Prothrombin Houston: A Dysprothrombin Identifiable by Crossed Immunoelectrofocusing and Abnormal Echis carinatus Venom Activation

By Ronald S. Weinger, Christine Rudy, Joel L. Moake, John D. Olson, and Philip L. Cimo

A 70-yr-old male with a lifelong history of easy bruising and posttraumatic bleeding had a prolonged prothrombin time and activated partial thromboplastin time. His plasma Stypven, Taipan, and Echis carinatus venom clotting times were prolonged. The presence of a dysprothrombin was confirmed by the discrepancy between plasma prothrombin coagulant activity and prothrombin antigen levels. His plasma prothrombin was capable of being completely absorbed onto and then eluted from barium sulfate. Crossed immunoelectrophoresis of his plasma prothrombin, and normal plasma prothrombin, into agarose containing rabbit anti-human factor II antibody were similar. Crossed immunoelectrofocusing, a procedure combining isoelectric focusing in disc gels with electroimmunoassay in the second dimension, demonstrated that the patient’s prothrombin antigen was more basic than normal. The eluate from barium sulfate absorption of patient plasma, when reacted with Echis carinatus venom (which directly cleaves prothrombin to thrombin) clotted purified fibrinogen at a rate slower than normal plasma eluate. SDS-slab gel electrophoresis revealed that the prothrombin present in the patient’s eluate was cleaved by Echis carinatus venom. These studies suggest that the coagulopathy of prothrombin Houston results from the generation of a dysfunctional thrombin.

IMMUNOLOGIC METHODS have greatly increased the understanding of congenital disorders involving the prothrombin (factor II) molecule. Dysprothrombinemia is now distinguishable from hypoprothrombinemia by measurement of plasma factor II antigen. Several patients have been reported whose plasma factor II coagulant levels are reduced in comparison to factor II antigen levels. This report describes a patient with an abnormal prothrombin whose plasma factor II antigen could be distinguished from normal factor II antigen by crossed immunoefirotefocusing. Venom coagulant studies of patient plasma and the barium sulfate eluate from patient plasma suggest that the defect is a result of the generation of a dysfunctional thrombin.

CASE REPORT

The patient is a 70-yr-old white male who was referred to the Gulf States Hemophilia Center because of an abnormal prothrombin time and activated partial thromboplastin time. He had a lifelong history of easy bruising, epistaxis, and posttraumatic bleeding. At the age of 65, following a transurethral prostatectomy, he required multiple whole blood and plasma transfusions. There was no history of bleeding in either parent, his two sisters, or in his daughter (deceased). Physical examination of the patient was normal. There was no clinical or laboratory evidence of hepatic disease.

MATERIALS AND METHODS

Coagulation studies were performed on plasma obtained from whole blood anticoagulated with either 1/9 volume of 3.8% sodium citrate, or 0.01 M sodium oxalate. Studies included platelet count and template bleeding time. The activated partial thromboplastin time, prothrombin time, thrombin time, and fibrinogen assay were performed by standard techniques. Inhibitors to coagulation factors were sought by measurement of the activated partial thromboplastin and prothrombin times of a 1:1 mixture of patient plasma and normal plasma immediately and following a 2-hr incubation at 37°C. Factor XI, IX, VIII, and X were measured by the one-stage partial thromboplastin time assay and factors VII and V by the one-stage prothrombin time assay using congenital deficient plasma substrates (George King, Overland Park, Kans.). Prothrombin was measured by both a one- and two-stage assay system. Factor X was measured by both the partial thromboplastin and prothrombin time one-stage assay. Russell viper venom (Stypven) was purchased from Burroughs Wellcome (Research Triangle Park, N.C.). The plasma Stypven clotting time was performed by the method of Macfarland using 0.5% inosithin (Associated Concentrates, Woodside, N.Y.) as a platelet substitute. Taipan snake venom was kindly supplied by Dr. Ethan Natelson (Houston, Texas). This venom was diluted 1:10,000 with 0.15 M sodium chloride, 0.01 M calcium chloride, 0.5% inosithin, and then added directly to citrated plasma, and the clotting time measured. Echis carinatus venom was purchased from Sigma Chemical Co. (St. Louis, Mo.). This venom was diluted to a concentration of 35 μg/ml with distilled water and then 0.05 ml of the venom solution was added directly to 0.20 ml of citrated plasma and the clotting time measured.

