Acquired Hypoprothrombinemia Due to Nonneutralizing Antibodies to Prothrombin: Mechanism and Management

By S. Paul Bajaj, Samuel I. Rapaport, Samantha Barclay, and Kenneth D. Herbst

A patient developed bleeding due to an acute acquired specific prothrombin deficiency. Unlike previously described patients, this patient had no evidence of an associated lupus anticoagulant. Prothrombin activity and antigen were decreased concordantly and the patient’s plasma did not neutralize the activity of added prothrombin or interfere with its measurement by electroimmunoassay. Nevertheless, immunoelectrophoresis and experiments using 125I-prothrombin revealed a prothrombin-binding antibody. The residual prothrombin in the patient’s plasma was in the form of a prothrombin–antibody complex. Administration of adrenal corticosteroids was associated with a rise in prothrombin activity and cessation of bleeding, but circulating prothrombin was still bound to the antibody. This suggests that nonneutralizing antibodies to prothrombin cause plasma prothrombin deficiency because of a rapid clearance of prothrombin–antibody complexes, which is slowed by adrenal corticosteroids. The antibody had a relatively low affinity for prothrombin (Kd 5 to 8 × 10^{-7}) and was transient. It is possible, therefore, that the antibody arose not to prothrombin itself, but to an antigen sharing an epitope with prothrombin.

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MATERIALS AND METHODS

Antibody Preparations

An antiserum against purified human prothrombin was prepared in a rabbit. A sevenfold dilution of the antiserum neutralized 50% of the prothrombin activity of normal plasma. On double immunodiffusion the antiserum gave a single line of identity with purified prothrombin and normal plasma. Goat antisera to human immunoglobulin heavy chain classes were purchased from Cappel Laboratories, Inc., Cochranville, Pa.

Purified Prothrombin

Human prothrombin was purified and radiolabeled with 125I as previously described.4-5 Radiospecific activity was 2 × 10^5 cpm/mg. Prothrombin fragment I and prothrombin I were prepared by treatment of prothrombin with thrombin.6

Prothrombin Assays

Specific prothrombin activity was measured in a one-stage assay as described earlier.4 Prothrombin antigen was measured by electroimmunoassay as described by Laurell.7

Immunoelectrophoresis

Immunoelectrophoresis was performed on a 2.4-mm thick slab of agarose. A 20-μL sample was applied to each well, and electrophoresis was carried out for six hours at 10 V/cm across the plate. Then, 150 μL of prothrombin antisera was added to each trough and allowed to diffuse for 36 hours.

Binding Studies With 125I-Prothrombin

The patient’s oxalated plasma of day 4 was treated with barium sulfate powder to remove any residual free prothrombin or prothrombin complexed with antiprothrombin antibody. After adsorption, no prothrombin antigen could be demonstrated in the plasma by electroimmunoassay. Fifty microliters of 125I-prothrombin (5 μg/mL in 0.05 mol/L TRIS-HCl, 0.15 mol/L NaCl, pH 7.4, containing 1 mg/mL of bovine serum albumin) was added to duplicate 12 × 75-mL glass tubes containing 50 μL of the absorbed plasma. After 16 hours at 4 °C, 175 μL of goat antiserum specific for a human immunoglobulin heavy chain class and 125 μL of normal goat serum (carrier) were added. After one hour at 4 °C, the tubes were centrifuged at 3,000 g for 20 minutes, the supernatants were aspirated, and the pellets were counted for radioactivity.

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In experiments to determine the saturation and binding affinity of the antibody, 50 μL of barium-treated patient's plasma or normal control plasma were incubated with 50 μL of increasing dilutions of 125I-prothrombin for 16 hours at 4 °C. Then, 175 μL of goat antiserum to human IgG and 125 μL of normal goat serum were added and the tubes treated as described. Counts in the pellets of control tubes, which were less than 10% of the total counts added to these tubes, were subtracted from the counts in pellets of tubes containing the patient plasma. The resulting corrected counts were used for calculations of saturation and binding affinity.

