Immunization of Rabbits Against Human Anti-Hemophilic Factor (AHF)

By Marvin D. Richards* and Theodore H. Spaet

NATURALLY OCCURRING acquired circulating anticoagulants which prevent the formation of thromboplastin have been described in humans.\(^1\)\(^-\)\(^3\) Reported cases have fallen into three major categories: hemophilies in whom the anticoagulant appeared subsequent to transfusion therapy, certain women in whom the disorder began following pregnancy, and patients with a variety of unrelated diseases who developed the anticoagulant during the course of their illness. On rare occasions the anticoagulant has occurred in otherwise normal individuals.\(^4\)\(^-\)\(^5\) The anticoagulants appearing in the three groups appear to be identical and have been characterized as globulins, possibly antibody in nature, which interfere with the early stages of blood thromboplastin formation by inactivating the anti-hemophilic factor.\(^2\)\(^-\)\(^3\),\(^8\)-\(^11\) It has been postulated by some investigators that these anticoagulants are the result of an immunization process.\(^1\)\(^-\)\(^4\) Others dispute the validity of such a hypothesis.\(^2\) It was felt that the production of such a circulating anticoagulant in a laboratory animal by immunization against AHF might shed light on the pathogenesis of this entity in humans.

METHODS

Unless otherwise stated, all blood was drawn in uncoated glassware using one part in nine of 0.1 M potassium oxalate as the routine anticoagulant. Serum was obtained from whole blood defibrinated with glass beads and incubated for three hours at 37 C. All clotting studies were performed in a 37 C. water bath. Hemophilic and PTC-deficient bloods were obtained from patients whose diagnoses had been verified by prothrombin consumption tests, thromboplastin generation tests, and cross-correction studies.

In the prothrombin consumption test, whole blood was added to the test substance to total 2 ml., the tube inverted once for adequate mixing and the blood allowed to clot at 37 C. for 3 hours. Nine volumes of the resultant serum were incubated for 30 minutes with 1 volume of heparinized 0.1 M potassium oxalate to destroy excess thrombin. Plasma and serum prothrombin times were then determined by the method of Ware and Strangnell.\(^12\) Prothrombin consumption was considered the difference between the original plasma prothrombin level and that of the serum.

The thromboplastin generation test was adapted from the original method of Biggs and Douglas\(^13\) as follows: One tenth ml amounts of platelet suspension, 10 per cent human serum, and various dilutions of adsorbed plasma were incubated for 1 minute and then recalcified with 0.1 ml of 0.025 M calcium chloride. At 2, 4, and 6 minutes subsequent to this recalcification, 0.1 ml aliquots of the generating mixture were added to 0.1 ml of substrate.

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which had been recalcified 15 seconds earlier with 0.1 ml. of 0.025 calcium chloride. Reagents were prepared as described by Biggs and Douglas\(^{18}\) with the following exceptions:

1. The source of AHF was oxalated plasma adsorbed with barium sulfate instead of citrated plasma adsorbed with aluminum hydroxide. Each ml. of plasma was adsorbed with the precipitate obtained when 0.1 ml. of barium chloride was mixed with 0.1 ml. of sodium sulfate both at molar concentrations. Adsorption was allowed to occur for 30 minutes at room temperature. (2) In some of the later studies human platelet suspensions were replaced by extracts of bovine platelets. Platelets obtained from oxalated beef plasma collected in silicone-coated glassware were washed free of plasma with saline. These platelets were then lysed by suspension in distilled water and repeated freezing and thawing. The particulate matter was removed by centrifugation at 3,000 r.p.m. for 30 minutes, and the supernatant fluid diluted with distilled water to a volume equal to one tenth that of the original plasma. This extract displayed platelet activity in the thromboplastin generation test and was found to be stable on storage at \(-20^\circ\) C. (3) Substrate was prepared from 10 percent normal human plasma in distilled water added to an equal amount of Ware-Strangnell beef substrate.

