Antithrombin III Deficiency: Decreased Synthesis of a Biochemically Normal Molecule

By Daniel R. Ambruso, Bruce D. Leonard, Roger D. Bies, Linda Jacobson, Wm. E. Hathaway, and E. Basil Reeve

A 29-yr-old white female has suffered from recurrent venous thromboses over the last 12 yr. Plasma antithrombin III (AT-III) levels were 48% of normal by immunoelectrophoresis and 96% by chromogenic assay. Three of four siblings and the father had similar AT-III levels without associated venous thromboses. Heparin-Sepharose chromatography demonstrated normal behavior of the patient’s AT-III. Her purified AT-III could not be distinguished from AT-III purified from a normal control either by SDS polyacrylamide gel electrophoresis or by crossed immunoelectrophoresis, and the heparin cofactor activity and the progressive antithrombin activity of both AT-III samples were identical. Turnover studies were made in the patient using her own purified AT-III labeled with 131I (*I). The results did not differ significantly from studies made with autologous *I-AT-III in two normal control women. Her fractional breakdown rate of 0.54 total plasma AT-III per day compared with 0.45 and 0.52 in the controls. These studies indicate that the patient synthesizes a normal AT-III molecule at half normal rates.

Antithrombin III-HEPARIN cofactor (AT-III) is a major inhibitor of procoagulant serine proteases. Diminished activity of this plasma protein can occur as an inherited disorder and often is associated with decreased amounts of apparently normal protein.1-3 Individuals affected with this hereditary deficiency are predisposed to thromboembolic disease.1-3 We report a patient with hereditary AT-III deficiency and recurrent venous thrombosis. Kinetic studies of thrombin inactivation by purified AT-III and protein turnover studies with autologous AT-III indicate that this patient has a normally functioning AT-III molecule synthesized at a rate of only 50% of normal.

MATERIALS AND METHODS

Coagulation Studies and Antithrombin III Assays

Plasma was separated by centrifugation at 1400 g for 30 min from blood drawn by the two-syringe technique. Nine volumes of blood were mixed with one volume of citrate anticoagulant (3 vol 0.1 mole/liter trisodium citrate to 2 vol 0.1 mole/liter citric acid). Prothrombin time, partial thromboplastin time, thrombin time, fibrinogen, fibrinogen-fibrin split products, fibrin monomer, and euglobulin lysis time were measured by standard techniques.3 Progressive antithrombin activity was determined in defibrinated plasma* and expressed as the ratio between the subject’s value and that of normal control plasma or of a standard pool of plasma tested simultaneously. AT-III-heparin cofactor activity was measured with a chromogenic substrate1 and AT-III antigen level was measured by rocket immunoelectrophoresis1 using a rabbit anti-human AT-III antibody purchased from Behring Diagnostics, Somerville, N.J., or prepared by us. Results were expressed in terms of percent of the value of a standard plasma pool. Crossed immunoelectrophoresis was performed on plasma as described previously.3

Antithrombin III Preparation

Antithrombin III was purified by a modification of methods described previously.4,5 Fresh citrate plasma (40 ml) was heat defibrinated, diluted 1/1 with Tris-citrate-NaCl buffer (0.01 mole/liter, 0.01 mole/liter, 0.145 mole/liter, pH 7.4; buffer 1) and applied at 0.5-1.0 ml/min to a 1 x 8 cm heparin-agarose column.7 After washes with the above buffer and a similar buffer (0.3 mole/liter in NaCl from which citrate was omitted), the AT-III was eluted by a linear salt gradient formed from two identical beakers containing 0.01 mole/liter, pH 7.4, Tris buffers with 0.15 and 1.3 mole/liter NaCl, respectively. The fractions containing AT-III as determined by rocket immunoelectrophoresis and heparin cofactor activity were separated, diluted to -0.3 M salt by adding buffer 1 from which the citrate was omitted and concentrated on a short, heparin-agarose column. SDS-PAGE showed homogeneous AT-III preparations (Fig. 1). Specific activity (activity of purified AT-III with A0.80 of 1.0 expressed as percent of activity of 1 ml of a standard citrated plasma pool) was 1200-1300%. For turnover studies, the heparin-agarose, sterilized during its preparation and not previously used, was washed with 100 vol of sterile, pyrogen-free isotonic saline before the application of the plasma. The buffers were made in sterile, pyrogen-free water, and these and all glassware were autoclaved.

