Chronic nonspherocytic hemolytic anemia has been observed in a recently described glucose-6-phosphate dehydrogenase (G6PD) variant, G6PDW. The mechanical properties of these erythrocytes and other G6PD variants were examined. The deformability of G6PD-deficient erythrocytes was normal, as determined by osmotic scan ektacytometry, and was not significantly affected by hemolytic crisis. In the common varieties of G6PD deficiency, the mechanical stability of the red blood cell (RBC) membrane was greater than normal, but G6PDW membranes were abnormally susceptible to shear-induced fragmentation. There was no evidence for a concurrent genetic defect in spectrin, because self-association constants and tryptic digests were normal. The fragility of G6PDW membranes appeared to be a consequence of oxidative damage to membrane thiol groups associated with a low glutathione (GSH) level in these RBCs. Associations among GSH level, thiol oxidation, and membrane instability were also found when a larger group of G6PD-deficient RBCs were examined. In normal erythrocytes, 1-chloro-2,4-dinitrobenzene was used to reduce GSH levels by 50%. Membrane thiol oxidation and membrane fragility both increased when these cells were kept at 4°C for 3 to 5 days. Our findings suggest that chronic depletion of GSH leads to the destabilization of membrane skeleton through oxidation of membrane protein thiols.

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Beyond 24 hours. For this reason, samples for ektacytometry were collected in acid citrate dextrose.

Lipid oxidation. The extent of endogenous lipid oxidation was estimated by measuring the level of malonyldialdehyde (MDA) in isolated membranes.

Protein oxidation. Oxidant damage to membrane proteins was estimated by determining the amount of solubilized membrane protein that failed to bind to a thiol-Sepharose column. One gram of dry weight thiol activated Sepharose 4B (Pharmacia, Uppsala, Sweden) was swollen in H2O and washed three times in gel buffer (0.2 mol/L NaCl, 0.2 mol/L NaPi, 2% SDS, 100 mg/L phenylmethysulfonfluoride [PMSF], 1 mmol/L EDTA, pH 8.4). After resuspension in 6 mL gel buffer, the Sepharose was transferred to a 10-mL glass column with a sintered glass frit and stopcocks at both ends (Econo-Column, Bio-Rad, Hercules, CA). The buffer was allowed to drain from the packed gel. Membrane protein (1.5 mg) was mixed with 1 mL gel buffer, solubilized at 95°C for 5 minutes, cooled, and added to the thiol-Sepharose in the column. The columns were flushed with nitrogen a number of times to remove oxygen, closed at both ends, and rocked in a nitrogen atmosphere at room temperature for 16 hours. The columns were then drained into a 15 mL plastic graduated centrifuge tube. The Sepharose was washed by adding 2 mL of gel buffer, inverting the column several times, and draining. The pooled eluates contain the unbound protein fraction.

After washing columns with 30 mL wash buffer (5 mmol/L NaPi, 2% SDS, 100 mg/L PMSF, 1 mmol/L EDTA, pH 8.4), bound proteins were released by the addition of 2 mL wash buffer to which 1 mL β-mercaptoethanol was added. After rocking for 1 hour at room temperature, the columns were drained. The β-mercaptoethanol step was repeated, and a final 1 mL wash without β-mercaptoethanol was pooled with the two β-mercaptoethanol washes. This fraction is the bound protein.

Both bound and unbound fractions are dialyzed separately against 1 L dialysis buffer (wash buffer containing 1% SDS), and the protein concentrations were determined. The bound fractions were dialyzed three times, unbound fractions were dialyzed twice. The ratio of unbound protein to total protein represents the degree of oxidation of the membrane proteins in the ghost samples. In our hands, the unbound fraction contained more protein than was found in the original description of the procedure. This may be attributable to the use of a protein assay (BioRad DC) that is not affected by residual SDS in the dialyzed fractions.

Lowering GSH levels with 1-chloro-2,4-dinitrobenzene (CDNB). To demonstrate that reduced GSH levels affect membrane protein oxidation and fragmentation time, CDNB was used to lower intracellular GSH concentrations. GSH levels were determined on whole blood samples collected in heparin. As described below (see Results), it was found that G6PD-deficient erythrocytes with a GSH level of 3 to 4 mmol/kg hemoglobin (Hb) had an increase in membrane protein oxidation and lowered stability. CDNB concentrations were determined empirically to reduce the intracellular GSH of normal erythrocytes to approximately 3 mmol/kg Hb. In our experiments, GSH levels of 3.3 to 4.2 mmol/kg Hb were achieved.

