Membrane Deformability and the Capacity for Shape Change in the Erythrocyte

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Erythrocytes must have the capacity to undergo marked membrane deformation and shape changes in order to circulate through capillaries and respond to a range of shear stresses. To study the interrelationships between membrane deformability and the capacity for shape transformation, we created rigid membranes using several agents and then examined the ability of these erythrocytes with rigid membranes to undergo amphiphath-induced shape change. We have previously shown that wheat germ agglutinin (WGA) and a monoclonal antibody to glycoporphin A (R-10) cause membrane rigidity as measured by ektacymotery. Experiments were therefore designed to produce comparably rigid membranes using WGA, R-10, and diamide, and then to test the ability of lysophosphatidylcholine to produce echinocytes, and primaquine to produce stomatocytes. We found that diamide treatment substantially blocked both types of shape change. In contrast, R-10 binding did not impair either primaquine- or lysophosphatidylcholine-induced shape change. Further, WGA blocked echinocyte transformation, as previously reported, but not stomatocytosis. Using reduced and unreduced gel electrophoresis and Triton extraction, we compared the biochemical changes associated with WGA-, diamide-, and R-10-induced rigidity, and found them to be different. We conclude that not all rigid cells are incapable of shape change, and therefore that decreased membrane deformability is not predictive of impaired capacity for shape change.

In order for the erythrocyte to repeatedly traverse 2 to 3 μm capillaries and respond to a range of shear stresses, it must have the capacity to undergo marked membrane deformation and shape change. An interrelationship between the cell’s capacity for shape change and the deformability of its membrane necessarily exists. Clinical disorders of the red cell such as α and β thalassemia and sickle cell anemia result in decreased membrane deformability; however, it is not clear in these instances whether there is a parallel loss of the capacity for shape transformation. An inability to alter shape could contribute to the early removal of these pathologic red cells from the circulation. In vitro studies have shown that when erythrocyte membrane proteins are crosslinked by the sulfhydryl oxidizing agent, diamide, the cells become nondeformable and, in parallel, incapable of undergoing amphiphath-induced shape change. Therefore, we were interested in determining whether erythrocytes rendered nondeformable by other means were also resistant to shape transformation. To address this question, we used diamide as well as two additional agents, the lectin wheat germ agglutinin (WGA) and a monoclonal antibody (MoAb) to glycoporphin A (R-10), which we have previously shown induce membrane rigidity. In the current studies, we first produced rigid membranes with WGA, R-10, and diamide. We then examined the effect on these erythrocytes of lysophosphatidylcholine (LPC), which normally induces the discocyte to stomatocyte transformation. We found that LPC and primaquine evoked different responses in the three types of rigid membranes. Diamide-treated erythrocytes resisted the ability of LPC to produce echinocytes and primaquine to produce stomatocytes. In contrast, R-10 did not impair the LPC- or primaquine-induced shape change, while WGA blocked LPC-induced echinocytosis, as previously reported, but not primaquine-induced stomatocytosis. The biochemical mechanisms by which R-10, WGA, and diamide induced membrane rigidity appeared different for each of the three agents. These results imply that different biochemical perturbations produce rigid membranes that differ from one another in their capacity for shape transformation. We conclude that a decrease in membrane deformability is not necessarily associated with an impairment in shape transformation.

Materials and Methods

Reagents. Diamide and primaquine were purchased from Sigma Chemical Co, St. Louis, MO; dextran from Pharmacia Fine Chemicals, Uppala, Sweden; WGA from Vector Laboratories, Inc, Burlingame, CA; lysolecithin from General Biochemicals, Laboratory Park, Chagrin Falls, OH; lysopalmitoyl phosphatidylcholine L-1-[PAL-mitoyl-14C]- and Biofluor from New England Nuclear, Boston, MA; Silicon Oil-Dow Corning from Wm. F. Nye, New Bedford, MA.

