Quantitative Studies on the Sensitivity to Complement Lysis of Red Cells Treated With Thiol-reactive Reagents

By NANCY W. STEAD and WENDELL F. ROSSE

The effect of reduced glutathione on the complement sensitivity of normal and paroxysmal nocturnal hemoglobinuria (PNH) red cells was determined quantitatively. The susceptibility to complement lysis was increased in normal and complement-sensitive and complement-insensitive PNH cells; the percentage increase in susceptibility was inversely related to the initial complement-sensitivity. P-chloromercuribenzenesulfonate (PCMBS) likewise increased the sensitivity of normal cells to lysis by complement; the effect with both compounds appeared to be due to reaction with membrane thiol groups. GSH-treated normal cells were similar to PNH cells in that the susceptibility to immune lysis occurred without concomitant increase in fixation of antibody or first component of complement. GSH-treated normal cells differed from PNH cells in that the membrane acetylcholinesterase activity was normal and the cells were not stable during preparation and short-term storage.

PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (PNH) is an acquired hemolytic anemia characterized by abnormal red cells, which are more susceptible to immune lysis than normal cells. These cells do not appear to fix significantly more antibody nor are more complement sequences initiated by a given amount of antibody than on normal cells. Thus, the greater immune hemolysis occurring in vitro probably results from an increased effectiveness of the complement that is fixed to the cell surface.

The increased sensitivity of these cells to complement may be demonstrated in several ways. Ham first showed that PNH cells were partially lysed in acidified normal serum and that this lysis was dependent upon the presence of complement. This has become the classic test for PNH. More recently, complement sensitivity of the PNH cell has been quantitated by the lysis of antibody-sensitized red cells by varying dilutions of complement. By this test, PNH cells are seen to consist of two populations, one of which is markedly sensitive to complement (complement-sensitive population) and the other of which is more nearly normal (complement-insensitive population).

Normal red cells incubated in concentrated solutions of sulfhydryl-containing compounds have been shown to resemble PNH cells in some in vitro tests. Sirchia first demonstrated that cells treated with AET (2-aminoethyliso-

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Thiouronium bromide or cysteine were lysed in acidified serum and that complement was necessary for this lysis. Subsequently, penicillamine, N-acetyl-cysteine and glutathione have also been reported to produce cells which lyse in acidified serum, and the chemical structure \(-\text{CH}_2\text{CH}_2\text{S}^-\) was thought to be the group responsible for the production of this reaction. However, other compounds lacking the \(\beta\)-amino group have been found to produce these cells when incubated at high pH. Sirchia and Dacie found that the sensitivity to complement of these PNH-like cells was as much as 20 times that of untreated cells.

The present experiments were designed to study quantitatively the effect of chemicals capable of reacting with sulfhydryl groups on normal cells. In addition, the effect of glutathione on PNH red cells was also studied. The similarities and differences between cells treated with sulfhydryl-reacting reagents and the complement-sensitive cells of PNH were compared and contrasted.

Materials and Methods

Buffers, Complement, Antibody, and Red Cells

Veronal-buffered saline (VBS) pH 7.4 was made according to the formula given in Reference 10. This was used as diluting buffer unless otherwise noted.

Complement. Fresh human serum was frozen at \(-90^\circ\text{F}\) and thawed just prior to use.

Antibody. Human anti-I antibody with great hemolytic potency was obtained from a patient with cold agglutinin disease and kindly provided by Dr. and Mrs. John Crookston of Toronto, Canada. This serum was stored at \(-20^\circ\text{C}\) and was used in all tests of complement sensitivity of human cells.

Red Cells. Human red cells were separated from serum by centrifugation of cold fresh blood or of defibrinated blood. The cells were stored at 4\(^\circ\text{C}\) in Alsever's solution and were washed thrice in VBS solution prior to use. Standard suspensions containing \(2.2 \times 10^8\) cells/ml were made according to the method given in reference 2.

Glutathione. Crystalline reduced glutathione (GSH) was purchased from Sigma Chemical Company, St. Louis, Mo.

Incubation of Red Cells

Red cells were incubated in glutathione, using a modification of the method of Kann et al. Human red cells were washed three times in VBS and the packed cells were adjusted to an hematocrit of 80 per cent. GSH was dissolved in distilled water on the day of the experiment, and the pH of the solution was adjusted to 8.0 with 5 N NaOH unless stated otherwise. Two volumes of GSH and one volume of cells were mixed in a 50-ml Erlenmeyer flask. GSH molarities in the text are those in this flask; final hematocrit was adjusted to 25. The flasks were covered with Parafilm (American Can Co., Neenah, Wis.) and incubated at 37\(^\circ\text{C}\) without shaking. Control cells were similarly treated except that isotonic saline was substituted for the GSH solution.