CASE REPORT

A 92-year-old white woman, who had never bled abnormally, was hospitalized in October 1982 with a diffuse bleeding diathesis. Six days earlier she noticed tenderness in her left calf and began taking small amounts of aspirin for pain relief. Four days before admission she began bleeding from the gums and, two days later, from the nose. On admission to the hospital she also had scattered ecchymoses, a hematoma of the left calf, and gross hematuria. The physical examination was otherwise normal.

The hemoglobin was 11.6 g/dL, the WBC count was 13,500/μL with 82% segmented neutrophils, and the platelet count was 230,000/μL. Direct antiglobulin (Coombs) test was weakly positive, total serum bilirubin was 0.6 mg/dL, and other liver function tests were normal. The peripheral blood smear was normal with no evidence of spherocytosis. VDRL and antinuclear antibody tests were negative.

Screening tests of hemostasis were bleeding time, six minutes; prothrombin time, 17 seconds (normal, ten to 12 seconds); activated partial thromboplastin time (APTT), 39 seconds (normal, 28 to 31 seconds). Fibrinogen concentration was 415 mg/dL.

Hematuria continued unchecked during the first four days of hospitalization. Four 20-mg doses of phytanidine (Aquamephyton) were given subcutaneously over three days. Seven units of fresh frozen plasma were also given with no noticeable effect on the severity of the bleeding. The prothrombin time increased from the 17-second range to the 22-second range. Six units of packed red cells were also given, to maintain the hemoglobin between 9 and 11 g/dL.

A specific prothrombin assay performed on plasma from day 4 was 6%. A screening test for an inhibitor of prothrombin activity was negative in that mixing equal parts of patient’s plasma and normal plasma corrected the patient’s prolonged prothrombin time and APTT (see Table 2). Nevertheless, large doses of adrenal corticosteroids (methylprednisolone, 80 mg/d in divided doses) were administered, beginning on the evening of the fourth hospital day. Over the next three days the patient also received an additional six units of packed RBCs and five units of fresh frozen plasma.

Within 36 hours of starting methylprednisolone, hemostasis improved, as evidenced by passage of blood clots from an indwelling bladder catheter and reduced bleeding from the gums and nose. By 60 hours after starting steroids (morning of day 7), the prothrombin time and APTT test results had returned to normal, and by 84 hours all abnormal bleeding had ceased.

Steroids were tapered rapidly and discontinued 15 days after they were started. A Coombs test performed in April 1983 was negative. A prothrombin time and APTT were normal in May 1983 and again in April 1984. The patient has not noticed further abnormal bleeding.

RESULTS

Coagulation Studies

Serial values for APTT and prothrombin time test results and for specific plasma prothrombin activity and antigen are summarized in Table 1. In all samples the plasma levels of prothrombin activity and of prothrombin antigen were concordant. Mixing the patient’s plasma of either day 1 (data not shown) or day 4 (Table 2) with an equal volume of normal plasma corrected both the prolonged APTT and the prolonged prothrombin time. The patient’s plasma also failed to neutralize the prothrombin activity of normal plasma (Table 2) or of added purified prothrombin.

In additional experiments, one part of normal plasma was incubated for one hour with either four parts or nine parts of barium-adsorbed patient plasma, and the levels of prothrombin antigen in the resultant mixtures, as measured by electrophoresis, were compared with the levels of prothrombin activity. The results were for the 1:5 mixture, 21% prothrombin antigen and 17% to 23% prothrombin activity; for the 1:10 mixture, 11% prothrombin antigen and 9% to 12% prothrombin activity. Thus, we could not demonstrate material in the patient plasma capable of interfering with measurement by electrophoresis of added prothrombin.

