Action of Alpha-Tocopherol Phosphate on Hemolysis in Paroxysmal Nocturnal Hemoglobinuria

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Paroxysmal Nocturnal Hemoglobinuria (PNH) is a relatively rare chronic hemolytic anemia, but its clinical and laboratory characteristics have been well covered in the recent literature. Crosby has published a comprehensive review of the literature on this subject, and Dacie has summarized much of the information available on the mechanism of hemolysis in this disease.

It is recognized that the erythrocytes are abnormal in PNH. The disease is characterized by the in vitro lysis of PNH cells by fresh serum, either PNH or normal, particularly at an acid pH. On the other hand, PNH serum will not lyse normal cells. However, PNH cells are not susceptible to lysis by serum which has been heated at 56°C for 30 minutes, and the addition of fresh guinea pig complement will not restore the activity. Ham has utilized these phenomena to devise the diagnostic laboratory test, which bears his name.

The factor in serum, which is necessary for lysis of PNH erythrocytes, has not been fully identified at this time. There is a marked parallel between the characteristics of this serum factor and complement, and there is evidence both for and against their identity. We have advanced evidence that at least two serum factors are concerned in PNH hemolysis and have suggested that they may prove to be components of complement. Recently, Crosby and Dameshek observed that the PNH hemolytic activity of serum increases with the elapse of time after coagulation of the blood. They also noted the high incidence of thrombosis in reported cases of PNH. These two observations led them to investigate a possible relationship between PNH hemolysis and the normal coagulation of blood. They demonstrated that the addition of either thrombin or thromboplastin to mixtures of PNH cells with fresh acidified serum enhanced hemolysis. These workers found that a relatively pure preparation of the serum accelerator globulin of Seegers and Ware would restore PNH hemolytic activity to serum, which had been inactivated by BaSO₄ or by heating at 50°C for 30 minutes. However, activity could not be restored in this manner to serum which had been heated at 56°C for 30 minutes. Further, this accelerator globulin preparation, dissolved in isotonic saline, lysed PNH erythrocytes. Therefore, Crosby and Dameshek concluded that the heat labile serum factor concerned in PNH hemolysis closely resembled the serum accelerator factor.

As a result of their experimental work, Crosby and Dameshek studied the action of dicumarol on a case of PNH. They discovered that it had an inhibitory effect, but that this occurred only when the prothrombin activity of the blood was markedly lowered. Further, the administration of this drug did not prevent either the development of a hemolytic crisis in association with an infection or
a hemolytic transfusion reaction. These workers concluded that dicumarol was not effective from a therapeutic standpoint in this disease.

These investigations of Crosby and Dameshek suggested to us that the action of another anticoagulant on PNH should be studied. Alpha-tocopherol phosphate ($\alpha$TPO$_4$), which has been described as an anticoagulant by Kay, Hutton, Weiss and Ochsner, was selected for this purpose. Its action has been explained by its ability to combine with thrombin and fibrinogen and to inactivate thrombin. Kay, Hutton and Weiss also demonstrated that an alpha-tocopherol, experimentally indistinguishable from alpha-tocopherol phosphate, was present in both the Ac-globulin of Seegers and Ware and in the alpha and gamma globulins of plasma. This group believes that the antithrombin found in serum may be identical with the naturally occurring alpha-tocopherol. If the theories of this group concerning the anticoagulant action of $\alpha$TPO$_4$ are correct, it may be assumed that this drug, by virtue of its antithrombic action, would tend to delay conversion of the inert plasma Ac-globulin to the active serum form. This assumption is predicated upon the theory of coagulation advanced by Seegers and Ware.

Since the work of Crosby and Dameshek suggested that serum accelerator globulin may lyse PNH cells, it appeared that $\alpha$TPO$_4$ might have therapeutic value in PNH. This idea was investigated by both in vitro and in vivo studies on a case of PNH.

**Summary of Case**

A 30 year old man of Latin American descent was admitted to this hospital for evaluation of an anemia, which had been present for at least six years and had been diagnosed elsewhere as aplastic in type. It was first detected while he was serving with the Army during World War II. Prior to this, he had apparently been well. There were no other relevant features in either his personal or his family history.

On admission he was found to be pale but well developed and slightly obese. He complained of moderate shortness of breath and some weakness.