Induction of membrane rigidity. Blood from normal volunteers was drawn into heparinized tubes after informed consent was obtained, according to protocols approved by the Human Experimentation Committees of Stanford University and the University of California, San Francisco. The erythrocytes were washed three times in 5 mmol/L Tris, 140 mmol/L NaCl (pH 7.4), and then resuspended in WGA (1.0 μg/mL) in phosphate-buffered saline (PBS), pH 7.4 or R-10 (60 μg/mL) in PBS, pH 7.4, or diamide (0.375 to 0.75 mmol/L) in 90 mmol/L KCl, 45 mmol/L NaCl, 44 mmol/L sucrose, 10 mmol/L NaPO4, pH 8.0. The WGA and R-10 samples were incubated for 30 minutes at room temperature, while the diamide samples were incubated for 1 hour at 37°C. Following incubation, one aliquot of each sample was assayed for deformability using the ektacytometer, while a second aliquot was treated with primaquine or LPC.

Measurement of deformability. The deformability of the red cells treated with R-10, diamide, and WGA was measured by ektacytometry, a laser diffraction method previously described. In
brief, suspended cells are exposed to an increasing shear stress (0 to 400 dyne/cm²) and the change in their laser diffraction pattern from circular to elliptical is measured. This photometric measurement produces a signal, designated deformability index, which quantitates cell ellipticity. By an automatic image analysis system, the deformability index is recorded as a continuous function of applied shear stress. The deformability index curve thus produced is a measure of the ability of the cells to deform.

**Assay of capacity for shape change.** Following the incubations in diamide, WGA, and R-10, 0.1 mL (Hct 40%) was taken from each sample and incubated in 0.5 mL of either 1.5 to 3.0 mmol/L primaquine in PBS, 4 to 10 μg LPC/mL PBS or PBS alone. After 5 minutes of incubation at 37°C with LPC and 30 to 60 minutes of incubation with primaquine, 20 μL aliquots were removed and placed in 200 μL of 1% glutaraldehyde in PBS for phase microscopic examination. The remaining samples were then washed once with 1% bovine serum albumin (BSA) in PBS, and 20 μL aliquots added to 200 μL of 1% glutaraldehyde in PBS for further phase microscopic evaluations. Microscopic evaluation of the fixed cells on poly L-lysine-covered slides was done under phase microscopy at 450× and 1,000× and the erythrocyte shapes were classified as described by Bessis. Photographs were taken using Panatomic X film (Eastman Kodak Co, Rochester, NY).

**Measurement of lysophosphatidylcholine uptake.** The uptake of 14C-LPC into control red cells and red cells pretreated with diamide, WGA, and R-10 was measured using 14C-palmitoyl LC-LPC. To obtain these measurements, the radioisotope was added to stock solutions of LPC to provide specific activities of 27,000 to 60,000 cpm/nmol. A 0.1 mL volume (Hct 40%) of R-10–WGA–diamide-treated and untreated control red cells was incubated at 37°C for 5 minutes in 0.5 mL (4 to 8 μg LPC/mL) of the radiolabeled LPC mixture. Following the incubation, samples were layered on 0.5 mL of silicon oil and centrifuged in the Eppendorf centrifuge for 10 minutes at full speed. The supernatant fraction was distinguished as a clear layer above the oil, while the red cells formed a distinct pellet below the oil. All the fractions were solubilized in 1 mL of Protosol:ethanol (1:2) at 37°C and then 0.3 mL of 30% H2O2 and 0.3 mL of 6 N HC1. The mixture was allowed to cool for 2 hours and then the radioisotopic activity was determined by liquid scintillation spectrometry.

