Studies on the Mechanism of in Vitro Acid Hemolysis in Paroxysmal Nocturnal Hemoglobinuria

By J. G. SCOTT, R. I. WEED and S. N. SWISHER

The erythrocytes of individuals suffering from paroxysmal nocturnal hemoglobinuria (PNH) show marked increase in susceptibility to lysis in fresh human serum at an acid pH.1-3 The mechanism of this hemolysis has not been clearly elucidated at the present time, but components of human complement, C',3-5 and Mg++6 are considered essential components of the hemolytic system. In some cases hemolysis in vitro occurs at physiologic pH, but hemolysis is markedly accentuated by acidification to pH values between 7.0 and 6.5. The addition of thrombin has also been shown to augment in vitro acid hemolysis.7 Dacie and Mollison8 demonstrated that the defect was intracorpuscular in nature. Although the defect is generally considered to reside in the erythrocyte membrane, electron microscopic studies to date have been inconsistent.9-15 The relationship of these in vitro observations to in vivo hemolysis remains obscure. The subject has recently been extensively reviewed by Hartmann and Jenkins.16

Studies from our laboratory have shown that the mechanism of erythrocyte lysis which results from the action of complement and hemolytic antibody depends on the size of functional "holes" which are produced in the red cell membrane.17,18 Thus, antibody-C' lysis of human red cells may occur by a direct mechanism if the initial defect produced is greater than the size of the hemoglobin molecule, or by an indirect mechanism wherein a hole smaller than hemoglobin is produced, permitting colloid osmotic swelling and subsequent lysis to take place. Both of these mechanisms have been observed in in vitro systems. The purpose of this paper is to determine whether acid hemolysis occurs by the direct mechanism or by a colloid osmotic mechanism. In addition, since the PNH erythrocyte has been shown by many investigators to show increased reactivity in certain isoimmune systems,4,19,20 it seemed important to determine whether this cell would be capable of reacting in a normal fashion—i.e., by colloid osmotic lysis—when treated with a rabbit anti-human red cell antibody and C'. Such a mechanism has previously been demonstrated for this particular antibody17 using normal human red cells.

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Materials and Methods

Red cells. Red blood cells* from patients with paroxysmal nocturnal hemoglobinuria were collected in sterile vacuum containers containing acid citrate dextrose solution (ACD), and stored at 4°C until used. In most cases cells were used within 1 to 2 days. Cells were washed three times in 1 per cent saline prior to use. In certain cases, PNH cells were spun at 15,000 g in small polyethylene tubes (diameter 3 to 4 mm.) for 10 minutes to collect the reticulocyte-rich upper layer which has been shown to have increased susceptibility to acid lysis.21-23

Complement. Blood was collected in sterile containers from hematologically normal female and male donors, allowed to clot at room temperature (approximately 23°C) for 1½ hours, and the serum separated by centrifugation at 1800 g for 30 minutes. The serum was either used immediately or kept frozen until used. Sera stored for any length of time appeared to lose considerable potency in producing acid hemolysis. Numerous compatible sera were tested for their ability to hemolyze PNH cells in the acid serum test. A wide variation was observed in the amount of hemolysis produced.24 The sera used in these experiments were selected for their ability to produce maximum hemolysis at pH 6.6.

Antisera. The rabbit antihuman red cell antibody used in some experiments was prepared as previously described.17

Macromolecules Added to Medium. (1): Well-characterized dextran fractions† were prepared by dissolving the dextran powder in 0.1 molar tris buffer made isotonic with sodium chloride. The dextran and buffer were warmed in a water bath to facilitate dissolving.

(2): When ovalbumin and bovine serum albumin§ were used, they were added directly to the serum samples.

Preparation of Samples. The bovine serum albumin and ovalbumin were added directly to the fresh serum and the pH of the mixture adjusted to 6.6 ± 0.1 with 0.3 N HCl before the addition of the test cells. Dextran solutions as prepared above were added to the fresh serum and the pH adjusted to 6.6 ± 0.1. The cells, after being washed three times, were added to the above mixture and incubated for 1½ hours at 37°C in a Dubnoff metabolic shaker. The final hematocrit ranged from 10 to 20 per cent. At the beginning and during the incubation, the pH was rechecked and adjusted if necessary to maintain a constant pH (6.6 ± 0.1). The amount of hemolysis in some experiments was increased to make the observations more reproducible by (1) the addition of Mg++ to a final concentration of 5 mM and (2) the use of reticulocyte-rich blood with increased susceptibility to acid lysis (Table 1). The pattern of hemolysis, however, remained unchanged in all cases. In the case of the dextrans, the amount of hemolysis in the final mixture was considerably less than that observed in the standard Ham’s test (Table 2).

