Isolation and Characterization of an Acquired Antithrombin Antibody

By Jeffrey H. Lawson, Beverly J. Pennell, John D. Olson, and Kenneth G. Mann

A 68-year-old man, following mitral valve replacement, presented with a low-grade chronic consumptive coagulopathy. Laboratory analysis showed mild fibrinolysis, minimal effect of coumadin therapy, and a prolonged thrombin time (>150 seconds using bovine Ila). When purified human Ila was used the thrombin time normalized to within 17 seconds of controls, suggesting a possible inhibitor of bovine Ila. An anti-Ila antibody was isolated by protein A-Sepharose (Sigma, St Louis, MO) chromatography followed by affinity chromatography using a bovine Ila-Sepharose column. The effects of this purified anti-Ila antibody on both bovine and human Ila procoagulant and anticoagulant functions were studied. The isolated immunoglobulin G (IgG) was observed to inhibit bovine Ila in all assays tested. This IgG was also able to slightly prolong fibrinogen clotting by human Ila. Using an enzyme-linked immunosorbent assay it was observed that the IgG bound to bovine Ila, bovine II, human Ila, but not to human II.

Further, binding was detectable at approximately 50-fold lower concentrations to bovine Ila (1 nmol/L IgG concentration) than to human Ila (50 nmol/L IgG concentration). The effect of the antibody on the reaction between Ila and AT III/heparin was investigated. Human Ila was found to be protected from AT III/heparin neutralization in the presence of this antibody. These results suggest that this patient developed an antibody that strongly binds to and inhibits the bovine Ila in all assays tested. However, the antibody only significantly affects human Ila neutralization by AT III/heparin, and has little effect on the human Ila procoagulant activity. These data suggest that the decreased effect of AT III/heparin on this patient’s Ila may have been a contributing factor in his coagulopathy. The exact cause of this antibody development is unclear, but the role of bovine topical thrombin used during cardiac valve replacement surgery is suspect.

T HROMBIN is a 37,000 molecular weight (mol wt) serine protease that is thought to be central in the regulation of blood coagulation. The generation of thrombin, from its zymogen precursor prothrombin, is required for the conversion of fibrinogen to fibrin. Because of thrombin’s central role in blood coagulation, its regulation, physiologic function, and biochemical properties have been the subject of intense investigation. One area of interest in this laboratory has been the regulation of thrombin generated from prothrombin via prothrombinase and the subsequent inactivation of thrombin by antithrombin III and other plasma protease inhibitors. The rate of thrombin generation relative to its rate of inactivation is a key regulatory event in blood coagulation.

Acquired inhibitors of thrombin have been reported sporadically throughout the literature. Loeliger and Hers reported a thrombin inhibitor isolated from the γ-globulin fraction of plasma from a patient with rheumatoid arthritis. Other reports of acquired thrombin inhibitors have been associated with autoimmune diseases, liver cirrhosis, and surgery (see Table 1). Recently, reports of antithrombin antibodies have suggested that these inhibitors may be associated with prosthetic cardiac valve replacement or to exposure to bovine topical thrombin during the surgical procedures.

Thrombin is commonly used as a topical therapeutic agent during cardiac and neurologic surgical procedures. Recent reports have raised questions about the use of agents such as bovine topical thrombin because of the potential associated immune responses to the crude nonhuman protein. It has been suggested that patients exposed to bovine topical thrombin may generate antibodies that cross-react with human thrombin. These reports have evaluated the immunologic reactivity of these antithrombin antibodies and their ability to grossly prolong the thrombin time in patient plasma; however, none of these reports have evaluated the significance of the purified antibody inhibitors on thrombin functions.

This report describes the characterization of an antithrombin antibody, isolated from a 68-year-old man with a synthetic mitral heart valve and evidence of a chronic low-grade consumptive coagulopathy. We report on the isolation and functional characterization of this antibody and suggest a possible role that this inhibitor may have played in this patient’s coagulopathy and difficulty with coumadin therapy.

CASE HISTORY

T.A. is a 68-year-old white man with a remote history of uncomplicated resection of a colon polyp, peptic ulcer disease, and a 105 pack per year history of cigarette smoking. In 1970, the patient suffered an inferior wall myocardial infarction that responded well to medical management. In November 1986 he presented with unstable angina, hypertension, and significant dyspnea on exertion. One month later he underwent coronary artery bypass graft (RCA) and mitral valve replacement. Postoperative recovery was unremarkable. In March 1987 the patient again presented with dyspnea on exertion, fatigue, and recurrent mitral regurgitation. That same month the patient again underwent mitral valve replacement surgery. At the time of surgery 10,000 U of bovine thrombin was used to control local hemostasis. The postoperative course was uncomplicated. The patient was managed medically on oral anticoagulants and diuretics until April 1988 when he again presented with clinical findings.