**Triton X-100 extraction of erythrocytes.** Erythrocytes were extracted with Triton by a previously described method. In brief, 2.4 mL of diamide- and WGA-pretreated cells (Hct 65%) were lysed in Triton X-100 (100 mg/10⁶ cells) in 140 mmol/L KCl, 20 mmol/L Hepes, 0.5 mmol/L MgCl₂, 1 mmol/L EGTA, 0.05 mmol/L CaCl₂, 2 mmol/L reduced glutathione, and 0.03 mmol/L phenylmethylsulfonyl fluoride. The resulting lysate was layered on a linear sucrose-density gradient (10% to 60%) and centrifuged for 1 hour at 22,000 rpm in a Beckman L3-50 ultracentrifuge (Beckman Instruments, Inc, Fullerton, CA). The visible band in each gradient was collected and resuspended in 140 mmol/L KCl, 20 mmol/L Hepes, 1 mmol/L EDTA, 2 mmol/L reduced glutathione, and 0.03 mmol/L phenylmethylsulfonyl fluoride (pH 7.4) and centrifuged for 30 minutes at 22,000 rpm. The pellet was then solubilized and analyzed by electrophoresis as described below.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.** Samples for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were solubilized in 0.5 mol/L Tris-Cl, pH 6.8, 1.25% sodium dodecyl sulfate, 0.38 mol/L dithiothreitol, and heated to 100°C for 2 minutes. They were then analyzed in the discontinuous system of Laemmli26 on slab gels, composed of a 10% acrylamide separating gel and a 3% acrylamide stacking gel. After electrophoresis, the gels were fixed and stained for protein with Coomassie blue or for carbohydrate with periodic acid-Schiff reagent.11

**RESULTS**

**Effect of membrane rigidity on cell shape change.** In order to measure the rigidity of the diamide-, R-10–, and WGA-treated cells, an aliquot from each sample was assayed by ektacytometry. The effects of diamide, WGA, and R-10 on red cell deformability are shown in Fig 1. In this figure, the deformability index is plotted as a function of the logarithm of the shear stress. The deformability of normal control cells, as shown in the left-hand curve, was normal in response to low shear stress. Cells pretreated with WGA required a greater amount of shear stress to produce the same degree of deformation. Since the lines are parallel, one can calculate that cells treated with WGA required fourfold greater shear stress than normal cells to reach equivalent deformation at all points along the curve, indicating that these cells were four times more rigid than control cells. Treatment of erythrocytes with 0.375 mmol/L diamide produced cells eight times more rigid; R-10 produced cells 10 times more rigid; and 0.75 mmol/L diamide produced cells 19.6 times more rigid than controls. These results show that a substantial increase in membrane rigidity was induced by each of these agents. It should be noted that WGA-induced rigidity is independent of ABO blood type and that, although higher concentrations of WGA have been shown to cause even greater rigidity,2 these concentrations could not be used in our experiments because they induce agglutination.

To test the capacity of these pretreated cells for shape change, we exposed them to either LPC or primaquine, agents classically known to induce echinocytosis and stomatocytosis, respectively.2,6,12 The response of the cells is depicted in Fig 2. Control cells became stomatocytic after treatment with 2 mmol/L primaquine, and echinocytic (stage 3) after treatment with LPC (4 μg/mL). For cells treated with the lower concentration of diamide (0.375 mmol/L), shape change was substantially inhibited. In contrast, erythrocytes pretreated with the antiglycophorin IgG, R-10, and ten times more rigid than controls, were as capable as control cells of responding to LPC and primaquine. Because of the concern that the addition of primaquine or LPC might affect the action of R-10, the deformability of both LPC- and primaquine-treated cells was remeasured by ektacytometry. Cells with R-10 alone, R-10 plus LPC, and R-10 plus primaquine all had similar degrees of rigidity. Hence, the rigidity induced by R-10 was not altered by the addition of either LPC or primaquine. The effect of WGA pretreatment is shown in Fig 2. As has been previously reported by Lovrien et al,7 the response of these cells to LPC was substantially inhibited. Seventy-five percent of the control cells became stage 3 echinocytes or spherocinocytes, whereas only 33% of the WGA-treated cells became stage 3 echinocytes. However, surprisingly, the WGA-treated cells were fully capable of becoming stomatocytic in response to primaquine.

