Increased Resistance to Membrane Deformation of Shape-Transformed Human Red Blood Cells

By Anne Chabanel, Walter Reinhart, and Shu Chien

Evidence shows that shape changes of the erythrocyte found in clinical conditions or induced by experimental manipulation are associated with alterations in erythrocyte rheology, which may have detrimental influences on the microcirculation. Abnormal rheologic behavior of non-discocytic, pathologic erythrocytes have been described in several hematological disorders, including sickle cell disease, hereditary elliptocytosis, hereditary spherocytosis, and hereditary stomatocytosis. Alterations in erythrocyte shape can also be induced by a variety of chemical agents. Treatment of fresh human erythrocytes with lysolecithin, olate, sodium salicylate (SA), alkaline medium, or the Ca²⁺-ionophore A23187 as well as ATP depletion, can lead to crenation, ie, formation of echinocytes. On the other hand, erythrocytes become cup-shaped, ie, stomatocytes, following treatment with chlorpromazine or local anesthetics, as well as from cholesterol depletion. These shape changes occur rapidly and are reversible if the distortion is not carried out to the spherocyte stage. These shape transformations might be expected to cause alterations in the rheological properties of erythrocytes through direct modification of the cell geometry (surface area/volume ratio) or through associated alterations of the membrane skeleton. Indeed, several groups have reported that drug-induced shape changes of erythrocytes are accompanied by alterations in the rheological properties of erythrocyte suspensions. Reinhart and Chien found that erythrocyte passage through a narrow channel is impaired for stomatocytes and facilitated for echinocytes; these changes in rheological behavior were explained by altered surface area/volume relationship, as the isovolumic shape transformations were accompanied by a loss of surface area in stomatocytes and a gain of surface area in echinocytes. Because microsieving measurements cannot clearly dissociate the contributions of the various determinants of cell deformability (cell geometry, internal cell viscosity, and membrane mechanical properties), the present experiments were performed to investigate directly the membrane mechanical properties of shape-transformed erythrocytes by using the micropipette aspiration technique. Indeed, the micropipette test is suited to the study of membrane mechanical properties since it has been shown that the viscous dissipation inside the RBC in a small deformation during aspiration into a micropipette is negligible under isotonic conditions.

Materials and Methods

RBC suspensions. Blood was drawn from healthy human volunteers into heparinized vacutainer tubes. The blood was centrifuged at 1,500 x g for 10 minutes, and the plasma and the buffy coat were removed. The erythrocytes were washed three times in a standard buffer used throughout this study; this buffer contained 0.85 g/dL of NaCl, 0.25 g/dL of bovine serum albumin (BSA) and 0.1 g/dL of EDTA; its pH was adjusted to 7.4 with 0.1 N Tris. The washed erythrocytes were resuspended in the buffer at a hematocrit of 10%.

Stomatocytes were produced in vitro with chlorpromazine hydrochloride (CP), and echinocytes were produced with SA. A calculated volume of the suspending medium of the 10% erythrocyte suspension was replaced by an equal volume of stock solution of CP (1.5 mmol/L) or SA (160 mmol/L). The suspensions were incubated for 20 minutes at room temperature and then used for measurements. ATP-depleted cells were obtained by incubation of whole blood under sterile conditions for 24 hours at 37°C. The depleted erythrocytes were resuspended in the autologous plasma to a hematocrit of 10%.

Micropipette technique. The micropipette technique has been described elsewhere. Micropipettes with an internal radius ($R_i$) of 0.45 to 0.70 μm were prepared with a micropipette puller (Narishige Scientific Instrument Laboratory, Tokyo). The micropipette was filled with the buffer solution and mounted on a micromanipulator (Narishige). The wide end of the micropipette was connected to a pressure-regulating system, which consisted of two reservoir bottles and a damping chamber. By adjustment of the relative heights of the reservoir bottles with a micrometer device, desired pressure levels were preset and then imposed on the micropipette by turning a stopcock. The applied pressure was measured with a transducer (model 23 BC, Statham Instrument, Oxnard, CA) and recorded with an amplifier-recorder system (Gould, Cleveland). A suspension

