Glucose-6-Phosphate-Dehydrogenase-Deficient Erythrocytes Have an Impaired Shape Recovery Mechanism

By Eytan Alhanaty, Michael Snyder, and Michael P. Sheetz

In the human erythrocyte, the maintenance of the biconcave disc shape is important for cell viability as well as cell function. Previous studies have indicated the involvement of the hexose monophosphate shunt in the recovery of discoid shape after perturbation of echinocytic agents. In glucose-6-phosphate-dehydrogenase-deficient (Gd-) erythrocytes, the shunt activity is significantly decreased; thus, it might be expected that the shape recovery rate of Gd- erythrocytes would be decreased. We show here that shape recovery rates in the presence of the shunt stimulator methylene blue are as much as fivefold lower in Gd- erythrocytes. We also show that the protease inhibitor, Nα-tosyl-1-phenylalanine-chloromethyl ketone, has no effect on shape recovery in Gd-, whereas it increases normal cell shape recovery rates by 10–30-fold at 50 μM and causes cupping at 200 μM (see companion article by Alhanaty et al.). These changes are not due to reticulocytosis, as other hemolytic disorders do not show such changes. Further, both chronic hemolyzing Gd- and A Gd- variants show similar abnormal shape recovery behavior, whereas the extent of hemolysis is quite different between variants. Thus, the activity of the hexose monophosphate shunt appears to have a dramatic effect on the rate of reversal of echinocytosis. The lack of shunt activity of Gd- cells would necessarily impair their ability to recover normal shape after perturbation.

THE PRESENCE OF abnormal cell shapes of human erythrocytes provides an indication of a major dysfunction and is normally associated with hemolysis. Studies of erythrocyte behavior in vitro have suggested that the erythrocyte is able to restore and preserve its normal shape following an external perturbation through a distinct shape control mechanism. The alterations of erythrocyte shape fall most often into two categories, either evaginated forms (echinocytes or crenated cells) or invaginated forms (stomatocytes or cupped cells). These changes can be produced by the addition of amphipathic compounds to cells in vitro. Upon shape perturbation of erythrocytes with a crenator agent, cells are capable of restoring their normal biconcave disc shape by a stomatocytic process. Experimental evidence has shown that a reversible alteration of the membrane itself occurs during shape recovery, rather than a passive redistribution of the crenator within the membrane (see accompanying article). The biochemical mechanism of the shape recovery is, however, unknown. Recent studies in our laboratory have suggested a correlation between hexose monophosphate shunt (HMP) activation and shape recovery, and it has been suggested that this shape recovery process is not a passive process, but rather is an energy-dependent one. Methylened blue and hydrogen peroxide activate the hexose monophosphate (HMP) shunt, by oxidizing NADPH diaphorase, which in turn oxidizes NADPH to NADP⁺, and by oxidizing glutathione (GSH), respectively. Interestingly, both are potent activators of the shape recovery process.

In the present work we take advantage of a genetic defect of human erythrocytes to probe further the HMP role in the control of the erythrocyte shape. Glucose-6-phosphate-dehydrogenase deficiency (Gd-) is a hereditary disorder in which the activity of glucose-6-phosphate-dehydrogenase is markedly reduced. This enzyme is the first step in the HMP shunt pathway, and therefore, in Gd- erythrocytes, this pathway can hardly be stimulated by methylene blue. If, indeed, the hexose monophosphate shunt plays a role in cell shape maintenance and restoration upon extracellular perturbation, then it would be expected that Gd- erythrocytes would have an impaired shape recovery mechanism. The experimental evidence presented in this article strongly supports this hypothesis and suggests a simple visual test for severe Gd- cells.