131I-Antithrombin III Preparation and Turnover Studies

Labeling with radioactive iodine (*I) was performed with iodine monochloride.4 Radioactive iodine unbound to AT-III was removed by washing the radiolabeled AT-III (*I-AT-III) on a small heparin-agarose column with buffer 1.1 SDS-PAGE showed homogeneous *I-AT-III preparations that ran identically with unlabeled AT-III (Fig. 1). Specific activity of AT-III preparations was unchanged by the iodination. Immediately before i.v. injection, the *I-AT-III preparations were passed through Millipore filters previously treated with a few milliliters of sterile 1 mg/ml human albumin solution (Armour Pharmaceutical) to prevent surface denaturation. About 0.2 mg *I-AT-III, pyrogen-free as shown by the Limulus test,4 containing 3-10 μCi 131I (New England Nuclear) was injected per study. For radioactive counting, plasma samples were handled as described previously1 and whole body counts were made in the

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ANTITHROMBIN III DEFICIENCY

Colorado State Health Department whole body counter and/or in a linear scanner. Careful tests of the linear scanner showed good agreement between the whole body counts and the difference between the total radioactivity injected and that excreted in the urine. Calculation of protein turnover from radioactivity data was performed by standard methods.7

Inactivation of Thrombin by AT-III

AT-III purified from the patient and a normal control were diluted with 0.01 mole/liter Tris buffer, 0.145 M NaCl, pH 7.5 to OD_{280} 0.03. Human thrombin (Lot H-1, Bureau of Biologics, Food and Drug Administration, Bethesda, Md.) was diluted to 45 U/ml in the same buffer. Human fibrinogen, obtained from KABI (Stockholm) was further purified by ammonium sulfate precipitation. One volume of saturated ammonium sulfate solution was mixed with 3 vol of fibrinogen (17 mg/ml in distilled water) and the precipitate was separated by centrifugation at 500 g for 5 min. The precipitate was suspended in 0.05 mole/liter Tris, 0.15 mole/liter NaCl, pH 7.4, and dialyzed against this buffer overnight at 4°C. The dialyzed fibrinogen was then diluted to 2.5 mg/ml with this buffer and stored at −70°C. Equal volumes of AT-III and thrombin solutions were mixed in plastic tubes at 37°C, and 0.1 ml samples were removed sequentially from the reaction mixture and added to 0.4 ml of the human fibrinogen at 37°C. The time to clotting was noted, and the percent thrombin activity was calculated by comparison with a standard curve obtained with serial dilutions of the thrombin solution. The results were expressed as the percent of initial thrombin activity remaining after incubation.

Informed consent for these studies was obtained from the patient and control subjects according to the requirements of the Human Research Committee of the University of Colorado Health Sciences Center.

CASE REPORT

S.H. is a 29-yr-old white female with a history of recurrent thromboses. The first episode occurred at 20 yr of age as an ileofemoral thrombosis with pulmonary embolus. She was on birth control pills at the time. Over the subsequent 5 yr she had several episodes of superficial venous thrombosis involving both legs and was treated with bed rest and short-term oral anticoagulant therapy.

We first evaluated her when she was 25 yr old. At that time she was not on anticoagulant medications and had no clinical evidence of thrombosis, and her prothrombin, partial thromboplastin and thrombin times were all within normal limits. Other studies then included: fibrinogen 240 mg/dl (normal range, 200-450 mg/dl), fibrinogen/fibrin split products <10 µg/ml, negative fibrin monomer, normal thromboelastogram, and egulobulin lysis time 135 min (normal range, 90-300 min). Progressive clotting AT-III activity was 0.17 (normal range, 0.66-1.38) and AT-III antigen by immunoelectrophoresis was 54% (normal range, 83%-117%).

During the next 4 yr, S.H. noted progressive swelling and pain in her right leg after periods of standing. At 27 yr of age she developed acute swelling of the right leg and tenderness over the right femoral vein. At this time she was 6-8 wk pregnant. A venogram documented thrombosis involving right femoral, internal, and external iliac veins. Thrombectomy and therapeutic abortion were performed, and she was started on coumadin. Subsequently, she has not developed any severe thrombotic episodes.

Although no other family members have had thromboembolic episodes, the patient’s father and three siblings demonstrated low levels of AT-III (Fig. 2). Plasma levels of cholesterol and triglycerides were normal in the propositus and D.H.Jr.

RESULTS

Coagulation and Immunochemical Studies

Measurements of plasma AT-III levels by progressive thrombin inhibition, interaction with chro-

![Fig. 1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels of S.H.’s purified AT-III (left) and AT-III purified from a normal subject (right). Both preparations are homogeneous. S.H.’s purified AT-III labeled with radioactive iodine ran exactly with normal AT-III on SDS PAGE.](image)

![Fig. 2. Plasma antithrombin III studies in the family of S.H. Progressive clotting AT-III activity (ratio) = AT-III act., heparin cofactor activity (percent) = AT-III chrom., AT-III by immunoelectrophoresis (percent) = AT-III Ag. S.H., her father, and 3 siblings show ~50% normal levels of plasma AT-III.](image)
Table 1. Plasma Antithrombin III Assays* in S.H. Obtained Over 4 yr

<table>
<thead>
<tr>
<th>Assay</th>
<th>Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progressive serum activity (0.66–1.38)†</td>
<td>0.26 ± 0.09 (6)‡</td>
</tr>
<tr>
<td>Chromogenic (84%–118%)†</td>
<td>56% ± 4% (7)‡</td>
</tr>
<tr>
<td>Antigen (83%–117%)†</td>
<td>48% ± 9% (7)‡</td>
</tr>
</tbody>
</table>

*Assays were performed as described in Materials and Methods.
†Normal range.
‡Numbers represent mean ± SD of assays performed during the 4-yr observation period. The number of determinations appears in parentheses.

