Mechanical Properties of Oxygenated Red Blood Cells in Sickle Cell (HbSS) Disease

By Gerard B. Nash, Cage S. Johnson, and Herbert J. Meiselman

Little data exist for the mechanical properties of individual irreversible or reversible sickle cells (ISC and RSC, respectively), nor is the process of ISC formation well understood. For oxygenated ISC and density-fractionated RSC, we have used micropipette techniques to measure cell surface area (SA) and volume (V), membrane shear elastic modulus (µ), time constant for viscoelastic shape recovery (tc), and hence to calculate membrane surface viscosity (η = µ · tc). Volume loss associated with increasing cell density was accompanied by a proportionately smaller surface area decrease; SA/V ratio thus increased for denser cells, with ISC having the highest values. Membrane area loss by fragmentation must thus be accompanied by an accelerated decrease in cell volume. ISC had relatively rigid membranes (µ 130% above normal controls) and tc close to normal values, so that their effective membrane viscosity was more than double control. RSC had viscoelastic properties close to control, but showed wider variation between sickle cell donors and within samples. Measurements on density-separated RSC showed that, on average, µ was nearly constant, but that tc was longer for the densest cells, with their η approaching ISC levels. A small subpopulation of RSC were found that had µ close to ISC values. Hypotonically swollen ISC (with internal hemoglobin concentration decreased to normal levels) retained their increased membrane stiffness but had markedly decreased tc, so that their η approached normal values. The results show that elevated hemoglobin concentration influences the viscoelastic behavior of ISC and RSC, but that an irreversible change in membrane elasticity also occurs for ISC. These data suggest that ISC formation occurs via a two-stage process: (1) accelerated volume loss leading to increased cytoplasmic and effective membrane viscosity; (2) a sharp rise in membrane rigidity, presumably linked to membrane structural alteration.

The hemoglobinopathy of sickle cell (HbSS) disease continues to be of both basic scientific and clinical interest. Upon deoxygenation, this hemoglobin (HbS) polymerizes to form long chains, and the erythrocytes undergo shape transformation to characteristic "holly leaf" and elongated forms with pointed spicules. When reoxygenated, reversible sickle cells (RSC) return to discoidal shape. However, even in oxygenated blood samples, there exists a population of irreversibly sickled cells (ISC) that retains elongated shape, although spicules are generally lacking. Sickling in vivo leads to vascular stasis and occlusion, and ISC are thought to be involved in the initiation of microvascular occlusion. The flow properties of HbSS blood are abnormal (see Chien for review), and thus the pathogenic process appears to be related to impaired red blood cell (RBC) deformability associated with changes in cell shape and internal state.

The cell membrane itself is also altered in ISC, as evidenced by their retention of a distorted shape, even in the oxygenated state when they no longer contain polymerized hemoglobin (Hb); this distortion also remains for membrane ghosts and protein skeletons prepared from ISC. In addition, ISC have elevated mean corpuscular hemoglobin concentration (MCHC) due to cellular dehydration arising from loss of effective ion regulation. However, the process of ISC formation is not well understood. It is not known whether they result from gradual alteration of RSC properties during repeated cycles of sickling or whether they form as a consequence of a single event of sequestration and prolonged hypoxia. Whatever the mechanism of ISC formation, it is clear that these cells, and also deoxygenated RSC, are relatively non-deformable: (1) the filterability of HbSS RBC is impaired and is further reduced upon deoxygenation; (2) the viscosity of sickle blood increases with decreasing PO2; (3) even in the oxygenated state, the viscosity of HbSS red cell suspensions is above normal, with the extent of the abnormality depending on the percentage of ISC present.

In contrast to viscometric and filtration measurements on cell suspensions, there exist relatively few direct observations of the mechanical properties of individual sickle erythrocytes. LaCelle and Kirkpatrick have demonstrated the reduced cellular deformability of ISC by measuring the pressure necessary to fully aspirate these cells into 3-μm diameter pipettes. Greater pressures were generally necessary for ISC compared to controls, although results were strongly dependent on the width of the ISC relative to the pipette. Both groups also

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found that ISC had relatively rigid membranes, as judged by the pressure required to aspirate small membrane tongues into micropipettes; no values were reported for the intrinsic mechanical properties of the membrane. Note that Havell et al. used relatively large micropipettes (3-μm diameter) to aspirate membrane portions from the cell edge rather than from the flat regions of the RBC; quantitative interpretation of these data is thus made difficult by the possible contribution of cell geometry to the measurements. For oxygenated RSC, membrane deformability (i.e., aspiration of small membrane tongues) was close to normal, although slightly above-normal pressures were required to fully aspirate RSC into micropipettes. In the deoxygenated state, RSC had greatly increased membrane rigidity and also impaired overall deformability. Smith et al. separated oxygenated ISC into two groups by Micropore filtration and tested the fractions by aspiration into 1.8–2-μm pipettes. The resulting membrane tongue length was greater for the post-filter ISC, thus leading them to suggest the existence of “hard” and “soft” ISC. However, interpretation of these results is difficult, since the aspiration pressure used (200 mm H2O) is far beyond that required to deform and measure the properties of the membrane itself, and the tongue length would thus be more closely related to the excess surface area of the membrane itself, and the tongue length would thus be more closely related to the excess surface area of the RBC. ISC mechanical behavior has also been examined using a laser-diffraction technique for analysis of deformation in a shear field. Results for the influence of osmotic swelling on ISC deformation were interpreted as showing that elevated MCHC, rather than abnormal membrane mechanical properties, is the primary cause of reduced ISC deformability. The present study was designed to measure selected membrane mechanical and cellular geometric properties of oxygenated HbSS erythrocytes and thus to quantify some of the factors that influence cell deformability in sickle cell disease. Blood from normal HbAA donors was also studied to provide control data. Using micropipette techniques, we have measured membrane shear elastic modulus (μ, the resistance to shear deformation at constant area) and the time constant for shape recovery after release of extended RBC (τc, viscoelastic time constant). The membrane surface viscosity η, which limits the rate at which deformation can occur, was calculated as the product of μ times τc. These viscoelastic properties were measured for both ISC and RSC, and the intra- and interdonor variability of HbSS RBC were examined. Density-separated RSC were also studied to see if they had density-dependent properties that approached those of ISC. Osmotically swollen ISC were measured to investigate the influence of MCHC on their viscoelastic behavior. Note that, for the present study, we consider hemoglobin that is bound to or interdigitated with membrane cytoskeletal proteins to be a part of the membrane structure. Thus, changes in MCHC might influence “membrane” as well as cytoplasmic properties. Finally, the surface area and volume of individual ISC and fractionated RSC were directly measured. The aim was to indicate whether volume loss is accompanied by loss of membrane area, and to test whether, as has been suggested, cell fragmentation plays a significant role in ISC formation.

