Development of Antibodies to Thrombin and Factor V With Recurrent Bleeding in a Patient Exposed to Topical Bovine Thrombin

By James L. Zehnder and Lawrence L.K. Leung

A 65 year old patient who was exposed to topical bovine thrombin during cardiac surgery developed markedly prolonged clotting times and a severe bleeding diathesis. Mixing studies with normal plasma failed to correct the clotting times. Platelet transfusions, immunosuppressive and immunomodulatory therapies were ineffective, but and in the resolution of clinical bleeding events. The patient’s purified IgG reacted with bovine thrombin by immunoblotting and enzyme-linked immunosorbent assay (ELISA). However, the IgG reacted minimally with human thrombin. In view of the severe bleeding, a coexisting inhibitor was sought. The patient’s factor V activity was 1% of normal and was not corrected by mixing with normal plasma, demonstrating the presence of an inhibitor against factor V. The patient’s IgG reacted with both bovine and human factor V. Immunoblotting localized the site of antibody binding to the light chain of activated bovine factor V. Detectable amounts of bovine factor V were found in commercial bovine thrombin preparations by ELISA. The data suggest that patients exposed to topical bovine thrombin may develop antibodies to thrombin and factor V. Anti-thrombin antibodies may mask coexisting factor V inhibitors responsible for clinical bleeding.

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CASE PRESENTATION

The patient K.Y. is a 65-year-old Japanese-American man who presented to Stanford Hospital in congestive heart failure for a second prosthetic porcine mitral valve replacement. The first valve had been placed 12 years previously uneventfully. Since his surgery, the patient had been maintained on coumadin. There was no history of lymphoid malignancy, autoimmune or hepatic disease. Coumadin was stopped 1 week before admission and presurgery laboratory values showed a normal PT and PTT. The patient underwent valve replacement, receiving a second porcine heterograft. The operation was complicated by a right ventricular tear and extensive blood loss. Fresh frozen plasma, cryoprecipitate and topical bovine thrombin (Thrombostat, Parke-Davis, Morris Plains, NJ) were used to achieve hemostasis. Postoperatively, the patient was treated with cefamandol and ceftriaxime. He did well until 1 week after surgery, when his PT, PTT and thrombin time (TT) began increasing. After therapy with large amounts of vitamin K and fresh frozen plasma, the clotting times continued to increase (Fig 1). On the twelfth hospital day the PT was 43 seconds, PTT 99.7 seconds, TT > 100 seconds. The patient suffered a hemothorax, associated with respiratory distress requiring intubation and a decrease in hematocrit from 37% to 32%. A chest tube was placed and 900 mL of bloody fluid drained from the thorax. The platelet count was 271,000/mm3; the Simplate bleeding time and reptilase time were normal (Table 1). A heparin effect was excluded by treatment with protamine and by an ion-exchange resin (Heparsorb, General Diagnostics, Organon Teknika Corp, Durham, NC). The PT, PTT and TT did not correct when mixed with an equal volume of normal plasma, demonstrating the presence of a circulating inhibitor. The initial interpretation was