Antithrombin III, an important inhibitor of coagulation proteases,12 plays a central role in hemostasis as

Fig. 3. Crossed immunoelectrophoresis of normal AT-III (A) and S.H.’s AT-III (B).

Fig. 4. Progressive antithrombin activity of AT-III purified from S.H. (broken line) and a normal subject (solid line) against human thrombin (see text for experimental details).
evidenced by the clinical association of thromboembolic disease with decreased levels of this plasma protein. Several varieties of inherited abnormality of AT-III have been described. Our patient demonstrates the commonest form, which is inherited as an autosomal dominant. In our patient's plasma, AT-III levels were 40%–50% normal and the AT-III exhibited no gross chemical abnormality. While some subjects with this abnormality, such as S.H., suffer recurrent severe attacks of venous thrombosis, others, such as the patient's father and siblings, are free from clinical disturbances of coagulation. In this article we provide additional evidence that the AT-III of patients with this deficiency is chemically normal and define the abnormality responsible for the low plasma AT-III level.

Biochemical properties of our patient's protein were characterized by normal crossed immunoelectrophoresis of S.H.'s plasma AT-III and normal heparin-agarose chromatography during purification of AT-III from S.H.'s plasma. As shown by SDS-PAGE (Fig. 3), the purified AT-III was homogeneous and ran with the same Rf as AT-III purified from a normal control. Specific activity, expressed as the percent heparin cofactor activity of a purified sample divided by the absorbance at 280 nm of 1 cm depth of the solution, was 1200% and ran at the high end of our normal AT-III range. Progressive antithrombin activity (Fig. 4) was indistinguishable from normal. Thus, any major chemical change in the AT-III molecule is unlikely.

In a steady state, synthetic rate, $S$, equals breakdown rate, $B$. Defining $V$ as plasma volume, $C$ as plasma AT-III concentration, and $k_b$ as the fraction of plasma AT-III broken down per day in a steady state:

$$S \text{ (mg/day)} = B \text{ (mg/day)} = k_b V C \quad (1)$$

For instance, with normal plasma AT-III level of ~0.13 mg/ml, S.H.'s measured plasma volume of 2400 ml and the average normal $k_b$ based on our human studies of about 0.5/day, $S = B = 156$ mg/day. Equation 1 can be rearranged to show the relation

$$S = \frac{B}{k_b V} \quad (2)$$

Table 2. Parameters From Turnover Studies Using Autologous $^{112}$I-AT-III in Two Healthy Control Women and Patient SH.

<table>
<thead>
<tr>
<th>Parameters Used in the Text and Fig. 6 Are Also Shown</th>
<th>Controls</th>
<th>Patient</th>
<th>Theoretical Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>R.Ho, M.S.</td>
<td>S.H.</td>
<td>S.H.</td>
<td></td>
</tr>
<tr>
<td>Plasma Ag AT-III antigen</td>
<td>99%</td>
<td>129%</td>
<td>50%</td>
</tr>
<tr>
<td>Control</td>
<td>S.H.</td>
<td>S.H.</td>
<td></td>
</tr>
<tr>
<td>$^{10}$I-AT-III parameters*</td>
<td>$C_1$</td>
<td>0.52</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>$r_1$</td>
<td>0.271</td>
<td>0.234</td>
</tr>
<tr>
<td></td>
<td>$C_2$</td>
<td>0.48</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>$r_2$</td>
<td>1.73</td>
<td>2.07</td>
</tr>
<tr>
<td>Fractional breakdown rate, day $^{-1}$</td>
<td>$k_b$</td>
<td>0.45</td>
<td>0.52</td>
</tr>
<tr>
<td>AT-III distribution: interstitial/plasma</td>
<td>0.65</td>
<td>1.46</td>
<td>1.13</td>
</tr>
</tbody>
</table>

$^{112}$I-AT-III plasma radioactivity is described by a two-exponential curve $C_1 e^{-r_1 t} + C_2 e^{-r_2 t}$. Fractional breakdown rate, $k_b$, was calculated from $[C_1/r_1 + C_2/r_2]^{-1}$. AT-III distribution was calculated allowing for the delay in $^{112}$I-AT-III catabolism.7,8
between $C$, $S$, and $k_B$, thus:

$$C = \frac{S}{k_B V} \quad (2)$$

This equation shows that if changes in $V$ are excluded, concentration ($C$) of plasma AT-III could be halved by halving $S$, doubling $k_B$, or changes in both $S$ and $k_B$. Turnover studies with *I-AT-III that reflect the behavior of S.H.'s native AT-III allow measurement of $k_B$. However, the *I-AT-III must be prepared from S.H.'s plasma, and the purification and iodination of plasma and whole body radioactivity curves, labeled $T$, fall much more steeply than those determined in the turnover studies.

In summary, we have described a patient with AT-III deficiency, severe thromboembolic complications, and an AT-III molecule that is biochemically normal but is synthesized at half the normal rate. This results in half the normal plasma AT-III level. Evaluation and classification of other patients by these methods will aid in understanding the pathophysiology of their diseases and their clinical management.

REFERENCES

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