MATERIALS AND METHODS

Donor Population

Blood samples were obtained from a total of 25 patients with homozygous HbSS disease, with approval of the University of Southern California Human Subjects Research Committee. Patients were defined by the usual hematologic criteria. Hemoglobin-S was identified by alkaline electrophoresis on cellulose acetate and confirmed by acid electrophoresis on citrate agar gel. Excluded from the study were patients in sickle cell crisis and those who had received transfusions within the prior 3 mo. The age of the patient group was 26 yr (range 4–43 yr). Table I summarizes the hematologic indices, obtained by standard clinical laboratory techniques, for these patients. Hemoglobin-F was measured by the Betke method of alkali denaturation and hemoglobin-A2 by DEAE-cellulose microchromatography. All patients were seen at the Comprehensive Sickle Cell Center, Los Angeles County–University of Southern California Medical Center, Los Angeles, CA. Control blood samples were obtained from healthy adult laboratory personnel (average age 31 yr, range 24–37 yr).

Blood and RBC Preparation

Blood from sickle donors was collected by venipuncture into heparin (5 IU/ml) and stored up to 16 hr at 4°C until used; measurements were completed within approximately 24 hr of venipuncture. Storage was necessitated by the withdrawal of blood at a late afternoon outpatient clinic. All cell preparations and measurements were made at room temperature (23±1°C). Part of each blood sample was density fractionated by a centrifugal method described in prior studies. Whole HbSS blood was centrifuged at 100 g for 5 min, and a portion of the plasma was removed and reserved for later use. The blood cells were resuspended in the remaining plasma to a hematocrit of 50%–70%. This suspension was loaded into narrow glass tubes (2 mm internal diameter by 70 mm long) and centrifuged at 12,000 g for 15 min. By cutting the tubes, the packed cell column was divided into four fractions: (1) T1, cells from the top 10% of the column; (2) T2, cells from the second 10%; (3) MID, cells from the middle 70%; (4) BOT, cells from the bottom 10%. These cell fractions were suspended at approximately 107/109 cells/ml.

Table 1. Hematologic Data for HbSS Donors*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent HbA2</td>
<td>3.7 ± 0.8</td>
<td>2.2–5.3</td>
</tr>
<tr>
<td>Percent HbF</td>
<td>7.1 ± 5.5</td>
<td>0.2–15.7</td>
</tr>
<tr>
<td>MCV (μm³)</td>
<td>89 ± 13</td>
<td>61–106</td>
</tr>
<tr>
<td>MCH (pg/cell)</td>
<td>29.7 ± 5.1</td>
<td>19.6–36.7</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>33.3 ± 1.2</td>
<td>30.3–36.4</td>
</tr>
</tbody>
</table>

*Data are for the 25 HbSS donors used in this study and were obtained by standard clinical laboratory techniques.
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75
cells/ml in phosphate-buffered saline (PBS, 0.122 M NaCl, 0.03 M KH$_2$PO$_4$ + Na$_2$HPO$_4$, 0.2 g/dl glucose, pH 7.44, 300 mosmole/kg) containing 0.2 g/dl human serum albumin. If unfractionated HbSS blood or control samples were to be studied, whole blood was diluted to a similar concentration with this medium. In one series of experiments, ISC were osmotically swollen in hypotonic media. For a similar concentration with this medium. In one series of experiments, ISC were osmotically swollen in hypotonic media. For these studies, the BOT fraction was divided and suspended in PBS with different NaCl concentrations (nominal osmolalities of 300, 250, and 150 mosmole/kg); actual buffer osmolalities were measured before use. All cell suspensions were equilibrated with room air (suspension $p_0$, 150–155 mm Hg).

To test whether the sickle blood fractionation was truly based on density, portions of the T2, MID, and BOT cells from two HbSS donors were mixed with isotonic Percoll (Pharmacia Fine Chemicals, Piscataway, NJ), having a density of approximately 1.11 g/cc, and centrifuged at 20,000 $g$ for 30 min. The RBC fractions appeared as bands at different levels in the spontaneously formed density gradients, and their mean densities were estimated by comparison to density-calibrated beads (Pharmacia Fine Chemicals) that had been centrifuged under identical conditions.