After the incubation, VBS was added to the flask, and the cells were collected and centrifuged at room temperature. After centrifugation, they were resuspended with difficulty, since they tended to aggregate. Considerable hemolysis occurred during the washing procedure, which was continued until the supernatant fluid was clear. The cells surviving were analyzed.

Parachloromercuribenzenesulfonate (PCMB) was dissolved in VBS at a concentration twice the final concentration desired. The pH was adjusted to 7.4, if necessary. Equal volumes of PCMB solution and standard red cell suspension \((2.2 \times 10^8\) red cells/ml)
were mixed in a flask and incubated at 37°C for the appropriate time. The final concentration of PCMBs was about 1/200 that of GSH used in these experiments. The cells were washed with VBS used in these experiments. The cells were washed with VBS until the supernatant fluid was clear and were resuspended in a standard suspension.

**Immunological and Other Tests**

The complement lysis sensitivity assay was done according to the method of Rosse and Dacie,² using a system in which the total volume was 1.5 ml. The method was modified in that the sensitized cells were added to the complement-containing solutions at 37°C; the mixture was then incubated at 0°C for 15 minutes, followed by an incubation at 37°C for 60 minutes. Complement lysis sensitivity titer (CLS H₅₀ titer) is defined as the reciprocal of the dilution of serum, 0.2 ml of which will lyse half the red cells in the assay.

CD (first component of complement) fixation and transfer test was done according to Borsos and Rapp,¹¹ as modified by Rosse and Sherwood.¹² The agglutination antibody assay was performed as described by Dacie and Lewis.¹³ Red cell acetylcholinesterase activity was determined by the method of Pilz.¹⁴ ¹⁵

**RESULTS**

Untreated and GSH-treated cells from normal donors were sensitized with anti-I and lysed with graded amounts of human serum as a source of complement. With both types of cells, a single straight line was derived when the logarithm of the complement concentration was plotted against the logarithm of the fraction of cells lysed divided by the fraction unlysed (Fig. 1, normal cells). The complement lysis sensitivity titer CLS H₅₀ of the GSH-treated cells, indicating that 1/3 to 1/5 the amount of serum was required to lyse 50 percent of these cells.

The increase in sensitivity is a function of the time of incubation (Fig. 2), glutathione or PCMBs concentration (Fig. 3), and pH of the reaction solution (Fig. 3). The relationship to pH is not plotted directly; rather, the concentration of thiol as mercaptide ion (R-S⁻) was calculated for given pH values at a constant concentration of GSH. The value of 8.7 for pKₐ of the thiol group¹⁶ was used in the Henderson-Hasselbach equation. The concentration of mercaptide ion is plotted against the change in complement lysis sensitivity in Fig. 3. Increasing the time of incubation, the concentration of GSH or PCMBs,
or the pH brought about an associated increment in nonimmune lysis of the cells. Thus, when cells were incubated to obtain maximum increase in complement sensitivity they became increasingly adherent, and resuspension became virtually impossible. The nonspecific lysis of these cells, either during the initial incubation with the sulfhydryl reagent or during the complement lysis sensitivity assay, likewise increased (see Table 1). This nonspecific lysis limited the degree to which the complement lysis sensitivity could be increased. Further, only those cells that survived this procedure could be analyzed. Optimum conditions for increasing the CLS titer while having a sufficiently low blank were determined to be 0.46 M GSH at pH 8.0 and incubation for 60 minutes.

When PNH cells were incubated with glutathione, the complement lysis sensitivity of both the complement-sensitive and the complement-insensitive cells was increased (Fig. 1). In each instance, the increase was greater for the complement-insensitive cells and the two populations became more nearly alike in their sensitivity to complement. The proportion of cells in each population remained the same, indicating that neither population was more susceptible to the nonimmune lysis.

The percentage change in the complement-sensitivity of the normal or PNH cells (both populations) is inversely related to the initial complement lysis sensitivity of the cell (Fig. 4). When the logarithm of the initial CLS H50 is plotted against the logarithm of the ratio of the CLS H50 of the untreated cells, a straight line results. The results from treatment of normal cells, complement-insensitive PNH cells and complement-sensitive PNH cells are fitted to this line.