Barium-sulfate-absorbed plasma was prepared from oxalated plasma. Prothrombin was eluted from the barium sulfate precipitate with 0.20 M sodium citrate, over 20 min at room temperature. Additional coagulation studies of the patients prothrombin were performed by dialyzing the barium sulfate eluates against phosphate-buffered saline (pH 7.4) and adjusting the prothrombin antigen concentration to 50% of that found in our plasma pool of 20 normal donors. Echis carinatus venom (125 μg/ml, final concentration) was then added to the eluates. After incubation at 37°C for varying time periods, aliquots were removed for measurement of fibrinogen clotting times and SDS-polyacrylamide slab gel electrophoresis. Fibrinogen clotting times were performed by adding 0.1 ml of the incubation mixture to 0.2 ml of a purified fibrinogen

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solution, \(^{14}\) 2.0 mg/ml final concentration, and measuring the clotting time. Also, at each time period, aliquots were removed, made 1 mM in disopropyl phosphorofluoridate (DFP), and subjected to slab gel electrophoresis (see below).

Factor II antigen was assayed by Laurell electroimmunoassay \(^{17}\) in 0.9% agarose (Fisher Scientific, Pittsburgh, Pa.) using 0.4% rabbit and anti-human factor II antibody (Behring Diagnostics, Sommerville, N.J.). A standard curve was constructed by assaying serial dilutions of a pool of normal citrated plasma collected from 20 normal donors.

Crossed immuno-electrophoresis of plasma factor II antigen was performed by a modification of the method of Montgomery et al. \(^{18}\) Oxalated plasma was electrophoresed in 8 cm x 10 cm glass plates coated with 16 ml of 0.9% agarose in 0.05 M barbital buffer (pH 8.6), 2 mM calcium lactate, at 100 V for 150 min. Second-dimension electrophoresis was performed at 12 mA for 18 hr into 0.9% agarose in 0.05 M barbital buffer (pH 8.6), 2 mM calcium lactate, which contained 0.4% anti-human factor II antibody. Crossed immuno-electrofocusing was by a modification of the method of Soderholm et al. \(^{19}\) Polyacrylamide gels (4%) were prepared in a gradient from pH 5.0 to pH 7.0, containing 3% (w/v) ampholyte (Brinkman pH isolytes, Brinkman Instruments, Inc. Westbury, N.Y.), 2% NP 40 (Patrick Data Laboratories, Ltd., Elmhurst, Ill.) and 9.0 M urea. Specimens were isolectric focused for 15 hr at 400 V at 4°C. A Corning Model 12 (Corning Glass Works, N.Y.) pH meter with Bio-Lyte Gel Pro pHiler (BioRad Laboratories, Richwood, Calif.) electrodes was used to measure the pH gradient across the isolectric focusing gel. Next, the polyacrylamide gels were placed into troughs prepared in an 8 cm x 10 cm glass plate coated with 16 ml of 0.9% agarose in 0.05 M barbital buffer, pH 8.6, which contained 0.4% anti-human factor II antibody. Crossed electrophoresis was then carried out at 10 mA per plate for 18 hr. Electrophoresis plates were dried and stained with Coomassie blue.

Normal human prothrombin was purified from normal plasma by the method of Bajaj and Mann \(^{20}\) as modified by Mann \(^{15}\). Sufficient plasma from our patient was not available to allow purification of his prothrombin. This necessitated using the barium sulfate eluates from small volumes of his plasma.

