Techniques for Demonstration of the Specificity of Circulating Anticoagulants Against Antihemophilic Factor (Factor VIII), With Studies of Two Cases Possibly Related to Diphenylhydantoin Therapy

By Man-Chiu Poon, Hidehiko Saito, Oscar D. Ratnoff, Walter B. Forman, and Jeffrey Wisnieski

Circulating anticoagulants against antihemophilic factor (AHF, factor VIII) occasionally appear to inactivate other clotting factors as well. Such an anticoagulant has been demonstrated in a patient under treatment with diphenylhydantoin who developed a hemorrhagic disorder. The studies to be described demonstrate two ways in which the specificity of the anticoagulant can be established. The detection of a similar circulating anticoagulant in a second patient treated with diphenylhydantoin suggests a causal relationship between administration of this drug and the evolution of the anticoagulant. This second patient, however, had been treated with penicillin, an agent previously associated with the evolution of circulating anticoagulants against AHF, 10 days before the onset of symptoms.

CASE HISTORIES

Patient 1

A 34-yr-old mentally retarded black male with microcephaly and a seizure disorder was admitted to University Hospitals of Cleveland on May 1, 1974, for effusion of the left knee secondary to trauma and anemia. He had been under treatment with diphenylhydantoin since he was 10 yr old, and at the time of admission was taking 400 mg/day; no history of ingestion of other drugs or alcohol was obtained. In February 1973, the one-stage prothrombin time and activated partial thromboplastin time were normal.

On admission, the pertinent physical findings included gingival hyperplasia and warm, tender
swelling of the left knee. The hematocrit was 24%, and stools were guaiac-positive. No lesions of the gastrointestinal tract were demonstrated by x-ray.

Aspiration of the left knee revealed sterile bloody fluid that did not contain crystals; the latex fixation test was negative. After aspiration, blood continued to ooze from the puncture site. The prothrombin time and thrombin time were normal, but the activated partial thromboplastin time was greatly prolonged, 102 sec compared to a control of 47 sec. The platelet count was 390,000/sq mm. A polyclonal increase in the concentration of serum IgG (1400 mg/dl) was detected; tests for antinuclear antibodies and LE cell preparations were negative.

A peripheral blood smear and bone marrow aspirate were compatible with iron-deficiency anemia; the serum folic acid concentration was 10 ng/ml (normal >3 ng/ml), and the concentration of diphenylhydantoin in serum was 9.6 mg/dl.

Because diphenylhydantoin was thought to be etiologically related to the genesis of the bleeding syndrome, therapy with this drug was discontinued, and the patient was thereafter treated with phenobarbital. The hemarthrosis resolved spontaneously.

In April 1976, the patient was readmitted with left knee hemarthrosis and upper gastrointestinal bleeding; his hematocrit was 30%. His symptoms resolved with conservative therapy, including transfusion of washed red blood cells. Again, no gastrointestinal lesions were demonstrated by X-ray, and the bleeding was attributed to gastritis.

Studies of the abnormality in clotting are described below.

Patient 2

A 64-yr-old white male with a seizure disorder was transferred from another hospital to the Cleveland Veterans Administration Hospital on April 1, 1976, because of a bleeding disorder and a rapidly falling hematocrit. On March 10, 1976, he had been found to have a tender hematoma of the right upper leg. In the ensuing days, he developed multiple ecchymotic areas and hematomas over the trunk and extremities. During the 3 days before admission, his hematocrit fell from 32% to 18%.

The patient had a lifelong history of alcohol abuse. In 1970, a ventriculojugular shunt had been inserted in treatment of low-pressure hydrocephalus. Thereafter, he had grand mal seizures, and treatment with diphenylhydantoin was instituted. At the time the bleeding disorder appeared, the patient was taking diphenylhydantoin, 300 mg/day, and Dimetapp (brompheniramine maleate, 4 mg; phenylephedrine hydrochloride, 15 mg; and phenylpropanolamine hydrochloride, 5 mg), two tablets/day.

The patient had two bouts of pneumonia, in June 1975 and February 1976, both successfully treated with penicillin. The second course of penicillin was administered from February 18, 1976 to March 1, 1976, without apparent side effects.