The thromboplastin inactivation test was performed as described by Spaet and Garner.\(^{14}\)

EXPERIMENTAL

1. Preparation of Purified Antihemophilic Factor (AHF) Antigen and Antiserum

Cohn fraction * was prepared from 150 ml. of fresh plasma obtained from a single normal human donor. A purified AHF fraction was prepared from this by the method of Spaet and Kinsell.\(^{15}\) This material migrated electrophoretically as a beta globulin and displayed approximately one-fourth of the AHF activity of the original plasma as assayed by the thromboplastin generation test. The AHF fraction was divided into aliquots equivalent to 82 mg. of fraction I or 11 ml. of the original plasma. These were stored at \(-20^\circ\) C. Gradual loss of activity occurred upon such storage, and by 12 weeks little AHF activity remained in the stored fractions.

Two types of antigenic preparations were tried: a saturated solution in saline and a water in mineral oil emulsion (Freund's adjuvant without the addition of killed mycobacteria).\(^{16}\) Varied doses of either antigen were injected intramuscularly into four 3.8 to 4.0 Kg. rabbits and the optimum time schedule determined by trial and error. The most effective antiserum was produced by three weekly injections of an AHF suspension (equivalent to 82 mg. of fraction I or 11 ml. of the donor plasma) in 0.5 ml. of buffered saline, pH 7.4, the blood being drawn 10 to 14 days after the third injection. Serum was prepared as described under Methods and stored at \(-20^\circ\) C. A significant antibody level was not obtained with any of the Freund's adjuvant preparations.

2. Antiserum Activity: Precipitin Tests

AHF antigen derived from 11 ml. of the original plasma was diluted with 1.1 ml. of normal saline buffered to pH 7.4 (tenfold antigen concentration representing 2.5 times the AHF activity of the original plasma). This was used as the initial dilution, and increasing dilutions of the antigen were tested against the AHF antiserum. Similar dilutions of the antigen were tested against normal

* Kindly prepared by Cutter Laboratories, Berkeley, California.
TABLE 1.—Precipitin Tests

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Fig. 1.—Inhibition of normal plasma AHF in the thromboplastin generation test. (A) Normal plasma plus BaSO₄ AHF antiserum, (B) normal plasma plus BaSO₄ normal human serum, (C) Normal plasma plus BaSO₄ normal rabbit serum, (D) normal plasma plus unadsorbed AHF antiserum, (E) normal plasma plus saline.

rabbit serum as a control. One-tenth ml. amounts of the antigen suspensions and sera were used. Readings were made immediately and expressed as 1 plus to 4 plus according to the degree of flocculation at the interface. Readings were also made subsequent to storage overnight at 10 °C, and judged 1 to 4 plus according to the amount of precipitate formed. Both methods gave identical results.

The A HF antigen gave strongly positive precipitin tests against the undiluted antiserum. Positive tests could be obtained up to antigen dilutions of 1:5,000 (table 1). Normal rabbit serum at all antigen dilutions failed to give a positive precipitin test.

3. Inhibition of Normal Plasma A HF Activity in the Thromboplastin Generation Test

The effect of the antiserum on the antihemophilic activity of normal human plasma was studied by means of the thromboplastin generation test. A series of thromboplastin generation tests was performed in which barium sulfate-adsorbed normal human plasma serving as the source of AHF was added to the AHF antiserum and to various controls as illustrated in figure 1. For each generation test
0.1 ml. of the 20 per cent plasma was combined with 0.1 ml. each of 10 per cent normal human serum, normal human platelet suspension, and 0.025 M. calcium chloride.

When the plasma AHF was diluted with saline, the generation of thromboplastin was normal (fig. 1 E). However, when barium sulfate-adsorbed AHF antiserum was added to the plasma, the resultant mixture displayed minimal AHF activity in the thromboplastin generation test (fig. 1 A). This type of generation curve is similar to that seen when hemophilic plasma is used. When the normal plasma was diluted with either barium sulfate-adsorbed normal human serum or barium sulfate-adsorbed normal rabbit serum, the AHF activity of the mixture remained normal and generation values similar to the saline control were obtained (fig. 1 B and C). Thus the barium sulfate-adsorbed AHF antiserum inhibited the AHF activity of normal plasma while normal human and normal rabbit sera did not.