The results observed with diamide- and R-10–treated erythrocytes imply that cells with similar degrees of decreased membrane deformability can have marked differ-
ences in their capacities to undergo shape change. Further, our observations following WGA binding imply that there can be an uncoupling of the bilayer response, since the introduction of primaquine into the inner bilayer resulted in unimpaired stomatocytosis, but the introduction of LPC into the outer bilayer failed to induce equivalent echinocytosis. 

Effect of antibody, WGA, and diamide on LPC uptake. To determine whether the observed differences in the response to R-10-, WGA- and diamide-treated cells to LPC was due to differences in uptake of the amphipath, we performed uptake experiments with radiolabeled LPC. As shown in Table 1, the diamide- and WGA-treated erythrocytes incorporated amounts of LPC comparable to control in Table I shown our observations following WGA binding imply that there

Fig 1. Effect of diamide, WGA, and R-10 on erythrocyte deformability. Erythrocytes exposed to diamide, WGA, and R-10 required significantly higher values of applied shear stress as compared with normal membranes to reach equivalent deformation. 

Effect of diamide and WGA on protein-protein associations. It has been shown previously that as a sulfhydryl oxidant, diamide induces the formation of intermolecular and intramolecular disulfide bonds, thereby increasing protein-protein associations. In order to determine whether this effect was indeed occurring at the concentrations of diamide producing 8 and 19 times increased rigidity, we compared diamide-treated membranes in the reduced and unreduced state by means of SDS-PAGE. As shown in Fig 3, the membranes treated with diamide contained high molecular weight complexes that disappeared when the membranes were reduced with dithiothreitol before electrophoresis. To examine whether WGA binding also causes the formation of high molecular weight complexes, WGA-treated membranes were analyzed by electrophoresis in the presence and absence of the reducing agent, dithiothreitol (Fig 3). In contrast to diamide, WGA did not induce any high molecular weight complexes. These results imply that the observed changes in membrane properties induced by diamide and WGA are effected through different molecular mechanisms.

DISCUSSION

Red cells in the circulation spend little time as discocytes. In the microcirculation, the red cell folds longitudinally and moves down the capillary by “tank treading,” while in the
arterial bed, applied shear stresses normally produce an ellipsoid. Therefore, shape alteration is a necessary physiologic process. There are several determinants of the intact red cell's ability to deform and recoil, including cytoplasmic viscosity, surface area-to-volume ratio, and membrane deformability. In this study, we examined the property of membrane deformability and its influence on the capacity of the red cell to undergo shape change.

In previous studies, we have shown that WGA, diamide, and the MoAb R-10 each decreases whole cell deformability by decreasing membrane deformability. Contributions to the decreased cellular deformability from either changes in

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**Fig 2.** Response of R-1O-, WGA-, and diamide-treated erythrocytes to primaquine and LPC. In control cells, primaquine (2 mmol/L) induced stomatocytes, and LPC (4 µg/mL) induced stage 3 echinocytes. Erythrocytes pretreated with the antiglycophorin IgG, R-10, and 10× more rigid than controls were as capable as control cells of shape change. WGA-treated cells became stomatocytic in response to primaquine; however, the response to LPC was substantially blocked. In cells treated with even the lower concentration of diamide, shape change was substantially inhibited.
The membranes were reduced with dithiothreitol before electrophoresis. WGA binding did not induce high molecular weight complexes.