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consistent with mitral regurgitation and abnormal liver function tests. The patient underwent his third mitral valve replacement and was again exposed to 10,000 U of bovine thrombin for control of local hemostasis. The postoperative period was complicated by recurrent epistaxis beginning on the ninth postoperative day. On postoperative day 15 the coumadin was discontinued because of persistent epistaxis. On postoperative day 22 a coagulation and fibrinolysis profile was performed (Table 2). The profile showed evidence of a mild consumptive coagulopathy and a marked prolongation of the thrombin time (bovine thrombin time). The patient recovered clinically and continues to take oral anticoagulants. In December 1988 the patient underwent an uncomplicated cholecystectomy. At the time of surgery the patient's thrombin time remained strikingly prolonged.

**Table 1. Acquired Inhibitors of Thrombin**

<table>
<thead>
<tr>
<th>Investigator</th>
<th>Date</th>
<th>Patient Age/Sex</th>
<th>Illness</th>
<th>Bleeding</th>
<th>Exposure to Bovine IgG</th>
<th>IgG Species Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loeliger and Hers³⁶</td>
<td>1987</td>
<td>45/M</td>
<td>RA</td>
<td>Yes</td>
<td>ND</td>
<td>Unknown</td>
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<tr>
<td>Hawiger et al³⁴</td>
<td>1984</td>
<td>73/F</td>
<td>SLE</td>
<td>Yes</td>
<td>ND</td>
<td>Bovine</td>
</tr>
<tr>
<td>Galanakis et al³⁵</td>
<td>1978</td>
<td>73/F</td>
<td>PVC/Procoainamide</td>
<td>Yes</td>
<td>ND</td>
<td>Unknown</td>
</tr>
<tr>
<td>Scully et al³⁶</td>
<td>1982</td>
<td>68/F</td>
<td>? SLE</td>
<td>Yes</td>
<td>ND</td>
<td>Bovine, human</td>
</tr>
<tr>
<td>Barthels and Heimberger²⁷</td>
<td>1985</td>
<td>68/F</td>
<td>Liver cirrhosis</td>
<td>Yes</td>
<td>ND</td>
<td>Bovine &lt; human</td>
</tr>
<tr>
<td>Gabriel et al³⁶</td>
<td>1987</td>
<td>66/M</td>
<td>Peptic ulcer, paraprotein</td>
<td>No</td>
<td>ND</td>
<td>? Bovine, human</td>
</tr>
<tr>
<td>Stricker et al³⁹⁰</td>
<td>1988</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flaherty et al⁰⁶</td>
<td>1989</td>
<td>91/F</td>
<td>Surgery (CABG)</td>
<td>No</td>
<td>Yes</td>
<td>Bovine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23/M</td>
<td>Seizures, surgery</td>
<td>No</td>
<td>Yes</td>
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<tr>
<td></td>
<td></td>
<td>17/M</td>
<td>Fallot tetralogy, surgery</td>
<td>No</td>
<td>Yes</td>
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<tr>
<td></td>
<td></td>
<td>68/F</td>
<td>PVD, surgery</td>
<td>No</td>
<td>Yes</td>
<td>Bovine</td>
</tr>
<tr>
<td>This report</td>
<td></td>
<td>68/M</td>
<td>Mitral stenosis, surgery</td>
<td>No</td>
<td>Yes</td>
<td>Bovine &gt; human</td>
</tr>
</tbody>
</table>

Abbreviations: lla, thrombin; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; RHD, rheumatic heart disease; IVDD, intravenous drug user; CABG, coronary artery bypass graft; PVD, peripheral vascular disease; PVC, premature ventricular contractions; ND, no data.

**MATERIALS AND METHODS**

**Materials.** Sepharose CL4B, protein A-Sepharose, Staph protein A-Protexase, 1-palmitoyl-2-oleyl phosphatidylserine (PS), 1-palmitoyl-2-oleyl phosphatidylcholine (PC), 2-mercaptoethanol, bovine serum albumin (BSA), fibrinogen, and heparin were obtained from Sigma (St Louis, MO). Chromogenic substrate S2238 and S2222 were obtained from Helena Laboratories (Beaumont, TX). Bovine thrombin-Sepharose was prepared by cyanogen bromide activation of Sepharose CL4B. The coupling ratio of thrombin to Sepharose was 1.5 mg of bovine thrombin per milliliter of Sepharose CL4B resin. Dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide (DAPA) was prepared as described. Phospholipid vesicles (PCPS) composed of 75% PC and 25% PS were prepared as described. Ninety-six-well microtiter (ELISA and Assay) plates were obtained from Costar (Cambridge, MA). All other reagents were of analytical grade.