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that the patient had a thrombin inhibitor that resulted in a prolonged TT as well as the proximal clotting times. Intravenous (IV) IgG, 0.8 g/kg was administered over 2 days without effect. Immunosuppressive therapy with 1 mg/kg/day of prednisone and 100 mg/day of oral cyclophosphamide was initiated. The patient's clotting times continued to increase, and on the 23rd hospital day he suffered a major hemorrhage into his left forearm, manifested by severe pain, tense edema and distal sensory loss. Over a 24-hour period the hemocrit decreased from 29.8% to 21.2%. At this time, the platelet count was 181,000, the PT 58 seconds and the PTT 160 seconds. A fluorescent anti-nuclear antibody (FANA) titer was >1:160, <1:640 dilution with a diffuse pattern. Complement levels were normal, the rheumatoid factor was <1:160, and a serum protein electrophoresis showed a normal pattern. At this point, because the patient was felt to have a life-threatening bleeding disorder due to an acquired clotting factor inhibitor, which was poorly controlled with low dose chemotherapy, higher dose combination chemotherapy was used, with cyclophosphamide 450 mg/d for 5 days IV, vincristine 2 mg IV and prednisone 80 mg/d for 5 days. Simultaneously, the patient underwent plasmapheresis, exchanging 1 plasma volume/day for 4 days in an attempt to lower the titer of the inhibitor. The patient was also treated with epsilon-amino caproic acid, 16 g/d for four days. The patient's clotting times decreased immediately after the first pheresis, to a PT of 30 seconds and PTT of 70 seconds, with cessation of bleeding (Fig 1). Over postoperative days 25 through 40, the patient's course was complicated by fever and neutropenia, for which he was empirically treated with broad spectrum antibiotics. No infectious agent could be identified and the patient became afebrile when his absolute neutrophil count returned to >500/mm³. The patient's platelet count was maintained at >100,000/mm³ by platelet transfusions. During this time the patient's clotting times progressively increased to a PT of 37.3 and PTT of 116.8 on day 41 and the patient suffered a hemorrhage into his left thigh. The hemocrit decreased from 37% to 25% over a 12-hour period. The platelet count was 265,000/mm³. The bovine TT remained >100 seconds, but the TT using human thrombin was normal. A repeat FANA was >1:40, <1:160. Clotting factor assays revealed a 1% functional activity of factor V, with an inhibitor noted to be present (Table 1). Plasmapheresis was initiated, with a prompt decrease in clotting times and cessation of bleeding. The patient was discharged from the hospital on day 57 with a PT

Table 1. Clotting Parameters of Patient K.Y.

<table>
<thead>
<tr>
<th>I. Day 12 (hemothorax)</th>
<th>TT</th>
<th>PT</th>
<th>PTT</th>
<th>RT</th>
<th>BT (min)</th>
<th>D-dimer</th>
<th>Fibrinogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>14.2</td>
<td>11.1</td>
<td>24.5</td>
<td>9.6-14</td>
<td>&lt;9</td>
<td>&lt;200 ng/dL</td>
<td>160-360 mg/dL</td>
</tr>
<tr>
<td>Patient &gt;100</td>
<td>43.8</td>
<td>99.7</td>
<td>12.7</td>
<td>7</td>
<td>500-1,000</td>
<td>383</td>
<td></td>
</tr>
<tr>
<td>Normal + buffer</td>
<td>15.2</td>
<td>12.7</td>
<td>33.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient + buffer &gt;100</td>
<td>40.8</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient + normal &gt;100</td>
<td>30.7</td>
<td>65.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient + platelets</td>
<td>63.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| II. Day 41 (thigh hemorrhage) | Clotting times >100 | 37.3 | 116.8 | 13.7 | 200-500 | 311 |
| Factor Assays (functional activity) | Factor II 108% | Factor V 1% | Factor VII 77% | Factor VIII 250% | Factor X 114% |

<table>
<thead>
<tr>
<th>Human and Bovine Thrombin Times</th>
<th>Bovine TT</th>
<th>Human TT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>17.8</td>
<td>29.7</td>
</tr>
<tr>
<td>Patient &gt;100</td>
<td>32.7</td>
<td></td>
</tr>
</tbody>
</table>

All clotting times are in seconds unless otherwise labeled.

Abbreviations: TT, thrombin time; PT, prothrombin time; PTT, partial thromboplastin time; RT, reptilase time; BT, bleeding time.
and PTT of 35.9 seconds and 83 seconds, respectively. When seen in follow-up on day 64 postoperative, the patient was noted to have a small right ankle hematoma, not associated with a significant drop in his hematocrit. The platelet count was 368,000/mm$^3$. Clotting times were PT 36 seconds and PTT 106 seconds. The patient was admitted, plasmapheresed again with prompt decrease in his clotting times and discharged on day 66. When last seen in follow-up on postoperative day 161, the PT was 13.3, PTT 33, TT $>100$ seconds and factor V activity was 80% of normal, with a weak factor V inhibitor noted to be present. He has had no further episodes of bleeding and has resumed his life as an almond farmer in the central valley of California.