**Microscope-Video and Micropipette Systems**

The microscope-video and micropipette systems were as recently described, except that, in the present study, a Zeiss 40 x, NA = 0.75, long working distance, water immersion objective was used. Also, micropipette tips were broken using a method based on that of Merril and Ainsworth. In this technique, a small sphere of low melting point solder glass (Corning Glass, Corning, NY) was electrically heated on a wire microfilament. Under low power microscopic observation, the pipette tip was advanced into the softened glass and the heater turned off. After cooling for a few seconds, the pipette was withdrawn and broke cleanly at its interface with the solder glass. Pipettes of various internal diameters could be prepared; using high-power light microscopy, all tips appeared smooth with their openings perpendicular to the pipette axes.

Red cell suspensions were placed in chambers made from standard glass microscope slides and coverslips, separated by U-shaped, 1-mm thick parafilm gaskets. These were inverted and the cells allowed to settle and attach to the coverslip for 15–30 min. The remaining suspension was then removed and the chambers flushed and finally filled with PBS containing 9% v/v autologous plasma; the plasma was appropriately diluted in the studies with hypotonic media. Chambers were then turned right side up, placed on the microscope stage, and the micropipette introduced through their open side.

**Determination of Viscoelastic Properties**

The viscoelastic behavior of the RBC was characterized by measuring their time constant for shape recovery ($t_c$) and membrane shear elastic modulus ($\mu$). The membrane surface viscosity was then calculated from the relation:

$$\eta = \mu \cdot t_c$$ (1)

Viscoelastic recovery of cell shape was measured and analyzed by the method of Hochmuth et al. Cells that were point attached to the chamber coverslip were tested by placing the micropipette tip (ID 1.2–1.6 $\mu$m) adjacent to their rims, opposite the point of attachment. A negative pressure was applied to the pipette and the cells were extended and then released by withdrawing the pipette. The presence of plasma in the medium prevented further attachment of cells to the coverslip during the extensional recovery and helped abolish sticking of cells to the pipette tip. From video recordings of the recovery process, the cell length/width ratio $(L/W)$ was measured, initially every 1/60 sec, but at longer intervals later in the recovery process. Using a model of a viscoelastic strip that has undergone uniform extension, Hochmuth et al. derived the following equation to describe the change in cell dimensions with time:

$$(L/W) - (L/W)_0 = \frac{(L/W)_0 + (L/W)_0^*}{(L/W) + (L/W)_0^*} \cdot \exp \left(-t/t_c\right)$$ (2)

where $t_c = \eta/\mu$. The ratio $L/W$ refers to the cell dimensions at time $t$ and the subscript 0 refers to the ratio at the first data point ($t = 0$) and * at its final value. Optimal values for both $t_c$ and $(L/W)_0^*$ were calculated numerically, using the method outlined by Hochmuth et al. to minimize the deviation of the experimental data from the model theory. Calculations were based on the $(L/W)$ values from the first 0.5 sec of the shape recovery process.

The membrane shear elastic modulus was measured by a method similar to that of Evans and LaCelle. After recording shape recovery, the same cell was detached from the coverslip and a small membrane tongue was aspirated into the micropipette from a flat portion of its surface. Tongue length $(L)$ was measured from video recordings at a series of increasing aspiration pressures $(P)$. Depending on pipette size and membrane stiffness, $P$ was generally in the range of $1–10$ mm H$_2$O, so that the ratio $L/R$ (radius) varied between approximately 1.5 and 3. The increase in $L/R$ as a function of $P$ was analyzed by linear regression to obtain the slope, which is proportional to $1/\mu$. This approach is a linear approximation to the theory developed by Evans for analysis of this system and is valid for $L/R > 1$.

In studies of density-separated RSC, the same micropipette was used for measurements on each fraction from a given donor; a single pipette was also used when comparing ISC suspended in media with varying osmolality. In all cases, measurements of RSC viscoelasticity were carried out on discoidal cells, and for ISC, cells were from the BOT fraction. Control RBC were discocytes from unfractionated blood. A series of control experiments was carried out, in which normal red cells were tested both before and after 24-hr storage at 4°C. The micropipettes used in these experiments were also stored overnight and reused.

**RBC Morphology and Geometry**

The morphology of cells in the density-separated sickle fractions was evaluated microscopically using suspensions fixed with 1% glutaraldehyde. In each fraction, at least 100 cells were classified as either: (1) DISCS, i.e., discocytes or cells with a nearly normal shape; (2) intermediate (INT) cells, i.e., irregularly or abnormally shaped; (3) ISM, i.e., elongated, sickle or double pointed cells. with an $L/W$ ratio of approximately 2 or greater. Cell dimensions (i.e., diameter $(D)$ for RSC and control cells and length $(L)$, and width $(W)$ for ISC) were measured from video recordings of the cells subjected to micropipette analysis. The surface area $(S_A)$ and volume $(V)$ of individual cells were measured by the micropipette method of Nash and Wyand. Using micropipettes with ID 1.5–1.8 $\mu$m and aspiration pressures of 2–6 mm H$_2$O, cells were forced to adopt a smooth outline with two distinct portions: a spherical part external to the pipette tip and a cylindrical part within the pipette. Measurement of the dimensions of the cell portions and of the pipette diameter enabled calculation of $S_A$ and $V$. The pipettes employed in this part of the study were slightly narrower than those previously used for measurements on normal erythrocytes. The smaller diameter was necessary because a significant proportion of ISC was found to be able to enter and transverse pipettes of diameter 1.8–2 $\mu$m; normal red cells cannot enter such pipettes except at very high pressures, which cause hemolysis. This ability of the ISC suggests that they have an $S_A/V$ ratio greater than normal RBC. Note that with the 1.5–1.8-m$\mu$m pipettes, the aspirated portion of the ISC conformed to the cylindri-
Table 2. Characteristics of Different HbSS Red Cell Fractions