**Proteins.** Human and bovine thrombin, human prothrombin fragment 1, human factor X, human factor Xa, bovine protein C, human antithrombin III, and rabbit lung thrombomodulin were prepared by previously reported methods. Normal human immunoglobulin G (IgG) was prepared by 50% ammonium sulfate precipitation of normal human serum followed by DEAE-cellulose chromatography in 0.02 mol/L Tris-HCl, 0.15 mol/L NaCl, pH 7.4 (TBS). Flow-through fractions containing lgs were collected and concentrated by 50% ammonium sulfate precipitation. The concentrated protein was resuspended in TBS, pH 7.4.

**Clotting assays.** Immunologic fibrinogen levels were assayed by the latex agglutination method using standard commercial fibrin degradation products (FDP) reagent latex beads coated with antifibrinogen antibody. Fibrin monomer was assayed by the method of Kisker et al. All other coagulation tests and assays were performed using commercially available clinical laboratory reagents by standard techniques.

**Isolation of the thrombin inhibitor.** Patient plasma (2.0 mL) containing the thrombin inhibitor was applied at 22°C to a 5-mL column of protein A-Sepharose equilibrated in 0.1 mol/L sodium phosphate buffer, pH 8.0. Washing continued with 0.1 mol/L sodium citrate buffer, pH 6.0. Specifically bound protein was eluted using 0.1 mol/L sodium citrate, pH 3.0. To minimize protein exposure to low pH, 2.0-mL fractions were collected into 0.6 mL of 1 mol/L Tris-HCl, pH 8.0. Antithrombin antibody isolation was
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The isolation of antithrombin activity was monitored by the inhibition of thrombin cleavage of fibrinogen in a fibrinogen clotting assay. Protein fractions that contained antithrombin immunoreactivity (by ELISA) were pooled, dialyzed against 0.02 mol/L Tris-HCl, 0.15 mol/L NaCl, pH 7.4 (TBS) and applied to a column of bovine thrombin-Sepharose that was equilibrated in the same buffer. The antibody and thrombin were allowed to incubate for 2 minutes at 22°C before the initiation of the reaction. The reaction was started by the addition of 100 µL of 400 µmol/L S2238. The rate of substrate hydrolysis by thrombin was monitored as the change in absorbance per unit time at 405 nm using a Molecular Devices Vmax spectrophotometer.

Thrombin activation of protein C was performed essentially as described by Haley et al.14 with the following modifications. Bovine or human thrombin (5 nmol/L) was premixed with various concentrations of the antithrombin antibody (5 nmol/L to 250 nmol/L), in HBS with CaCl₂ (5 mmol/L), pH 7.4 for 2 minutes at 22°C. Thrombomodulin (20 nmol/L) and PCPS (200 pmol/L) were then added to the reaction and incubated for 2 minutes at 22°C. The reaction was started by the addition of protein C (2 µmol/L) to the mixture. The final volume for each reaction was 200 µL. At designated time points 15 µL of the reaction mixture was removed and added to 65 µL of stopping solution containing antithrombin III (4 µmol/L), Heparin (6 U/mL), and DAPA (3 µmol/L). This solution was made to rapidly inactivate any thrombin in the reaction, but to allow for the activated protein C to be measured by chromogenic substrate hydrolysis. Activated protein C was assayed by the hydrolysis of substrate S2238. The change in absorbance at 405 nm/unit time was monitored by Molecular Devices Vmax spectrophotometer.

The thrombin inhibition of antithrombin III/heparin assay was designed to test the effect of the isolated antithrombin antibody on the inhibition of thrombin by antithrombin III and heparin. The reaction was performed by mixing thrombin (10 nmol/L) with various concentrations of either normal human IgG or the isolated antithrombin IgG all diluted in HBS with CaCl₂ (5 mmol/L), pH 7.4. The antibody and thrombin were allowed to incubate for 5 minutes at 22°C before the addition of antithrombin III (final concentration 20 nmol/L) and heparin (final concentration 6 U/mL). The final volume of this mixture was 100 µL. After the addition of antithrombin III/heparin, the reaction was incubated at 22°C for 3 minutes, then 200 µL of 200 µmol/L S2238 was added to the mixture and thrombin activity was evaluated by hydrolysis of the thrombin substrate S2238. The rate of S2238 hydrolysis per unit time was recorded at 405 nm by a Molecular Devices Vmax spectrophotometer. In a control experim shotgun factor Xa was substituted for thrombin, and the effect of the antithrombin antibody (100 nmol/L) on the factor Xa-AT III/heparin reaction was evaluated. All experimental conditions were identical to those described above except the chromogenic substrate S2222 was used to quantify factor Xa activity. The rate of S2222 hydrolysis was recorded at 405 nm by a Molecular Devices Vmax spectrophotometer.