In order to demonstrate that \textit{Echis carinatus} cleared our patient's prothrombin, the barium sulfate eluates from normal and patient plasma, and highly purified normal prothrombin, were incubated with venom (125 ug/ml, final concentration). Following a 30-min incubation at 37°C, the mixture was dissolved in 2.25 M urea, 0.01 M H\textsubscript{3}PO\textsubscript{4}, 0.3% SDS, and analyzed on 8% polyacrilamide slab gels. \(^{25}\) Slab gel electrophoresis was at 200 V for 150 min. Gels were then stained with Coomassie blue, dried and photographed.

<table>
<thead>
<tr>
<th>Table 1. Routine Coagulation Studies</th>
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<tbody>
<tr>
<td><strong>Test</strong></td>
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<tr>
<td>Platelet count (cu mm)</td>
</tr>
<tr>
<td>Template bleeding time (min)</td>
</tr>
<tr>
<td>Activated partial thromboplastin time (sec)</td>
</tr>
<tr>
<td>Prothrombin time (sec)</td>
</tr>
<tr>
<td>Thrombin time (sec)</td>
</tr>
<tr>
<td>Inhibitor screen</td>
</tr>
<tr>
<td>PT system</td>
</tr>
<tr>
<td>aPTT system</td>
</tr>
<tr>
<td>Fibrinogen (mg/dl)</td>
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*PT, Prothrombin time.
†aPTT, activated partial thromboplastin time.

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**RESULTS**

Routine coagulation studies in the patient are summarized in Table 1. Of significance are the modest prolongations of the activated partial thromboplastin and prothrombin times. Tests for the presence of a circulating anticoagulant were negative. Special hemostasis tests are shown in Tables 2 and 3. Specific factor assays of factors V, VII, VIII, IX, X, and XI were normal. Factor II coagulant level by the one- and two-stage assay was 5.1% and 9.7%, respectively, of that found in the normal plasma pool. Plasma Stypven, Taipan venom, and \textit{Echis carinatus} venom clotting times were prolonged in comparison to normal plasma. When \textit{Echis carinatus} venom was incubated with the barium sulfate eluate prepared from patient plasma and which contained 50% of the prothrombin antigen found in our normal plasma pool, the subsequent fibrinogen clotting times were prolonged at all

<table>
<thead>
<tr>
<th>Table 2. Special Hemostasis Tests</th>
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<tbody>
<tr>
<td><strong>Test</strong></td>
</tr>
<tr>
<td>Factor II — coagulant level (%)*</td>
</tr>
<tr>
<td>One-stage assay</td>
</tr>
<tr>
<td>Two-stage assay</td>
</tr>
<tr>
<td>Factor V (%)</td>
</tr>
<tr>
<td>Factor VII (%)</td>
</tr>
<tr>
<td>Factor VIII (%)</td>
</tr>
<tr>
<td>Factor IX (%)</td>
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<tr>
<td>Factor X (%)</td>
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<tr>
<td>Factor XI (%)</td>
</tr>
<tr>
<td>Factor II — immunoassay (%)*</td>
</tr>
<tr>
<td>Stypven—inosithin clotting time (sec)</td>
</tr>
<tr>
<td>Taipan snake venom clotting time (sec)</td>
</tr>
<tr>
<td>\textit{Echis carinatus} venom clotting time (sec)</td>
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*%: percent of control values in normal pooled plasma from 20 donors.

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**Table 3. Coagulant Activity of Plasma Eluates With \textit{Echis carinatus}**

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Normal Eluate* (sec)</th>
<th>Patient Eluate* (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min)</td>
<td>1</td>
<td>15.5</td>
</tr>
<tr>
<td>5</td>
<td>9.8</td>
<td>54.1</td>
</tr>
<tr>
<td>15</td>
<td>8.6</td>
<td>55.4</td>
</tr>
<tr>
<td>30</td>
<td>7.1</td>
<td>58.0</td>
</tr>
<tr>
<td>60</td>
<td>8.0</td>
<td>54.2</td>
</tr>
</tbody>
</table>