<table>
<thead>
<tr>
<th>Sample</th>
<th>MCV† (μm)</th>
<th>MCH (pg/Cell)</th>
<th>MCHC (g/dl)</th>
<th>DISCS</th>
<th>INT</th>
<th>ISC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated cells</td>
<td>91 ± 11 (26%)</td>
<td>32.0 ± 7.7</td>
<td>35.3 ± 4.5</td>
<td>52 ± 10</td>
<td>36 ± 6</td>
<td>12 ± 7</td>
</tr>
<tr>
<td>T1</td>
<td>111 ± 24 (38%)</td>
<td>30.5 ± 8.9</td>
<td>26.6 ± 5.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T2</td>
<td>98 ± 19 (26%)</td>
<td>32.0 ± 7.4</td>
<td>30.6 ± 2.8</td>
<td>51 ± 10</td>
<td>43 ± 8</td>
<td>6 ± 6</td>
</tr>
<tr>
<td>MID</td>
<td>86 ± 12 (24%)</td>
<td>30.8 ± 6.0</td>
<td>34.5 ± 2.7</td>
<td>53 ± 9</td>
<td>36 ± 6</td>
<td>11 ± 7</td>
</tr>
<tr>
<td>BOT</td>
<td>79 ± 12 (26%)</td>
<td>32.3 ± 7.0</td>
<td>39.8 ± 3.8</td>
<td>33 ± 6</td>
<td>33 ± 8</td>
<td>34 ± 6</td>
</tr>
</tbody>
</table>

†Figures in parentheses are the average coefficients of variation (CV) for the cell volume distributions; for normal donors, CV = 18 ± 1.2% (n = 5) for unfractionated blood.

RESULTS

Table 2 presents hematologic and morphological characteristics for unfractionated and density-separated sickle erythrocytes. The T1 fraction included a pale band at the top of the packed cell column, which was wider than the buffy coat found for similarly treated normal blood. This fraction was rich in white blood cells, reticulocytes, and platelets, and included cell fragments and membrane ghosts; the T1 fraction was thus not subjected to micropipette or detailed morphological analysis. RBC in the other fractions were classified into three morphological groups (see Materials and Methods). The BOT sample was enriched with ISC and had fewer discocytes than either the other fractions or the unfractionated cells; these contained an approximately constant proportion of 50% discocytes. The ISC were mostly thin and flattened, with peripheral margins of thicker cytoplasm. RSC also often appeared thinner than normal discocytes. Variability in cell size within HbSS samples was indicated by the large coefficients of variation (CV) obtained when cell volume distributions were measured with the computerized orifice-type system; for unfractionated HbSS samples, the CV were approximately 45% greater than for controls. The MCV was smaller for cells further down the cell column. However, MCH was essentially independent of position in the column, so that, as expected, MCHC was greatest in the BOT fraction. Note that, whereas MCH varied greatly between donors, MCHC values were more constant; this observation agrees with the pooled clinical data shown in Table 1. Density measurement in Percoll gradients verified that denser cells were located in the lower fractions. For the two HbSS samples tested, the BOT cells had an average density of approximately 1.140 g/cc, compared to 1.113 g/cc for the MID and 1.106 g/cc for the T2 fraction.

Direct micropipette measurement on individual cells also showed that cell volume (V) decreased progressively with cell density (Table 3). There was a parallel but proportionately smaller decrease in surface area (SA), so that the SA/V ratio was greatest in the BOT fraction (i.e., 25% greater than in the T2 fraction). Data for the different cell types within the BOT fraction indicated that even for RBC of comparable density, V, SA, and SA/V ratio varied with cell morphology. Both SA and V were slightly less for ISC than DISC, with INT values lying between these two shapes; V changes again exceeded those for SA, so that ISC had the greatest SA/V ratio.

Membrane viscoelastic properties of ISC, RSC, and control RBC are presented in Table 4; also shown are cellular dimensions and control RBC membrane properties, both before and after 24-hr storage at 4°C in heparinized plasma. Dealing first with the size and storage results, we note that: (1) RSC discs had a diameter slightly larger than control RBC (0.02 > p > 0.01), and ISC were highly asymmetric (mean L/W = 2.0 ± 0.1); (2) there were no meaningful differences in

Miscellaneous Techniques

Solution osmolalities were determined by freezing point depression (Model 2007, Precision Systems, Inc., Sudbury, MA). Mean cell volume (MCV) and sample coefficient of variation (CV) in isotonic PBS and, for the swollen BOT samples, in hypotonic PBS, were measured electronically as previously described, using a computerized Electrozone Celloscope (Particle Data Inc., Elmhurst, IL). Mean corpuscular hemoglobin (MCH) was determined from Celloscope cell counts and cyanmethemoglobin measurement of hemoglobin concentration. Sickle RBC were sonicated for 5 min in the cyanmethemoglobin reagent solution to ensure complete hemolysis. Mean corpuscular hemoglobin concentration (MCHC) was calculated as MCH/MCV x 100. For all experimental measurements, statistical comparisons were carried out using an unpaired two-tailed t test.