Determination of Hemoglobin and Potassium Loss. Hemoglobin determinations were carried out using the cyanomethemoglobin method of Crosby, Munn, and Furth.25 Potassium was measured with the Baird Atomic Flame Photometer as described previously.17 The loss of hemoglobin and potassium were calculated as previously described.17

Results

During the incubation, when no protective macromolecule was added to the incubation mixtures, Hb and K+ loss tended to parallel one another. The 1½ hour incubation period was chosen to assure maximal hemolysis, although in

*Some of the PNH cells used in these experiments were provided through the courtesy of Dr. Charles E. Mengel. Department of Medicine, Duke University, Durham, North Carolina.
† Obtained from Pharmacia Fine Chemicals, Uppsala, Sweden; Rochester, Minnesota.
‡ Obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.
§ Obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.
Table 1.—The Effect of High Speed Centrifugation on the Reticulocyte Concentration and the Increased Susceptibility of Reticulocytes to Acid Hemolysis

<table>
<thead>
<tr>
<th>Layer</th>
<th>Reticulocyte Count (%)</th>
<th>Hemolysis (%) at pH 6.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top</td>
<td>60</td>
<td>72</td>
</tr>
<tr>
<td>Bottom</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>Whole blood</td>
<td>14</td>
<td>53</td>
</tr>
</tbody>
</table>

Table 2.—The Effect of Dilution with Isotonic Buffer on Acid Hemolysis of PNH Erythrocytes

<table>
<thead>
<tr>
<th>Fresh Human Serum</th>
<th>Hemolysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluted</td>
<td>52.0</td>
</tr>
<tr>
<td>1:2</td>
<td>20.0</td>
</tr>
<tr>
<td>1:4</td>
<td>7.0</td>
</tr>
<tr>
<td>1:8</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Fig. 1.—The effect of the addition of Dextran 40 on the loss of Hb and K⁺ induced by acid hemolysis of PNH erythrocytes.

some cases hemolysis was observed almost immediately. Normal red cells incubated at pH 6.6 in fresh compatible serum showed negligible loss of Hb or K⁺ during the 1½ hour incubation. When PNH cells were incubated at pH 6.6 in inactivated human serum, negligible loss of Hb or K⁺ was noted. When PNH cells were incubated at pH 7.2 in fresh human serum, there was usually very little hemolysis observed.

The effect of adding Dextran 40 to the incubation mixture is shown in
Fig. 2.—The effect of the addition of Dextran 150 on the loss of Hb and K+ induced by acid hemolysis of PNH erythrocytes.

Fig. 3.—The effect of Dextran 40 on the loss of Hb and K+ induced by the action of rabbit antihuman red cell antibody and guinea pig C' on PNH erythrocytes.

Figure 1. Dextran 40 is a well-characterized fraction of dextran with a molecular weight of 26,200 and an effective diffusion radius greater than Hb. When Dextran 40 was added to the medium in a concentration calculated to balance
Fig. 4.—The effect of Dextran 10 on the loss of Hb and K+ induced by the action of a rabbit antihuman red cell antibody and guinea pig C' on PNH erythrocytes.

the intracellular osmotic pressure of the cell, Hb and K+ loss were unaffected. With higher concentrations of the Dextran 40—i.e., 4.0 mM—there was some decrease in hemolysis observed, but in no case was Hb loss significantly less than K+ loss, thus indicating inhibition of the lytic reaction rather than colloid osmotic protection. Similar experiments were carried out with other macromolecules, including ovalbumin, bovine serum albumin and Dextran 10. As anticipated from the results of the Dextran 40 studies, similar failure of osmotic protection was observed.

Figure 2 shows a similar experiment where Dextran 150 was the added macromolecule. Dextran 150 has a molecular weight of 95,000. In this case it can be seen that marked inhibition of both Hb and K+ loss is observed at concentrations far below that expected to provide osmotic protection. The effect in this case is thus shown to be related to inhibition of the C' system rather than to colloid osmotic protection.

In order to study the pattern of reactivity of PNH erythrocytes exposed to a hemolytic antibody and C' at pH values 7.2 ± 1, studies were carried out with these cells exposed to a hemolytic rabbit antihuman red cell antibody employed in previous studies of normal human red cells. Both human serum and guinea pig serum were used as a source of C'. In the case of the guinea pig C', no spontaneous hemolysis was observed at pH 6.6. Figure 3 shows the results of treating PNH cells with this rabbit antihuman red cell antibody and guinea pig complement when Dextran 40 is added to the incubation mixture in increasing amounts. The addition of Dextran 40 in a concentration
calculated to balance the internal osmotic pressure of the cell almost inhibited Hb loss, while K⁺ loss remained unchanged. Identical results were obtained when bovine serum albumin was used in place of the Dextran 40. These findings indicate osmotic protection. On the other hand, the addition of Dextran 10, as demonstrated in Figure 4, failed to show any alteration of hemoglobin or potassium loss, indicating lack of any protective effect. Similar results were obtained when ovalbumin was used in place of the Dextran 10. When human C' was used in place of guinea pig C', there was more hemolysis observed than with normal RBC but evidence of osmotic protection was clear.