MATERIALS AND METHODS

Blood

Human erythrocytes were drawn into heparinized tubes. Cells were then washed 3 times in HEPES-Ringer buffer [120 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgSO₄, 18 mM N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid (HEPES), 2 mM Na₂HPO₄, and 10 mM glucose] adjusted to pH 7.4. Washed packed red cells were finally suspended to 1% hematocrit in HEPES-Ringer buffer. Cells were normally used within 4–8 hr after drawing, but no differences in behavior were noted in samples up to 24 hr. Human erythrocytes were collected from normal healthy controls. In addition, blood samples were drawn from patients with homozygous Gd- Worcester, associated with chronic nonspherocytic hemolytic anemia; a patient with the heterozygote carrier state of Gd- Worcester; 2 patients with homozygous primaquine-sensitive Gd- A⁺; 1

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heterozygous Gd- A- carrier; 3 patients with hereditary xerocytosis; and from 4 patients with hereditary xerocytosis.

**Tos-pheCH₂Cl Pretreatment of Erythrocytes**

Normal and abnormal erythrocytes were incubated in HEPES-Ringer buffer (0-4°C) in the presence of tos-pheCH₂Cl (usually 50 μM final concentration) for 1 hr. Then, cells were washed 4 times in tos-pheCH₂Cl-free HEPES-Ringer buffer (0-4°C). Cells were finally suspended to 1% hematocrit and incubated at 37°C. After 10 min of thermal equilibration, cells were treated either with 1 mM 2,4-dinitrophenol (DNP) or 15 μM pyrenebutyric acid (PBA). The shape recovery process was then followed.

**Shape Recovery Process**

Cells were incubated at 37°C in the presence of the crenator agent. At various time intervals, aliquots were fixed with 2% (final concentration) glutaraldehyde and allowed to stay at 4°C for at least 1 hr before counting under a darkfield microscope. At least 300 cells were counted for each determination in a blind fashion. The recovery rate was calculated as the percentage of cells that recovered their shape from crenated to biconcave disc forms per minute in the first 10 min.

**Chemical**

2,4-Dinitrophenol (DNP), methylene blue, and N-α-tosyl-phenylalanine-chloromethyl ketone (tos-pheCH₂Cl) were obtained from Sigma Chemical Co. (St. Louis, MO) and 1-pyrenebutyric acid (PBA) from Eastman Organic Chemicals (Rochester, NY). DNP and PBA were recrystallized from ethanol three times before use.

**RESULTS**

After washing, the morphology of Gd- erythrocytes appeared normal. When 2,4-dinitrophenol (DNP), a crenator agent, is added to the cells (1 mM final concentration), normal as well as Gd- became crenated to the same extent (95%-100% crenation). The recovery to the normal discoid morphology did occur in both control and deficient cells, but at a slightly slower rate in the Gd- cells (see Table 1 and Fig. 1). When methylene blue was added to the cell suspension, the recovery rate of control cells was dramatically increased relative to the Gd- cells (Fig. 1). For example, with 100 μM methylene blue, there was a more than 30-fold increase in the shape recovery rate of control cells, whereas in Gd- cells, only a 7-fold increase was found. Methylene blue alone did not alter cell shape at these concentrations during parallel incubations. During incubations for as long as 24 hr, the shape recovered cells maintained the discoid morphology in the presence of DNP. Thus, although an amphipath-induced shape change occurs normally in Gd- cells, the shape recovery process appears to be slow, particularly in the presence of methylene blue.

It has been previously shown that tos-pheCH₂Cl is a selective and potent activator of the shape recovery process (Alhanaty and Sheetz, see companion article). After treatment with 50 μM tos-pheCH₂Cl, the shape recovery rate of control cells was increased by 12-fold, but that of Gd- cells was unaltered (see Fig. 2). No

**Table 1.** Shape Recovery Rates of Normal and Hereditary Abnormal Erythrocytes Before and After Tos-PheCH₂Cl Pretreatment

<table>
<thead>
<tr>
<th>Variant</th>
<th>Recovery Rate% Discs/min ± SD</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>- tos-pheCH₂Cl</td>
</tr>
<tr>
<td>GD- erythrocytes</td>
<td></td>
</tr>
<tr>
<td>Chronic hemolyzing</td>
<td>(9)*</td>
</tr>
<tr>
<td>Black</td>
<td>(3)</td>
</tr>
<tr>
<td>Control erythrocytes—Gd-</td>
<td></td>
</tr>
<tr>
<td>Carrier of chronic hemolyzing Gd-</td>
<td>(2)</td>
</tr>
<tr>
<td>Hereditary spherocytosis</td>
<td>(4)*</td>
</tr>
<tr>
<td>Xerocytosis</td>
<td>(3)</td>
</tr>
<tr>
<td>Normal</td>
<td>(17)</td>
</tr>
</tbody>
</table>