In April 1975, the activated partial thromboplastin time was normal. In May 1975, reduced concentrations of serum IgA (19 mg/dl) and IgM (37 mg/dl) were demonstrated, while that of IgG (800 mg/dl) was normal.

On admission, the patient was semicomatose and hypothermic (temperature 34.4° C). He had numerous areas of confluent ecchymoses, most evident over the trunk and lower extremities. The liver was palpable 3 cm below the costal margin. Blood oozed from earlier venipuncture sites. No other pertinent physical abnormalities were demonstrated. The patient’s hematocrit was 16%, the WBC was 12,200/cu mm with a normal differential count, and the platelet count was 590,000/cu mm.

Arterial blood gas analysis revealed metabolic acidosis. The cerebrospinal fluid was unremarkable. Many leukocytes and white blood cell casts, and mixed gram-positive and gram-negative bacilli were present in urine. Mild abnormalities of hepatic function were demonstrated. A test for antinuclear factor was positive at a 1:100 dilution, with a peripheral pattern; an LE test was not performed. Direct and indirect Coombs’ antiglobulin tests were negative. The prothrombin time (patient 18 sec, control 13 sec) and activated partial thromboplastin time (patient 127 sec, control 30 sec) were prolonged. After blood transfusion and parenteral vitamin K therapy, the prothrombin time shortened within 24 hr to 15 sec (control 13 sec), but the activated partial thromboplastin time remained abnormally long.

The patient was treated with gentamicin, oxacillin, penicillin, and methylprednisolone sodium succinate for presumptive urinary tract infection and sepsis, and was transfused with whole blood. He rapidly deteriorated and died on April 3, 1976 after a period of hypotension of 20 hr.
At autopsy, the patient had purpuric lesions of the skin and gastrointestinal tract; no ulcer was found. Diffuse acute centrilobular necrosis of the liver was present. No evidences of vasculitis nor of lupus erythematosus were detected.

Studies of the clotting defect detected in postmortem serum are described below.

**MATERIALS AND METHODS**

Pooled normal plasma and plasma from patient 1, and from patients with congenital clotting factor deficiencies, were prepared as described earlier. Serum was obtained from patient 2 four hr postmortem and was adsorbed for 10 min with tricalcium phosphate dihydrate (10 mg/ml) and heated at 60°C for 1 hr to reduce the amount of thrombin and other heat labile factors that might interfere with clotting assays. Citrated Fletcher trait and Fitzgerald trait plasmas were kindly supplied by Dr. Charles Abildgaard, University of California at Davis, and Dr. Robert Waldmann, Henry Ford Hospital, Detroit, Mich., respectively.

Partially purified AHF was prepared from lyophilized cryoprecipitate (Factorate, Armour Pharmaceutical Co., Phoenix, Ariz.) as described earlier. Partial inhibition of AHF was performed from lyophilized cryoprecipitate (Factorate, Armour Pharmaceutical Co., Phoenix, Ariz.) as described earlier.

Assays for clotting factors were performed by earlier methods. One unit of clotting factor activity or antigen was that amount detected in 1 ml of pooled normal plasma.

Qualitative detection of circulating anticoagulants against factors participating in the intrinsic clotting pathway was performed by incubating mixtures of the plasma to be tested and normal plasma at ratios of 9:1 and 1:1 in 10-mm x 75-mm disposable glass tubes for 1 hr at 37°C. The mixtures were then diluted with barbital-saline buffer (0.025 M sodium barbital, 0.125 M sodium chloride, pH 7.4) 1:100 with respect to normal plasma. Residual clotting factor activity was measured in specific assays in comparison to similar mixtures prepared from plasmas incubated separately for 1 hr at 37°C and then mixed in the same proportions, and to normal incubated plasma, diluted 1:100 in the buffer. A delay in clotting of 5 sec or more of mixtures incubated together compared to these components incubated separately and mixed just before assay suggested the presence of a circulating anticoagulant. Differences in the clotting time between mixtures incubated together and separately suggested progressive inactivation of the clotting factors under test.

Quantitative assay for circulating anticoagulants in plasma, plasma fractions, or serum was performed as described earlier. One unit of inhibitor was defined as twice the concentration of the inhibitor preparation that inactivated 50% of the AHF in pooled normal plasma after incubation of an equal mixture of the test preparation and normal pooled plasma at 37°C for 1 hr.