The appropriate amount of CDNB was added to duplicate samples of 4 mL of whole blood immediately after drawing (treated sample). These specimens, along with an additional 4 mL whole blood to which no CDNB has been added (untreated sample), were placed in the refrigerator for 3 days. GSH levels were determined on the day the sample was drawn, 1 hour after the addition of CDNB to verify that GSH was lowered appropriately, and after 3 days. Fragmentation tests were run on the blood samples before CDNB addition, and after 3 days of storage at 4°C on both CDNB treated and untreated specimens. Thiol binding columns were performed in duplicate on untreated and treated ghosts after 3 days of incubation.

RESULTS

Clinical and hematologic findings. These are summarized in Table 1. The two siblings with G6PD WaYne have CNSHA and both have undergone splenectomy (one after cholecystitis and pancreatitis). The presplenectomy and postsplenectomy hematologic data are shown. The child with G6PD Huron has mild CNSHA frequently exacerbated by intercurrent viral infections.

The G6PD A- individuals were identified because of prior hemolytic episodes and the G6PD deficiency was verified by the clinical (service) laboratory. G6PD levels in the remaining subjects were determined in the authors' laboratory. In all individuals, GSH was measured in our laboratory using freshly drawn samples. In all, 22 individuals with A- were examined. Complete studies, including thiol column chromatography and ghost fragmentation assays, were performed on 7 of these 22. Four individuals with the Mediterranean type were identified, but complete studies were only possible on one.

Ektacytometry. Osmotic scan ektacytometry (Fig 1A) of G6PD A- erythrocytes during the steady state showed an increased EI at 290 mOsm. In one case, there was a left shift of the low osmolality minimum, indicating an excess of membrane (high surface/volume ratio). During hemolytic crises, when peripheral smears showed numerous dense pyknotic cells with precipitated Hb, little change was seen in the osmoscans (Fig 1B), except for an increase in the minimum EI value at low osmolality that can be attributed to increased cell heterogeneity. In three cases, we were able to examine the same individual during hemolytic crisis and in the steady state, which confirmed that there was no change in cell deformability. Hemolytic crisis was triggered in all 3 children by the accidental ingestion of naphthalene mothballs.

Osmoscans of G6PD WaYne erythrocytes resembled other G6PD-deficient RBCs, except that the EI was very high at isotonicity (data not shown). This was not a consequence of the elevated reticulocyte level, because an equal level of reticulocytosis in other patients with chronic anemia did not show this alteration in the osmocan. For example, ektacytometric scans of G6PD Huron samples with equally high reticulocytosis resembled the common G6PD-deficient varieties.

Membrane fragmentation assays. Significantly, G6PD WaYne membranes were also abnormal in their ability to resist shear forces in the ektacytometer. Membrane stability was determined as the half time to fragmentation (t½), and was markedly decreased in the two siblings with G6PD WaYne and chronic hemolysis (Fig 2). This reduction in membrane stability was especially striking in light of the observation that all the G6PD A- variants, both during steady state and during acute hemolytic episodes, as well as G6PD Huron exhibited a significant increase in t½ (Table 1).

Spectrin in G6PD WaYne subjects. Initial attempts to explain the reduced membrane stability focussed on the possibility of a concurrent spectrin defect in the G6PD WaYne fam-
RBC MEMBRANE STABILITY IN G6PD DEFICIENCY

Table 1. Relationships of Membrane Stability to G6PD Activity, GSH Levels, and the Percentage Unbound on Thiol Binding Columns

