Paroxysmal Nocturnal Hemoglobinuria: The Role of Antibodies in the Diagnostic Thrombin Test

By William H. Crosby and Naomi R. Benjamin

An abnormality of the red cells of paroxysmal nocturnal hemoglobinuria (PNH) is responsible for their short life span in the circulation and their lysis in vitro by certain factors present in normal plasma or serum. The hemolytic system in vitro is characteristically more active when the serum is made somewhat acid, about pH 6.8. The addition of thrombin to the serum is also capable of increasing the hemolytic activity. These two phenomena, enhancement of hemolysis by acidification and by thrombin, are the basis of several diagnostic tests for the disease.\(^1\)\(^2\)

Although several studies dealing with these phenomena have been published, it is not yet known how either of the tests works.\(^3\)\(^-\)\(^7\) Hinz recently proposed a mechanism whereby the thrombin preparations may enhance PNH hemolysis.\(^8\) He demonstrated that thrombin of bovine origin was contaminated with heterophil antibodies and that these antibodies were capable of acting as immune hemolysins to hemolyze PNH red cells independent of the PNH hemolytic system. When these antibodies were absorbed by preliminary incubation of the thrombin solutions with normal human red cells, the material would no longer enhance hemolysis in the thrombin test. It is known that PNH red cells are exceptionally susceptible to hemolysis by various kinds of hemolytic antibodies,\(^9\) and therefore it seemed reasonable to conclude that the thrombin test was positive in PNH because of the presence of the contaminating antibodies in the thrombin preparations.

We found in thrombin the antibodies described by Hinz. After we had removed the antibodies, the “absorbed thrombin” was used in the thrombin test with red cells of several patients with PNH. The tests were positive in every case. This report deals with the basis of the discrepancy.

Methods and Materials

PNH red cells were obtained from three patients with signs of the disease and histories of several years’ duration.* The red cells were washed with isotonic saline solution at least three times before use. Five per cent suspensions of the cells were prepared by measuring in appropriate volume of washed, packed cells directly into the incubation mixture. Normal red cells were prepared in the same way and used in appropriate controls and for preparing the thrombin.

Sera were prepared from blood drawn into siliconized or oiled syringes and centrifuged in silicone immediately without anticoagulants. The supernatant plasma was transferred to glass, and when it had clotted the fibrin was squeezed out to get the serum. Serum and cells were kept cold until used.

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Thrombin was prepared by dissolving 5000 U. of powder (Parke Davis Co., Thrombin Topical, of bovine origin) in 4 or 5 ml. of fresh human serum of a blood group compatible with that of the red cells to be used. To absorb the antibodies, a portion of the solution was added to an equal volume of normal red cells, freshly drawn, washed and packed by centrifugation. This cell suspension was incubated at 37 C. for 20 minutes, then centrifuged and the supernatant serum transferred to another equal volume of red cells. The incubation was repeated, this time at 4 C. The cells were changed again and a third and final incubation was carried out at 4 C. for 20 minutes and then at 37 C. Serum was used instead of saline to dissolve the thrombin because it has been observed that some antibodies are fixed by red cells only in the presence of active complement.1

Three tests performed on the absorbed thrombin demonstrated the completeness of absorption of the antibodies. (1) Microscopic inspection of a 2 per cent suspension of red cells in the thrombin after the final incubation revealed no agglutination. (2) A Coombs test performed on these red cells with rabbit antiovine serum was negative. (3) Addition of absorbed thrombin to a suspension of PNH red cells in fresh serum at pH 7.6 did not result in any hemolysis. In such a suspension unabsorbed bovine thrombin caused hemolysis due to the heterophil antibodies. The acid-serum PNH system was inactive at this pH.

Absorption of "acid antibodies" (antibodies active at low pH) was performed by acidifying serum thrombin material with N/3 HCl. After adding normal erythrocytes to absorb the antibodies, the pH of the cell suspension was measured to ensure that it was 6.8. This acid absorption was in addition to the others described above. It did not modify the characteristics of the thrombin material in the tests described below.