RESULTS

Clotting assays. The thrombin inhibitor was initially detected by a prolongation in the bovine thrombin time (>150 seconds) in a standard coagulation profile. The patient’s coagulation test profile is listed in Table 2. These data are representative of a patient with an ongoing low-grade consumptive coagulopathy, with evidence of mild fibrinolysis. To rule out possible heparin contamination of the plasma that would artificially prolong the thrombin time, the plasma was adsorbed with triethylamino ethyl (TEAE) to remove any contaminating heparin. The bovine thrombin monitored by absorbance at 280 nm and an enzyme-linked immunosorbent assay (ELISA) for antibody binding to bovine thrombin. The isolation of antithrombin activity was monitored by the inhibition of thrombin cleavage of fibrinogen in a fibrinogen clotting assay. Protein fractions that contained antithrombin immunoreactivity (by ELISA) were pooled, dialyzed against 0.02 mol/L Tris-HCl, 0.15 mol/L NaCl, pH 7.4 (TBS) and applied to a column of bovine thrombin-Sepharose that was equilibrated in the same buffer. The flow rate was 0.5 mL/min, and 2.0 mL fractions were collected. The column was washed with the same buffer, and the antithrombin antibody was eluted with 0.1 mol/L glycine, 0.5 mol/L NaCl, pH 2.8. The low pH eluate was collected in tubes containing 0.3 mL of 1 mol/L Tris-HCl pH 8.0, to minimize the protein exposure to low pH conditions. The absorbance at 280 nm of the fractions was determined. Antithrombin immunoreactivity of the fractions was determined by ELISA. Antithrombin activity of the protein peaks separated by chromatography on the thrombin-Sepharose column was determined by the inhibition of thrombin cleavage of fibrinogen in a standard fibrinogen clotting assay (see below).

Electrophoresis. The proteins that eluted from the thrombin-Sepharose column at pH 2.8 in 0.1 mol/L glycine buffer were analyzed by electrophoresis on a 5% to 15% polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS-PAGE). Ten micrograms of the isolated protein from the thrombin-Sepharose column was brought to a final concentration of 2% (wt/vol) SDS, 0.01 mol/L Tris-HCl, pH 6.8, 10% glycerol, and 2% (vol/vol) 2-mercaptoethanol, heated for 5 minutes at 90°C, and analyzed by SDS-PAGE as described by Laemmli.23 Protein bands were visualized by staining the gels with Coomassie Brilliant Blue and destaining by diffusion.

Antithrombin ELISA. Antibody purification, antibody specificity, and relative binding affinity of the antibody to bovine and human thrombin were determined using an ELISA. Typically, 10 µg/mL of purified antigen (human or bovine prothrombin, thrombin, or prothrombin fragment 1) were coated on to 96-well microtiter plates (Costar 3639) for 1 hour at 37°C in 30 mmol/L sodium carbonate buffer, pH 9.5. Unbound protein was removed by washing the plates three times with 0.02 mol/L phosphate, 0.15 mol/L NaCl, pH 7.4 (PBS) containing 0.05% Tween 20. Plates were blocked by the addition of 1% BSA in Tris-buffered saline (TBS), pH 7.4 for 2 hours at 37°C. Unbound BSA was removed by washing. Plates were then incubated with Staph protein A-peroxidase, 2 µg/mL in 0.1% BSA in TBS for 45 minutes at 37°C. Unbound protein-A peroxidase was removed by washing. The bound protein-A peroxidase conjugate was detected by adding the substrate o-phenylenediamine at a concentration of 0.4 mg/mL in 8 mmol/L citrate phosphate buffer, pH 5.0. Microtiter plates were incubated in the dark for 5 minutes at room temperature. Substrate hydrolysis was stopped by the addition of 50 µL of 2 mol/L H₂SO₄ and the absorbance of each well at 490 nm was determined by a Molecular Devices Vmax spectrophotometer.

Thrombin assays. The effect of the antithrombin antibody on the thrombin cleavage of fibrinogen was assayed in a purified fibrinogen clotting system. Clotting assays were performed essentially as described by Lundblad et al.13 The assay was conducted by mixing 150 µL of standard fibrinogen reagent consisting of fibrinogen (5 µmol/L), calcium chloride (5 µmol/L), polyethylene glycol (4.95%), and NaCl 0.9%, all in imidazole buffer (20 mmol/L) pH 7.4, with 150 µL of antithrombin antibody (200 nmol/L) diluted in the same buffer. This mixture was incubated for 2 minutes at 22°C before 10 µL of thrombin (2 National Institutes of Health [NIH] thrombin units to 0.125 NIH thrombin units) was added to the reaction mixture. The reaction was timed by standard tilt tube clotting assay and the end point was recorded in seconds at the first visible formation of a clot in the test tube. The rate of the antithrombin antibody on thrombin hydrolysis of the artificial substrate S2238 was monitored. Typically, 10 µL of thrombin (final concentrations 2 nmol/L to 130 nmol/L) in 0.02 mol/L HEPES, 0.15 mol/L NaCl, pH 7.4 (HBS) was added to 190 µL of antithrombin antibody solution (100 nmol/L) diluted in the same buffer. The antibody and thrombin were allowed to incubate for 2 minutes at 22°C before the initiation of the reaction. The reaction was started by the addition of 100 µL of 400 µmol/L S2238. The rate of substrate hydrolysis by thrombin was monitored as the change in absorbance per unit time at 405 nm using a Molecular Devices Vmax spectrophotometer.