4. Nature of the Anticoagulant: Mechanism and Site of Action

Both normal human and rabbit sera markedly shorten the recalciﬁed clotting time of normal human plasma. Such activity may be related to the accelerating effect of convertin, or may result from small quantities of residual thrombin, remaining in the serum. Minute amounts of thrombin are known to markedly accelerate the first stage of clotting. The anti-AHF effect of unadsorbed antiserum tended to be masked by this normally present “thromboplastic-like” effect (fig. 1D). However, this “thromboplastic” activity could be removed from the serum by barium sulfate adsorption (fig. 1 A). Such adsorption would remove any thrombin or convertin present in the antiserum. This adsorption enhanced the anti-AHF activity of the antiserum in the thromboplastin generation and prothrombin consumption tests. Therefore, in all clotting studies designed to show anti-AHF activity, the antiserum was barium sulfate adsorbed. Barium sulfate adsorption of serum was accomplished as with plasma.

Normal serum has antithrombin activity. The antithrombin content of the AHF antiserum was compared to that of normal rabbit serum using a modiﬁcation of the method of Quick. A quantity of 0.8 ml. of thrombin (50 units/ml.) was combined with 0.2 ml. of the serum to be studied, incubated at room temperature, and the remaining thrombin activity tested immediately and after two, five, ten, and ﬁfteen minutes of incubation. The effects of barium sulfate-adsorbed AHF antiserum and normal rabbit serum on both human and beef thrombin were observed. The AHF antiserum and normal rabbit serum displayed similar antithrombin activity. Therefore, the anticoagulant effect of the antiserum cannot be attributed to its antithrombin content.

Normal serum has the ability to inactivate thromboplastin of both blood and tissue origin. The antiserum and control sera were examined for their antithromboplastin activity by the thromboplastin inactivation test of Spae and Garner. Blood thromboplastin was generated by the usual thromboplastin generation test. At four minutes, thromboplastin activity was determined by testing an aliquot of the generating mixture. At five minutes, thromboplastin generation was stopped by the addition of a citrated test serum which was allowed to incubate with the formed thromboplastin for 30 minutes, at which
time thromboplastin activity was again determined. The inactivating abilities of the sera were compared to that of a citrated saline control. By this method normal human serum, unadsorbed and barium sulfate-adsorbed normal rabbit serum, and unadsorbed and barium sulfate-adsorbed AHF antiserum were studied. The results are illustrated in figure 2.

All of the sera tested demonstrated antithromboplastic activity. The antithromboplastic activity of the AHF antiserum was no greater, in fact less, than that of normal human or normal rabbit serum. Apparently this inactivation of thromboplastin is proportional to the duration of incubation and is minimal in the first few minutes.

To study the effect of incubation on the inhibitory activity of the antiserum, the following experiment was performed. A normal control thromboplastin generation test was done, using 20 per cent barium sulfate-adsorbed normal human plasma in saline for the AHF source. This was repeated, substituting plasma diluted with AHF antiserum instead of saline, immediately starting the generation test. In the third test the barium sulfate-adsorbed plasma and the antiserum were preincubated for 15 minutes at 37 C. before the generation test was performed. The inhibiting effect of the antiserum on thromboplastin generation occurred immediately, was not dependent on previous incubation, and was not enhanced by incubation (fig. 3). The anticoagulant activity of the antiserum differed from the antithromboplastic effects of control rabbit and normal human sera, which were shown to be minimal in the first few minutes of incubation and which increased in proportion to the incubation time (fig. 2). When added prior to the formation of thromboplastin, the antiserum markedly interfered with production of thromboplastin (fig. 1 A). When added subsequent to the formation of thromboplastin, it had little anticoagulant effect (fig. 2 D). Thus it acted not as an antithromboplastin but rather by inactivation of a necessary precursor of thromboplastin.
5. Specific Activity Against AHF

In the following studies the effect of a fixed concentration of antiserum on increasing concentrations of AHF was observed. Fresh plasma was obtained from a single normal human donor. This was barium sulfate-adsorbed as previously described and divided into two aliquots: the first served as a control and the second as a source for a euglobulin fraction. The euglobulin fraction was prepared by diluting the plasma aliquot to 20 times its original volume with distilled water and then adjusting the pH to 5.4 by the addition of 1 per cent acetic acid. The precipitate formed at this point was dissolved in a volume of isotonic saline buffered to pH 7.4, equal to one-fifth the original plasma aliquot volume. The AHF activity of this euglobulin preparation was approximately five times that of the control plasma aliquot as determined by thromboplastin generation studies. Thromboplastin generation tests were performed using as the plasma (AHF) source the mixtures illustrated in figure 4.