Imidazole buffer pH 6.8, molarity 0.75, was prepared according to the method of Mertz and Owen11 and used according to the instructions of Hinz et al.8,12

In preparing the incubation mixtures for the thrombin test, the serum was first acidified by the addition of five per cent of N/3 HCl to lower the pH to approximately 6.8. The packed red cells were added and mixed with the serum; the pH was not appreciably changed by addition of the red cells. Finally, the suspension was divided into the various tubes for incubation; in these tubes had been placed the various additives such as thrombin. After mixing, the tubes were incubated in a water bath at 37 C. for 15 minutes, then centrifuged and the serum removed to be analyzed for hemoglobin concentration.13

RESULTS

Absorption of heterophil antibodies.—The thrombin test was performed with the use of a solution of 5000 U. of bovine thrombin in 4 ml. of normal compatible serum. After the heterophil antibodies had been removed by incubation with human red cells, the thrombin solution was divided and part of it was heated to 56 C. for 30 minutes. Some of the solution was heated without having had the antibodies removed, and some of the serum was heated without thrombin. In each tube 0.5 ml. of acid-serum red cell suspension was mixed with 0.02 ml. of additive (table 1).

<table>
<thead>
<tr>
<th>Table 1.—The Effect on the Thrombin Test of Absorption and Heating of the Thrombin Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Results (mg. Hb./100 ml.)</td>
</tr>
<tr>
<td>Acidified fresh serum plus PNH cells</td>
</tr>
<tr>
<td>Cell suspension plus thrombin dissolved in serum</td>
</tr>
<tr>
<td>Cell suspension plus absorbed thrombin</td>
</tr>
<tr>
<td>Cell suspension plus heated thrombin</td>
</tr>
<tr>
<td>Cell suspension plus absorbed, heated thrombin</td>
</tr>
<tr>
<td>Cell suspension plus serum without thrombin</td>
</tr>
<tr>
<td>Cell suspension plus heated serum without thrombin</td>
</tr>
</tbody>
</table>
COMMENT. As previously reported, the heating of thrombin did not remove its activity in the PNH test. But this experiment also indicates that absorption of the heterophil antibodies did not eliminate the effect of thrombin in the PNH hemolytic system. With "absorbed thrombin" and even absorbed, heated thrombin, the thrombin test was strongly positive.

The thrombin test using human thrombin.—A preparation of thrombin prepared from human plasma (Cutter Laboratories) was used in the thrombin test. About 2 mg. of the dry material was added to a hemolytic reaction with group-O PNH red cells. It made the serum rather turbid, but it definitely increased the hemolysis. A human thrombin marketed by Ortho Pharmaceutical Corporation under the trade name Fibrindex proved to be unsatisfactory, completely inactivating the hemolytic system. Several years ago a similar failure of the thrombin test was reported from Algeria: the thrombin in this case was a French product, Thrombase Roussel, dissolved in a glycol base which inactivated the hemolytic system.

The original work on this procedure done in 1949 involved a test of group-O PNH red cells with a human thrombin prepared by The Upjohn Company. The test was positive.

The effect of dilution on the thrombin test.—With the use of absorbed thrombin, the thrombin test was done in acidified normal serum which has been diluted with isotonic sodium chloride solution. Dilution did not alter the pH. The serum was obtained by allowing plasma to clot at room temperature.

In a similar experiment, MgCl₂ was added to the saline to achieve a concentration of 2 mEq. per liter. This increased the hemolysis in tubes 3 and 4, but the amount of hemolysis was the same in both tubes. In table 2, it is apparent that dilution of the thrombin test with saline abolished the effect of thrombin. However, the effect of dilution with saline was not the same in every serum tested. With some sera thrombin would still increase hemolysis if dilution was carried only to 50 per cent. It was also found that thrombin was sometimes ineffective when the diluted serum used was strictly fresh, but six hours later portions of the same serum were diluted and tested again with thrombin, and this time the thrombin test was positive.