The increase in sensitivity to complement lysis could be due to an increase in antibody binding, an increase in the number of complement sequences initiated, or to an increase in the efficiency of completion of complement
Table 1.—The Effect of GSH on the Stability of Red Cells as Shown by Nonimmune Lysis During Incubation

<table>
<thead>
<tr>
<th>Concentration GSH Used</th>
<th>Complement Sensitivity Units</th>
<th>Per Cent Lysis During Initial Incubation and Washing</th>
<th>Per Cent Lysis 1 hr. 37°C</th>
<th>Per Cent Lysis 12 hr. 0°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td>0.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.30</td>
<td>4.2</td>
<td>1.3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.42</td>
<td>9.3</td>
<td>2.3</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>0.84</td>
<td>10.8</td>
<td>34.0</td>
<td>15.0</td>
</tr>
<tr>
<td>PNH</td>
<td></td>
<td>21</td>
<td>0.15</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>0.30</td>
<td>S* 3.2</td>
<td>S 34</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>I† 8.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* S, complement-sensitive cells.
† I, complement-insensitive cells.

The complement-sensitive cells comprised 70 per cent of the total population.

sequences, once begun. The reaction with antibody, as estimated by agglutination, and the initiation of complement sequences, as estimated by Cl fixation, was determined for GSH-treated normal cells and their paired controls (Table 2). Although the CLS titer was increased 4-fold by GSH treatment, there was no difference in agglutination titer or in the fixation of Cl between the treated and untreated cells.

The complement-sensitive cells in PNH appear to lack acetylcholinesterase. The AChE activity of normal cells before and after GSH treatment was compared (Table 3). The AChE content of the complement-sensitive cells of two patients with PNH are likewise shown; the complement sensitivity of this population in these patients was nearly the same as that of the GSH-treated cells.*

**DISCUSSION**

The PNH-like cells have been made with a number of thiol-containing compounds, many of which contain a β-amino group. Kann et al. proposed three mechanisms by which such compounds (glutathione in particular) might react with membrane constituents: (1) Glutathione might be oxidized in the presence of iron and molecular oxygen to yield hydrogen peroxide,

![Fig. 4](https://example.com/figure4.png)

**Fig. 4.**—The fractional increase in complement sensitivity brought about by incubation of normal and PNH cells with GSH (0.46 M, pH 8.0, incubated at 37°C for 60 min) as a function of the initial sensitivity to complement.

*These patients belong to a subgroup of patients with PNH, the red cells of whom are characterized by a less marked increase in CLS of the complement-sensitive cells than most patients with this disorder. Further details will be published.
Table 2.—Comparison of GSH-treated and Untreated Normal Cells With Respect to Agglutination and Fixation of the First Component of Complement (Cl) by Cold Agglutinin

<table>
<thead>
<tr>
<th>Donor</th>
<th>Complement Sensitivity (CL5 Hay Units)</th>
<th>Agglutination Titer (Reciprocal of Extinction Dilution)</th>
<th>Cl Fixation (Molecules Cl Fixed per Cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. B.</td>
<td>No GSH</td>
<td>3.3</td>
<td>1600</td>
</tr>
<tr>
<td></td>
<td>+ GSH</td>
<td>14.5</td>
<td>3200</td>
</tr>
<tr>
<td>G. E.</td>
<td>No GSH</td>
<td>3.3</td>
<td>800</td>
</tr>
<tr>
<td></td>
<td>+ GSH</td>
<td>15.0</td>
<td>800</td>
</tr>
<tr>
<td>M. J.</td>
<td>No GSH</td>
<td>3.0</td>
<td>3200</td>
</tr>
<tr>
<td></td>
<td>+ GSH</td>
<td>15.3</td>
<td>1600</td>
</tr>
<tr>
<td>N. M.</td>
<td>No GSH</td>
<td>2.6</td>
<td>400</td>
</tr>
<tr>
<td></td>
<td>+ GSH</td>
<td>13.3</td>
<td>400</td>
</tr>
</tbody>
</table>

* Normal human red cells were incubated 60 min at 37°C in VBS or in 0.472 M reduced glutathione (GSH), pH 8.0.

leading to lipid peroxidation; however, it has been subsequently shown that the reaction also occurs under strictly anaerobic conditions.\(\text{17}\) (2) The oxidation of reduced glutathione might yield intramembrane disulfide bridges. (3) Reduced glutathione might form mixed disulfides with membrane thiol groups. Stickney et al. have shown that radioactive glutathione is firmly bound to the membrane of the PNH-like cells, suggesting that the third mechanism was the most likely.\(\text{18}\) Our data also suggest that reaction with membrane thiol groups is important since PCMBS could only react with membrane thiol groups and could not partake in the other suggested reactions.