*Plasma was absorbed with barium sulfate. Eluates were prepared with 0.20 M sodium citrate, dialyzed against phosphate-buffered saline and adjusted to contain 50% of the factor II antigen found in our normal donor plasma pool. \textit{Echis carinatus} venom (125 µg/ml, final concentration) was added to each eluate. At the indicated time intervals, 0.1-ml aliquots were removed and added to 0.20 ml of purified fibrinogen solution (2.0 mg/ml, final concentration) and the fibrinogen clotting time measured. Venom alone did not clot the purified fibrinogen solution.
Fig. 1. SDS-polyacrylamide slab gel electrophoresis of purified normal prothrombin and barium sulfate eluates of plasma. Eluates were prepared, incubated at 37°C for 30 min with *Echis carinatus* venom and then electrophoresed into 8% polyacrylamide gels in SDS. (A) Purified normal human prothrombin, (B) purified normal prothrombin incubated with venom, (C) eluate from patient plasma, (D) patient eluate after incubation with venom, (E) normal plasma eluate, and (F) normal plasma eluate after incubation with venom. *Echis carinatus* venom was not visible when electrophoresed alone (not shown). The protein band corresponding to purified prothrombin in patient and normal eluate is no longer present after venom incubation.

Time periods compared to that of normal plasma eluate (Table 3). *Echis carinatus* venom alone did not clot purified fibrinogen solution.

Slab gel electrophoresis revealed purified normal factor II, and the factor II present in the barium sulfate eluates of normal and patient plasma were cleaved in the presence of *Echis carinatus* venom (Fig. 1). Also, when patient and normal plasma eluates were incubated with *Echis carinatus* venom for subsequent fibrinogen clotting time measurements (described above), slab gel electrophoresis of the mixtures revealed that the protein band corresponding to purified prothrombin was no longer present after 5 min of incubation with the venom.

Results of the Laurell electroimmunoassay of plasma factor II antigen are presented in Table 2 and Fig. 2. The patient's plasma contained 51% of the factor II antigen found in normal plasma. The factor II antigen present in patient plasma was completely removed after absorption with barium sulfate. The eluate of the barium sulfate precipitate prepared from patient plasma and normal plasma contained greater than 90% of the factor II antigen present in the unassembled plasmas. In comparison, the eluate from the barium sulfate precipitate of plasma from a chronically coumarinized patient contained 6% of the factor II antigen present in the unassembled coumarinized plasma.

The migration of patient plasma factor II antigen on crossed immunoelectrophoresis was similar to that of normal plasma factor II antigen (Fig. 3). However, the partially purified factor II antigen from the barium sulfate eluate of patient plasma electrofocused at a more basic isoelectric point than the factor II antigen present in the barium sulfate eluate of normal plasma (Fig. 4A and B). When the eluates from
patient plasma and normal plasma were combined and then subjected to crossed immunoelectrofocusing, there were two antigen peaks (Fig. 4C). These peaks correspond to the isoelectric focusing positions of the individually immunoelectrofocused eluates.

**DISCUSSION**

Normal prothrombin is a glycoprotein of approximately 70,000 molecular weight. \(^{13,22}\) Prothrombin is synthesized on hepatic ribosomes, and via a posttranslational, vitamin-K-dependent microsomal carboxylase reaction, acquires up to ten \(\gamma\)-carboxyl groups on glutamic acid residues near amino terminal end of the molecule. \(^{23-25}\) \(\gamma\)-Carboxyglutamic acid groups allow normal prothrombin to absorb onto barium sulfate, bind calcium, and associate with phospholipid. \(^{26,27}\) The prothrombin molecule secreted by the liver cells of humans receiving vitamin-K antagonists possess fewer \(\gamma\)-carboxyglutamic acid groups than normal prothrombin. \(^{24,26,28}\) In these abnormal prothrombins, the rate at which thrombin is generated from the carboxy-terminal portion of prothrombin, by the normal activation system (factors Xa, Va, calcium, and phospholipid), is markedly reduced. \(^{27,28}\) In addition, rat hepatic prothrombin precursors are more basic proteins than normal prothrombins. \(^{27,29}\) This may be related to the lower sialic acid content of precursor prothrombin. \(^{29}\) Removal of sialic acid residues from normal plasma prothrombin produces a more basic protein, \(^{29}\) shortens its in vivo survival, \(^{30,32}\) but does not affect its coagulant properties. \(^{31}\)

