Paroxysmal Nocturnal Hemoglobinuria

Mechanism of the Enhancement of Hemolysis by Bovine Thrombin

By Stuart F. Blum and Frank H. Gardner

With the technical assistance of Dominica Paci

The erythrocytes of patients with paroxysmal nocturnal hemoglobinuria (PNH) are uniquely susceptible to hemolysis in normal human serum in the absence of demonstrable antibody. The degree of hemolysis is increased if the serum in which the cells are incubated is slightly acidified, and this phenomenon is the basis for the diagnostic test for the disease. In 1950 Crosby found that the addition of bovine thrombin to the test serum likewise enhanced the lysis of the PNH erythrocytes, and he proposed that the coagulation system was intimately involved with the PNH hemolytic system. This proposal has been disputed, and a controversy has developed concerning the significance of the enhancement of the lysis of PNH erythrocytes by bovine thrombin. The present studies were undertaken to define the role, if any, of thrombic clotting activity in enhancing PNH lysis.

Methods

Whole blood from three patients with PNH was obtained aseptically and stored in ACD-A anticoagulant at 4 C. for no more than 7 days. The suitability of such stored cells for use in studies of PNH lysis has been reviewed. The erythrocytes were washed 5 times in isotonic saline prior to use. Serum was obtained from normal donors and from patients with polycythemia vera requiring phlebotomy. Whole blood was allowed to clot at 37 C. for 30 min., and the serum was separated and stored at -80 C. Crude bovine thrombin (Upjohn Topical Thrombin) was obtained commercially. The acid hemolysis test was performed by adding 0.05 ml. of a 20 per cent suspension of red cells to 1 ml. of compatible serum, the pH of which had been adjusted to 6.5 with N/3 HC1. The mixture was covered with 2 drops of mineral oil to prevent pH rise due to loss of CO2 during incubation, and the mixture was incubated at 37 C. for 30 min. After centrifugation the optical density of the supernatant serum was determined at 541 mJ. Hemolysis, 100 per cent, was measured by freezing and thawing the mixture three times and then determining the optical density of the supernatant serum. Hemolysis accentuated by thrombin was determined similarly with the exception that 50 NIH units of thrombin in lyophilized form was added to each milliliter of serum prior to acidification. This addition of thrombin in lyophilized form prevented any dilution of the acidified serum.

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Supported in part by grants (TI-AM-5241-06 and ROI-AM-00964-11) from the United States Public Health Service and by a United States Public Health Research Career Program award (HE-K3-14-927) from the National Heart Institute.

First submitted Oct. 20, 1966; accepted for publication Feb. 21, 1967.

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Blood, Vol. 30, No. 3 (September), 1967
Diisopropylfluorophosphate (DFP) inactivated thrombin was prepared by dissolving the thrombin at a concentration of 50 U/mL in imidazole buffer and then adding sufficient DFP to obtain a $1 \times 10^{-5}$ M solution of DFP. After 15 minutes at room temperature the solution was dialyzed overnight at 4°C against phosphate buffered saline, pH 7.4. A control sample of thrombin in imidazole buffer without DFP was similarly prepared and dialyzed.

Thrombic clotting activity was evaluated by a standard test.

The esterolytic activity of the thrombin solutions was measured by the ability to hydrolyze tosylarginylmethylester (TAME) in a standardized test procedure.

Gel filtration chromatography of crude thrombin was performed on a 200 × 2 cm. column of Sephadex G-200 crosslinked dextran gel. The protein fractions were eluted with 1 M sodium chloride.

Immunoelectrophoresis was performed on 25 mm. × 75 mm. glass microscope slides. Rabbit antihuman serum was obtained commercially.

Thrombin absorbed with normal human red cells was prepared by dissolving the thrombin at a concentration of 50 U/mL in phosphate buffered saline, pH 7.4, and then adding one-tenth volume of washed packed group A red cells from a normal donor. The suspension was incubated at 37°C for 30 min., centrifuged, and the thrombin solution added to another tenth volume of cells and the suspension incubated at 4°C for 30 min. This process was repeated four times and aliquots were taken at the end of each set of incubations for assay of clotting and PNH enhancing activity.

Mercaptoethanol treatment of the hemolysis enhancing thrombin fraction (tube 30, Fig. 1) eluted from the Sephadex column was performed by adding sufficient mercaptoethanol to make the solution 0.1 M in mercaptoethanol. After standing for 30 min. at room temperature, sufficient iodoacetic acid was added to make the solution 0.1 M in iodoacetic acid. The solution was then dialyzed overnight at 4°C against phosphate buffered saline.