From these studies we concluded that neither the “lupus anticoagulant” nor a specific antibody capable in vitro of neutralizing prothrombin activity or of

Table 1. Serial Coagulation Test Results

<table>
<thead>
<tr>
<th>Day</th>
<th>Hours After Corticosteroids Were Begun</th>
<th>APTT (s)</th>
<th>Prothrombin Time (s)</th>
<th>Prothrombin Activity (% of Normal)</th>
<th>Prothrombin Antigen (% of Normal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>—</td>
<td>39</td>
<td>17</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>4 (evening)</td>
<td>0</td>
<td>66</td>
<td>22</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>5 (morning)</td>
<td>12</td>
<td>59</td>
<td>19</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>7 (morning)</td>
<td>60</td>
<td>30</td>
<td>12</td>
<td>50</td>
<td>48</td>
</tr>
<tr>
<td>8 (morning)</td>
<td>84</td>
<td>29</td>
<td>11</td>
<td>60</td>
<td>62</td>
</tr>
<tr>
<td>49</td>
<td>—</td>
<td>28</td>
<td>11</td>
<td>130</td>
<td>120</td>
</tr>
</tbody>
</table>

Normal values for APTT and prothrombin time are 28 to 31 seconds and ten to 12 seconds, respectively.
Table 2. Absence of Anticoagulant Activity in the Patient Plasma

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Clotting Time (s)</th>
<th>APTT</th>
<th>Prothrombin Time</th>
<th>Prothrombin Biological Activity (% of Normal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient (Pt)</td>
<td>66</td>
<td>22</td>
<td>28</td>
<td>6</td>
</tr>
<tr>
<td>Normal (N)</td>
<td>28</td>
<td>11</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>Pt/N (1:1)</td>
<td>28</td>
<td>12</td>
<td>55</td>
<td></td>
</tr>
</tbody>
</table>

Patient plasma of day 4 was mixed with equal parts of normal plasma and incubated for 30 minutes at 37 °C before testing.

interfering with the measurement of prothrombin antigen was present in the patient plasma.

Demonstration by Immunoelectrophoresis of a Material in the Patient Plasma Binding Prothrombin

Immunoelectrophoresis revealed a difference between the electrophoretic mobility of prothrombin in normal plasma and prothrombin in the patient plasma samples of days 1 through 8 (Fig 1). Purified prothrombin in buffer (well 1), prothrombin in normal plasma (wells 2 and 10), prothrombin in normal plasma diluted 1:10 to reduce its prothrombin concentration to that approaching the plasma concentration in early patient plasma samples (well 3), and prothrombin in the patient plasma after recovery (well 9)—all migrated further toward the anode than did prothrombin present in the patient plasma samples of days 1 through 8 (wells 4 through 8). Thus, the residual prothrombin in the patient plasma on days 1 through 8 circulated bound to a material that retarded its electrophoretic mobility. One should note particularly that the prothrombin in the patient plasma was still complexed with binding material on day 7 (well 7), at which time the prothrombin level had risen to 50%, and also on day 8 (well 8), at which time all abnormal bleeding had ceased.

Because of the faintness of the precipitin lines given by the low concentration of prothrombin present in the patient’s early samples, immunoelectrophoresis was also performed after adding exogenous purified prothrombin to the patient plasma samples and to normal control plasma (Fig 2). The electrophoretic mobility of purified prothrombin added to buffer (well 1), to normal plasma (well 3), or to the patient plasma after recovery (well 9) was similar to the electrophoretic mobility of endogenous prothrombin in normal plasma (wells 2 and 10). In contrast, prothrombin added to the patient plasma samples of days 1 through 8 (wells 4 through 8) gave elongated precipitin lines indicative of a mixture of prothrombin with normal mobility and prothrombin with reduced mobility. The increased intensity of slowly migrating prothrombin after addition of exogenous prothrombin (compare wells 4, 5, and 6 of Figs 1 and 2) suggests that the patient plasma samples of days 1, 4, and 5 contained an excess of prothrombin-binding material.