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Table 3. Surface Area and Volume of HbSS Red Cells*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume (μm³)</th>
<th>Surface Area (μm²)</th>
<th>SA/V Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2</td>
<td>117 ± 18 (17%)</td>
<td>156 ± 14 (10%)</td>
<td>1.37 ± 0.08 (9%)</td>
</tr>
<tr>
<td>MID</td>
<td>91 ± 2 (19%)</td>
<td>139 ± 5 (10%)</td>
<td>1.56 ± 0.04 (11%)</td>
</tr>
<tr>
<td>BOT†</td>
<td>77 ± 2 (16%)</td>
<td>129 ± 2 (8%)</td>
<td>1.71 ± 0.04 (11%)</td>
</tr>
</tbody>
</table>

Different cell types in BOT

DISC 82 ± 6 (17%) 134 ± 4 (8%) 1.68 ± 0.08 (12%)
INT 79 ± 4 (14%) 130 ± 3 (9%) 1.67 ± 0.04 (8%)
ISC 71 ± 3 (16%) 125 ± 4 (7%) 1.79 ± 0.05 (11%)

*Data represent mean ± standard deviation of means from experiments on blood from 3 donors; in each experiment 20 or more cells of each type were measured. Figures in parentheses are average CV for the samples.
†Data for BOT were calculated from data for the 3 cell types measured separately, by assuming that they occurred in equal proportions (see Table 2).

μ, τc, or η between control RBC on the day of blood withdrawal and after 24-hr storage. The latter results support the validity of comparing stored HbSS cells to fresh control cells and, by inference, suggest that use of stored HbSS blood does not vitiate the data obtained for these RBC.

The membrane mechanical properties of ISC were significantly different from those of control RBC (Table 4). The ISC membranes were much more rigid (130% increase in μ), but, on average, their shape recovery times were close to normal. Thus, the calculated ISC membrane viscosity (i.e., η = μ · τc) was also elevated; the approximately 150% increase in η was highly significant. On average, RSC were less abnormal than ISC, in that the RSC had μ values only slightly greater than controls (20% mean increase) and had calculated membrane viscosity that did not significantly differ from normal. It should be noted that the range of μ values for individual control and RSC donors overlapped, and thus, some HbSS donors had RSC discocytes with normal mechanical properties, whereas others had RSC with slightly elevated elastic modulus. The HbSS donors represent a more variable population than controls, as demonstrated by the large standard deviations for the mean μ and η values. Also, as shown by the average coefficients of variation given in Table 4, there tended to be greater variability in membrane properties within cell samples for both ISC and RSC.

The apparent heterogeneity of RSC mechanical properties was examined in a series of experiments on density-fractionated RSC (i.e., discocytes from the T2, MID, and BOT fractions); the results are summarized in Table 5. In general, the elastic modulus did not vary significantly between fractions, but the time constants, and hence the calculated membrane viscosity, were greater for the BOT cells. Thus, T2 and MID fractions had equal μ and τc, and only the BOT sample showed increases in either parameter. Again, however, there was variability between donors; for one donor, neither μ nor τc differed between any fractions. This variability was probably due to interdonor differences in the proportion of very dense cells. Thus, our BOT fraction, which consistently sampled the 10% most dense RBC for a given donor, would itself contain RBC of variable density. Note that, during the micropipette tests, a small number of rigid cells were encountered within the RSC population. These cells had membrane properties approximating those of ISC and were mainly responsible for biasing μ values toward higher levels for the BOT fraction, where they were more frequently found. However, the RSC population as a whole does not appear to gradually approach ISC rigidity.

Table 4. Mechanical Properties of ISC, RSC, and Control Red Cells*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Shear Elastic Modulus (10⁻¹ dyn/cm)</th>
<th>Time Constant (sec)</th>
<th>Calculated Surface Viscosity (10⁻² dyn·sec/cm²)</th>
<th>Dimensions (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISC†</td>
<td>12.9 ± 2.5 (36%)</td>
<td>0.161 ± 0.037 (37%)</td>
<td>20.8 ± 6.2 (50%)</td>
<td>L = 12.6 ± 0.8</td>
</tr>
<tr>
<td>(n = 7)</td>
<td></td>
<td></td>
<td></td>
<td>W = 6.3 ± 0.5</td>
</tr>
<tr>
<td>RSC†</td>
<td>6.7 ± 0.8 (20%)</td>
<td>0.131 ± 0.025 (16%)</td>
<td>8.9 ± 2.2 (23%)</td>
<td>D = 8.8 ± 0.3</td>
</tr>
<tr>
<td>(n = 10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls §</td>
<td>5.6 ± 0.4 (11%)</td>
<td>0.144 ± 0.017 (16%)</td>
<td>8.1 ± 1.1 (24%)</td>
<td>D = 8.5 ± 0.2</td>
</tr>
<tr>
<td>(n = 10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls II (n = 3)</td>
<td>5.6 ± 0.4 (11%)</td>
<td>0.132 ± 0.023 (16%)</td>
<td>7.4 ± 1.4 (24%)</td>
<td></td>
</tr>
<tr>
<td>Fresh</td>
<td>5.6 ± 0.4 (11%)</td>
<td>0.135 ± 0.014 (16%)</td>
<td>7.9 ± 1.4 (24%)</td>
<td></td>
</tr>
<tr>
<td>Stored 24 hr</td>
<td>5.8 ± 0.5 (11%)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

*Data represent mean ± standard deviation of means from n donors; figures in parentheses are the average CV for the samples. For controls and RSC, 8 cells were measured in each donor sample; and for ISC, 6 cells were measured per donor. The RSC data are mainly from measurements on the MID fraction (8 donors) although for 2 donors, RSC from unfractionated blood were studied. L, ISC length; W, ISC width; D, disc diameter.
†The differences between ISC and Control I shear elastic modulus and surface viscosity are significant (p < 0.001).
§Controls I are for 10 donors whose RBC were measured on day of blood withdrawal. Controls II are for 4 donors whose red cells were measured both on the day of blood withdrawal and after 24-hr storage at 4°C in heparinized plasma.
Table 5. Mechanical Properties of Density-Fractionated RSC*