COMMENT. With some sera it is possible to dilute out the thrombin effect by addition of saline to the test mixture of PNH red cells in acidified serum. This is not related to reduction of the Mg²⁺ concentration. Perhaps it results from alteration of the factor against which thrombin is effective.

The effect of imidazole on the thrombin test.—The dilution test described above was repeated with the use of normal serum to which had been added 10 per cent of imidazole in the manner described by Hinz et al. In this

<table>
<thead>
<tr>
<th>Tube</th>
<th>Acid serum</th>
<th>Saline</th>
<th>PNH red cells</th>
<th>Absorbed thrombin (mg. Hb./100 mL)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>-</td>
<td>0.025</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>-</td>
<td>0.025</td>
<td>50 U.</td>
<td>95</td>
</tr>
<tr>
<td>3</td>
<td>0.25</td>
<td>0.25</td>
<td>0.025</td>
<td>50 U.</td>
<td>35</td>
</tr>
<tr>
<td>4</td>
<td>0.25</td>
<td>0.25</td>
<td>0.025</td>
<td>50 U.</td>
<td>30</td>
</tr>
</tbody>
</table>
experiment, the mixtures of serum, saline and imidazole were prepared, the
PNH red cells were added and mixed, and then half of each mixture was placed
in a paired tube to which the absorbed thrombin had been added (table 3).

A similar experiment was performed with two other normal sera. The buffer,
saline and thrombin were measured into tubes. In another tube, the acid
serum was prepared, PNH red cells were added and mixed and the suspension
was subdivided (table 4).

COMMENT. In the experiment, table 3, addition of 10 per cent of imidazole
buffer to normal serum did not affect the results of the thrombin test; the
amount of hemolysis was reduced, but the test was still positive. However,
when the test mixture was diluted with saline, the amount of hemolysis dimin-
ished until it was impossible to make a distinction by visual inspection
(serum A) or until there was actual inhibition by thrombin (serum B). The
experiment in table 4 showed that 10 per cent imidazole inhibited hemolysis
and the addition of thrombin to the incubation mixture caused further inhibition.
Note that these two experiments (tables 3 and 4) differ with regard to the
moment at which the PNH red cells were added. In the latter experiment,
the thrombin and imidazole were mixed together, the acid serum and the
cells were mixed together and then the two mixtures were combined. Under
these circumstances, the imidazole appears to have had a greater inhibitory
effect than it had when PNH cells were measured into the incubation mixture
just before the addition of thrombin (table 3).

DISCUSSION

This series of experiments utilized absorbed thrombin, imidazole buffer
and dilution with saline in order to reproduce the conditions under which
Hinz had performed the thrombin test. With his results he was able to con-
clude "that all, or most, of the effects on PNH hemolysis of thrombin prepara-

Table 3.—Effect of Imidazole on the Thrombin Test Diluted with
Saline. Results with Two Normal Sera

<table>
<thead>
<tr>
<th>Acid serum</th>
<th>Imidazole buffer</th>
<th>Saline</th>
<th>PNH red cells</th>
<th>Results (mg. Hb/100 ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum A</td>
<td>Serum B</td>
<td></td>
<td></td>
<td>No thrombin</td>
</tr>
<tr>
<td>0.5</td>
<td>—</td>
<td>—</td>
<td>0.025</td>
<td>90</td>
</tr>
<tr>
<td>0.45</td>
<td>0.05</td>
<td>—</td>
<td>0.025</td>
<td>60</td>
</tr>
<tr>
<td>0.40</td>
<td>0.05</td>
<td>0.05</td>
<td>0.025</td>
<td>50</td>
</tr>
<tr>
<td>0.35</td>
<td>0.05</td>
<td>0.1</td>
<td>0.025</td>
<td>40</td>
</tr>
<tr>
<td>0.30</td>
<td>0.05</td>
<td>0.15</td>
<td>0.025</td>
<td>30</td>
</tr>
<tr>
<td>0.25</td>
<td>0.05</td>
<td>0.2</td>
<td>0.025</td>
<td>15</td>
</tr>
</tbody>
</table>