MATERIALS AND METHODS

Enzyme-linked immunosorbent assays (ELISA). Purified human factor V, bovine factor Va and murine monoclonal antibodies against human factor V and the light chain of bovine factor Va (BFV-4) were gifts of Dr Kenneth G. Mann, University of Vermont. Bovine thrombin preparations were obtained from Parke-Davis (Thrombostat) and Armour Pharmaceutical Company (Kankakee, IL) (Thrombinar). Results with both thrombin preparations were similar; data presented was done using the Parke-Davis product. Purified human thrombin was a gift from Dr Marc Shuman, University of California, San Francisco. Proteins were coated onto 96-well microtiter plates at a concentration of 5 $\mu$g/mL with a 0.1 mol/L carbonate buffer, pH 9.5, incubated 12 hours at 4°C and washed three times with phosphate-buffered saline (4.3 mmol/L Na$_2$HPO$_4$, 1.4 mmol/L KH$_2$PO$_4$, 2.7 mmol/L KCl, 137 mmol/L NaCl, pH 7.4) and 0.05% Tween 20 (PBST). Antibody was added to the wells and the plate incubated at 37°C for 1 hour. Monoclonal antibodies were used at a concentration of 5 $\mu$g/mL. Purified patient IgG was used at concentrations varying from 0.01 to 12 mg/mL. A control human purified IgG was used in all experiments at identical concentrations. Plates were washed thrice with PBST. In assays performed with human IgG as the primary antibody, the second antibody added was protein A-alkaline phosphatase (Cappel, Cooper Biomedical, Inc, Malvern, PA) or goat anti-human IgG-alkaline phosphatase (Tago, Inc, Burlingame, CA). In experiments where the first antibody was a murine monoclonal, rabbit anti-mouse IgG conjugated to alkaline phosphatase (Bio-Rad Laboratories, Richmond, CA) was used. All conjugated antibodies were used at a 1:2,000 dilution. After application of the secondary antibody, the plates were again incubated at 37°C for one hour, washed, and alkaline phosphatase substrate (Sigma 104) was added. Absorbance at different time points was monitored at 405 nm using a TiterTek Multiskan ELISA spectrophotometer (Flow Laboratories, Inc, McLean, VA). All assays were done in triplicate.

Immunoblots. Commercial bovine thrombin preparations, purified human and bovine factor V were electrophoresed on 7.5% SDS-polyacylamide gels. Separated proteins were then electrophoretically transferred to polyvinylidene difluoride membranes (Immobilon, Millipore Corp). The membranes were soaked in 5% gelatin, 1% BSA for one hour to block nonspecific binding sites. Membranes were then probed with murine monoclonal antibodies against intact human factor V, light chain of bovine factor Va, a control murine anti-CD20 monoclonal antibody (IF5, obtained from Dr Robert Negrin, Stanford University), patient’s purified IgG, or control human IgG. Patient and control IgG were used at a concentration of 50 $\mu$g/mL. Monoclonal antibodies were used at a concentration of 9 $\mu$g/mL. After extensive washing, a second antibody conjugated to alkaline phosphatase (rabbit anti-mouse IgG for murine monoclonals, protein A or goat anti-human IgG for purified human IgG) was incubated with the membranes. Following extensive washing, the membranes were developed with NBT/BCIP (nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, Promega Corporation, Madison, WI) substrates.

Clotting times. Prothrombin time, partial thromboplastin time, human and bovine thrombin times, reptilase time, clotting factor assays and inhibitor screens were performed by the coagulation laboratories at Stanford University Hospital according to standard protocols. The thrombin time at Stanford Hospital is routinely done using bovine thrombin.

Plasmapheresis. Plasmapheresis was performed using Cobe 2997 or Cobe Spectra devices (Cobe Laboratories Inc, Lakewood, CO). Cellular elements were separated from the plasma by differential centrifugation and reinfused with fresh frozen plasma. The first 200 mL of the patient’s plasma removed by the machine was saved for analysis and the remainder discarded. One plasma volume per day was exchanged for three to five days, until the patient’s clotting times stabilized. The procedure was performed on three occasions as outlined in Fig 1.