Shortly after admission, two transfusions of compatible blood were administered. Both of these produced violent reactions with chills, fever, hemoglobinuria and jaundice. The hemoglobinuria persisted for several days after the transfusions. Despite the administration of 2 units (1000 ml.) of whole blood, the patient's hemoglobin did not rise.

**Laboratory Data**

- R.B.C. 1.4 million per cu. mm.; Hgb. 6.0 g (Haden-Hauser); hematocrit 17; M.C.V. 121; M.C.H. 44.5; M.C.H.C. 35 per cent; corrected sedimentation rate 44 (Wintrobe); reticulocyte count 7.0 per cent; red cell osmotic fragility normal; W.B.C. 5,200 per cu. mm. with 35 per cent neutrophils and 65 per cent lymphocytes; platelets 116,000 per cu. mm.; Kahn precipitin test negative; hemolytic index 50 (normal 11-20); hemosiderin in urine sediment; Hegglin-Maier test positive; complete Ham test positive; Crosby test positive for PNH; direct Coombs test positive; neither cold nor warm agglutinins in the serum; methemalbumin present in the serum; normoblastic hyperplasia of both aspirated and trephined sternal marrow.

On the basis of a consistently positive Ham test, the diagnosis of paroxysmal nocturnal hemoglobinuria was established. During the time spent in the hospital, hemoglobinuria was present only after each of the blood transfusions. The patient did not recollect passing dark urine at any other time.

Since this time, the patient has been followed for a period of eighteen months. The anemia has persisted and the Ham test has always been positive except for the instances reported
below. Hemoglobinuria has not occurred but hemosiderin is consistently present in the urine, as is methemalbumin in the serum.

**Materials and Methods**

**Serum:** Blood obtained by venipuncture was allowed to clot at room temperature, and the serum was removed from the clot in the usual manner.

**Red blood cells:** Venous blood was added to approximately 25 volumes of isotonic saline (0.85 per cent NaCl) and the red cells were sedimented by centrifugation at 1500 r.p.m. The cells were washed three times in large volumes of saline and the saline discarded. The packed cells thus obtained were used in the acid hemolysis test without resuspension in saline, thereby eliminating any dilution of the serum.

**Alpha TPO₄ solutions:** Alpha-tocopherol phosphate (Epsilan Phosphate, Warren Teed Products) was dissolved in isotonic saline and diluted with saline to contain 2.0, 4.0, and 8.0 mg. αTPO₄ per ml.

**Acid hemolysis test:** The acid hemolysis test was based upon Ham's method. Only acidified mixtures were used, since hemolysis so obtained was generally greater than in non-acidified mixtures. For the test, one volume of serum (1.0 ml.) was acidified by the addition of 0.05 volume N/3 HC1. αTPO₄ solutions were then added in 0.50 volume. Whenever αTPO₄ was used, a control tube was set up which contained a volume of isotonic saline equal to the volume of solution added to the test mixture. The final pH of the test mixture was measured using the Beckman Model G pH Meter and ranged from 6.5 to 6.8. After the addition of 0.1 volume of packed red cells, prepared as shown above, to the serum, the mixture was incubated at 37 C. for 30 minutes. Following incubation, the mixture was centrifuged to sediment the red cells and the degree of hemolysis was recorded as one plus to four plus.

**Coomb's test:** Red cells were washed three times with isotonic saline and resuspended in saline to 2 per cent concentration. One drop (0.05 ml.) of this suspension was placed in a test tube with two drops (0.10 ml.) of undiluted antiglobulin serum (various commercial preparations). The mixtures were incubated at 37 C. for 30 minutes and centrifuged at 1500 r.p.m. for 2 minutes. They were examined macroscopically, while still in the test tube, with the aid of a concave mirror, and then transferred to a glass slide and examined microscopically.