**DISCUSSION**

The studies reported here indicate that in the case of PNH erythrocytes the “hole” produced in the red cell membrane by acid hemolysis has an effective radius greater than that of hemoglobin—i.e., greater than 32.5 A—sufficient to permit direct egress of hemoglobin without the necessity for prior colloid osmotic swelling. In these experiments the size of the acid-induced membrane defects is defined in terms of the size in solution of molecules which do or do not protect the cell against colloid osmotic lysis.¹⁷

Other investigators demonstrated that commercially available dextran (molecular weight 75,000–300,000) were able to inhibit acid hemolysis in vitro⁶,²⁶,²⁷ as well as in vivo.²⁶,²⁷,²⁹ The mechanism of this inhibition appears to be due to the combination of the high molecular weight dextrans with a component or components of the C’ system.⁵ Pillemer,¹¹ however, demonstrated that some dextrans exhibit this ability to inactivate C’ or some components of the C’ system while still others are inactive. In the experiments reported here, the dextran compounds that were active in this inhibition were all of high molecular weights, whereas the low molecular weight clinical dextrans (molecular weight 75,000) were entirely inactive in the concentrations studied. Our findings lend support to these earlier observations—i.e., the lack of inhibition of the hemolytic system by the well-characterized low molecular weight dextrans (Dextran 40) and marked inhibition of the hemolytic reaction by the high molecular weight dextran fractions (Dextran 150).

When PNH erythrocytes were treated with a hemolytic antibody and C’, the defect produced in the red cell membrane appeared to be identical with that seen in the case of normal red cells; that is, it was possible to provide protection against colloid osmotic lysis indicating a membrane defect smaller than 32.5 A. The antihuman rabbit serum produced no greater hemolysis of the PNH red cells than normal cells when guinea pig serum was the source of C’,⁴ and the reactivity in this system was identical with normal cells. In the presence of human serum the amount of hemolysis observed with the PNH erythrocytes was greater than that seen with normal red cells. The pattern of reactivity with the dextrans was, however, identical.

These findings are in contradistinction to the observations of Rosse et al.,³¹ who studied the effects of a variety of hemolysins and human C’ on PNH and normal erythrocytes as well as acid hemolysis of PNH erythrocytes. They
found in all cases what appeared to be membrane defects or “holes” 80 to 100 Å in diameter. These were the same for both PNH and normal erythrocytes treated with the various hemolysins, and were also identical in the case of PNH erythrocytes treated with acid. However, these authors have not used a rabbit antihuman red cell antibody as was employed for the present studies. They have also suggested that more holes are induced by the action of human C’ and antibody than when guinea pig C’ is used, thus providing an explanation for the failure of guinea pig C’ to support acid hemolysis. Although previous studies did not show any difference in the mechanism of action of C’ from different species in this experimental system, the possibility remains that failure of protection against osmotic hemolysis may be related to coalescence of several holes, each having an effective diffusion radius smaller than hemoglobin.

The studies of Yachnin suggest that PNH erythrocytes have the ability to fix C’ (β1c globulin) directly, perhaps due to a difference involving the number of membrane sites available for binding. Indeed, the studies of Rosse and Dacie indicate that the increased sensitivity of PNH erythrocytes to antibody lysis is in fact due largely to a greater efficiency of each complement complex at the red cell surface. The relationship of these findings to acid hemolysis is not clear, since no antibody has yet been identified.

It is generally presumed that the plasma pH never reaches the levels required for maximal acid hemolysis in vitro. However, Murphy has shown that the pH in the spleen may fall as low as 6.8. In addition, using the values given by Hong for urinary carbon dioxide, and assuming a normal plasma bicarbonate, the pH, as calculated from the Henderson-Hasselbach equation, may fall below 7.0 in some areas of the renal medulla. Thus, although the whole body pH never reaches levels of 6.8–7.0, it may in selected areas of the circulation.

It is clear that the in vitro observations described here fail to explain in vivo mechanisms. They do, however, provide insight into the nature of the membrane defect or structural abnormality which appears to be present in the PNH erythrocyte.

**Summary**

1. The mechanism of acid hemolysis of PNH erythrocytes appears to be a direct mechanism, with the production of an initial “hole” in the membrane sufficiently large to permit the direct egress of hemoglobin.

2. PNH erythrocytes react like normal red cells with rabbit antihuman red cell antibody and human or guinea pig C’ at pH 7.2 to produce a membrane defect which is less than 32.5 Å in effective diffusion radius.

3. Inhibition of acid hemolysis of PNH erythrocytes by high molecular weight dextrans (Dextran 150) is also associated with inhibition of K⁺ loss, indicating inhibition of complement.

**SUMMARIO IN INTERLINGUA**

1. Il pare que le mecanismo del hemolysce acide de erythrocytos in paroxysmic hemoglobinuria
globinuria nocturne es un mecanismo directe, con le production de un "lacuna" inicial in le membrana sufficientemente grande pro permitter le egresso directe de hemoglobina.

2. Erythrocytos de paroxysmal hemoglobinuria nocturne reage como erythrocytos con anticorpore anti erythrocytos human ab coniolios e C' de porco de India a pH 7, 2, resultante in le production de un defecto membranose que possede un efficae radio diffusional de minus que 32, 5 A.

3. Le inhibition del hemolyse acide de erythrocytos in paroxysmal hemoglobinuria nocturne per dextranos de alte peso molecular (Dextran 150) es etiam associate con le inhibition del perdita de K', lo que indica un inhibition de complemento.

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