Because of the higher concentration of endogenous bound prothrombin in the samples of days 7 and 8 (50% and 60%, see Table 1 and Fig 1), supplemental experiments were performed in which these samples were treated with barium sulfate powder to remove endogenous prothrombin. Then, exogenous prothrombin, 150 μg/mL final concentration, was added and the plasma samples were subjected to immunoelectrophoresis. Elongated precipitin lines were obtained that were indistinguishable from those shown in Fig 2. Thus, the patient plasma still contained substantial amounts of prothrombin-binding material at a time when the prothrombin concentration had risen and her hemostatic function had essentially returned to normal.
Evidence That the Prothrombin-Binding Material Is IgG

Experiments in this and the following sections were performed with the patient plasma of day 4, which had been treated with barium sulfate powder to remove endogenous prothrombin. In the first set of experiments, 125I-prothrombin was incubated with patient plasma in a series of tubes to which specific goat anti-human heavy chain antiserums were added to precipitate the different classes of immunoglobulin. As can be seen from Table 3, only precipitates formed with anti-IgG contained 125I counts substantially above the control value. Thus, the prothrombin-binding material in the patient plasma was IgG.

Binding of the Antibody to Prothrombin Fragments

Thrombin cleaves prothrombin into an NH2-terminal segment, fragment 1, and a COOH-terminal segment, prethrombin 1. Binding of the patient’s antibody to these fragments was studied by immunoelectrophoresis. As seen in Fig 3, prethrombin 1 added to adsorbed patient plasma (well 2) had the same electrophoretic mobility as prethrombin 1 added to adsorbed normal plasma (well 1). In contrast, fragment 1 added to adsorbed patient plasma (well 4) gave an elongated precipitin line as compared with fragment 1 added to adsorbed normal plasma (well 3). This indicates that the patient’s antibody binds to an epitope or epitopes confined to the NH2-terminal segment of the prothrombin molecule.

Saturation Curve and Scatchard Analysis of Binding of 125I-Prothrombin Binding to the Patient’s Antiprothrombin Antibody

Figure 4 is a saturation curve obtained by adding increasing concentrations of 125I-prothrombin to an equal volume of the patient’s adsorbed plasma. The maximal binding of approximately 55 μg/mL of prothrombin observed in this equal parts assay mixture means that the patient’s undiluted plasma is capable of binding 110 μg/mL of prothrombin. The Kd (dissociation constant) for prothrombin–antiprothrombin antibody binding, which is equal to the molar concentration of free prothrombin at 50% antibody saturation, is calculated from this curve as 8 × 10⁻⁷ mol/L.

On Scatchard analysis (Fig 5) these data gave a straight line, which suggests that the patient’s antibody was of a single-affinity class. Extrapolation of this line to the X-axis gives a concentration of 120

| Table 3. Immunoglobulin Class of the Patient’s Antiprothrombin Antibody |
|--------------------------|----------------|
| Class of Goat Anti-Human | B/Bo         |
| Immunoglobulin Antiserum |              |
| IgA                      | 0.10         |
| IgD                      | 0.06         |
| IgM                      | 0.13         |
| IgE                      | 0.08         |
| IgG                      | 0.63         |
| Control (nonimmune goat serum) | 0.08 |

Barium-treated patient plasma of day 4 was incubated with 125I-prothrombin followed by precipitation of antigen–antibody complexes with class-specific goat anti-human immunoglobulin. Bo represents total 125I counts in the tube and B represents counts in the pellet.
we have recently shown that the hypoprothrombinemia in this syndrome stems from the presence of a nonneutralizing antibody to prothrombin, which is not demonstrable in clotting assays and is recognized only by immunochemical techniques. The reason formation of an antibody reacting with prothrombin should be linked to formation of antibodies causing the lupus anticoagulant phenomenon is unknown.

