Variable Deformability of Irreversibly Sickled Erythrocytes

By Clark M. Smith II, John F. Kuettner, David P. Tukey, Steven M. Burris, and James G. White

The relationship of altered deformability of irreversibly sickled cells (ISC) to their morphological and physiologic characteristics was evaluated in this study. ISC obtained by differential centrifugation and density gradient sedimentation were separated and enriched into hard and soft populations on the basis of their ability to pass through Nuclepore filters with an average pore size of 3.0 μm. Measurement of deformability by micropipette and Nuclepore elastimetry documented marked differences of erythrocyte rigidity in the two populations. Yet ISC morphology, intracellular viscosity determinants (mean cell hemoglobin concentration, density profile gradient, hemoglobin composition), and surface area geometry (mean cell volume, osmotic fragility) were similar in populations of hard and soft ISC. The similarity in intracellular viscosity and surface area geometry suggests that an intrinsic membrane alteration is responsible for the difference in deformability of filtration separated populations of ISC. Hard ISC may be more important to the pathophysiology of sickle cell disease than soft ISC, though ISC counts on peripheral blood smears would not be of help in distinguishing the two populations.

Irreversibly sickled cells (ISC) are commonly present in blood samples from patients homozygous for sickle hemoglobin (HbSS). ISC fail to resume the biconcave, discoid form of normal erythrocytes when sickled blood is fully reoxygenated, remain spindle shaped on peripheral blood smears, and appear dense due to an increase in mean corpuscular hemoglobin concentration (MCHC). Biochemical studies have shown that ISC are deficient in surface membrane, contain reduced levels of adenosine triphosphate (ATP), have lost water and cations, possess elevated levels of calcium, and are more sticky and less deformable than either normal cells or fully oxygenated HbSS erythrocytes.

Many attempts have been made to relate the number of ISC found on blood smears to the frequency and the severity of vaso-occlusive episodes in patients with sickle cell anemia. Although ISC may well be involved in morbid clinical manifestations of sickle cell disease, it has not been possible to establish direct correlations between the numbers of ISC on smears and the time of onset of crises, the severity or duration of particular episodes, or the eventual outcome of the illness.

The reason why ISC fail to herald the onset of crises or serve as an index of disease severity has remained obscure. Recent investigations have suggested that ISC represent a heterogeneous, rather than a uniform population of damaged HbSS erythrocytes. Deformability of ISC measured by different techniques was noted to decrease in proportion to the severity of shape distortion and increase in MCHC. However, the magnitude of the heterogeneity and its relationship to other biochemical and physiologic characteristics of ISC was not clarified.

The present study was undertaken to evaluate the heterogeneity of ISC populations in blood from patients with HbSS disease. Blood samples enriched in ISC by differential centrifugation and density gradient sedimentation were separated into hard and soft populations on the basis of their ability to pass through Nuclepore filters with an average pore size of 3.0 μm. Measurement of deformability by micropipette and Nuclepore aspiration revealed marked differences of erythrocyte rigidity in the two populations of ISC. Hard ISC may be more important in the pathophysiology of sickle cell disease than soft ISC, even though ISC counts on peripheral blood smears would not be of help in distinguishing the two populations.

MATERIALS AND METHODS

Cell Samples

Whole blood was obtained from adult patients with homozygous sickle cell anemia after informed consent in accord with the Committee on the Use of Human Subjects in Research of the University of Minnesota. Every patient had experienced typical vaso-occlusive crises and was verified as homozygous for sickle hemoglobin by isoelectric focusing electrophoresis, Coulter counter cell sizing, and quantitative HbA₂ determination. Fetal hemoglobin was less than 10% in each subject. All were in good health at the time of blood donation and had not been transfused for at least 6 mo. Blood was drawn into heparinized syringes, and red cells were separated from leukocytes and platelets by differential centrifugation at 4°C, washed 3 times, and resuspended in phosphate-buffered saline (PBS) containing 10.0 g/liter albumin, 1.0 g/liter glucose at pH 7.4. The red cells were oxygenated with moistened room air at 500 ml/min for 15 min. ISC were defined as cells with approximate axial to width ratios of greater than 2:1 by light microscopy after oxygenation. Suspensions of washed red cells were treated as described in the following sections.