The normal control generation using 20 per cent plasma in saline is shown by figure 4 C. A 20 per cent solution of the euglobulin fraction (fivefold plasma AHF) gave a similar generation curve within the normal range (fig. 4 D). When barium sulfate-adsorbed AHF antiserum was substituted for saline, the 20 per cent plasma failed to produce normal thromboplastin, and a hemophilic curve was produced (fig. 4 A). The euglobulin fraction plus barium sulfate-adsorbed AHF antiserum displayed relatively normal AHF activity and produced a generation test almost within the normal range (fig. 4 B). These studies demonstrated the ability of excess AHF to overcome the inhibitory effect of the antiserum on thromboplastin generation.

To study further the specificity of the antiserum, its effects on the correction of hemophilic blood by normal plasma and of PTC-deficient blood by normal
serums were investigated. Whole blood was obtained from a known hemophilic and from a known PTC-deficient patient. Serum and fresh plasma were obtained from a single normal human donor. The same antiserum was used throughout.

The deficient prothrombin consumption of the hemophilic whole blood could be corrected by the addition of 1.25 per cent by volume normal human plasma. Combining the plasma with an equal amount of AHF antiserum markedly reduced this corrective ability.

The minimal prothrombin consumption of the PTC deficient whole blood was restored to normal by the addition of 0.5 per cent by volume normal human serum. Combining the human serum with an equal amount of AHF antiserum did not alter its corrective ability.

Hemophilic blood is deficient only in its AHF content. Normal plasma corrected the poor prothrombin consumption of the hemophilic blood by supplying a small quantity of AHF. When the antiserum prevented this correction, it did so in the presence of an excess of all clotting factors except AHF. PTC-deficient blood has an adequacy of all clotting factors except PTC. Its deficient prothrombin consumption could be corrected by a small quantity of normal serum even in the presence of the antiserum. Thus the antiserum does not inactivate PTC. It inactivates AHF and can be overcome by an excess of AHF.

The specificity of the antiserum was further demonstrated by the positive precipitin tests against the purified AHF antigen (table 1) and against barium sulfate-adsorbed fraction I (containing AHF, fibrinogen and labile factor). Purified PTC fractions (containing no AHF) gave negative precipitin tests.
6. Species and Individual Specificity

The inhibitory effect of the antiserum on the plasma AHF of various species was investigated using the thromboplastin generation test. Experiments were performed as described previously, using as AHF sources the following barium sulfate-adsorbed plasmas: 20 per cent human, 5 per cent beef, 20 per cent rat, 10 per cent dog, and 20 per cent rabbit. The above concentrations were found to give approximately equal AHF activity in thromboplastin generation studies. The effects of dilutions made in saline were compared with those made in barium sulfate-adsorbed AHF antiserum. The results are illustrated in figure 5. In addition, various human plasmas were tested. There were no significant differences between the original plasma from which the antigen was made and fifty random normal human plasmas. A typical generation showing inhibition of human AHF by the antiserum is shown in figure 5 A. Marked inhibition of both beef and rat AHF was also demonstrated. Plasmas from six normal rats were investigated and all were equally inhibited, a representative generation being shown by figure 5 C. Two different beef plasmas were studied: both were markedly inhibited (fig. 5 B). Generations performed with six normal dog plasmas and six normal rabbit plasmas displayed no significant AHF inhibition by the antiserum (fig. 5 D and E) when compared to the saline controls (fig. 5 G and I).

7. Physical Properties of the Anticoagulant

The antiserum was stable at room temperature for 24 hours. There was no loss of activity after storage at -20 C for three months. Heating to 65 C. for eight
minutes did not destroy antiserum anti-AHF activity. The active component remained in the serum after dialysis against saline, and activity was enhanced after serum was adsorbed with barium sulfate for 30 minutes. The active component was precipitated by one-fourth to one-third saturation of the serum with ammonium sulfate, and the anticoagulant was not ether extractable. The positive precipitin tests identify the anticoagulant as an antibody, and the physical properties are compatible with those of antibodies.

**Discussion**

In certain patients the acquired circulating anticoagulants have been shown to be gamma globulins which gave positive precipitin tests against AHF.\(^3\)\(^6\)\(^7\) Such data support the antibody nature of the anticoagulant but fail to establish AHF as the immunizing agent. Until the present study, the antigenicity of AHF was never established. Our data demonstrate that human AHF is antigenic, and that the antibody produced against it has anticoagulant activity.