Techniques used to demonstrate the specificity of the circulating anticoagulant in the plasma of patient 1 included assay of the various clotting factors after progressive dilution of the plasma, and after partial inhibition of the circulating anticoagulants with partially purified AHF. Partial inhibition of the circulating anticoagulant in patient 1’s plasma was achieved by incubating 0.1 ml of his plasma with 0.9 ml of partially purified AHF (AHF functional activity of 4.5 units/ml, antigen 12.8 units/ml, 140 µg protein/ml) devoid of other clotting factor activity at 37°C for 2 hr. Thereafter, assays for specific clotting factors were performed after further dilution with buffer in comparison to a control of 0.1 ml of the patient’s plasma and 0.9 ml buffer, similarly incubated.

Preparation of a globulin fraction of plasma or serum, and neutralization of circulating anticoagulant activity by heterologous antiserum in these fractions, was performed as reported earlier. Rabbit antiserum against human IgG (λ-chain specific) was purchased from Behring Diagnostics (Somerville, N. J.). Rabbit antisera against human kappa and lambda light chains, kindly provided by Dr. George Bernier, Case Western Reserve University, Cleveland, Ohio, were prepared and characterized as described earlier.

**RESULTS**

**Patient 1**

In May 1974, the AHF procoagulant titer of patient 1 was <0.01 units/ml (Table 1), and a circulating anticoagulant directed against this agent was demonstrable in qualitative tests. The anticoagulant was of high potency,
Table 1. Clotting Factor Activities in Patient 1's Plasma

<table>
<thead>
<tr>
<th>Clotting Factors</th>
<th>Functional Activity</th>
<th>Plasma Not Incubated With AHF*</th>
<th>Plasma Incubated With AHF†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal Range</td>
<td>(1/20)</td>
<td>(1/200)</td>
</tr>
<tr>
<td>Hageman factor (factor XII)</td>
<td>0.35–1.83</td>
<td>0.06</td>
<td>0.57</td>
</tr>
<tr>
<td>Fletcher factor (plasma prekallikrein)</td>
<td>0.50–1.50</td>
<td>0.23</td>
<td>0.58</td>
</tr>
<tr>
<td>Fitzgerald factor (high MW kininogen)</td>
<td>0.46–1.32</td>
<td>0.10</td>
<td>0.30</td>
</tr>
<tr>
<td>PTA (factor XI)</td>
<td>0.50–1.56</td>
<td>0.12</td>
<td>0.40</td>
</tr>
<tr>
<td>Christmas factor (factor IX)</td>
<td>0.65–1.85</td>
<td>0.52</td>
<td>0.90</td>
</tr>
<tr>
<td>AHF (factor VIII)</td>
<td>0.46–1.75</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Stuart factor (factor X)</td>
<td>0.65–1.85</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Procaccelerin (factor V)</td>
<td>0.45–1.65</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td>Prothrombin (factor II)</td>
<td>0.65–1.50</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Factor VII</td>
<td>0.55–0.85</td>
<td>0.86</td>
<td></td>
</tr>
</tbody>
</table>

*Procoagulant activity measured by a modification of the activated partial thromboplastin time or one-stage prothrombin time, as appropriate. The patient's plasma was diluted 20-, 200-, or 1000-fold before addition to the substrate mixture; its concentration in the final assay mixture was 1/80, 1/800, or 1/4000 respectively.

†One-tenth milliliter of the patient's plasma was incubated with 0.9 ml buffer or partially purified AHF (functional activity 4.5 units/ml, antigen 12.8 units/ml, protein 140 μg/ml) at 37°C for 2 hr. Thereafter, the incubation mixture was further diluted with buffer (final dilution 1/200 with respect to plasma) before addition to the substrate mixture for assay of functional activity.

measuring 500 units/ml in a quantitative assay. The titers of Hageman factor (factor XII), plasma prekallikrein (Fletcher factor), high MW-kininogen (Fitzgerald factor), plasma thromboplastin antecedent (PTA, factor XI), and Christmas factor (factor IX), all factors that participate earlier than AHF in the intrinsic pathway, appeared to be decreased in routine assays in which the patient's plasma was diluted only 20-fold, presumably because the anticoagulant inactivated AHF in the substrate plasmas. When the patient's plasma was diluted 1000-fold before assay, the titers of all clotting factors except AHF were normal. Similarly, when the action of the anticoagulant was partially blocked by incubation of the patient's plasma with a preparation containing AHF, assays for individual clotting factors other than AHF gave normal results. These data suggested that the circulating anticoagulant was specific against AHF, and that the results of routine assays for other factors participating in the early steps of the intrinsic pathway were spurious.