The effect of the antithrombin antibody on thrombin hydrolysis of the artificial substrate S2238 was monitored. Typically, 10 µL of thrombin (final concentrations 2 nmol/L to 130 nmol/L) in 0.02 mol/L HEPES, 0.15 mol/L NaCl, pH 7.4 (HBS) was added to 190 µL of antithrombin antibody solution (100 nmol/L) diluted in the same buffer. The antibody and thrombin were allowed to incubate for 2 minutes at 22°C before the initiation of the reaction. The reaction was started by the addition of 100 µL of 400 µmol/L S2238. The rate of substrate hydrolysis by thrombin was monitored as the change in absorbance per unit time at 405 nm using a Molecular Devices Vmax spectrophotometer.
time after TEAE adsorption was still found to be greater than 150 seconds. Mixing studies of the patient’s plasma showed it was capable of prolonging the thrombin time of normal plasma.

Thrombin times are routinely performed using (Pacific Hemostasis) bovine thrombin in the clotting assay. However, when human thrombin was substituted for bovine thrombin in the assay it was observed that the clotting time normalized to within 17 seconds of control. It was also observed that a 35% ammonium sulfate precipitate from this patient’s plasma (resuspended in PBS, pH 7.4), was capable of prolonging bovine thrombin times in 1:1 mixing studies (data not shown). These data suggested that this patient’s plasma contained an inhibitor of thrombin (bovine) and that the inhibitor was in the IgG fraction of plasma.

Isolation of the thrombin inhibitor by affinity chromatography. The thrombin inhibitor was isolated by affinity chromatography of the patient’s plasma on protein A-Sepharose followed by bovine thrombin-Sepharose. When the protein pools that were isolated by chromatography on the protein A-Sepharose column were evaluated for antithrombin activity it was observed that only the protein fractions that eluted from the protein A-Sepharose column at pH 3.0 in 0.1 mol/L citrate buffer were able to inhibit bovine thrombin activity in a purified fibrinogen clotting assay.

Further, only this protein peak exhibited antithrombin antibody binding to bovine thrombin by ELISA. This Ig fraction was pooled and applied to a column of bovine thrombin-Sepharose. The elution profile and immunoactivity of the second chromatographic step is shown in Fig 1. From 2 mL of starting plasma 570 μg of affinity purified antithrombin IgG was isolated. The protein product, which eluted at pH 2.8, had electrophoretic mobility similar to that of human plasma IgG when analyzed by SDS-PAGE. The nonreduced preparation of the protein migrated with an apparent mol wt of 150,000. When the protein was analyzed under reducing conditions, two bands were observed with apparent mol wts of 50,000 and 25,000, respectively (Fig 1). This two-step purification of plasma yielded a pure preparation of protein consistent with identification of IgG.

Fibrinogen clotting assay. The two peaks of protein that were separated by chromatography on bovine thrombin-Sepharose were assayed for antithrombin activity in a fibrinogen clotting assay (Fig 2). This figure illustrates that the IgG which bound to the thrombin-Sepharose column was able to significantly prolong the fibrinogen clotting time when the reaction was initiated with bovine thrombin. When less than 1 NIH unit of bovine thrombin was added to the reaction in the presence of the isolated IgG, no clot was observed even after 300 seconds (Fig 2A). This figure also

![Figure 1](https://www.bloodjournal.org/)

*Fig 1. Chromatography of the patient’s IgG on bovine thrombin-Sepharose. Fractions isolated by Staph protein A-Sepharose chromatography that exhibited antithrombin immunoactivity by ELISA were pooled, dialyzed against 0.02 mol/L Tris-HCl, 0.15 mol/L NaCl pH 7.4, and applied to a column of bovine thrombin-Sepharose (see Materials and Methods). The isolation of the antithrombin antibody was monitored by absorbance at 280 nm (O) and ELISA for antibody binding to bovine thrombin (A). The majority of the patient’s IgG is not retained on the bovine thrombin-Sepharose column, whereas the IgG greatly enriched in bovine thrombin binding eluted from the column at pH 2.8 in 0.1 mol/L glycine buffer. The material that specifically bound to the thrombin-Sepharose column was analyzed by SDS-PAGE (5% to 15% polyacrylamide gradient). Protein bands were visualized by staining with Coomassie Brilliant Blue. Lane 1, nonreduced preparation of the isolated anti-thrombin IgG; lane 2, nonreduced normal human IgG; lane 3, reduced preparation of the isolated antithrombin IgG (2% 2-mercaptoethanol); lane 4, reduced preparation of normal human IgG. The nonreduced preparation of the isolated antithrombin IgG migrated at an apparent mol wt of 150,000. The reduced preparation of the isolated material migrated as two protein bands with apparent mol wts of 50,000 and 25,000, respectively. This is identical to the electrophoresis pattern that was observed for isolated normal human IgG.*