<table>
<thead>
<tr>
<th>Variant</th>
<th>Hb (g/dL)</th>
<th>PCV</th>
<th>MCV (fl)</th>
<th>Reticulocyte Count (%)</th>
<th>G6PD Ru/g Hb</th>
<th>GSH (μmol/g Hb)</th>
<th>t% Control</th>
<th>Unbound Fraction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6PD Wav</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S.H.</td>
<td>14.1</td>
<td>43.8</td>
<td>104.2</td>
<td>7.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postsplenectomy</td>
<td>13.3 ± 0.2</td>
<td>41.8 ± 0.3</td>
<td>118 ± 2</td>
<td>14-30</td>
<td>0.6</td>
<td>3.3 ± 0.6</td>
<td>75 ± 5</td>
<td>23.7</td>
</tr>
<tr>
<td>3.H.</td>
<td>12.5</td>
<td>37.5</td>
<td>100.8</td>
<td>4.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postsplenectomy</td>
<td>12.1 ± 0.9</td>
<td>37.9 ± 2.6</td>
<td>111-124</td>
<td>11.8 ± 0.8</td>
<td>0.7</td>
<td>3.5 ± 0.4</td>
<td>72 ± 8</td>
<td>24.0</td>
</tr>
<tr>
<td>G6PD Wav</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J.B.</td>
<td>11.3 ± 0.6</td>
<td>33.7</td>
<td>98.1</td>
<td>12.3 ± 3.5</td>
<td>0.7</td>
<td>6.0 ± 0.6</td>
<td>131 ± 7</td>
<td>17.8</td>
</tr>
<tr>
<td>G.M.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.7</td>
<td>6.8</td>
<td>170</td>
<td>19.0</td>
</tr>
<tr>
<td>G6PD A- (n = 7)</td>
<td>11.5 ± 1.9</td>
<td>34.3 ± 5.1</td>
<td>83.1 ± 11.6</td>
<td>3.4 ± 1.8</td>
<td>4.1 ± 1.4</td>
<td>6.5 ± 0.8</td>
<td>127 ± 22</td>
<td>18.3 ± 1.3</td>
</tr>
<tr>
<td>G6PD Med (n = 1)</td>
<td>14.5</td>
<td>38.9</td>
<td>81.98</td>
<td>0.5-1.5</td>
<td>12 ± 0.5</td>
<td>6.24 ± 0.1</td>
<td>(100%)</td>
<td>19.4 ± 1.1</td>
</tr>
<tr>
<td>Control</td>
<td>13.3-17.1</td>
<td>38.9-49.7</td>
<td>81-98</td>
<td>0.5-1.5</td>
<td>12 ± 0.5</td>
<td>6.24 ± 0.1</td>
<td>(100%)</td>
<td>19.4 ± 1.1</td>
</tr>
</tbody>
</table>

The values shown are means ± SE.
* The fraction of solubilized membrane protein that did not bind to thiol-sepharose columns.

ily. Many inherited hemolytic anemias are characterized by a variant spectrin with an altered spectrin self-association site that weakens the membrane skeleton.16,17 These binding defects lower the ghost fragmentation time, which is highly sensitive to the stability of the spectrin-actin network.16,18 However, when the state of spectrin association in 0°C extracts from G6PD Wav erythrocytes was examined, spectrin tetramer content was found to be normal (data not shown).

Furthermore, two-dimensional gel electrophoresis of partial tryptic digests of spectrin6 did not show any abnormal peptide fragments (data not shown). We also examined membranes from G6PD Wav and a subset of the G6PD A- patients by these methods without detecting any alterations in spectrin.

Oxidant damage to membranes. A distinctive feature of G6PD Wav erythrocytes was a strikingly low intracellular

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Fig 1. (A) Osmotic gradient scans on 6 G6PD A- individuals during the steady state. (==) Control. (B) Three G6PD A- individuals during acute hemolytic crisis. (==) Control.
GSH level (Table 1). In view of the protective role of GSH, evidence for oxidant damage in G6PD WaYne RBC membranes was sought. However, no evidence for in vivo lipid peroxidation was found, because no MDA was detected in fresh erythrocytes from any G6PD individual, including G6PD WaYne. However, G6PD WaYne erythrocytes were abnormally susceptible to in vitro lipid peroxidation, because large amounts of MDA were generated when the cells were exposed to 2.5% H₂O₂.¹³

Membrane proteins, in contrast, were oxidized in G6PD WaYne RBCs, as shown by the increased amount of protein that did not bind to thiol-Sepharose columns (Table 1). The finding of reduced t½, membrane protein oxidation, and low GSH in G6PD WaYne suggested a relationship among these factors. GSH has a general role in defending the erythrocyte against oxidant attack, and others have shown that membrane protein oxidation can lead to weakening of the spectrin-actin network.¹⁹-²²