**Experimental Results**

As a preliminary to the in vivo investigations, αTPO₄ was added to the acid hemolysis test mixture in 1.0, 2.0, and 4.0 mg. amounts. The final pH of these mixtures was always 6.5 to 6.8. It was found that the addition of 1.0 mg. of αTPO₄ diminished hemolysis and that the addition of 2.0 mg. abolished it (table 1). The results with 4.0 mg. of αTPO₄ were variable and inconsistent. These in vitro studies were considered sufficiently encouraging to warrant a clinical trial on a case of PNH. As hemoglobituuria was not present at this time, it was decided to follow the in vivo action of the drug by means of the acid hemolysis test and the antithrombin level. The antithrombin level of the patient's serum, estimated by the method of Kay and associates, was found to be $\frac{1}{16}$ as compared with a normal control of $\frac{1}{32}$. The drug (Epsilan Phosphate, Warren Teed Products) was administered in 100 mg. amounts intramuscularly at 8 hour intervals and concurrently one daily intravenous injection of 10.0 ml. of 10 per cent calcium gluconate was given. Twenty-four hours after this procedure was initiated, changes were noted in the acid hemolysis test. In the mixture containing PNH cells and PNH serum, there was 1 plus hemolysis, while prior to this time 4 plus hemolysis had been obtained consistently. In the mixture containing PNH cells and normal fresh serum, there was no hemolysis. The antithrombin level of the patient at this time was $\frac{1}{32}$ with
a control giving an identical reading. Forty-eight hours after the initiation of this study, hemolysis was absent in both mixtures, and it was found that PNH cells after incubation with PNH serum were agglutinated by antiglobulin serum. After 72 hours hemolysis was still absent and the Coombs test was strongly positive. These results are summarized in Table 2. The injections of $\alpha$TPO$_4$ proved to be extremely painful and produced a local and systemic reaction with elevation of the body temperature. Therefore, after 72 hours, the oral preparation of $\alpha$TPO$_4$ (Epsilan-M capsules, Warren Teed Products, 300 mg. every 8 hours) was substituted while continuing the daily intravenous administration of calcium gluconate. Twenty-four hours after this innovation, the antithrombin level had fallen to 1/16 with the control remaining at 1/32. After 48 hours, there was 2 plus hemolysis in the test mixtures. In view of the patient's profound anemia and the apparent inefficacy of oral $\alpha$TPO$_4$, it was decided to discontinue the drug at this time.

### Table 1. The Effect of Alpha-Tocopherol Phosphate on Hemolysis in Vitro

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Serum (1.0 ml.)</th>
<th>N/3 HCl (0.05 ml.)</th>
<th>$\alpha$TPo$_4$ (0.5 ml.)</th>
<th>Saline (0.5 ml.)</th>
<th>Cells (0.1 ml.)</th>
<th>Incubation</th>
<th>Hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NS +</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>PNHC</td>
<td>37 C.-30</td>
<td>3+</td>
</tr>
<tr>
<td>2</td>
<td>NS +</td>
<td>-</td>
<td>1.0 mg.</td>
<td>-</td>
<td>PNHC</td>
<td>new minutes</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>PNHS +</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>PNHC</td>
<td>4+</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>PNHS +</td>
<td>-</td>
<td>1.0 mg.</td>
<td>-</td>
<td>PHNC</td>
<td>2+</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>PNHS +</td>
<td>-</td>
<td>2.0 mg.</td>
<td>-</td>
<td>PNHC</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Key:
- NS = normal serum
- PNHS = PNH serum
- PNHC = PNH cells
- + = added
- - = not added
- Final pH of all mixtures: 6.5 to 6.8.

### Table 2. The Effect of Intramuscular Alpha-Tocopherol Phosphate on Acid Hemolysis Test with Coombs Tests on Sedimented Cells

<table>
<thead>
<tr>
<th>Test No.</th>
<th>Treatment of Patient</th>
<th>(Column 1) Acid Hemolysis Test Using PNH Serum and PNH Cells</th>
<th>(Column 2) Acid Hemolysis Test Using Normal Serum and PNH Cells</th>
<th>(Column 3) Coombs Test on Sedimented and Washed Cells from Column 1</th>
<th>(Column 4) Antithrombin Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Untreated</td>
<td>4+</td>
<td>3+</td>
<td>N.D.*</td>
<td>1/16</td>
</tr>
<tr>
<td>2</td>
<td>24 hours on intramuscular $\alpha$TPO$_4$</td>
<td>1+</td>
<td>0</td>
<td>N.D.</td>
<td>1/32</td>
</tr>
<tr>
<td>3</td>
<td>48 hours on intramuscular $\alpha$TPO$_4$</td>
<td>0</td>
<td>0</td>
<td>1+</td>
<td>1/32</td>
</tr>
<tr>
<td>4</td>
<td>72 hours on intramuscular $\alpha$TPO$_4$</td>
<td>0</td>
<td>0</td>
<td>2+</td>
<td>1/32</td>
</tr>
<tr>
<td>5</td>
<td>48 hours after $\alpha$TPO$_4$ discontinued</td>
<td>2+</td>
<td>1+</td>
<td>1+</td>
<td>1/16</td>
</tr>
</tbody>
</table>