The anticoagulant produced in the rabbit by immunization with AHF is similar in mechanism of action and in studied physical properties to those anticoagulants reported to occur in humans.\(^2\)\(^6\)\(^11\) Both specifically inactivate AHF and prevent subsequent thromboplastin formation. Both anticoagulants have been identified as globulins. They both are heat stable, and storage stable when frozen. Neither of the anticoagulants is dialyzable, adsorbable on barium sulfate, or ether extractable. Since the anticoagulant in the rabbit is known to be an antibody and is similar in properties to acquired circulating anticoagulants in humans, it is conceivable that such anticoagulants in humans are also antibody in nature and may be the result of an immune mechanism.

A study of the types of patients developing such anticoagulants reveals the highest incidence among hemophiliacs.\(^1\)\(^2\) These patients had received repeated transfusions of blood plasma and Cohn's fraction I. Once the anticoagulant has occurred in a hemophilic, further transfusion aggravates the hemorrhagic tendency. When transfusions are withheld, the anticoagulant gradually decreases and may eventually disappear.\(^7\) This rise and subsequent diminution of anticoagulant activity is indeed suggestive of an immune reaction since antibodies are known to diminish in similar fashion in the absence of continued immunization.

If one postulates an immune process in these cases, certain aspects are puzzling. The actual number of hemophiliacs developing the anticoagulant is small despite the many who receive multiple transfusions. Also, the occurrence of the anticoagulant does not seem to be related to the number of transfusions any given patient has received. One of our patients has received daily plasma transfusions for over a year and a half without developing an anticoagulant. Undoubtedly, individuals vary in their ability to produce antibodies. Those who develop antibodies may be "hyper-reactors" who respond to minimal antigenic stimuli; whereas the average patient may require prolonged and repeated exposure. However, another explanation is possible. Human AHF may vary in its protein structure. It is conceivable that a common form and one, or more, less common forms exist. Indeed such differences of protein structure have been described in human hemoglobins.\(^26\) In a patient whose AHF is the uncommon type, repeated transfusions with the more common and incompatible type might lead to im-
munization, although repeated transfusions with the compatible type would be harmless. In our study species differences in AHF were demonstrated. Similarly, Spaet and associates have reported species specificity of AHF antibodies that developed in 2 of their patients. The presence of antigenic differences in the structure of human AHF remains speculative, however, and was not confirmed by a survey of 50 normal subjects in the present study.

Immunization by transfusion could account for antibody production irrespective of the underlying disease process. However, the anticoagulant has occurred subsequent to pregnancy in the absence of transfusions, and has arisen de novo in the course of certain chronic diseases and even in patients with no known underlying primary condition. In the majority of these cases the anticoagulants have been similar in action and in physical properties to those produced by AHF immunization. Possibly in these cases "autoimmunization" occurs analogous to that encountered in acquired hemolytic anemia, idiopathic thrombocytopenic purpura, and certain leukopenias. Thus, the patients with anticoagulants would develop antibodies against their own AHF by a mechanism comparable to that in which the other groups become immunized against their own blood cellular elements. The term "autoimmune hemophilia" proposed by Spaet and Kinsell seems appropriate for such a condition.

In the present study small amounts of anticoagulant were best determined by the thromboplastin generation test. The prothrombin consumption method was considerably less sensitive and therefore less reliable. Similar results have been reported by others in patients with low titer circulating anticoagulants.

**SUMMARY**

Antibodies against human AHF were produced in the rabbit. These antibodies acted as anticoagulants in vitro by inactivating AHF and preventing normal thromboplastin formation. Species specificity and physical properties of the anticoagulant are described. It is postulated that similar anticoagulants occurring in humans are also antibodies produced by AHF immunization.

**SUMMARIO IN INTERLINGUA**

Anticorpores contra human factor anti-hemophilic (FAH) esseva producite in conilios. Iste anticorpores ageva como anticoagulantes in vitro per inactivar FAH e per prevenir le formation normal de thromboplastina. Le specificitate del anticoagulante pro varie species et suas caracteristicas physic es describite. Nos postula que anticoagulantes comparabile que occurre in humanos es equalmente anticorpores producete per immunisation a FAH.

**REFERENCES**


Immunization of Rabbits Against Human Anti-Hemophilic Factor (AHF)

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