**RESULTS**

1. The Site of Action of Thrombin. Whereas Crosby had suggested that thrombin activated serum factors enhancing PNH lysis, Kirchmayer maintained that the thrombin affected the PNH cells themselves. To test this point, a suspension of PNH red cells in a 50 U/mL thrombin in saline solution was preincubated at 37°C for 30 min. This procedure strongly agglutinated the cells. These cells were then washed 3 times in saline and their lysis in a standard acid hemolysis test was compared with the lysis in a standard thrombin test of both unincubated cells and cells which had been incubated in saline for 30 min, at 37°C and washed 3 times in saline. The results confirm the findings of Kirchmayer and clearly indicate that maximal enhancement of hemolysis was obtained by preincubating the cells with the thrombin and that no further increment in hemolysis was obtained by adding the thrombin to the cell-serum mixture (Table 1). The experiments were repeated using a 5 per cent suspension of PNH cells, and similar results were obtained. These data imply that the entire effect of the bovine thrombin is on the erythrocytes themselves without any contribution due to activation of serum factors.

2. To evaluate whether the action of the thrombin on the cells was an enzymatic effect, DFP inactivated thrombin was prepared and tested for its ability to enhance PNH lysis. Although the DFP-treated thrombin was devoid...
Fig. 1.—Sephadex G-200 chromatography of crude bovine thrombin. The PNH hemolysis enhancing activity and the thrombic clotting activity are clearly separated.

Table 1.—Sensitization of PNH Cells to Acid Hemolysis by Prior Incubation with Crude Bovine Thrombin

<table>
<thead>
<tr>
<th></th>
<th>% Hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells preincubated with thrombin, then tested for acid hemolysis</td>
<td>44.2</td>
</tr>
<tr>
<td>Cells preincubated with saline, then thrombin-tested</td>
<td>44.0</td>
</tr>
<tr>
<td>Routine thrombin test</td>
<td>43.2</td>
</tr>
<tr>
<td>Cells preincubated with saline, then tested for acid hemolysis</td>
<td>27.4</td>
</tr>
<tr>
<td>Routine acid hemolysis test</td>
<td>27.7</td>
</tr>
</tbody>
</table>

of clotting and esterolytic activity, it was as effective as untreated thrombin in enhancing PNH lysis (Table 2).

3. To further define the factor or factors in crude bovine thrombin responsible for the enhancement of PNH lysis, thrombin which had been absorbed with normal human red cells was tested. Although the absorption removed none of the clotting activity, each step of the absorption removed a significant quantity of the PNH enhancing factor until, after 4 absorptions, the thrombin was devoid of enhancing activity (Table 3). It was noted that in the initial absorption the normal red cells were strongly agglutinated, but the degree of agglutination diminished with each subsequent absorption step and in the final step there was no agglutination.

4. To effect physical separation of the thrombic clotting activity and the PNH hemolysis enhancing activity, a thrombin solution was fractionated by gel filtration. Assays of the clotting activities and the PNH enhancing activities of the effluent fractions showed clear separation of these activities (Fig. 1). The PNH enhancing activity was eluted with the void volume of the column, indicating, according to the manufacturer of the gel, a molecular weight greater than 200,000. The thrombic clotting activity was eluted later, indicating a smaller-sized molecule.
Table 2.—Enhancement of PNH Hemolysis by DFP Inactivated Thrombin

<table>
<thead>
<tr>
<th>Clotting Time</th>
<th>TAME Units</th>
<th>% Hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 2000 seconds</td>
<td>0</td>
<td>32.2</td>
</tr>
<tr>
<td>Control dialysed thrombin</td>
<td>7 seconds</td>
<td>31.5</td>
</tr>
<tr>
<td>Routine acid hemolysis test</td>
<td></td>
<td>18.4</td>
</tr>
</tbody>
</table>

Table 3.—Effect of Absorption by Normal Red Cells on the PNH Enhancing Activity of Crude Bovine Thrombin

<table>
<thead>
<tr>
<th>% Hemolysis</th>
<th>Clotting U./ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unabsorbed thrombin</td>
<td>41.9</td>
</tr>
<tr>
<td>One absorption</td>
<td>23.8</td>
</tr>
<tr>
<td>Two absorptions</td>
<td>19.0</td>
</tr>
<tr>
<td>Three absorptions</td>
<td>14.8</td>
</tr>
<tr>
<td>Four absorptions</td>
<td>12.9</td>
</tr>
<tr>
<td>Routine acid hemolysis test</td>
<td>13.6</td>
</tr>
</tbody>
</table>

5. To define the characteristics of the PNH enhancing factor, a fraction from the Sephadex column containing peak enhancing activity was treated with mercaptoethanol, which by disrupting disulphide bonds inactivates 19S and aggregated 11S antibodies. This treatment completely destroyed the PNH enhancing activity of the fraction, providing further evidence of the macromolecular nature of the enhancing factor (Table 4).

6. Immunoelectrophoresis of whole crude bovine thrombin revealed that the preparation appeared to contain most, if not all, the proteins of bovine serum (Fig. 2). Immunoelectrophoresis of the fractions eluted from the Sephadex column showed that partial separation had been achieved.