Other G6PD variants. We found variations in GSH levels among G6PD-deficient individuals in our patient population. It therefore seemed possible that the relationship between intracellular GSH, membrane protein oxidation, and ghost fragmentation times found in G6PD WaYne might also hold for other types of G6PD deficiency. Thiol columns, GSH, and membrane stability were therefore determined for a group of G6PD-deficient individuals. Significant correlations were found among all of these values (Fig 3). The amount of oxidized membrane protein was inversely related to GSH (Fig 3A), and increased when intracellular GSH decreased to less than 5 μmol/g Hb. In low GSH RBCs with oxidized membrane proteins, membrane stability was also compromised (Fig 3B). Intracellular GSH levels were well correlated with the t½. It is of interest that t½ was higher than normal in membranes from the common G6PD variants. These erythrocytes are characterized by low G6PD levels but near normal GSH levels. Finally, membrane thiol oxidation was well-correlated with membrane instability (Fig 3C).

As others have reported, we found no correlation between GSH and the level of G6PD activity.

SDS gel electrophoresis. Membranes from nearly all of the G6PD-deficient erythrocytes were also analyzed by standard SDS gel electrophoresis. In every case, a normal pattern of membrane proteins was found. We did not detect any of the high molecular weight aggregates reported by others, even in G6PD WaYne RBCs, which had the lowest GSH levels found in our patient population.

In vitro reduction of GSH. To obtain direct experimental evidence for a role in GSH in protecting membrane proteins against oxidation and in maintaining membrane stability, we used CDNB to lower GSH levels in normal RBCs. Intraerythrocytic GSH can be converted to an irreversible adduct, 2,4-dinitrophenyl-S-glutathione, by reaction with CDNB.¹² The conjugate remains undegraded in the erythrocyte. CDNB was added in amounts sufficient to reduce GSH in normal erythrocytes to the level observed in G6PD WaYne RBCs. After 2 to 4 days of storage at 4°C, the membranes from these GSH-depleted cells had diminished shear resistance and an increase in protein thiol oxidation (Table 2). When GSH was reduced to undetectable levels by the addition of 4 mmol/L CDNB, the t½ of membrane ghosts was increased after 3 days at 0°C. However, there was extensive Hb oxidation in these cells.

**DISCUSSION**

In most cases of G6PD deficiency, hemolysis is episodic and linked to exposure to oxidizing agents. Some G6PD deficiencies have chronic hemolysis of varying severity, without a clear underlying pathogenesis. G6PD WaYne is a recently described new variant with a unique mutation in the G6PD gene involving nucleotide 769 (C-G substitution) causing a substitution of glycine for arginine at amino acid 257.²⁴ The results reported here suggest that the chronic hemolysis found in G6PD WaYne can be explained as the consequence of low GSH and membrane protein oxidation that destabilizes the cell membrane.

The erythrocyte has multiple defenses against oxidation, which may protect different components of the cell. For example, Scott et al.²⁵ eliminated GSH in normal erythrocytes by reaction with CDNB and found no increase in the rate of Hb oxidation by exogenous hydrogen peroxide. They suggested that NADPH is the significant antioxidant for the protection of intracellular Hb, at least against exogenous peroxides. In contrast, we found that membrane protein thiols became oxidized when GSH was lowered to a similar extent with CDNB. Eaton²⁶ proposed that catalase and NADPH protect the erythrocyte against acute high exogeous levels of peroxide, whereas GSH is needed to protect the cell against the low levels that are continuously generated endogenously. Our data, which indicate that low GSH, but not low levels of G6PD itself, is associated with protein damage and mechanical instability in the membrane, are consistent with this proposal.