Repeat studies in April 1976 demonstrated that the titer of functional AHF was still <0.01 units/ml, while the titer of the circulating anticoagulant had decreased to 114 units/ml.

The polyclonal nature of the circulating anticoagulant in patient 1 was reported previously.10

Patient 2

Only postmortem serum was studied in patient 2. A weaker (30 units/ml) but specific inhibitor of AHF was found. Its action was time and temperature dependent, since inhibitory activity was observed in qualitative tests only after the serum had been incubated for 1 hr with normal plasma at 37°C.
The circulating anticoagulant in the serum of patient 2 was completely neutralized by heterologous antiserum to IgG, and partially neutralized by either anti-kappa or anti-lambda antiserums, indicating that the anticoagulant was a polyclonal immunoglobulin.

DISCUSSION

Circulating anticoagulants directed against AHF, antibodies against this clotting factor, have been described in a minority of patients with severe classic hemophilia who had been transfused with preparations containing AHF. They have also been found on rare occasions in a variety of circumstances, appearing, for example, in postpartum women, in elderly patients without overt disease, in patients with lupus erythematosus, and in individuals sustaining hypersensitivity reactions to penicillin.\(^{10}\)

A few cases have been reported\(^{11-13}\) in which the patient’s plasma appeared to have inhibitory activity directed not only against AHF, but also against other factors participating in the intrinsic pathway of clotting. The studies reported herein suggest the possibility that potent inhibition of AHF may interfere with the measurement of other clotting factors participating in the intrinsic pathway of coagulation by inactivating AHF in the substrate plasmas used in specific assays. Two techniques are described to circumvent this difficulty. When plasma containing a potent anticoagulant against AHF is diluted 1000-fold before addition to the test mixture, interference with the assay procedure is decreased, and relatively normal titers of factors other than AHF are obtained.

Even more effective in displaying the true specificity of the anticoagulant is the absorption of the antibody by addition of a partially purified preparation of AHF. Under these circumstances, the titers of all factors other than AHF are normal and, in the case of Hageman factor, the accuracy of the titer has been confirmed immunologically.

The patient in whom these studies were performed had been under treatment with diphenylhydantoin, an agent that to our knowledge has not previously been associated with the appearance of circulating anticoagulants against AHF. In this patient, the titer of the circulating anticoagulant decreased after therapy with diphenylhydantoin was discontinued. A second patient is described in whom a weaker anticoagulant was detected. This patient, too, had been under treatment with diphenylhydantoin. In this case, a causal relationship between the administration of this drug and the genesis of the anticoagulant was less obvious, as shortly before the onset of symptoms he had been treated with penicillin, an agent thought to be related to the evolution of antibodies against AHF.\(^{11}\)

Moreover, the positive antinuclear antibody test makes it impossible to rule out the presence of systemic lupus erythematosus, which may be complicated by circulating anticoagulants. Systemic lupus erythematosus, however, may be induced by diphenylhydantoin therapy, and one such case terminated in azotemia with hemorrhagic complications.\(^{12}\) Attempts to block circulating anticoagulant activity in vitro with diphenylhydantoin were unsuccessful because of the insolvability of this drug. Among the many adverse reactions attributed to the use of diphenylhydantoin and related agents, a number have been associated with disordered immune mechanisms. Thus, the induction of benign\(^ {13}\) or malignant\(^ {14}\) changes in lymphoid tissues, or of syndromes resembling serum sickness\(^ {15,16}\) or
lupus erythematosus\textsuperscript{12,17} and depression of cellular and humoral immunity\textsuperscript{18,19} have been described repeatedly.

Similar to other circulating anticoagulants against AHF, both in hemophiliacs and nonhemophiliacs,\textsuperscript{10,20} those observed in these two patients were polyclonal IgG antibodies.

ACKNOWLEDGMENT

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