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Shear Elastic Modulus (10^3 dyne/cm)</th>
<th>Time Constant (sec)</th>
<th>Calculated Surface Viscosity (10^-1 dyne - sec/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2</td>
<td>6.2 ± 0.4 (14%)</td>
<td>0.127 ± 0.026 (19%)</td>
<td>7.8 ± 1.4 (25%)</td>
</tr>
<tr>
<td>MID</td>
<td>6.5 ± 0.6 (17%)</td>
<td>0.123 ± 0.024 (12%)</td>
<td>7.9 ± 1.3 (21%)</td>
</tr>
<tr>
<td>BOT</td>
<td>7.1 ± 1.1 (16%)</td>
<td>0.206 ± 0.057 (36%)</td>
<td>15.0 ± 6.0 (40%)</td>
</tr>
</tbody>
</table>

*Data represent mean ± standard deviation of 4 experiments; figures in parentheses are average CV for the samples. In each experiment, 8 cells were measured per fraction.

although the calculated membrane viscosity for RSC in the BOT fraction was close to ISC values (Table 4).

Data for ISC viscoelasticity as a function of suspending medium osmolality are shown in Table 6. In addition, relative volume-osmolality data are tabulated for the BOT fraction. The latter data allow insight into the swelling behavior of ISC and dense RSC and were analyzed using the osmotic swelling equation proposed by Ponder:39

$$V/Viso = A\left(Tiso/T\right) + B,$$

where Viso and V are the mean cell volumes at isosmotic (Tiso) and experimental (T) osmolalities. The computed regression equation was $V/Viso = 0.43\left(Tiso/T\right) + 0.56$, thus indicating that these cells exhibit much smaller volume increases with decreasing tonicity than do either unfractionated or density-separated normal RBC.26 This decreased osmotic response probably arises because cellular water represents a relatively smaller fraction of the internal contents of the dense HbSS cells, with hemoglobin itself occupying a larger proportion of their greatly reduced cell volume.

ISC membrane rigidity was not significantly altered by osmotic swelling; the membrane shear elastic modulus remained approximately constant over the range of 304–150 mosmole/kg (Table 6). However, swollen ISC had markedly reduced recovery times (33% mean decrease at 209 mosmole/kg and 42% decrease at 150 mosmole/kg). Thus, the apparent membrane viscosity of ISC decreased with swelling and, at 150 mosmole/kg, approached RSC and control values (Table 4). Note that the RBC shape recovery process is driven by the membrane elasticity, $\mu$, and that, in general, its rate is limited by the membrane viscosity, $\eta$.28 Thus, if both $\mu$ and $\eta$ are similarly elevated above normal, then $t_c$ will remain nearly constant (e.g., as for ISC). If $\mu$ alone is elevated, then $t_c$ will be shorter (e.g., as for maximally swollen ISC); if $\eta$ alone is increased, then $t_c$ will be longer (e.g., as for dense RSC). However, since the dependence of ISC and, possibly, dense RSC time constants and calculated $\eta$ values on MCHC is not clearly understood (see Discussion), it seems expedient to refer to increases in “effective” membrane viscosity for these cells.

It should be noted that both the use of pressure versus membrane deformation data to obtain $\mu$ values and the calculation of $\eta$ values from equation 1 assume that the membrane behavior is essentially elastic rather than plastic. Ideally, when measuring membrane tongue length versus aspiration pressure, hysteresis should be absent in the loading and unloading phases. In practice, however, some hysteresis occurs even for normal RBC. This results in an uncertainty of 10%–15% in the measured $\mu$ values and is probably due to friction between membrane and pipette surfaces.40

In the present study, during ISC elasticity measurements, cells were subjected to an initial aspiration pressure ($P_i$) that was subsequently increased in steps. Consequently, membrane deformation increased. Upon return of the pressure to $P_i$, 76% of this increased deformation was recovered (mean from 20 ISC). In addition, when the pressure was finally zeroed, residual deformation was rarely observed. Other workers have noted incomplete recovery and “permanent buckled distortion” (i.e., plastic deformation) after release of ISC membrane tongues from micropipettes.20,22 Presumably the small deformations ($L_t/R \leq 3$) used in this study, which were applied for relatively short periods (about 1–3 min), were not sufficient to frequently induce such behavior. Membrane buckling, as described by Havell et al.,21 was

Table 6. Mechanical Properties of Hypotonically Swollen ISC*

<table>
<thead>
<tr>
<th>Medium Osmolality (mosmole/kg)</th>
<th>Shear Elastic Modulus (10^3 dyne/cm)</th>
<th>Time Constant (sec)</th>
<th>Calculated Surface Viscosity (10^-1 dyne - sec/cm)</th>
<th>Relative Volume (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>304 ± 2</td>
<td>12.2 ± 2.7</td>
<td>0.143 ± 0.022</td>
<td>17.3 ± 1.3</td>
<td>1.00</td>
</tr>
<tr>
<td>209 ± 3</td>
<td>11.1 ± 1.4</td>
<td>0.096 ± 0.012</td>
<td>10.4 ± 0.8</td>
<td>1.17 ± 0.03</td>
</tr>
<tr>
<td>150 ± 3</td>
<td>11.1 ± 0.7</td>
<td>0.084 ± 0.013</td>
<td>9.0 ± 1.1</td>
<td>1.44 ± 0.08</td>
</tr>
</tbody>
</table>

*Data represent mean ± standard deviation of means for n experiments. Swollen and isotonic ISC were compared on 4 occasions, but elastic modulus was measured only in three of these experiments; thus, surface viscosity values are for $n = 3$. Six ISC were measured in each sample at each osmolality.