IgG purification. Heat-inactivated patient and control plasma was dialyzed against a large volume of 0.02 mol/L K$_2$HPO$_4$, pH 8.0 for 12 hours at 4°C. Plasma samples were then loaded onto a DEAE Affi-Gel Blue (Bio-Rad) column that had been equilibrated with the same buffer, and eluted at 45 mL/hour with the eluate monitored at A280. The first peak eluted, representing the IgG fraction and confirmed by SDS-PAGE, was pooled and precipitated with 50% NH$_4$SO$_4$, reconstituted with H$_2$O and dialyzed for 12 hours against the 0.02 mol/L phosphate buffer with 0.02% sodium azide.

RESULTS

Demonstration of anti-bovine thrombin inhibitor. At initial presentation, a circulating heparin was excluded as the cause of the patient’s prolonged clotting times by failure to correct the clotting time by adsorbing the patient’s blood with an ion-exchange resin (HeparSorb) or by treatment with protamine. Mixing studies were then performed (Table 1). The PT, PTT, and TT failed to correct with normal plasma, suggesting the presence of a circulating inhibitor. Other causes of a prolonged thrombin time include hypofibrinogenemia, dysfibrinogenemia, paraproteins or disseminated intravascular coagulation (DIC). The fibrinogen level was within normal limits. The D-dimer fibrin breakdown product was 500 to 1,000 ng/dL. However, the reptilase time, a measure of the conversion of fibrinogen to fibrin by a snake enzyme that is independent of the thrombin mechanism, was normal. Therefore paraproteinaemia, dysfibrinogenemia or significant DIC was unlikely. The initial hypothesis was that the patient had developed an inhibitor to thrombin, which caused prolongation of all clotting times. In light of recent reports of postoperative surgical patients developing anti-bovine thrombin antibodies, thought to be secondary to the use of topical bovine thrombin intraoperatively as a local hemostatic agent, we compared human and bovine thrombin times. ( Routinely, the thrombin time is done using bovine rather than human thrombin). The bovine thrombin time remained very prolonged, whereas the human thrombin time was not significantly greater than control, suggesting that the patient’s anti-thrombin antibody was primarily directed against bovine thrombin with little or no cross-reactivity to human thrombin (Table 1). This finding was confirmed by purifying the patient’s IgG and showing by ELISA (Fig 2) and immunoblotting (data not shown) that the patient’s IgG was
strongly reactive with bovine thrombin, with no cross-reactivity to human thrombin.

**Demonstration of an inhibitor to human and bovine factor V.** Because the patient had a life-threatening bleeding disorder and the anti-thrombin activity was directed against bovine and not human thrombin, it seemed unlikely that the basis for the bleeding could be due to an anti-thrombin antibody. Therefore other clotting factors involved in the final common pathway of both the PT and PTT were assayed. As depicted in Table I, the functional activity of factor V was 1% of normal, while factor X and other clotting factors were normal. The factor V activity was not corrected by adding normal plasma, consistent with the presence of an antibody against factor V. The antibody was functional at 25°C, and there was no significant change in activity after one hour incubation at 37°C. By ELISA, both the anti-thrombin and anti-factor V antibodies were IgG, with IgM activity equivalent to control levels (data not shown). The presence of an anti-factor V antibody was directly demonstrated by an ELISA showing that the patient’s IgG reacted strongly with bovine and human factor V (Fig 3). Immuno-blots using the patient’s purified IgG to probe purified bovine Va showed that the patient’s IgG specifically reacted with the light chain of bovine Va (Fig 4).

**Demonstration of factor V in bovine thrombin preparations.** Hypothesizing that crude bovine thrombin preparations might also contain factor V and be a source of immunogen, we investigated whether immunoreactive material in the preparations could be detected with the anti-bovine Va antibody. As shown in Fig 5, by ELISA commercial thrombin preparations were reactive with the anti-Va monoclonal, consistent with the presence of factor V in the bovine thrombin preparations. Results were similar with both commercial thrombin preparations; data shown is reactivity with Thrombostat (Parke-Davis).