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of erythrocytes at a hematocrit level of ~0.05% was placed in a small round chamber located on the stage of a Nikon inverted microscope (Ehrenreich Photo-Optical, Garden City, NY). The erythrocytes were viewed with the use of a 100 x objective and a 20 x eye piece. The image was recorded with a video camera and tape recorder system (Panasonic, Division of Matsuchita Electric of America, Franklin Park, IL). The micropipette tip was manipulated for positioning at the surface of the erythrocyte membrane. A small portion of the erythrocyte was aspirated by a preset negative pressure for 20 seconds. The length of the aspirated tongue and the radius of the pipette were measured on a television screen. The membrane extensional rigidity, which reflects the steady-state resistance to deformation, was calculated from the relationship between the stress applied, \( (\Delta P)R_p \), and the strain induced, \( D_{pm}/R_p \) with \( \Delta P \) the applied negative pressure, \( R_p \) the internal radius of the micropipette, and \( D_{pm} \) the maximum length of the aspirated portion within the micropipette. When the aspiration pressure was removed, the deformed erythrocyte segment in the micropipette decreased in length with time, and the cell recovered its original shape. All measurements were made at room temperature (21 to 24 °C). For each experiment, the same micropipette was used to test shape-transformed RBCs and control RBCs. Statistical analysis of the data was performed by comparison of the slope of the linear regression using a paired Student's \( t \) test or by analysis of variance followed by the Newman-Keuls test.

Scanning electron microscopy. RBC specimens were fixed in 1.0% glutaraldehyde in cacodylate buffer. After two cacodylate buffer rinses, the specimens were processed through an ethanol dehydration series from 30% to 100% ethanol. The cells were resuspended in 100% ethanol and gently dropped onto a glass coverslip that had been cleaned with ethanol. After being air-dried, the coverslip with cells was glued onto aluminum stubs (JEOL USA, Electron Optics Division, Peabody, MA) with silver conductive paint. The stubs with samples were coated with gold-palladium in a sputterer (Hummer I; Technics, Alexandria, VA), and the specimens were viewed and photographed in a scanning electron microscope (JEOL35; JEOL USA) at 25 kV.

RESULTS

Bessis' nomenclature was used to classify erythrocytes according to their shape. Thus, a discocyte is a normal biconcave RBC with a smooth contour, echinocyte I is a flat erythrocyte with an irregular contour, echinocyte II is a flat erythrocyte with one or several spicules, and echinocyte III is an ovoid or spherical cell with numerous spicules evenly distributed over its surface. Stomatocytes are bowl-shaped cells with a single concavity (stoma); the concavity is large and shallow in stomatocytes I, deep and narrow like a token-slit in stomatocytes II, and deep and folded in stomatocytes III. Spherostomatocytes have a smaller diameter and small irregular invaginations. The morphology corresponding to this classification is illustrated in Fig 1.

Erythrocytes incubated with 25 or 100 \( \mu \)mol/L of CP produced stomatocytes I through III. Incubation of erythrocytes with 20 mmol/L of SA or depletion of their ATP content for 24 hours at 37 °C produced echinocytes over the whole range from echinocytes I to spherocytic RBCs. The micropipette test was carried out on every type of echinocyte. Their mechanical behavior can be compared by plotting the steady-state deformation (\( D_{pm} \)) normalized for the pipette radius (\( R_p \)) against the stress parameter (\( \Delta P \)). When the aspiration was performed at a spicule site, the length of the protrusion inside the pipette prior to the application of aspiration pressure was subtracted from the length of the tongue induced by the negative pressure to give the steady-state deformation (\( D_{pm} \)). Figure 2 shows the results for the echinocytes. The slope (K) of the stress-strain relationship was obtained by linear regression analysis of the data on \( D_{pm}/R_p \) vs (\( \Delta P \)). The extensional rigidity was calculated as \( 1/0.245 K \); the value of 0.245 was based on the model described in ref. 20. Because the geometry at the point of aspiration is not an infinite plane, in shape-transformed RBCs we could not readily use the mathematical model to derive values for the intrinsic membrane elastic modulus; ie, due to the geometry of the cell, the micropipette test cannot distinguish between an effect on the membrane elastic modulus itself and a consequence in overall cell geometry that might generate local membrane tensions. By analogy with the work of Evans and colleagues and Nash and co-workers, who studied sickle cells (another type of abnormally shaped RBC) by micropipette test, we used values for the extensional rigidity to allow simple comparison of the resistance to deformation of shape-transformed RBCs and control discocytes. Extensional rigidity significantly and progressively increased with the degree of echinocytosis (Table 1).
RBC SHAPE AND MEMBRANE DEFORMABILITY

Fig 2. Strain \( \frac{D_{\text{mem}}}{R} \)-stress \( \Delta P \times R \) relationship for discocytes (C, O), and echinocytes type I (E1, O), type II (EII, A), and type III (EIII, O). Lines represent linear regressions computed from \( n \) data pairs for each group (\( n \geq 55 \)). Data points were grouped according to \( \Delta P \times R \) with mean \( \pm \) SEM shown for each group.

