and the ghost mechanical properties were determined in a fragmentation assay. Controls were AA erythrocytes from the top layer of parallel Stractan gradients. This layer included at least 95% of all the normal erythrocytes applied to the gradient.

Fig 3. Membrane deformability data for ghosts of Stractan-fractionated sickle erythrocytes. Two representative experiments are shown. The MCHC (and percent ISC) of the separated fractions were as follows: (A) □, 29.0 (0); ●, 30.4 (18); ○, 35.6 (25); and (B) ○, 30.2 (2); ●, 32.7 (24); ○, 36.0 (28); △, 39.0 (37). Each indicates control data for AA erythrocytes; MCHC, 33.4. Few erythrocytes were obtained in the dense Stractan layers and the ramp assays (Fig 1B) were very noisy. El and rpm values were read from the experimental curve and plotted on a semilog scale. Linear regression lines of the data are shown.
and a-thalassemia without any apparent effects on the vascular endothelium: Possible mechanism relative contributions of the various alterations found in mechanical properties can explain the hemolytic process of increases in ghost rigidity are seen in iron deficiency anemia with specific regard to the increased membrane rigidity in ISC ghosts, it can be noted that slight membrane lesions to the loss of mechanical stability will the destruction of red cells: The biophysics and biology of sickle cell disease. This is consistent with the clinical observation that ISC counts are only weakly correlated with the degree of hemolysis. In another erythrocyte abnormality, hereditary spherocytosis, the membrane has a diminished spectrin content, although the spectrin itself appears to be normal. The reduction in spectrin is correlated with a shortened t^{1/2} in the ghost fragmentation test. From the data of Chasis et al, it can be deduced that lowering t^{1/2} to 65% of normal (the value we found in ISC-rich fractions) requires about a 20% reduction in spectrin content. Examination of the clinical data, however, reveals that a reduction in spectrin content of 20% is associated only with a mild hemolytic anemia. Since all the RBCs are affected in hereditary spherocytosis, while we find that only a subset of the RBCs have a reduced t^{1/2} in sickle cell anemia, we conclude that the membrane instability of ISC, while real, is not a major contributor to the severe hemolysis of sickle cell disease. This is consistent with the clinical observation that ISC counts are only weakly correlated with the degree of hemolysis. Nevertheless, dense sickle erythrocytes do have a weakened membrane structure, and, thus, it is possible that this facilitates their intravascular fragmentation.

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