†Differences between ISC at 304 versus 209 or 150 mosmole/kg were significant for both time constant and surface viscosity ($p < 0.01$).

‡Volume data are MCV for the BOT fraction and are expressed relative to the volume in isotonic PBS.
carefully avoided. During shape recovery measurements, no residual (i.e., plastic) deformation was evident for any cells. Thus, although some nonideality probably exists in ISC viscoelastic behavior, we conclude that comparison of normal and HbSS RBC membrane properties by the present techniques is valid. We also note that the unequal geometry of the ISC and control RBC should not greatly influence comparative measurements using the micropipette techniques. ISC were sufficiently broad and flat (cell width at least six times the pipette radius) to allow μ measurement and had shapes approximating the model of a rectangular membrane strip originally used to develop the theory for viscoelastic shape recovery.28

DISCUSSION

The abnormal rheology of HbSS blood and its importance in the pathogenesis of sickle cell disease have been widely documented.6,7,12-19 To better understand the cellular basis of this disorder, we have measured specific membrane mechanical and cellular geometric properties of both reversible (RSC) and irreversible (ISC) sickle cells in the oxygenated state. Previous studies on single cells have demonstrated that ISC have impaired cellular deformability and relatively rigid membranes.20,22 We have quantified this abnormality by obtaining values for two intrinsic membrane viscoelastic properties (i.e., shear elastic modulus μ, surface viscosity η). ISC were found to have a shear elastic modulus approximating double that for controls. By measuring time constants for viscoelastic shape recovery, we calculated that their membrane surface viscosity was similarly elevated. On the other hand, RSC generally had membrane properties close to normal, although RSC from some HbSS donors had slightly increased membrane rigidity (i.e., increased μ). Interdonor variation in membrane mechanical properties was greater for sickle cell patients than for controls. Also, within RSC samples, μ varied more than in normal red cell populations, even though the RSC tested were cells with nearly normal, biconcave morphology. Both intradonor and interdonor variability in HbSS cell size, shape, and MCHC have previously been reported.2,4,12 Thus, it is evident that even for individual donors, there is great heterogeneity in mechanical properties in HbSS RBC populations. Therefore, we note that measurements of averaged mechanical properties might not fully reflect either the presence of abnormal cells or the magnitude of their abnormality.

ISC have very high MCHC, which has been suggested to be an important determinant of their rheologic behavior.6,27 In the present study, we found that osmotic swelling of ISC, such that their MCHC approached normal levels, did not significantly modify their abnormal membrane stiffness (Table 6). However, swollen ISC had markedly reduced shape recovery times, so that their calculated membrane viscosity approached control values (Table 6). These reductions in tc and η were presumably mediated by decreases in internal Hb concentration and viscosity. For normal cells, the cytoplasmic viscosity has been calculated to have minimal influence on energy dissipation during extensional shape recovery, and thus has little effect on tc;28 extensional recovery then reflects the membrane characteristics alone. However, in a previous study,29 we have shown that shrinkage of normal cells beyond a certain level (i.e., so that MCHC approaches 40 g/dl) leads to a rapid increase in tc. Although the viscosity of Hb solutions rises sharply at concentrations above those physiologically normal,43 the observed increase in tc could not be explained solely by a rise in internal viscosity.29 We thus suggested that a reversible, concentration-dependent Hb/membrane interaction (i.e., a reversible change in membrane structure) had increased the membrane viscosity. A similar interaction might explain the dependence of ISC viscoelasticity on internal Hb concentration. Both ISC (Table 6) and normal RBC29 show a similar nonlinearity in their relation between tc and induced volume change. On the other hand, ISC MCHC is likely to be near 45 g/dl,27 and thus, both their hemoglobin concentration and cytoplasmic viscosity would be higher than the levels induced in our earlier study of shrunk normal cells. Hence, it is possible that the calculated ISC membrane viscosity is elevated either because of Hb/membrane interaction (i.e., a change in a true membrane property) or as an effect of greatly increased intracellular viscosity. Because of this uncertainty, it may be better to refer to "effective" membrane viscosity for cells with abnormally high MCHC.

Density-separated RSC were measured to test whether their mechanical properties changed as a function of cell density and to gain insight into the processes of ISC formation and in vivo cell aging. RSC discs in the T2 and MID fractions had equal membrane properties; only BOT cells differed (Table 5). On average, these densest RSC had increased tc and hence higher effective membrane viscosity, with the latter nearly equaling ISC values. However, average μ values were essentially constant for all RSC fractions, although a few discs, which were mostly present in the BOT fraction, did have membrane rigidity comparable to ISC. Thus, in general, the RSC membrane elastic modulus did not gradually approach ISC levels. These changes in membrane mechanical properties can be compared to those found for similarly separated normal RBC.26,29,44 First, neither RSC nor normal RBC

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show density-dependent changes in their $\mu$ value. Second, both cell types have recovery times that are density-dependent, but, whereas normal cells show gradual increases in $tc$ with density, only RSC in the BOT fraction had elevated $tc$. For normal cells, this increase in $tc$ is a consequence of a gradual irreversible change in membrane viscosity during aging; it does not result from altered MCHC.29 On the other hand, the abrupt change in $tc$ for the BOT RSC is probably linked to elevated internal Hb concentration. Note that the BOT fraction had an MCHC greater than that observed for comparably separated normal RBC, and thus, these dense RSC probably had intracellular Hb concentration in the region we have previously found to alter membrane viscoelasticity.29 The Hb-independent changes in membrane viscosity referred to above, which are seen during aging of normal cells,29 may not develop sufficiently to be apparent in our measurements on sickle cells, as erythrocyte lifespan is greatly shortened in HbSS disease.45 If such Hb-independent changes had occurred, ISC would be expected to exhibit irreversible changes in $\eta$, even after osmotic swelling; no such irreversible changes were found (Table 6).