**Table 1. Uptake of Radiolabeled LPC by Erythrocytes**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>nm LPC/mL Packed Erythrocytes</th>
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<tbody>
<tr>
<td>Control erythrocytes</td>
<td>105</td>
</tr>
<tr>
<td>R-10–treated erythrocytes</td>
<td>98</td>
</tr>
<tr>
<td>Control erythrocytes</td>
<td>93</td>
</tr>
<tr>
<td>R-10–treated erythrocytes</td>
<td>151</td>
</tr>
<tr>
<td>Control erythrocytes</td>
<td>155</td>
</tr>
<tr>
<td>Diamide-treated erythrocytes</td>
<td>162</td>
</tr>
<tr>
<td>(0.5 mmol/L)</td>
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</tr>
<tr>
<td>Diamide-treated erythrocytes</td>
<td>164</td>
</tr>
<tr>
<td>(1.0 mmol/L)</td>
<td></td>
</tr>
<tr>
<td>Control erythrocytes</td>
<td>146</td>
</tr>
<tr>
<td>WGA-treated erythrocytes</td>
<td>139</td>
</tr>
<tr>
<td>(1.0 μg/mL)</td>
<td></td>
</tr>
<tr>
<td>WGA-treated erythrocytes</td>
<td>148</td>
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<tr>
<td>(1.5 μg/mL)</td>
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Cytoplasmic viscosity or surface area-to-volume ratio were excluded in these studies by directly measuring the deformability of ressealed membranes. These earlier results enabled us to perform the current studies on intact red cells with the knowledge that R-10, diamide, and WGA were each influencing whole cell deformability by decreasing membrane deformability.

Erythrocyte membrane deformability can be described as a composite of various membrane material properties. The properties that characterize a membrane with elastic behavior include extensional modulus, bending modulus, area compressibility modulus, and coefficient of surface viscosity in shear. The decreased membrane deformability observed in WGA-, R-10–, and diamide-treated erythrocytes resulted in part from an increase in the extensional modulus. In addition, these agents may well affect membrane deformability by altering the area compressibility and bending stiffness of the membranes differentially. In the normal cell, primaquine intercalates into the inner monolayer of the erythrocyte lipid bilayer resulting in stomatocytosis, while LPC intercalates into the outer monolayer inducing an echinocyte. In order for the membrane to form a positive curvature, as in echinocyte formation, the outer layer of the membrane must expand while the inner layer compresses. Likewise, in order for the membrane to form a negative curvature, such as in a stomatocyte, the inner aspect of the membrane must expand while the outer aspect compresses. Therefore, area compressibility and bending stiffness are important properties involved in discocyte to stomatocyte and discocyte to echinocyte shape transformations. In order for LPC or primaquine to intercalate into either of the two monolayers, that particular monolayer must be capable of undergoing expansion. We suggest that WGA, R-10 antibody, and diamide affect the expansibility of the two mono-layers differently, and thereby influence the localization of primaquine and LPC. The lectin, WGA, binds not only to the N-acetylglucosamine residues on glycoporphin, but can also self-associate, thus producing a lattice at the outer surface of the red cell. Such a lattice might well interfere with the expansion of the outer monolayer, thereby inhibiting the intercalation of LPC into this leaflet of the bilayer. However, such a lattice might not inhibit the expansion of the inner monolayer and would permit intercalation of primaquine. Additionally, a WGA lattice might well change the bending stiffness of the membrane, opposing outward bending but not affecting inward bending. Consistent with this model, we observed that WGA does not inhibit primaquine-induced stomatocytosis but does impair LPC-induced echinocytosis. The sulfhydryl oxidizing agent, diamide, induces multiple protein-protein associations involving components on both the inner and outer portion of the red cell membrane. These covalent protein-protein associations would not only interfere with extensional deformation, but might also decrease area compressibility of both lipid monolayers, thereby impairing both echinocytosis and stomatocytosis. In contrast, the anti-glycoporphin antibody R-10 induces an increased association of the integral membrane protein glycoporphin A with the spectrin network in the cytoskeleton. R-10, therefore, may increase the extensional rigidity of the spectrin network, producing membrane rigidity, but allow the normally observed intercalation of LPC and primaquine into the outer and inner monolayers, respectively, and not alter area compressibility or bending stiffness. That such is the case is suggested by the observed ability of rigid R-10–treated...
The data presented here show clearly that the relationship between shape change and membrane deformability is a complex one, and that decreased membrane deformability is not predictive of impaired capacity for shape change.

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References

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