From the Departments of Pediatrics, Family Practice, Laboratory Medicine, and Pathology, University of Minnesota Health Science Center, Minneapolis, Minn.

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Address reprint requests to Clark M. Smith II, Box 342, Mayo Memorial Building, University of Minnesota Health Science Center, 420 Delaware Street S.E., Minneapolis, Minn. 55455.

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ISC Enrichments

ISC enrichment was performed by two techniques that resulted in different sickle populations. Washed packed red cells were centrifuged at 10,000 g for 30 min at 30°C and the bottom 10% of cells was saved. This population was designated as a "moderate ISC" sample and contained ISC counts of 39%-64%. The second procedure utilized a dextran 40 (Sigma Chemical Co., St. Louis, Mo.) discontinuous density gradient with centrifugation at 90,000 g for 90 min at 4°C. The published procedure was modified by elimination of two of the standard density layers, dextran 40, 24 g/dl, and dextran 40, 32 g/dl. The dense bottom layer of the gradient yielded a population with ISC counts greater than 80% and was designated "marked ISC." The cells were washed three times and resuspended in ice-cold standard buffer to remove dextran.

Micropipette and Nuclepore Membrane Elastimetry

The micropipette aspiration procedure established in our laboratory was described in detail in an earlier publication. Red cells were aspirated into micropipettes with internal diameters of 3.0-3.2 μm, and the pressure required for an entire cell to enter the pipette was recorded. This micropipette elastimetry value was termed the passage pressure (P) in cm of H₂O. Red cells were also aspirated into smaller micropipettes with internal diameters of 1.8-2.0 μm at a constant 20 cm H₂O pressure. The length of cells aspirated into narrower micropipettes was determined and referred to as the micropipette tongue extension (Tₚ). Each P and Tₚ study was based on the mean of 15-20 individual cell aspiration measurements. Micropipette elastimetry to evaluate P was performed on unperturbed washed sickle erythrocytes. Both P and Tₚ were determined on discocytes and ISC in unfiltered samples of moderate ISC and after Nuclepore filtration separation into hard and soft populations.

An alternate assessment of whole cell deformability was used in the marked ISC experiments and was based on aspiration of cells into Nuclepore membranes (Nuclepore Corporation, Pleasanton, Calif.) with average pore size of 2.0 μm at a constant 20 cm H₂O pressure. The apparatus used was described in detail in an earlier publication. Erythrocytes were fixed with 0.1% and then 3.0% glutaraldehyde while in the Nuclepore membrane under constant pressure. The membrane was dissolved away with chloroform, the cells critical point dried, and the tongue extensions measured on the same calibrated video cassette as the micropipette data. Each Nuclepore tongue extension (Tₚ) study was based on the mean of 30-40 individual tongue extension measurements. Nuclepore elastimetry, Tₚ, was carried out exclusively on unfiltered samples of marked ISC and after Nuclepore filtration separation into enriched soft and hard populations.

Nuclepore and micropipette elastimetry of normal red cells gave comparable tongue extensions at lower pressure (<5 cm H₂O) and smaller pore/internal pipet diameter (1.0 μm). Comparison of the two methods with normal red cells is not possible at 2.0 μm diameter and 20 cm H₂O pressure because of the limiting thickness of the Nuclepore membrane. Under these conditions, many normal red cells have an extension length that exceeds the 10 μm thickness of the Nuclepore membrane resulting in bulb formation at their tip. These cells cannot be accurately measured for Tₚ extension length. Of note, marked ISC samples processed by Nuclepore elastimetry did not form bulbs despite having tongue extension measurements that exceeded 10 μm on calibrated video cassette. Nevertheless, the differences in tongue extension length between moderate ISC (Tₚ) and marked ISC (Tₚ) could be due to disparities in both cell population and elastimetry method.