* Number of blood samples examined.
† The endpoint of the shape recovery assay was the discoid shape that these cells normally have when fresh.
‡ Experimental details are described in Materials and Methods.
response to tos-pheCH₂Cl in Gd⁻ cells was observed even after pretreatment with 200 μM tos-pheCH₂Cl, whereas control cells will become stomatocytic in time after treatment with such high concentrations. Thus, this stimulator of the shape recovery process is totally ineffective in Gd⁻ erythrocytes.

The slow recovery process, and the unresponsiveness to stimulators of shape recovery in Gd⁻ deficient erythrocytes, does not reflect a permanent rigidification or fixation of the cells in their crenated form, as at any time during incubation, upon washing away the crenator agent, cells immediately regain their biconcave morphology. These effects are not related to the crenating compound, DNP, because similar results have been obtained with another crenator agent, pyrenebutyric acid, at 15 μM. In the absence of a crenator agent, normal as well as Gd⁻ cells preserve their biconcave disc shape regardless of whether or not they have been pretreated with 50 μM tos-pheCH₂Cl or incubated with or without methylene blue. Moreover, reducing the shape recovery rates of control cells to values comparable to those of Gd⁻ deficient erythrocytes (by increasing the concentration of the crenator agent) did not affect their capability to respond to tos-pheCH₂Cl, indicating that the lack of response of Gd⁻ deficient cells to tos-pheCH₂Cl is not due to their slower recovery rate compared to normal cells.

In order to examine the possibility that reticulocytosis or other factors associated with hemolytic anemia caused this change, the cells from a number of other hemolytic anemias and from different Gd⁻ variants were tested. Three samples of the A⁻ variant of Gd⁻ (black variant) were tested and found to be unresponsive to tos-pheCH₂Cl (Table 1). In contrast to the chronic hemolizing Gd⁻ cells, which contained 8%-12% reticulocytes, the A⁻ variant cells had 1.0%-1.5% reticulocytes. The glucose-6-phosphate dehydrogenase activity was 6% of normal in the A⁻ cells and was undetectable with the chronic hemolizing variant. Hereditary spherocytic and xerocytic cells with reticulocytes, 12% and 4%-6%, respectively, both responded to tos-pheCH₂Cl treatment with a dramatic increase in their shape recovery rates. Erythrocytes from carriers of both the A⁻ and chronic hemolizing variants of Gd⁻ were found to have normal recovery rates and responded normally to tos-pheCH₂Cl (glucose-6-phosphate-dehydrogenase activity was half of normal in both cases). With the blood from heterozygotes, it was perhaps expected that half of the cells would, and half would not, respond to tos-pheCH₂Cl treatment because of clonal expression of the enzyme. To test whether these results indicated that all cells from heterozygotes were capable of responding to tos-pheCH₂Cl or that a factor was transmitted from the normal to the abnormal cells, a 1:1 mixture of normal and homozygous Gd⁻ cells was treated with tos-pheCH₂Cl and shape recovery was analyzed. It was found that 40% of the cells recovered very rapidly in the mixture, which supports the ideas that tos-pheCH₂Cl sensitivity is not transmittable and that, in the blood cells from heterozygotes, there is sufficient enzyme to respond to tos-pheCH₂Cl. The lack of response to tos-pheCH₂Cl, therefore, correlates most closely with a very low activity of glucose-6-phosphate dehydrogenase and not any other discernible factor.