Discussion

Since the initial description of the effect of bovine thrombin in enhancing the lysis of PNH erythrocytes, this phenomenon has been the subject of considerable study. Crosby felt that this action implied involvement of the coagulation mechanism in the PNH lytic system, but Hinz suggested that contamination of the thrombin preparations with heterophil antibody was responsible for the enhancement of hemolysis. Hinz has shown that an antibody coating a PNH cell increased the cell's liability to lysis. Subsequent studies of the mechanism of thrombic enhancement of PNH lysis failed to clarify the situation. Similarly, studies of the ability of normal red cells to absorb the PNH enhancing activity yielded conflicting results; Hinz found that all, Crosby almost none, Auditore none, and Jenkins almost all of the PNH enhancing activity could be absorbed by normal human red cells. Evidence was accumulating, however, which tended to disprove any role of thrombic clotting activity in the PNH hemolytic system. Auditore showed that highly purified bovine thrombin had lost most of its PNH enhancing activity. This finding was confirmed by Jenkins, who purified thrombin by gel filtration and ion exchange chromatography and found it to have no PNH enhancing
Table 4.—Abolition of PNH Enhancing Activity of Mercaptoethanol

<table>
<thead>
<tr>
<th>Mercaptoethanol treated thrombin fraction (Tube 30, Fig. 1)</th>
<th>20.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated thrombin fraction (Tube 30, Fig. 1)</td>
<td>35.5</td>
</tr>
<tr>
<td>Routine acid hemolysis test</td>
<td>21.3</td>
</tr>
</tbody>
</table>

Fig. 2.—Immunoelectrophoretic analysis of crude bovine thrombin and of the PNH enhancing fraction (Fraction 30) and the clotting fraction (Fraction 55) obtained by Sephadex G-200 chromatography. The crude bovine thrombin is very heterogeneous. Partial separation of the components has been achieved.

activity at a concentration of 20 NIH units. Jenkins also has shown that whereas PNH cells surviving acid hemolysis bear only the $\beta_{1C}$ globulin component of complement on their surface, PNH cells surviving thrombin accentuated hemolysis bear both $\beta_{1C}$ and $\beta_{1G}$ globulins. The presence of both complement components is additional evidence for the participation of antibody in the accentuation of PNH lysis by crude thrombin.

As referred to previously, Crosby felt that thrombin affected serum factors enhancing PNH lysis, but the studies of Kirchmayer indicated that the effect of the thrombin was on the PNH cells. We have confirmed Kirchmayer’s findings and have clearly shown by preincubating the PNH cells alone with the thrombin that essentially all the enhancing effect is obtained by this preincubation and that the addition of the thrombin to the incubation mixture results in no further enhancement of the hemolysis. These data indicate that the effect of the thrombin is solely on the PNH erythrocytes.

We have investigated this effect of the crude thrombin on the red cells by physical and chemical separation of the thrombic clotting and PNH enhancing activities. Through DFP inactivation of enzyme activity, absorption of PNH enhancing activity with normal red cells, and by gel filtration on Sephadex, we have been able to clearly separate the two activities and to show that the enzymatic thrombic clotting activity plays no role in enhancing PNH lysis.
The crude bovine thrombin preparation has been shown by immunoelectrophoresis to contain most, if not all, the proteins of bovine serum. The lack of retardation of the enhancing factor on the Sephadex column and the inactivation of its activity by mercaptoethanol provide evidence of the macroglobulin nature of this factor. These data support the contention that the enhancement of PNH lysis by bovine thrombin results from contamination of the thrombin by high molecular weight heterologous antibody, and the data negate any role of thrombic clotting activity in the PNH lysis system.

SUMMARY

1. Crude bovine thrombin contains most, if not all, the proteins of bovine serum.
2. The effect of crude bovine thrombin in enhancing the lysis of paroxysmal nocturnal hemoglobinuria erythrocytes has been shown to be an effect on the erythrocytes themselves.
3. By enzyme inactivation, absorption technics and Sephadex chromatography the thrombic clotting activity has been separated from the PNH enhancing activity nullifying any role of thrombic clotting activity in the enhancement of PNH hemolysis by crude bovine thrombin.

SUMMARIO IN INTERLINGUA

1. Crude thrombina bovin contine le plus grande parte si non le totalitate del proteina de sero bovin.
2. Esseva demonstrate que le promotion causate per crude thrombina bovin in le lyse de erythrocytos ab patientes con paroxysmic hemoglobinuria nocturne es un effetto del erythrocytos mesme.
3. Per inactivation enzymatic, technicas de absorption, e chromatographia a Sephadex, le activitate coagulatori thrombic esseva separate ab le activitate promovente paroxysmic hemoglobinuria nocturne, nullificante omne rolo de activitate coagulatori thrombic in le promotion de hemolyse de paroxysmic hemoglobinuria nocturne per crude thrombina bovin.

REFERENCES

9. Auditore, J. V., Hartmann, R. C., and
Paroxysmal Nocturnal Hemoglobinuria: Mechanism of the Enhancement of Hemolysis by Bovine Thrombin

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