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Fig 2. Fibrinogen clotting assay. The effect of the antithrombin antibody on the thrombin cleavage of fibrinogen was assayed in a purified fibrinogen clotting system. Assays were conducted by mixing 150 μL of standard fibrinogen reagent with 150 μL of antithrombin antibody (200 nmol/L) diluted in the same buffer. This mixture was incubated for 2 minutes at 22°C before 10 μL of various concentrations of thrombin were added to the reaction mixture. The reaction was timed by standard tilt-tube clotting assay and the end point was recorded in seconds at the first visible formation of a clot in the test tube. Panel (A) illustrates the effect of these same pools of protein when bovine thrombin was used in a fibrinogen clotting assay. This panel shows the clotting time of the following reagents: the protein that specifically bound to the thrombin-Sepharose column, (○); the protein that did not bind to the thrombin-Sepharose column, (△); bovine thrombin standard, (●). When the pool of protein that specifically bound to the thrombin-Sepharose column was mixed with bovine thrombin, no clot was observed in any test tubes that contained less than 1 U of bovine thrombin in the reaction, even after 300 seconds. Panel (B) illustrates the effect of the material isolated by affinity chromatography on the thrombin-Sepharose column when bovine thrombin was used in a fibrinogen clotting assay. This panel shows the clotting time of the following reagents: the protein that specifically bound to the thrombin-Sepharose column, (○); the protein that did not bind to the thrombin-Sepharose column, (△); bovine thrombin standard, (●).

Fig 3. Chromogenic assay for thrombin activity. The effect of the antithrombin antibody on thrombin hydrolysis of the artificial substrate S2238 was monitored. Thrombin (final concentrations 2 nmol/L to 130 nmol/L) in HBS, pH 7.4 was added to the antithrombin antibody solution (100 nmol/L) diluted in the same buffer. The antibody and thrombin were incubated for 2 minutes at 22°C before the addition of substrate. The reaction was started by the addition of 100 μL of 400 μmol/L S2238. The rate of substrate hydrolysis by various thrombin mixtures (bovine thrombin standard, [○]; human thrombin standard, [●]; bovine thrombin + IgG, [△]; human thrombin + IgG, [△]) was monitored as the change in absorbance per unit time at 405 nm using a Molecular Devices Vmax spectrophotometer.
bovine thrombin. These data also suggest that the antibody is not bound directly to the active site region of bovine thrombin.

**ELISA for antithrombin antibody binding.** An ELISA was developed to further evaluate the characteristics of the isolated antithrombin antibody. The isolated antibody was able to recognize bovine thrombin, bovine prothrombin, and human thrombin in this assay. No antibody binding was detected when human prothrombin was used as the antigen. As controls for this experiment human prothrombin fragment 1 was used as a nonspecific antigen and normal human IgG was used as a nonspecific Ig.

To further characterize the relative binding of the antithrombin antibody to bovine and human thrombin, the isolated IgG was tested over a wide concentration range in the ELISA assay (Fig 4). It was observed in this assay that the antithrombin IgG recognized bovine thrombin at approximately a 50-fold lower antibody concentration (1 nmol/L IgG) than human thrombin (50 nmol/L IgG). These data suggest that this IgG is able to recognize both bovine and human thrombin, but that there are distinct differences in relative binding affinities of the antibody to the two different species of thrombin. This difference in relative binding affinity to the two species of thrombin may explain why a pronounced inhibition of bovine thrombin activity was observed in all of functional assays described in this report, whereas human thrombin activity appears to be only slightly affected by the presence of this antibody. However, these data should be interpreted with some caution due to the solid-phase nature of the ELISA assay described here. This solid-phase assay design was chosen because of the limited amount of the antithrombin IgG that was available for study (the entire study was performed with less than 6 mL of patient plasma). It was felt that the most judicious use of this rare antithrombin antibody was a simple solid-phase ELISA to attempt to establish the relative level of binding of the IgG to both bovine and human thrombin. However, this assay design only provides an approximation of the relative binding of the antithrombin IgG to bovine and human thrombin, and the exact difference in antibody binding to bovine and human thrombin in solution is unknown.

