Antithrombin "Chicago": A Functionally Abnormal Molecule With Increased Heparin Affinity Causing Familial Thrombophilia

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A family with a high incidence of spontaneous thromboembolism over four generations has been investigated. The propositus is a 21-yr-old male with a history of thrombophlebitis. Medical histories of 46 family members were obtained. Twelve of these individuals have experienced deep venous thromboses and/or pulmonary emboli. Seven members of the kindred, with a prior history of thrombotic phenomena, were investigated in detail. These subjects were found to have normal plasma concentrations of immunoreactive antithrombin (mean 96%), decreased plasma levels of progressive antithrombin activity (mean 50%), and greatly reduced amounts of plasma heparin cofactor activity (mean 42%). The abnormal antithrombin ("Chicago") was found to elute from heparin-Sepharose at a higher ionic strength than normal inhibitor. The functionally defective antithrombin molecules exhibit a reduced ability to neutralize thrombin in the presence or absence of heparin (approximately 10%-20% of normal). The molecular defect of this protease inhibitor thus appears to be distinct from those of previously described abnormal antithrombins.

The coagulation cascade is composed of a series of linked proteolytic reactions that ultimately lead to the generation of thrombin. At each stage of this mechanism, a parent zymogen is converted to a serine protease, which catalyzes a subsequent zymogen-serine protease transition. Once evolved, thrombin releases two pairs of fibrinopeptides from fibrinogen, which permits the resulting fibrin to polymerize into an insoluble clot. Alternatively, this serine protease may be inhibited by its natural antagonist, antithrombin, via the formation of a stable enzyme-inhibitor complex. Although other plasma proteins are able to inactivate thrombin, antithrombin is the inhibitor of primary physiologic importance. Other serine proteases of the coagulation system that can be neutralized by this inhibitor include factors IXa, Xa, Xla, and XIIa. Each of these enzyme-inhibitor interactions is dramatically accelerated by heparin.

At the present time, antithrombin is the only inhibitor of the coagulation system for which a congenital deficiency is highly correlated with thrombotic disease. The first such kindred was described in 1965 by Egeberg.

Norwegian family possessed inhibitor levels that averaged approximately 50% of normal by both immunologic and functional methods. Over the next 17 yr, a number of similar families with this "classical" type of antithrombin deficiency has been reported in the medical literature. However, recently, several families have been described in which affected individuals exhibit normal antithrombin levels on immunologic examination, yet possess lowered functional levels of the inhibitor. This rarer deficiency state is produced by a discrete molecular defect within the protease inhibitor and results in the presence of both normal and abnormal antithrombin molecules within the blood. This article describes another such family with a high incidence of spontaneous thromboembolism and an abnormal antithrombin distinct from those previously characterized. We have separated the mutant antithrombin moiety from its normal counterpart. Heparin binds to this molecule (antithrombin "Chicago") with increased avidity, but is unable to activate the protease inhibitor to an appreciable extent. Furthermore, antithrombin "Chicago" possesses minimal ability to function as a progressive inhibitor of thrombin.

Materials and Methods

Column Chromatographic Materials

Sepharose 4B was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Heparin was bound to cyanogen-bromide-activated Sepharose 4B by the method of Porath et al. The attachment process was conducted at 4°C in 0.1 M sodium bicarbonate, pH 8.5. Approximately 2.7 mg of heparin was added per milliliter of "packed" Sepharose 4B.

Chemicals

All chemicals were reagent grade or better.

Heparin

Heparin of porcine origin utilized in preparing the affinity matrix was obtained in crude form from Diosynth, Inc. (Chicago, IL).
was further purified by cetylpyridinium chloride precipitation. The specific activity of the preparation was 121 USP U/mg. Heparin (Panhepin), purchased from Abbott Laboratories (North Chicago, IL), was utilized in the functional assays for antithrombin activity.

Proteins

Bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO). Nonimmune rabbit IgG, nonimmune goat IgG, and donkey anti-goat IgG were obtained from Miles Laboratories (Elkhart, IN). Hirudin was bought from Pentapharm (Basel, Switzerland). Human thrombin, human thrombin–antithrombin complex, human antithrombin, and human factor X, were purified by techniques established in our laboratory.

Measurement of Protein Concentration

Protein concentrations were determined by absorbance readings at 280 nm. The molar extinction coefficients for human thrombin, human thrombin–antithrombin complex, human antithrombin, and human factor X, were assumed to be 16.2, 10.0, 6.5, and 11.6, respectively.