**DISCUSSION**

The process of shape control in the erythrocyte appears to be a complex phenomenon. Extensive characterization of this phenomenon in our laboratory has revealed that shape recovery does not involve fluctuations in adenosine triphosphate (ATP) levels,6,7 divalent cations,8 or cell volume (Alhanaty and Sheetz, unpublished results). The process can be understood by suggesting that a stomatocytic process is activated by crenation and stops when the discoid form is reached.6,8 Although the normal rate of this process is quite slow, it is dramatically activated by activators of the HMP shunt.9 There is no effect of shunt activators on the initial or final shapes of erythrocytes in these studies. It is only the rate of shape change that is affected. Erythrocyte membrane oxidation causes membrane rigidification and increased resistance to shape change13; therefore, the oxidation effects of the HMP shunt activators could not have produced the increased shape recovery rates.

There are a variety of ways in which activators of the HMP shunt could stimulate shape recovery. The absence of shunt activity in Gd⁻ cells makes it possible to eliminate some of those mechanisms. To do this, it is worthwhile to compare the striking differences in recovery rates of Gd⁻ and normal cells with other differences in the cellular properties of Gd⁻ and control cells. Only the glucose-6-phosphate-dehydrogenase activity,14 NADPH/NADP⁺ ratio,15 and GSH stability16 are markedly abnormal in Gd⁻ cells, whereas lipid content,17 glycolytic activity,18 deformability,19 and viability upon storage in blood bank preservation solution are nearly normal.

If methylene blue stimulates shape recovery in normal cells by reducing the NADPH/NADP⁺ ratio, then, in Gd⁻ cells, where NADPH production is already impaired, the effect on shape recovery should be greater. In fact, the effect of methylene blue is considerably less in Gd⁻ cells. Thus, the reducing potential or NADPH/NADP⁺ ratio does not correlate
with shape recovery rate. A similar argument can be used to rule out the possibility that a drop in GSH level causes an increase in shape recovery rates.

The major functional change in Gd cells that correlates with the difference in the rates of morphological change is the activity of the HMP shunt. When the HMP shunt pathway is stimulated, morphological changes occur more rapidly. If the HMP shunt activity is diminished by lower glucose-6-phosphate dehydrogenase activity, then shape recovery cannot be stimulated. The residual enzyme activity in the A- variant cells will allow some shunt activity, but there is little increase in shape change rate. A likely explanation for these observations is that the increased rate of morphological change depends on the buildup of an intermediate associated with the HMP shunt. It is conceivable that sugar phosphates (e.g., ribulose-5-phosphate) will increase in concentration upon HMP shunt stimulation, but would be lower in G6PD deficiency. Further experiments are underway to test this hypothesis.

The molecular basis of the tos-pheCH2Cl's effect is not known. It is capable of reacting with cellular sulfhydryl groups and causes a drop in GSH levels. In normal cells, tos-pheCH2Cl causes an increase in HMP shunt activity by 2–4-fold, whereas methylene blue increases activity by 20–30-fold (Alhanaty and Sheetz, unpublished observations). This extent of shunt activation is insufficient to explain tos-pheCH2Cl's effects. Because the increased shape recovery rate after tos-pheCH2Cl treatment also requires shunt activity, it is possible that it may act by raising levels of products through inhibition of one of the later steps in the HMP shunt.

Among the various hemolytic abnormalities examined, Gd cells were the only ones that showed a clearly abnormal shape recovery process. Recovery in hereditary spherocytic erythrocytes proved to be insignificantly different from normal, indicating that abnormalities in final shapes can occur independently of abnormalities in the shape recovery process. The apparently normal behavior of the hereditary spherocytosis and xerocytic cells indicates that reticulocytosis does not alter the shape recovery process. Thus, unusual shape recovery behavior of Gd cells appears to be a distinct property of the metabolic defect and may provide a simple method for testing for Gd erythrocytes.

During circulation in vivo, the erythrocyte encounters a number of environmental changes that may alter its shape. As altered shapes are hemolyzed more readily, this phenomenon would be expected to contribute to increased hemolysis in Gd cells. Because alterations in cell shape have not been observed in hemolytic crises of Gd individuals, further studies are required to determine whether or not the compromised shape recovery process in Gd cells compromises their viability upon stress.

ACKNOWLEDGMENT
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REFERENCES


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