Table 4.—Effect of Imidazole on the Thrombin Test Using Two Normal Sera

<table>
<thead>
<tr>
<th>PNH cells in acid serum</th>
<th>Imidazole buffer</th>
<th>Saline</th>
<th>50 U. absorbed thrombin</th>
<th>Results (mg. Hb/100 ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum C</td>
<td>Serum D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.9</td>
<td>—</td>
<td>0.1</td>
<td>—</td>
<td>220</td>
</tr>
<tr>
<td>0.9</td>
<td>—</td>
<td>0.1</td>
<td>+</td>
<td>270</td>
</tr>
<tr>
<td>0.9</td>
<td>0.1</td>
<td>—</td>
<td>—</td>
<td>190</td>
</tr>
<tr>
<td>0.9</td>
<td>0.1</td>
<td>—</td>
<td>+</td>
<td>130</td>
</tr>
</tbody>
</table>
tions available commercially are unrelated to their activity for clotting, but are due to their content of antibodies to red cells or to antibody-like substances." Hinz used a dilute acid, a dilute acid suspension, a dilute solution of thrombin and further diluted the serum in the reaction mixture by the addition of imidazole buffer. He did not report the exact dilution in the various experiments, but stated in general that "The total amount of diluent added to serum was always kept below 50 per cent of the original serum volume because further dilution resulted in marked decrease in hemolysis of PNH red cells."

When we had approximated Hinz's experimental conditions we were able to confirm his results, and we believe that we have established the reasons for his inability to demonstrate thrombic activity in the PNH hemolytic system using "absorbed" thrombin. Dilution, especially dilution with the imidazole buffer, was responsible for the discrepancies between his work and ours. The removal of heterophil antibodies from the preparations of bovine thrombin had little effect on the results of the thrombin test when the test was done without the addition of imidazole and saline.

The reason for failure of the thrombin test in diluted serum is unknown. It is possible that dilution is able to dissociate or destroy the factor in the PNH system against which thrombin acts. If thrombin acts on an inhibitor and dilution destroys that inhibitor, thrombin would no longer increase hemolysis. The "anticomplementary" effect of thrombin may be responsible for its inhibiting PNH hemolysis under these conditions.

It is pointed out that the evidence for a relation between coagulation and the PNH hemolytic system does not reside entirely or even mainly in the phenomenon of thrombin's enhancement of hemolysis in vitro.

**Summary**

1. The thrombin test for paroxysmal nocturnal hemoglobinuria (PNH) consists in the demonstration that PNH hemolysis in vitro is increased by the addition of a small amount of thrombin. This ability of thrombin is not substantially changed by preliminary removal of the heterophil antibodies which are present in commercial preparations of bovine thrombin, provided the test is performed in the way it was originally described.

2. Dilution of the serum with saline and the addition of imidazole buffer abolish the phenomenon of thrombin enhancement of PNH hemolysis.

3. Certain commercial thrombin preparations contain additives which inactivate the PNH hemolytic system. These materials are unsuitable for use in the thrombin test.

**Summario in Interlingua**

1. Le test a thrombina pro hemoglobinuria paroxysmic nocture (HPN) consiste in le demonstration que le hemolyse de HPN in vitro es augmentate per le addition de un micre quantitate de thrombina. Iste effecto de thrombina non es marcatemente alterate per le previe elimination del anticorpore heterophile que es presente in preparatos commercial de thrombina bovin, providite que le test es effectuate secundo le directivas in su description original.
2. Le dilution salin del sero e le addition de imidazol como tampon aboli le phenomeno del effecto promotori exercite per thrombina in le hemolyse de HPN.

3. Certe preparatos commercial de thrombina contine additivos que causa le inactivation del sistema hemolytic de HPN. Tal materiales non es appropriate a! uso in le test a thrombina.

REFERENCES


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