DeSandre et al.\(\text{8}\) have suggested that the substrate with which these reagents react must be other than membrane thiol groups. Reaction of normal cells with n-ethylmaleimide or DTNB \((5,5'\text{ dithiobis}(2\text{-nitrobenzoic acid})\), substances that react with intra- and extracellular -SH groups, did not make

Table 3.—The Effect of Treatment of Normal Cells With Reduced Glutathione (GSH) on Their Acetylcholinesterase Content

<table>
<thead>
<tr>
<th>Donor</th>
<th>Complement Sensitivity</th>
<th>Red Cell Acetylcholinesterase</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. S.</td>
<td>No GSH</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>+ GSH *</td>
<td>10.8</td>
</tr>
<tr>
<td>A. T.</td>
<td>No GSH</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>+ GSH</td>
<td>12.0</td>
</tr>
<tr>
<td>M. G.</td>
<td>PNH</td>
<td>10 †</td>
</tr>
<tr>
<td>A.W.</td>
<td>PNH</td>
<td>11.2 †</td>
</tr>
</tbody>
</table>

* Normal human red cells were incubated 60 min at 37°C in 0.46 M reduced glutathione (GSH). The acetylcholinesterase content and complement lysis sensitivity were determined on the surviving cells.
† Analysis of the complement-sensitive population only.
PNH-like cells and did not alter the ability of 2-mercaptoethylamine or AET to make the cells PNH-like. However, it is not clear from their paper the extent to which membrane thiols had been blocked by these compounds.

The data of Canellos et al. suggest that an abnormality of the membrane thiol structure may exist in PNH cells. They found that a portion of PNH cells were lysed by lower concentrations of PCMBs than normal cells, even in the absence of complement. They implied, but did not prove, that these susceptible cells were the complement-sensitive cells. However, they could not detect a reduction in the total number of membrane sulfhydryl groups in PNH cells but could in AET-treated cells.

Normal cells incubated in glutathione and PCMBs undergo changes other than augmented susceptibility to immune lysis. The cells tend to lyse spontaneously on incubation, and the amount of spontaneous lysis that occurs parallels the increase in complement sensitivity. Jacobs and Jandl have shown that reaction of membrane thiols results in ionic disequilibrium and consequent lysis of the cell. GSH and PCMBs probably react with membrane thiols, thus increasing spontaneous lysis. Untreated PNH cells, on the other hand, do not undergo more spontaneous lysis than normal cells. If, then, the increase in complement sensitivity in both PNH cells and GSH or PCMBs-treated cells is due to inactivation of membrane thiols, the defect in these thiols is more selective in the PNH cells than in the treated normal cells.

The glutathione-treated cells are distinctly different from PNH cells in another important respect. Membrane-bound acetylcholinesterase is undetectable in the complement-sensitive PNH cells but is slightly increased in the GSH-treated cells. Kann and Mengel and DeSandre et al. found a modest (up to 50%) reduction in the cholinesterase content in sulphydryl-reagent treated cells, which in the latter group did not parallel the increase in lysis in acidified serum. The levels of acetylcholinesterase which they report are far in excess of that seen in the PNH cells of comparable complement sensitivity.

The increase in complement sensitivity following incubation with glutathione appears to be due to an increased efficiency of the complement sequence. No evidence of increase in antibody adsorption or increase in the number of initiated complement sequences was found. In this respect, these cells resemble complement-sensitive cells in PNH, although the degree of complement sensitivity (10–15 CLS H50 units) is far less than the usual sensitivity of complement-sensitive PNH cells (30–50 CLS H50 units).

The complement sensitivity of both the complement-sensitive and complement-insensitive cells of PNH is also increased by incubation with glutathione. The fractional increase is less for these two types of cells and is in fact inversely related to the initial complement sensitivity. The line relating the logarithm of the initial sensitivity of normal and both types of PNH cells to the logarithm of the fractional increase is straight, suggesting that normal and both types of PNH cells form a continuous spectrum with regard to the effect of glutathione. Extrapolation of the line to the point where the line intercepts a ratio of 1.0 intersected at an initial CLS H50 of about 120, suggesting that no effect of GSH would be seen if cells had an initial CLS H50 of 120. Since the most
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sensitive PNH cell has a CLS H50 of about 45, this prediction is not experimentally evaluable.

These data do not delineate whether or not the lesion induced by glutathione is the same as that present in complement-sensitive PNH cells. Both lesions may be the result of changes in membrane protein structure involving the loss of membrane thiol groups. If so, certainly the lesion is more selective in PNH cells and may involve the loss of membrane protein, including acetylcholinesterase. Alternatively, the lesion in PNH, and the lesion induced by thiol compounds may be totally unrelated except that both result in an increased sensitivity to the lytic action of complement. In any event, detailed understanding of the lesion(s) leading to complement sensitivity in these two types of cells would help elucidate the mechanism of resistance to complement lysis in normal red cells.

REFERENCES

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