In contrast with G6PD WaYne, the common varieties of G6PD deficiency have normal GSH levels and no evidence...
for membrane thiol oxidation, yet their membranes have an increased, rather than a diminished, $\bar{t}_\frac{1}{2}$. A similar phenomenon has been reported for the thalassemias. In both $\alpha$- and $\beta$-thalassemias, membranes have bound globin and have suffered substantial amounts of oxidative damage, yet membranes from $\alpha$-thalassemia RBCs have increased $\bar{t}_\frac{1}{2}$ values, whereas $\beta$-thalassemic membranes have lowered $\bar{t}_\frac{1}{2}$. In $\alpha$-thalassemia, functional ankyrin sites on the membrane are diminished and sulfhydryls are oxidized in $\beta$-spectrin and ankyrin, whereas protein 4.1 is oxidized in $\beta$-thalassemia. Moreover, even exogenous low molecular weight oxidants can have differential effects. The very similar oxidizing agents phenylhydrazine and methylhydrazine have opposite effects on membrane fragility, and we have found that CDNB can either decrease or increase $\bar{t}_\frac{1}{2}$ depending on the degree of GSH depletion. In sickle cell anemia, there is substantial membrane protein oxidation and loss of functional ankyrin molecules, with only a marginal shortening of $\bar{t}_\frac{1}{2}$. The effect of oxidation on membrane stability appears to depend on the detailed properties of the oxidizing agent and the specific target within the membrane. A model to explain how Hb could oxidize different membrane components was recently proposed by Jarolim et al.

Oxidants can increase membrane fragility by damaging $\beta$-spectrin or protein 4.1 with a consequent defective formation of the spectrin-4.1-actin complex. We were unable to note a specific increase in protein 4.1 in the unbound fraction on the thiol columns, but this method may not detect small changes. Protein 4.1 has seven cysteine residues and all may have to be oxidized to eliminate binding to thiol-sepharose.

Osmotic gradient ektacytometry showed that the whole cell deformability of freshly obtained G6PD-deficient erythrocytes was normal both in the steady state (Fig 1A) and during hemolytic crisis (Fig 1B), although micropipette as-
Table 2. The Effect of GSH Depletion on Membrane Properties

<table>
<thead>
<tr>
<th>Experiment No</th>
<th>CDNB Added (nmol/L)</th>
<th>Days at 4°C</th>
<th>GSH (umol/g Hb)</th>
<th>Unbound Fraction (%)*</th>
<th>(% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>7.70</td>
<td>17.2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>3.70</td>
<td>19.0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3.90</td>
<td>19.2</td>
<td>0.83</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>3.00</td>
<td>19.6</td>
<td>0.78</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>6.30</td>
<td>16.5</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
<td>3.85</td>
<td>20.0</td>
<td>0.79</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>0</td>
<td>6.95</td>
<td>16.7</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1.15</td>
<td>3</td>
<td>3.40</td>
<td>19.6</td>
<td>0.71</td>
</tr>
</tbody>
</table>

On day 1, GSH was assayed in fresh normal erythrocytes and an appropriate amount of CDNB was added. They were then stored at 4°C for 3 to 5 days, when GSH, membrane thiols, and fragmentation time were determined. Four experiments were performed. The starting values are shown in the first row of each set.

* The fraction of solubilized membrane protein that did not bind to thiol-Sepharose columns.

Piration studies have indicated that the G6PD membrane is rigid.23,35 This can be rationalized by recent studies that show that Heinz bodies (the end result of oxidant damage to Hb) cause focal membrane rigidity, but that whole cell deformability is not affected until the Heinz bodies cover the entire membrane endface,36 as might occur after exposure to naphthalene.27 Membrane rigidity has also been attributed to the presence of high molecular weight disulfide bonded aggregates of spectrin and hemoglobin,22,35,36 but we did not find such aggregates in membranes from our patients, in agreement with the findings of Coeter and Zait.40

A further implication of our studies is that GSH levels less than 4 nmol/kg Hb will be associated with chronic hemolytic anemia. Additional evidence for this proposal is found in a number of rare defects in glutathione metabolism, including deficiencies in glutathione synthetase (which may be associated with S-oxoprolinuria in severe instances) and gamma-glutamylcysteine synthetase. With only one reported exception, all are associated with chronic life-long hemolysis.11,42 Similarly, some strains of sheep have low intraerythrocytic GSH and a shortened RBC life span.43

We tentatively conclude that, in some G6PD deficiencies with low GSH levels, components of the membrane essential for mechanical stability are oxidized. The resulting membrane instability may contribute to the chronic hemolytic state.

ACKNOWLEDGMENT

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Oxidant damage to erythrocyte membrane in glucose-6-phosphate dehydrogenase deficiency: correlation with in vivo reduced glutathione concentration and membrane protein oxidation

RM Johnson, Y Ravindranath, M el-Alfy and G Jr Goyette