Nuclepore Differential Filtration

Sickle erythrocytes were separated into subpopulations dependent on their ability to pass through Nuclepore filters. Moderate ISC and marked ISC samples were adjusted to a 1% hematocrit in PBS, 10.0 g/liter albumin, 1.0 g/liter glucose at pH 7.4. The red cells were slowly pumped at 0.76 ml/min by an infusion pump (Harvard Instrument Co. #755, Willis, Mass.) through Nuclepore membranes with 3 μm average pore diameters. The membranes were precut to 47 mm diameter and had a pore density of 2 × 10⁶/sq cm. The pressure generated across the membrane by the erythrocyte filtration was monitored with a pressure transducer (Statham Model P 23AA) and recorded (Model 7 polygraph, Grass instrument, Quincy, Mass.). At specific times, flow was stopped in the positive-pressure system and cells, which had collected in the prefiltre chamber, were removed and the membrane changed. The optimal time for harvesting prefiltre and postfiltre erythrocytes was experimentally found to be after 5-10 ml of red cell suspension had been pumped or at a time the pressure differential across the membrane exceeded 5 cm H₂O. The procedure was repeated until prefiltre and postfiltre populations were obtained in sufficient numbers for study. The separated prefiltre population was typically 5%-20% by volume of the postfiltre population.

In some experiments, the prefiltre and postfiltre populations were reliftred a second time to determine if gross deformability differences had been achieved. The cells were reliftred in a standard filtration deformability assay without manipulation of the filter chamber or separation into further subpopulations. The prefiltre populations from both the "moderate" and "marked" ISC prepara- tions had filtration pressures at least 2-3 times the postfiltre population. Unseparated samples had intermediate filtration pressures. Refiltiration of the original prefiltre population with a second filter chamber manipulation and separation resulted in greater enrichment of nonfilterable "hard" cells. This provided evidence that low pressure filtration did not artificially create soft cells and that the prefiltre population was a relative and not absolute enrichment in hard cells. The loss of sample yield and excessive time requirement for two Nuclepore separation procedures were prohibitive for subsequent biochemical and rheologic study. The present investigation utilized one Nuclepore differential filtration separation and resulted in prefiltre populations significantly enriched for nonfilterable hard cells.

Scanning Electron Microscopy

Prefiltre and postfiltre populations were fixed for scanning electron microscopy by the sequential addition of 0.1% glutaraldehyde followed by 3.0% glutaraldehyde, both in Whites saline, pH 7.3. Further processing and critical point drying were accomplished by standard techniques described in previous reports.

ISC Characteristics

Unseparated and separated populations of marked ISC were further characterized. MCHC was calculated from simultaneously measured hemoglobin concentration (measured as cyanmethemoglobin) and microhematocrit. A dextran 40 gradient was modified to incorporate higher density layers to allow comparison of the very dense prefiltre and postfiltre ISC. The ISC density profile gradient consisted of dextran 40 layers of 35, 36, 37, and 38 g/dl; its least dense layer corresponded to the standard gradients' most dense layer. Unincubated osmotic fragility was measured on the cells in standard buffer by the method of Beutler. Mean cell volume (MVC) was calculated from the microhematocrit and Coulter counter red cell count. Hemoglobin composition was evaluated by
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thin-layer isoelectric focusing electrophoresis with an LKB Multi-
phor using LKB polyacrylamide ampholines with pH gradient
6.0-9.0. New methylene blue reticulocyte staining was accom-
plished by a standard technique.2

Statistics
Mean and standard deviation (SD) were determined by standard
methods, and differences between means were tested for signifi-
cance by Student's t test. Results in the text are provided as mean ±
1 SD.

RESULTS
Micropipette Elastimetry of Sickle Erythrocytes
Micropipette elastimetry of oxygenated washed sickle erythrocytes prior to enrichment or separation revealed marked heterogeneity in passage pressure. \( P \) varied from \(<1.0\) cm H\(_2\)O to 200 cm H\(_2\)O with occasional cells failing passage through the 3.0 \( \mu \)m diameter pipettes. Initial attempts to attribute this heterogeneity in ability to deform to differences in the degree of sickle morphologic distortion were unsuccess-
ful. Many oxygenated ISC did not have \( P_s \) greater than their neighboring discoid sickle cells. Although the more distorted cells tended to be less deformable,
both plump and thin ISC had either markedly increased or normal aspiration pressures.