Collection of Blood Samples

Venipunctures were performedatraumatically using a two-syringe technique. The anticoagulant employed was sodium citrate, 38 mg/ml. The ratio of anticoagulant to blood employed was 0.1:0.9 (v/v). After collection of blood samples, platelet-poor plasma (PPP) was obtained by centrifugation at 4°C for 15 min at 1,600 g. When possible, tests were performed on fresh plasma samples; otherwise, aliquots of plasma were stored at –80°C prior to use. Platelet-rich plasma (PRP) was prepared from fresh citrated blood by centrifugation at room temperature for 10 min at 250 g. Specimen collection and informed consent procedures were approved by the institution's Human Experimentation Committee.

Normal ranges for antithrombin and plasminogen in both the immunologic and functional assays were derived from data on 22 control subjects; the mean value was taken as 100%. This population consisted of healthy laboratory and medical personnel who gave a negative history for thrombosis or bleeding, and who were not taking any medications, including oral contraceptives. A normal plasma pool was then constructed by pooling equal volumes of plasma from these control subjects.

Coagulation Studies

Prothrombin time, activated partial thromboplastin time, and thrombin time were determined by standard coagulation techniques using a fibrometer (Baltimore Biological Laboratory, Baltimore, MD) and commercially available reagents. Coagulation factor analyses were carried out by one-stage assay on substrate plasma deficient in the respective coagulation factor. Euglobulin lysis time was performed using PPP as described.

Bleeding time, platelet count, platelet factor 3, and platelet adhesiveness to glass beads were measured as previously described. Aggregation of platelets by adenosine diphosphate (ADP) (Sigma), epinephrine (Parke-Davis, Detroit, MI), bovine tendon collagen (Sigma), and ristocetin (Lenau Kongevi DK 1850, Copenhagen, Denmark) was assessed with an aggregometer (Chronolog, Havertown, PA) as described.

Immunoreactive plasminogen was measured by radial immunodiffusion (RID) employing commercially available antiserum and agar plates (M-Partigen, Calbiochem-Behring Corp., LaJolla, CA). A functional assay of plasminogen was performed fluorimetrically using a commercial kit (Dade Division, American Hospital Supply Corp., Miami, FL). Results of both assays were converted to percent of normal based on values obtained with the normal plasma pool.

α-1-Antitrypsin and α-2-macroglobulin were measured by radial immunodiffusion and rocket immunoelectrophoresis, respectively, using the appropriate antisera.

Preparation of Antisera

Antithrombin antiserum. Crude serum raised in goats immunized with human antithrombin was purchased from Atlantic Antibodies (Scarborou, ME) and was incubated at 56°C for 30 min to remove residual fibrinogen as well as destroy complement components. The IgG fraction was then obtained by ammonium sulfate precipitation and was demonstrated to be monospecific for species containing antithrombin, as outlined in a prior communication.

Thrombin–antithrombin complex antiserum. This antiserum was raised in rabbits and processed according to techniques outlined earlier.

Sheep anti-rabbit IgG serum was obtained as previously described.

Antithrombin Assays

Immunologic. Plasma antithrombin levels were measured on 19 members of the kindred by radial immunodiffusion, utilizing a goat antiserum (Calbiochem-Behring Corp.) and by a radioimmunoassay described below. Inhibitor levels of fractions from the heparin-Sepharose column were determined only by radioimmunoassay. This assay is able to accurately detect antithrombin down to a level of 0.05 nM. Radiolabeling of human antithrombin was carried out by the chloramine-T method of Greenwood et al. The initial reaction mixtures were composed of 50 μl of radiolabeled antithrombin (approximately 5,000 cpm), 50 μl of various concentrations of unlabeled antithrombin standard or unknown sample, as well as 100 μl of the specific antibody population. The tubes were mixed and then incubated at 4°C for 18 hr. All of the reagents had been extensively diluted in 0.155 M NaCl and 0.005 M EDTA in 0.0255 M sodium phosphate, pH 7.4, with 0.6% (w/v) sodium azide and 3.0% (w/v) BSA added. The antibody concentration selected had previously been shown to precipitate 33%-50% of 125I-labeled antithrombin when used in the absence of competing antigen. The second antibody system was then added to each tube. This consisted of 100 μl donkey anti-goat serum and 50 μl nonimmune goat IgG.

The relative amounts of goat IgG as well as anti-goat IgG sera were chosen to give the maximal precipitation of radiolabeled antigen. Incubation was carried out for 18 hr for an additional 18 hr. The tubes were centrifuged at 4°C for 10 min at 6,450 g and washed 3 times at 4°C with 0.2 ml of 0.155 M NaCl, 0.005 EDTA in 0.025 M sodium phosphate, pH 7.4, with 0.2% (w/v) BSA added. The resultant precipitates were quantitated for 125I counts.