* N.D.: not done because of hemolysis.
† Note that these tests represent the action of Coombs serum on sedimented and washed cells from the acid hemolysis tests (Column 1) and not on untreated cells direct from the patient.
The in vitro and in vivo studies on the action of αTPO₄ on PNH have produced interesting results. When αTPO₄ was added directly to the acid hemolysis mixture, lysis of PNH cells did not occur or was considerably reduced. Furthermore, lysis progressively decreased in the modified Ham tests performed concurrently with the intramuscular administration of αTPO₄.

The mechanism of inhibition of lysis in either the in vitro or the in vivo studies is not understood at this time. However, several possibilities must be considered. The action of the drug in either instance may have occurred as a result of its antithrombic action with subsequent diminution in the amount of available thrombin. It must be recalled that Seegers and Ware¹¹ have postulated that the presence of thrombin is necessary for the conversion of plasma Ac-globulin to the active serum form. Thus αTPO₄, by virtue of its antithrombic action, may be considered to reduce available serum Ac-globulin. During the intramuscular administration of the drug, the antithrombic activity of the patient’s blood increased to normal levels, while, prior to this time and also during the period of oral administration, the level was lower than that of normal controls. Hence these studies with αTPO₄ may be considered to lend some support to the thesis of Crosby and Dameshek¹⁰ that the serum accelerator globulin is the factor in serum which lyases PNX erythrocytes. On the other hand, we have described elsewhere that the addition of phosphate ions alone to the modified Ham test inhibits lysis of PNH erythrocytes. The phosphate ions present in αTPO₄ may have been responsible for the inhibition of lysis in the in vitro studies. However, it is unlikely that enough phosphate ions were administered to the patient to have so affected his metabolism. A further possibility to be considered is that during these studies, the PNH cells had become spontaneously resistant to lysis by acidified serum. It is well recognized that the cells do vary spontaneously in their susceptibility to lysis. However, the in vitro experiments were controlled by running modified Ham tests without the addition of αTPO₄ simultaneously with the test mixtures. These controls were positive at all times. On the other hand, the intramuscular αTPO₄ may have exerted some nonspecific effect. Some support for this idea comes from the fact that these injections were accompanied by fever and pain, while the ineffective oral administration did not produce these side effects. It must be concluded that at this time it is not possible to explain the action of αTPO₄ on PNH hemolysis.

It is of particular interest that, during the intramuscular administration of αTPO₄, the patient’s cells ceased to be lysed by fresh acidified serum, and that these cells after sedimentation gave a positive Coombs test (table 2). At no other time did the red cells, following incubation with fresh acidified serum, give a similar reaction with antiglobulin serum. It is felt that this reaction does not necessarily imply that the cells are coated with an incomplete antibody but rather that they have reacted in some manner with a nonspecific serum globulin. Under the conditions of the experiments described in this paper, PNH hemolysis has been inhibited. As a therapeutic agent in PNH, αTPO₄ appears to have no value at this time. The intramuscular route of administration alone gave promise of any ameliorative effect. Since these injections had to be dis-
continued, the possible action of the drug on the course of the disease could not be gauged.

SUMMARY

1. The action of alpha-tocopherol phosphate (αTPO₄), an anticoagulant, on PNH hemolysis was studied by both in vitro and in vivo means. It was found that the addition of αTPO₄ to the modified Ham test inhibited lysis of PXH erythrocytes. During the intramuscular administration of αTPO₄, the susceptibility of the erythrocytes to lysis by fresh acidified serum progressively decreased. These cells, after incubation with the serum, gave a positive Coombs test.

2. The possible significance of these findings is briefly discussed.

3. It is concluded that αTPO₄ has no therapeutic value in PNH.

REFERENCES

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