There was no significant difference in membrane rheological behavior between the echinocytes induced by SA or ATP depletion (Table 1). Less stress was required to deform an echinocyte between the spicules than at a spicule site (Table 1). The intercept on the strain axis (ordinate) at zero stress decreased with the degree of echinocytosis (Fig 2).

Because it was often difficult under light microscopy to determine the degree of stomatocytosis, stomatocytes I through III were grouped together in the analysis. Figure 3 compares the elastic behavior of stomatocytes I through III and spherostomatocytes induced by different concentrations of CP to that of control discocytes. The value of extensional rigidity for the control cells in the stomatocyte set of experiments (Table 2) was different from that in the echinocyte set of experiments (Table 1). These two sets of experiments were performed on different days with different blood donors. The overall range of values for extensional rigidity for the control cells was \( 3 \times 10^{-3} \) to \( 12 \times 10^{-3} \) dynes/cm. The stress needed to obtain a comparable \( D_{\text{mem}}/R \) was approximately three times greater for stomatocytes I through III than for discocytes, and it was \( \sim 30 \) times greater for spherostomatocytes than for discocytes (Fig 3). Values for the extensional rigidity are shown in Table 2.

These data show that both the discocyte–echinocyte and the discocyte–stomatocyte transformations resulted in increases of membrane extensional rigidity. The membrane rheological behavior of erythrocytes that had been transformed to echinocytes by SA (20 mmol/L) and subsequently returned to a discocytic shape by addition of CP (2.5 \( \mu \)mol/L) was also studied (Fig 4). With the insertion of both of these drugs in the erythrocyte membrane, the rheological behavior of the membrane returned to normal as the discocytic shape was recovered.

**DISCUSSION**

This study demonstrates that membrane deformability is decreased in shape-transformed erythrocytes (both echinocytes and stomatocytes) as assessed by the micropipette technique. Although no data exist on stomatocytes, several groups have studied echinocytes. Leblond, using a micropipette with a diameter of 1.5 \( \mu \)m, found a decreased membrane deformability in echinocytes induced by oleate, high pH, aged plasma, and ATP depletion, which is in agreement with our results. Baker, measuring erythrocyte deformabil-

### Table 1. Comparison of Membrane Elasticity Between Discocytes and Echinocytes

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Cells Tested</th>
<th>Extensional Rigidity* (10^(-3) dynes/cm)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Discocytes</td>
<td>55</td>
<td>3.8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Echinocytes I</td>
<td>25</td>
<td>5.3 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Echinocytes II</td>
<td>55</td>
<td>6.8 ± 0.4</td>
<td>&lt;0.05†</td>
</tr>
<tr>
<td>Echinocytes III</td>
<td>53</td>
<td>9.6 ± 1.0</td>
<td>&lt;0.05†</td>
</tr>
<tr>
<td>Comparison of different sites on echinocytes II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between spicules</td>
<td>16</td>
<td>4.5 ± 0.3</td>
<td>&lt;0.05‡</td>
</tr>
<tr>
<td>At spicules</td>
<td>55</td>
<td>6.8 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Comparison of two methods for producing echinocytes III</td>
<td>36</td>
<td>7.6 ± 0.7</td>
<td>NS‡</td>
</tr>
<tr>
<td>Salicylate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP depletion</td>
<td>17</td>
<td>8.9 ± 1.1</td>
<td></td>
</tr>
</tbody>
</table>

*Values are mean \( \pm \) SEM.

†P values are based on an analysis of variance followed by the Newman-Keuls test for the different types of echinocytes as compared with discocytes.22

‡P values are based on Student's t test (two-sided).21

### Table 2. Comparison of Membrane Elasticity Between Discocytes and Stomatocytes

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Cells Tested</th>
<th>Extensional Rigidity* (10^(-3) dynes/cm)</th>
<th>P values†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Discocytes</td>
<td>50</td>
<td>7.5 ± 0.4</td>
<td>—</td>
</tr>
<tr>
<td>Stomatocytes I–III</td>
<td>94</td>
<td>29.8 ± 1.7</td>
<td>0.01</td>
</tr>
<tr>
<td>Spherostomatocytes</td>
<td>16</td>
<td>163.9 ± 20.4</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*Values are mean \( \pm \) SEM.