**Thrombin activation of protein C.** The effect of the antithrombin antibody on thrombin activation of protein C was evaluated (Fig 5). When bovine thrombin (5 nmol/L) was treated with the antithrombin IgG (5 nmol/L) a decreased rate of protein C activation was observed. When 20 nmol/L IgG was added to the reaction, bovine thrombin activation of protein C was completely blocked. This is in contrast to the results that were observed when human thrombin was used as the antigen.

![Fig 4](image-url) **Antithrombin antibody binding to bovine and human thrombin by ELISA.** Purified antigen, 10 µg/mL, (human or bovine prothrombin, thrombin, or prothrombin fragment 1) was coated onto Costar 96-well microtiter plates for 1 hour at 37°C. Unbound protein was removed by washing. Plates were blocked by the addition of 1% BSA in TBS, pH 7.4 for 2 hours at 37°C. Unbound BSA was removed by washing. Purified IgG (antithrombin IgG or normal human IgG) was added to the microtiter plates such that final concentrations ranged from 10 pmol/L to 0.8 µmol/L (all dilutions were made in TBS pH 7.4) for 1 hour at 37°C. Unbound antibody was removed by washing. Staph protein A-peroxidase was then added to the plates, 2 µg/mL in 0.1% BSA in TBS, pH 7.4 for 45 minutes at 37°C. Unbound protein A-peroxidase was removed by washing. Bound protein A-peroxidase was detected by adding the substrate o-phenylenediamine at a concentration of 0.4 mg/mL in 8 mmol/L citrate phosphate buffer, pH 5.0. Substrate hydrolysis was stopped after 5 minutes by the addition of 50 µL of 2 mol/L H₂SO₄, and the absorbance of each well at 490 nm was determined by a Molecular Devices Vmax spectrophotometer. The above figure shows antibody binding to: bovine thrombin, (○); human thrombin, (●); nonspecific IgG binding to bovine thrombin, (△).

![Fig 5](image-url) **The antithrombin antibody effect on the thrombin activation of protein C.** Thrombin activation of protein C was performed by mixing thrombin (5 nmol/L) with the antithrombin antibody (5 nmol/L to 250 nmol/L). The reaction was treated with the antithrombin IgG (5 nmol/L) and DAPA (200 µmol/L) were then added to the reaction and incubated for 2 minutes at 22°C. The reaction was started by the addition of protein C (2 µmol/L) to the mixture. The final volume for each reaction was 200 µL. At designated time points 15 µL of the reaction mixture was removed and added to 85 µL of stopping solution containing antithrombin III (4 µmol/L), heparin (6 U/mL), and DAPA (3 µmol/L). This solution was made to rapidly inactivate any thrombin in the reaction but to allow for the activated protein C to be measured by chromogenic substrate hydrolysis. Human and bovine thrombin activation of protein C was assayed by the hydrolysis of substrate S2238 at each antithrombin IgG concentration. The change in absorbance at 405 nm/unit time was monitored by a Molecular Devices Vmax spectrophotometer. Data are plotted as a percent of bovine thrombin (○) or human thrombin (●) activation of protein C as a function of increasing antithrombin IgG concentrations. The percent of protein C activation at each antibody concentration was calculated by comparing the rate of protein C activation by bovine or human thrombin in the presence of the isolated antithrombin IgG to the rate of protein C activation by bovine or human thrombin in the presence of normal human IgG.
thrombin (5 nmol/L) was treated with the antithrombin IgG (5 nmol/L to 250 nmol/L). As Fig 5 illustrates, no decrease in the rate of human thrombin activation of protein C was observed over the entire concentration range of antithrombin IgG tested. These data suggest that the antithrombin IgG binds to bovine thrombin in solution in such a way that either the thrombin-thrombomodulin reaction or the thrombin-protein C reaction is inhibited. It is interesting to note that the antithrombin IgG did not affect the human thrombin activation of protein C even at a 50-fold excess of IgG to thrombin. This would suggest that either the IgG does not bind to human thrombin in solution at the concentrations tested, or that the antithrombin IgG when complexed with human thrombin does not inhibit the reaction with either thrombomodulin or protein C in vitro.

**Human thrombin inhibition by antithrombin III/heparin.** To test if this antithrombin antibody was able to block the reaction (inhibition) of antithrombin III (AT III) and heparin with thrombin, various concentrations of IgG were incubated with thrombin before the addition of AT III/heparin. After the addition of the AT III/heparin to the reaction, human thrombin function was evaluated by hydrolysis of the S2238 substrate. These data (Fig 6) illustrate that human thrombin is protected from AT III/heparin inhibition as a function of increasing antibody concentration. It was observed that 50% of human thrombin function was retained at an antibody concentration of 50 nmol/L. This represents a fivefold excess of antibody to antigen. Control experiments with normal human IgG added to the reaction mixture showed no protection of thrombin from AT III/heparin inhibition. We were unable to perform similar experiments using bovine thrombin for the following reason. The methods used to quantitate thrombin inhibition by AT III/heparin are dependent on the rate of thrombin hydrolysis of substrate S2238. However, as Fig 3 illustrates, the antithrombin IgG partially inhibits S2238 hydrolysis by bovine thrombin. Thus, the evaluation bovine thrombin inhibition by AT III/heparin is complicated by two simultaneous inhibitory processes of bovine thrombin hydrolysis of S2238. Due to the limited amount of antithrombin IgG at our disposal we have been unable to deconvolute these two inhibitory reactions. We do speculate however, that the antithrombin IgG blocks bovine thrombin inhibition by AT III/heparin.