The patient described in the present report differs from our previously described patients in that she developed acute acquired hypoprothrombinemia in the absence of an associated lupus anticoagulant. The well-documented syndrome. We have recently shown that the hypoprothrombinemia in this syndrome stems from the presence of a nonneutralizing antibody to prothrombin, which is not demonstrable in clotting assays and is recognized only by immunochemical techniques. The reason formation of an antibody reacting with prothrombin should be linked to formation of antibodies causing the lupus anticoagulant phenomenon is unknown.

The patient described in the present report differs from our previously described patients in that she developed acute acquired hypoprothrombinemia in the absence of an associated lupus anticoagulant. The
findings in this patient establish that nonneutralizing antibodies to prothrombin may arise by mechanisms independent of the lupus anticoagulant phenomenon. Moreover, the prothrombin antibodies arising in four patients studied to date in this laboratory with the "acquired hypoprothrombinemia-lupus anticoagulant syndrome" have all reacted with epitopes on the COOH-terminal segment of the prothrombin molecule, whereas the prothrombin antibody in the present patient reacted with an epitope on the NH2-terminal segment of the prothrombin molecule.

The mechanism triggering production of the prothrombin antibody in this patient is unknown. She had no other evidence suggestive of a systemic disorder of immune function except for an unexplained weakly positive Coombs test that was not associated with evidence of hemolysis. The prothrombin antibody developed in the absence of a recognized event, such as an acute infection or ingestion of a drug, that might have exposed her to foreign antigenic determinants. Nevertheless, because the Kd of 5 to 8 x 10^-7 mol/L indicates that the antibody had only a weak affinity for prothrombin, and because the antibody was transient, we suspect that the antibody arose in response to an antigen other than prothrombin that shares an epitope with prothrombin. A similar mechanism may have been responsible for the acute, transient (two to three weeks) episode of severe hypoprothrombinemia in a patient reported by Karpatkin et al10 in 1962, although tests for a nonneutralizing antibody were not carried out.

The small amount of residual prothrombin activity measured in the plasma samples of days 1, 4, and 5 (6% to 12% of normal) represents the activity of prothrombin circulating as a prothrombin-antiprothrombin antibody complex (see wells 4, 5, and 6 of Fig 1). The reduced prothrombin concentration of the patient plasma of these days presumably reflects accelerated intravascular clearance of this prothrombin-antiprothrombin antibody complex. Within 60 hours of beginning adrenal corticosteroid therapy, the prothrombin activity of the patient plasma had risen to 50% of normal, yet all prothrombin in the plasma was still in the form of a prothrombin-antiprothrombin antibody complex (well 7, Fig 1). Moreover, the plasma still contained substantial amounts of free antiprothrombin antibody. The rise in plasma prothrombin concentration could have resulted either from an increased release of prothrombin into plasma (with resultant increased formation of a prothrombin-antiprothrombin antibody complex) or from a decreased clearance of prothrombin-antiprothrombin antibody complex. Because steroids are thought to exert their therapeutic effect in many autoimmune disorders by impairing mononuclear phagocytic activity, the latter seems the most likely explanation for the observed findings. If so, the findings support the hypothesis that acute acquired prothrombin deficiency due to nonneutralizing antibodies stems from the rapid clearance of antibody-bound prothrombin.

The growing evidence that acute acquired specific hypoprothrombinemia results from nonneutralizing antibodies to prothrombin raises the possibility that similar antibodies cause acute, otherwise unexplained specific deficiencies of other clotting factors.9 In our patient, administration of adrenal corticosteroids was associated with rapid improvement in the clinical course. If nonneutralizing antibodies impair hemostasis because of accelerated clearance of antigen-antibody complexes, then the administration of large doses of adrenal corticosteroids would seem indicated for all patients with bleeding due to such antibodies. Nonneutralizing antibodies can not be detected by simple mixing tests for inhibitors, but require specialized technique taking at least 24 hours. Therefore, in a patient bleeding seriously, one may need to begin adrenal corticosteroid therapy, possibly supplemented by fresh frozen plasma, before the diagnosis is established.

REFERENCES


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