†P values are based on an analysis of variance followed by a Newman-Keuls test for the different types of stomatocytes as compared with discocytes.22
chlorpromazine (+

Fig 4. Normalized membrane deformation (\(\Delta D_{m}/R_p\)) at two different deforming stresses (\(\Delta P \times R_p\)) for control discocytes (C), echinocytes produced by 20 mmol/L of salicylate (SA), and after biconcave shape recovery with the addition of 2.5 mmol/L chlorpromazine (SA + CP). The vertical bars represent SEM.

ity by aspiration in Nuclepore filters with 0.6-μm pores, found that ATP-depleted erythrocytes were 16% less deformable than fresh cells. Meiselman and co-workers,27 however, using the micropipette test, reported that the decreased membrane deformability of ATP-depleted erythrocytes was not statistically significant. In these two last studies, the authors did not report any correction for the length of the spicule at rest inside the micropipette or the filter pore. The absence of such correction would lead to an overestimation of the length of the pressure-induced deformations and an underestimation of the elastic modulus for the echinocytes; this may explain the lesser effects seen in their results as compared with the present data. When we subtracted the mean spicule length from the pressure-induced pseudopod length, using Baker’s technique with Nuclepore filters,26 the membrane deformability of echinocytes also decreased (W. Reinhart, A. Chabanel, and S. Chien, unpublished observations, April 1985) to an extent similar to that found by the micropipette technique in the present study. Leblond6 reported that ATP-depleted cells were much more rigid than drug-crenated cells. Although we also observed a greater rigidity of the ATP-depleted echinocytes as compared with the echinocytes induced by SA, the difference was not statistically significant. Determining the deformability of resealed human erythrocyte ghosts in the ektacytometer, Shrier38 found that the echinocytic ghosts were more deformable than the discocytic ghosts, whether the ghosts were prepared from discocytic or echinocytic erythrocytes. He concluded that acquisition of the discocytic shape in ghosts is accompanied by an increase in membrane rigidity. The reason for the discrepancy between his results and ours is not clear. One possible explanation is that the influence of the cellular geometry is dominant over that of membrane deformability in measurements performed with the ektacytometer. In that case, Schrier’s results are consistent with the results of Reinhart and Chien18 on filtration through narrow pores, in which cell geometry is a major determinant of filterability; they found that echinocyte passage through narrow pores is facilitated rather than retarded as compared with that of discocytes. Meiselman and co-workers reported that the suspension viscosity of echinocytes induced isovolumically by 2,4-dinitrophenol, 2,4,6-trinitrophenol, or SA33 and by ATP depletion15 was higher than that of normal cell suspensions at low shear rates but nearly normal at high shear rates. Because it was believed that change in cellular deformability would be reflected by a change in suspension viscosity at high shear rate, they concluded that altered morphology, rather than erythrocyte membrane deformability, was the cause of the rheologic changes. Minor or moderate changes in membrane rheology, however, may be detected by suspension viscosity measurements only at low or medium shear rates.29 Considered thus the viscometric results obtained by Meiselman and Baker15,17 are in accord with our micropipette measurements in showing that shape transformation of erythrocytes results in an increased resistance to membrane extension.

This increase in resistance to membrane extension could be the consequence of (a) changes in the molecular organization of the membrane due to drug insertion or ATP depletion and/or (b) changes in the physical properties due to modification of the cell shape. Several groups have reported changes of the protein–lipid interface induced by CP,36–38 suggesting that interaction between the drugs (CP or SA) and the erythrocyte membrane constituents may lead to the increase in membrane rigidity. When both drugs were incorporated to cause the recovery of normal biconcave cell shape, we found that membrane deformability also returned to normal. This is in agreement with the results of Meiselman,17 who showed that both the morphology and rheology of 2,4-dinitrophenol-treated erythrocytes could be returned toward those of fresh control erythrocytes by addition of the antagonist chlorpromazine. Although we cannot rule out the possibility that when both drugs are added they have mutually compensating effects in their interaction with the membrane constituents, our data seem to support the concept that erythrocyte morphology per se has an important relation to the membrane mechanical properties. Leblond6 has proposed that the increased resistance to deformation observed in echinocytes may reflect the presence of new forces whose action is to produce foldings on the cell surface and hence to resist extension into the micropipette in response to pressure aspiration. We found that the intercept on the strain axis at zero stress decreased with the degree of echinocytosis (Fig 2), indicating that increasing stresses are required to reach a given extensional deformation. This might reflect an increase in the resting membrane tension as proposed by Leblond.6 Although we do not know if the spicule sites are predetermined, the present data show that the formation of spicules on an erythrocyte results in regional variations of physical properties on the membrane.

In conclusion, the present data demonstrate that changes in local membrane properties associated with overall erythrocyte shape transformation affect its membrane deformability. The decrease in erythrocyte membrane deformability following shape transformation could be of importance in the microcirculation when the erythrocyte must deform to negotiate its entrance into narrow capillaries.
REFERENCES


Increased resistance to membrane deformation of shape-transformed human red blood cells [published erratum appears in Blood 1987 Sep;70(3):893]

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