To be certain that effect of the isolated IgG was specific for the thrombin-AT III/heparin reaction, a control experiment was performed in which the antithrombin IgG was added to human factor Xa before the addition of AT III/heparin. In this experiment the inhibitory IgG was able to block AT III/heparin inhibition of thrombin but had no effect on the AT III/heparin inhibition of human factor Xa (data not shown). These data indicate that this antibody inhibits the thrombin/AT III reaction specifically by interacting with thrombin, thus neutralizing the anticoagulant effect of AT III/heparin. This experiment also illustrates that an inhibitor of AT III or heparin was not coincidentally purified by our antibody isolation procedure.

**DISCUSSION**

This report describes an antithrombin antibody isolated from a 68-year-old man with a synthetic mitral valve and evidence of a low-grade chronic consumptive coagulopathy with mild fibrinolysis (Table 2). This patient showed only mild recurrent epistaxis in the postoperative period and had a negative bleeding history. The high titer thrombin inhibitor was shown to bind both bovine and human thrombin. When the functional significance of this inhibitor was evaluated by in vitro assays the antibody was observed to inhibit all functions of bovine thrombin. Similar studies conducted using human thrombin led to the observation that this antibody only slightly inhibited fibrinogen clotting by human thrombin while markedly inhibiting the reaction between human thrombin and AT III/heparin.

It is impossible to test whether this antibody has an effect in this patient in vivo; however, our in vitro studies suggest that this antibody might have played a role in the development of this patient's consumptive coagulopathy. We have calculated, from our purification procedure, that the antithrombin IgG concentration in plasma is at least 1.2 μmol/L. This concentration of inhibitor would, most likely, be in vast excess to the amount of thrombin generated during any hemostatic event. Thus, thrombin produced via prothrombinase would likely be bound by the antithrombin antibody in vivo. The antibody-human thrombin complex displays a decreased rate of thrombin inhibition by AT III/heparin. This decrease in the rate of thrombin inhibition by AT III/heparin may allow the prolongation of functional proco-
agulant thrombin to continue to circulate in the blood. It is conceivable that small amounts of thrombin were generated in association with this patient's synthetic heart valve, and that this thrombin was not effectively neutralized by AT III/heparin leading to an augmentation of the coagulopathy ordinarily present in the postoperative period. This hypothesis is consistent with this patient's coagulation profile listed in Table 2.

This study lends credence to a growing body of literature, which suggests a link between patient exposure to bovine topical thrombin and the development of antithrombin antibodies (Table 1). Recent reports have described antithrombin antibodies derived from patient plasma. Flaherty et al have suggested that antithrombin antibodies are the result of iatrogenic immunization of these patients with a hemostatic agent, such as bovine topical thrombin, used during surgery. The patient described here had undergone three prior mitral valve replacement surgeries and had been exposed to bovine thrombin during two of the three procedures. He subsequently developed a strong antibody response to bovine thrombin in all assays tested. Because bovine and human thrombin share 85% amino acid sequence homology, it is likely that the antithrombin antibody characterized in this report was originally directed against bovine thrombin and that this IgG immunologically cross-reacts with a lesser affinity to human thrombin.

Of the eight patients with antithrombin antibodies described in recent reports (and this report), all developed antibodies that recognize bovine thrombin. Of these eight patients four (50%) also developed antibodies that recognized human thrombin. The functional effect of these antibodies on human thrombin is uncertain, but at least one of the three patients described by Stricker and Corash appeared to have an antibody that significantly prolonged the human thrombin time (>120 seconds). One of the eight patients described in these reports developed clinically significant bleeding. The possibility of low-grade thrombosis in all of these patients, except the patient described here, is unknown.

Because thrombin is an extremely active agonist molecule (procoagulant, anticoagulant, growth factor, mitogen, vasoconstrictor, and chemo-attractant) the ultimate effect of these antithrombin antibodies in vivo is difficult to evaluate. This report and the reports cited provide evidence that warrants an in-depth review of the use of bovine topical thrombin as a therapeutic agent. Further, we propose that the immunologic potential of bovine topical thrombin should be evaluated and the general incidence of subclinical antibody formation in those patients who have been exposed to bovine topical thrombin be reviewed.

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