**DISCUSSION**

Patient K.Y., who was status post 2 cardiac surgeries, developed antibodies to bovine thrombin as well as to bovine and human factor V associated with recurrent, severe bleeding episodes. Patients with prolonged thrombin times following surgery have been recently reported by two groups.4-6.
Several of these patients have had an isolated prolonged TT with normal PT and PTT, suggesting an antibody primarily directed against bovine thrombin. Other patients have had long TT in association with prolonged PT and PTT, with cross-reactivity of the antibody to human thrombin. Our patient appears to be an example of a patient’s developing an anti-bovine thrombin antibody in response to immunization with topical thrombin, without significant cross-reactivity to human thrombin. The profound inhibition of bovine thrombin activity suggests that the antibody binds near or sterically interferes with a site necessary for catalytic activity that is not present in human thrombin. Of note, none of the reported patients with anti-thrombin antibodies had a clinically significant bleeding diathesis, except for one with a coexistent anti-factor V antibody. This antibody was not characterized in terms of specificity and reactivity to bovine factor V.

The etiology of acquired factor V inhibitors has remained unclear. Recently, Nesheim et al characterized a human anti-factor V antibody and summarized 45 reported cases of acquired factor V inhibitors. Of six patients who had antibodies to human factor V without a previous history of surgery and who were tested for reactivity with bovine factor V, all were unreactive. In contrast, of six patients who had a previous history of surgery and antibodies to human factor V, all had significant reactivity to bovine factor V. Thus, there was a striking association between postsurgical acquired anti-human factor V antibodies and cross-reactivity to bovine factor V. This suggested to us that patients may have been exposed to a source of bovine factor V intra- or peri-operatively, leading to immunization against bovine factor V, with the resultant anti-bovine V antibody cross-reacting with human factor V, leading to bleeding. Bovine thrombin is widely used as a topical hemostatic agent in many surgical procedures. Our investigation has shown that commercially available bovine thrombin preparations contain detectable amounts of factor V. It is plausible that our patient and the other postsurgical patients who had antibodies to human and bovine factor V developed these antibodies as a response to antigenic challenge by bovine factor V contaminating the bovine thrombin preparation. This patient also received blood transfusions and factor replacement, which have been associated with factor V inhibitors. Whether these agents play a causative role in the development of anti-factor V antibodies is unclear. The patient also had a transiently positive FANA, and it is possible that the clotting inhibitor was a manifestation of an autoimmune phenomenon. Although we were unable to document exposure to topical bovine thrombin during the patient’s previous cardiac surgery, the short interval between exposure to the thrombin preparation and development of the inhibitors is consistent with a prior sensitization.

It is interesting to speculate that the basis for the low functional activity of factor V and the severe bleeding diathesis in this patient is secondary to an antibody’s interfering with the cell-surface assembly of the prothrombinase complex. The patient’s antibody is directed against the light chain of bovine Va, which is the site of phospholipid binding and factor Xa binding. We are currently investigating the effect of the patient’s antibody on cell surface prothrombinase assembly and activity.

A variety of therapies have been used in the treatment of acquired inhibitors to clotting factors, including combination chemotherapy, steroids and high dose intravenous immunoglobulin. All of these modalities were attempted without success in our patient. Platelet transfusion therapy has been reported to be of benefit in treating patients with factor V inhibitors, thought to be due to the high concentration of platelet factor V that may be relatively inaccessible to a circulating inhibitor. However, multiple platelet transfusions in our patient had no effect on the patient’s clotted times or clinical bleeding. On the other hand, plasmapheresis was effective in managing this patient’s bleeding diathesis. Our patient’s bleeding risk was correlated with a PTT > 100 seconds; on the three occasions that he achieved a PTT > 100 seconds, the patient had major bleeding events. While interpretation of the response to the first plasmapheresis is complicated by the use of previous and concurrent chemotherapy, the patient responded reproducibly and rapidly to subsequent phereses without concomitant chemotherapy, with a prompt decrease in clotting times and resolution of bleeding on three occasions. Like other patients with IgG inhibitors, the effect of plasmapheresis was transient, probably because of post-pheresis re-equilibration of serum IgG with the extravascular compartment.

This patient demonstrates that acquired inhibitors to bovine thrombin may mask other inhibitors or clotting factor deficiencies. As bleeding with bovine thrombin inhibitors is unusual, patients who develop an elevated thrombin time, PT and PTT and a bleeding diathesis after surgery should be investigated for evidence of a coexisting inhibitor to factor V.

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Development of antibodies to thrombin and factor V with recurrent bleeding in a patient exposed to topical bovine thrombin

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