Nuclepore Separated Sickle Erythrocytes
The \( P \) of the Nuclepore-separated moderate ISC erythrocyte suspension is shown in Fig. 1 (top). \( P \) was significantly increased in the prefilter ISC and disco-
ocyte populations. The micropipette \( T_m \) of Nuclepore-
separated moderate ISC samples is shown in Fig. 1 (bottom). The prefilter ISC and discocytes had significantly decreased \( T_m \) in comparison to the postfilter
cells. The \( P \) and \( T_m \) measurements of the unseparated moderate ISC samples included the range of deform-
ability observed in the separated preparations, suggesting no significant filtration procedure injury.

Nuclepore elastimetry \( T_n \) of the Nuclepore-sepa-
rated marked ISC suspensions is shown in Fig. 2. The marked ISC prefilter cells had significantly decreased \( T_n \) in comparison to the unseparated and postfilter erythrocytes. The unseparated sample more closely resembled the deformability behavior of the postfilter due to the smaller percentage of hard prefilter erythro-
cytes in the unseparated preparation. The range of \( T_n \) of the unseparated ISC encompassed the \( T_n \) determi-
ations of the separated prefilter and postfilter cells, mitigating against major filtration induced artifact or injury.

ISC Characteristics
The erythrocyte morphology of ISC from the Nuclepore-separated moderate and marked ISC suspensions did not show major differences. In all

Fig. 1. Micropipette elastimetry differences were present between prefilter and postfilter sickle cells from Nuclepore-
separated moderate ISC preparations. The passage pressure (\( P \))
in cm H\(_2\)O (top) and extension length (\( T_m \)) in \( \mu \)m (bottom) of
discocyte and ISC are compared between the prefilter and post-
filter populations. Each bar graph is the mean ± 1 SD of 100
independent micropipette aspiration measurements from 6 Nucle-
pore separations. The narrow bars on the two vertical axis are the
means of 100 aspirations of control hemoglobin-A red cells.

Fig. 2. Nuclepore elastimetry tongue extension (\( T_n \)) of unsepa-
rated, prefilter, and postfilter ISC from marked ISC preparations.
The \( T_n \) of the prefilter ISC are significantly less than the postfilter
ISC (\( p < 0.05 \)). Each bar graph is the mean ± 1 SD of 140
independent Nuclepore cell aspiration measurements from 5
Nuclepore separations.
cases, plump and thin ISC were present in both the prefilter and postfilter populations in ratios similar to the unseparated suspension. As illustrated in the scanning electron micrographs of marked ISC prefilter and postfilter cells in Fig. 3, similar degrees of shape distortion were present in the separated populations. Certain differences in erythrocyte morphology were apparent, however, between the separated populations from the moderate ISC filtration separations. The prefilter population was higher in ISC percentage (65% ± 5%) than the postfilter (45% ± 7%) or unseparated sample (49% ± 6%), and the prefilter discocytes were more spherostomatocytic than the postfilter discocytes.

The separated populations from the marked ISC preparations were then analyzed for differences in cellular composition and geometry. MCHC and dextran 40 density profile gradients of prefilter, unseparated, and postfilter ISC were similar. As shown in Fig. 4, no significant differences were present in either density parameter between the experimental populations. In addition, no differences were observed between the isoelectric focusing hemoglobin composition of the prefilter (HbF 6.2% ± 1.5%, HbS 90.5% ± 2.1%) and postfilter ISC (HbF 6.5% ± 1.7%, HbS 90.1% ± 1.8%) as shown in Fig. 5. The MCV of the prefilter ISC was 68 ± 6 fl and was not different from the postfilter ISC MCV of 69 ± 4 fl. Supravital staining of the suspensions showed only an occasional cell with intracellular inclusions in either separated population. As shown in Fig. 6, the osmotic fragilities of the separated and unseparated samples in decreasing salt concentration were similar. Although the mean hemolysis of prefilter ISC was somewhat greater than that of postfilter or unseparated ISC at salt concentrations between 0.40 and 0.60 g/dl, the differences were statistically insignificant (p > 0.25).
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Fig. 5. Hemoglobin composition of Nuclepore-separated prefilter and postfilter ISC. Thin-layer isoelectric focusing patterns from two ISC separations illustrate the similarity between prefilter and postfilter ISC.