Dysprothrombinemias are rare causes of congenital coagulopathy. Seven families, with eight abnormal prothrombins were recently reviewed by Shapiro and McCord. \(^{32}\) These cases are distinguishable from true hypoprothrombinemia by the presence of plasma prothrombin antigens whose coagulant activities were markedly reduced in one- or two-stage assays. Prothrombins Cardeza, \(^{1}\) Barcelona, \(^{2}\) San Juan I, \(^{3}\) and Padua \(^{4}\) appear to possess intact thrombin-active sites, as they are completely activated by _Echis carinatus_ venom or Staphlocoagulase (a nonenzymatic activator of normal prothrombin). Prothrombin Molise \(^{5}\) and Quick \(^{7}\) fail to activate with _Echis carinatus_ or Taipan venoms but are both absorbable onto barium salts. In addition, prothrombin Quick gives rise to dysfunctional thrombin. \(^{7,33}\)

The abnormality of the patient reported here is characterized by an excess of plasma prothrombin antigen level (measured by Laurell electroimmunoassay) compared with plasma factor II coagulant activity. Patient prothrombin antigen could be absorbed completely onto, and then eluted from, barium sulfate. Two-dimensional crossed immunoelectrophoresis of patient prothrombin, in the presence of calcium, had a similar mobility to normal prothrombin.

Clotting times, utilizing Stypven, Taipan, and _Echis carinatus_ venoms as thromboplastins yielded additional information. Stypven generates thrombin, from prothrombin, through the venom’s activations of factors X and V in the presence of phospholipid and calcium—analogous to the normal activation of prothrombin. \(^{14}\) Taipan venom directly generates thrombin and prothrombin fragments identical to
those generated by the normal activation system with the reaction accelerated by calcium and phospholipid.2734 Echis carinatus venom activates prothrombin directly and results in a thrombin identical to that produced by normal activation.3536 Cleavage of our patients prothrombin by Echis carinatus venom was demonstrated on SDS-slab gel electrophoresis of plasma barium sulfate eluates. In addition, when similar quantities of patient prothrombin and normal prothrombin antigens were activated by this venom, subsequent fibrinogen clotting times of patient prothrombin was prolonged.

These findings imply that the abnormality reported here does not result from defective γ-carboxylation of patient prothrombin. The observation that his prothrombin antigen isoelectricfocused at a more basic pH than normal prothrombin is also consistent with normal γ-carboxylation, as the isoelectric focusing point of non-γ-carboxylated prothrombin is reported to be identical to that of normal prothrombin.2937 Results of SDS-slab gel electrophoresis suggest that the cleavage of patient prothrombin peptide bond Arg 273-Thr 274 occurs.3238 We are, however, unable to exclude defective cleavage of his intrathrombin peptide bond, Arg 322-Ile 323, which is unassociated with a molecular weight change but is necessary for the generation of functional thrombin. This latter possibility seems unlikely, however, since Echis carinatus was able to generate functionally effective thrombin from patient plasma eluates as measured by fibrinogen clotting times.

The patient's reduced plasma level of factor II antigen may reflect a heterozygous state for true hypoprothrombinemia. An alternate possibility is that the abnormality demonstrated on crossed immunoelectrofocusing reflects an abnormal carbohydrate content of patient prothrombin, which results in shortened in vivo survival3032 and reduced factor II plasma levels.

Similar defects may be present in the dysprothrombins Molise6 and Quick.7,33 However, those reports do not describe either isoelectric focusing positions or crossed immunoelectrophoresis patterns of their patient's prothrombin. Unfortunately, we were unable to obtain quantities of his plasma sufficient to purify his prothrombin. As a result, it is not clear whether the abnormalities described here are identical to those previously reported. We, therefore, identify our patient as having dysprothrombin Houston.

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