ISC and density-separated RSC were also subjected to micropipette analysis of surface area and volume (Table 3). In agreement with electronic sizing measurements, denser cells had decreased volume, whether ISC or RSC. Surface area was also less for the denser cells, but their $SA/V$ ratio was greater; ISC had the largest $SA/V$ ratio, but all cells in the BOT fraction had high values (Table 3). Loss of membrane fragments has been observed for HbSS cells in their deoxygenated state, and it has been suggested that fragmentation plays a role in ISC formation.30,46 Fragmentation would cause membrane area to decrease but, on its own, it would lead to a decrease rather than an increase in cellular $SA/V$ ratio. Thus, to explain our observed $SA/V$ values, fragmentation must be accompanied by an independent volume loss. Membrane loss is a component of normal cell aging, but for normal RBC, the $SA/V$ ratio is essentially independent of cell age.25,26 It is thus the rate of volume loss that is preferentially accelerated for HbSS cells; as noted above, the BOT fraction had MCHC greater than for equivalently separated normal RBC.29 This volume loss may result from abnormally fast leakage of potassium ions and water, associated with elevation of internal calcium (i.e., enhanced Gardos effect).10 However, volume loss is not the only factor in ISC formation. Although elevated MCHC evidently influenced the effective membrane viscosity of ISC, RSC in the BOT fraction had undergone similar MCHC and effective membrane viscosity changes, yet remained discoidal. ISC, in addition, have altered membrane elasticity. Thus, considering the evidence for the influence of MCHC on RSC and ISC shape recovery rates, the irreversible nature of ISC membrane rigidity, and the failure to demonstrate intermediate RSC membrane elasticity alterations, there appear to be two stages in ISC formation: (1) abnormally rapid cellular dehydration leading to increased effective membrane viscosity (increasingly apparent when MCHC rises above a certain level); (2) a sharp discrete rise in membrane rigidity.

Abnormal ISC membrane elasticity is probably due to an irreversible alteration in membrane structure, distinct from a reversible, concentration-dependent Hb/membrane interaction that may influence membrane viscosity. It is generally agreed that RBC membrane elasticity is determined by its underlying protein structure,53,47 and additional evidence for ISC membrane changes exists, inasmuch as ghosts and Triton-extracted cytoskeletons derived from ISC retain a deformed shape.9 This ISC membrane abnormality presumably develops after cells have been released into the circulation, but the mechanism is not known. Disagreement exists as to the possible existence of abnormal membrane protein content in HbSS disease.48–50 Calcium levels are elevated in ISC,51 but concentrations do not appear to be high enough to promote calcium-dependent, enzymatic crosslinking of membrane proteins.11 High molecular weight protein aggregates have also not been detected in HbSS red cells.50 Abnormally strong Hb/membrane binding has been demonstrated for deoxygenated HbS,52 and ghosts formed from ISC have elevated levels of residual bound hemoglobin.53 It is thus possible that irreversible binding of HbS, perhaps in a polymerized or denatured state, alters ISC membrane rigidity and cell shape. However, changes in membrane elasticity are not necessarily accompanied by distortion of cell shape, since, in this study, a small percentage of RSC (i.e., discocytes) had rigid membranes.

The RBC mechanical properties measured in this study are important determinants of in vivo blood flow, where erythrocytes undergo repeated dynamic deformation.21 The abnormal viscous and elastic properties of ISC and some RSC are expected to be of particular importance in flow through the microcirculation, where cells must enter and flow through narrow vessels with diameters comparable to cellular dimensions. Note that since such cells generally have large $SA/V$ ratios and decreased volumes, their deformability should not be limited by these geometric factors. Clark et al.27 have suggested that “altered membrane properties are not the primary determinant of decreased deformability” of ISC, but rather that cellular dehy-
MECHANICAL PROPERTIES OF HbSS RED CELLS

hydration appears "to contribute in a major way to their abnormal rheologic behavior." Although our MCHC-membrane viscosity results support the latter suggestion, we have also shown that ISC have increased intrinsic membrane rigidity; this membrane abnormality is also likely to influence their rheologic behavior. Nevertheless, while the deformability of ISC is clearly impaired, there is uncertainty regarding the role of these cells in initiating vascular occlusion. The proportion of ISC in the circulation of a given HbSS patient is relatively constant and does not increase during sickle cell crisis.\(^{15}\) Noguchi and Schechter\(^ {16}\) have recently presented evidence that "undeformed" (i.e., discoidal) cells may contain some polymerized Hb, even at relatively high \(p_O_2,\) and suggested that these cells would have difficulty in passing through the microcirculation. Thus, we conclude that abnormal rheology of sickle blood may reflect the existence of poorly deformable RSC as well as the presence of ISC.

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Mechanical properties of oxygenated red blood cells in sickle cell (HbSS) disease

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