DISCUSSION

The present investigation has demonstrated that morphological methods are unable to distinguish ISC that have become stiffened from soft ISC, which are nearly as deformable as normal biconcave erythrocytes. Havell et al. previously reported that membrane rigidity was quantitatively increased in more severely deformed ISC in comparison to mildly poikilocytic or discoid cells. Our preliminary studies of whole cell deformability also demonstrated wide variation in elastic properties among sickle erythrocytes. However, it revealed only a slight tendency toward correlation with morphological distortion. Therefore, we developed a procedure for separating hard ISC from soft ISC by filtration through a 3 µm diameter Nuclepore membrane. The procedure was successful in separating a hard prefilter population from a soft postfilter population of ISCs. Whole cell micropipette and Nuclepore elastimetry of the isolated populations demonstrated significant differences in \( P, T_m, \) and \( T_N \) between hard prefilter and soft postfilter samples. However, there were no differences that could be defined in the morphology of the prefilter and postfilter ISC examined by light microscopy or in the scanning electron microscope. Thus, major differences in deformability exist among populations of ISC that are indistinguishable by morphological criteria.

The cellular deformability of erythrocytes is strongly influenced by intracellular viscosity, the surface area to volume ratio, and intrinsic membrane viscoelasticity. ISC have undergone several intracellular and surface membrane changes that influence deformability. ISC are shrunken cells that have lost water and volume as well as cations and have increased MCHC and intracellular viscosity. ISC have also taken up calcium, acquired an altered membrane cytoskeleton, redistributed membrane sialic acid and phospholipids, and have developed decreased membrane viscoelasticity. The relative contributions of these alterations to ISC deformability have been difficult to determine.

The micropipette and Nuclepore elastimetry systems used in these investigations assess whole cell deformability and might be influenced by any of the aforementioned deformability factors. The Nuclepore filter separated hard and soft ISC populations did not differ in MCHC, density profile gradient, or hemoglobin composition. Neither MCV nor osmotic fragility were discrepant between the separated ISC populations, mitigating against significant surface area to volume
ratio contributions to the deformability difference. The lack of alteration of intracellular viscosity or surface area volume parameters between the Nucleapore-separated hard and soft ISC populations points toward changes in membrane viscoelasticity as the basis for the deformability difference. A membrane elastimetry device is being established in the laboratory that will allow direct measurement of this parameter in future studies.

On the basis of available biochemical and deformability information, a schema for the evolution of sickle erythrocyte stiffness may be proposed. As the sickle cell progresses to the ISC and undergoes cation, water, and volume loss, a decrease in deformability ensues that correlates with the degree of morphological distortion and increase in MCHC. Clark, Mohandas, and Shohei have shown that normal whole cell deformability may be restored to ISC that have been rehydrated in hypotonic solutions to lower MCHC. These ektacytometer studies suggested that altered membrane viscoelasticity was not the primary determinant of decreased ISC deformability. Our observations suggest that as the result of a single critical event or accumulation of threshold events, a membrane deformability lesion(s) occurs that significantly hardens some ISC beyond the degree of their morphological distortion or change in their MCHC. This subpopulation of hard ISC may not be recognizable by ektacytometry because of its small proportion. An intriguing alternative possibility is that the membrane lesion can be reversed mechanically or biochemically by hypotonic stress. It is anticipated that the study of Nucleapore-separated ISC will provide new insights into the sequence of these events.

The percentage of ISC within individual patients is remarkably constant and has not been useful in predicting the frequency or severity of vaso-occlusive episodes. There has been a relationship, however, between ISC count and reduced red cell survival, conjunctival blood vessel abnormalities, and occurrence of spontaneous leg ulcers. These clinical impressions combined with the laboratory evidence that ISC have a faster sickling rate, increased adhesion, and reduced deformability continue to suggest a potential role for ISC in the pathogenesis of the disease. Verification of major deformability heterogeneity among ISC leads to the speculation that a smaller subpopulation of hard ISC may be more important to the pathophysiology of the disease than soft ISC. A peripheral blood smear ISC count would not be useful in defining this subpopulation among the ISC present on the smear, which may explain why total ISC counts do not correlate with the clinical severity of disease. The small group of patients examined in the study exhibited variations in the hard ISC fraction from ~5% to 20% of the total ISC filtered. Investigations are in progress to discover a more simple means of quantitating hard ISC to allow future studies on their possible correlation with clinical severity of sickle cell disease.

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