Crossed immunoelectrophoresis of antithrombin. Two-dimensional crossed immunoelectrophoresis was performed on the plasma of the propositus and his father. The first dimension was run in 0.9% agarose (FMC Corporation, Rockland, ME) at 400 V for 4 hr at room temperature. The gel was then cut in two parts, and the part containing the separated proteins was retained. A solution of 0.9% agarose containing 12.5 μl of antithrombin antiserum (Calbiochem-Behring Corp.) was poured into the site of the discarded gel. Electrophoresis in the second dimension was resumed at 100 V for 16 hr at room temperature. A similar experiment was carried out with heparin at a concentration of 17 U/ml incorporated into the gel of the first dimension.

Heparin cofactor assay for antithrombin. For plasma assays, an amidolytic assay utilizing the chromogenic substrate, Tos-Gly-Pro-Arg-pNA (Chromozym TH, Boehringer Mannheim, Indianapolis, IN) was employed, with one modification. The heparin concentration in the diluent buffer was reduced from 3 U/ml to 0.5 U/ml.
In order to assay fractions from the heparin-Sepharose column, a standard curve was prepared by adding 25, 50, 75, 100, 125, and 150 µl of human antithrombin at a concentration of 2,500 nM to a series of tubes. These standards were added to a buffer solution (0.01 M Tris, 0.15 M NaCl, pH 8.3) to obtain a final volume in each tube of 3 ml. Test samples were diluted appropriately with buffer, as judged by their antithrombin immunoreactivity. Exactly 400 µl of standard or sample was then admixed with 20 µl of heparin (10 U/ml) and incubated for 90 sec at 37°C. A volume of 100 µl of human thrombin (15 NIH U/ml of 0.15 M NaCl) was added, and a stopwatch was started. After 30 sec, 300 µl of substrate-polybrene solution (0.75 mM Tos-Gly-Pro-Arg-pNA, 0.3 mg/ml polybrene; Abbott Laboratories) was added. Exactly 60 sec later, 300 µl of glacial acetic acid was added and the contents thoroughly mixed. Absorbances were read at 405 nm against a blank containing 400 µl of buffer, 300 µl glacial acetic acid, and 400 µl distilled water.

The levels of antithrombin in test samples were derived from the linear relationship between concentration and absorbance.

Progressive antithrombin assay. For plasma measurements, progressive antithrombin activity was assessed in a two-stage clotting assay using a commercial kit (Ortho Diagnostics, Inc., Raritan, NJ) as well as by amidolytic assay. The latter method was that described by Abildgaard et al., with the following modifications.

The chromogenic substrate employed was Tos-Gly-Pro-Arg-pNA (Boehringer Mannheim) rather than Benzoyl-Phe-Val-Arg-p-nitroaniline (Ortho Diagnostics S-2160) at a concentration of 0.75 mM. This change necessitated shortening the reaction time after substrate addition to 30 sec prior to quenching with glacial acetic acid.

In order to assay fractions from the heparin-Sepharose column, a standard curve was prepared with human antithrombin at a concentration of 2,500 nM, as described above. These standards were added to a buffer solution [0.01 M Tris, 0.15 M NaCl, pH 8.3, which contained 0.1% (w/v) of BSA] to attain a final volume in each tube of 2 ml. Test samples were diluted appropriately with buffer. Exactly 500 µl of standard or sample was then admixed with 20 µl of polybrene (1.25 mg/ml) and prewarmed for 2 min at 37°C. A volume of 100 µl of human thrombin (15 NIH U/ml in 0.15 M NaCl) was added to each tube. After 480 sec, 500 µl of substrate solution (0.75 mM Chromozym TH) was added. Exactly 30 sec later, 300 µl of glacial acetic acid was added, and the contents were thoroughly mixed. Results were obtained in a manner entirely analogous to that described for the heparin cofactor assay.

For quantitating progressive antithrombin activity below 30 nM in column fractions, a standard curve was prepared by making serial dilutions of human antithrombin starting at 100 nM in buffer. A volume of 20 µl of polybrene (0.30 mg/ml) was added to 100 µl of standard or sample. The samples were prewarmed at 37°C for 2 min, and 20 µl of thrombin, at a concentration of 1,000 nM, was added. After incubation for 8 min at 37°C, 60 µl of hirudin (200 U/ml) was added to prevent further thrombin–antithrombin complex formation. A double-antibody type radioimmunoassay was then performed for thrombin–antithrombin complex, as previously described.

Antifactor X assay. Plasma antifactor X activity in the presence of heparin was determined utilizing the chromogenic substrate Bz-Ile-Glu-Gly-Arg-pNA (Ortho Diagnostics S-2222). The method was that described by Odegard et al. except that activated human factor X rather than bovine enzyme was utilized.

Analysis of Data

Estimation of relative immunoreactivity, computation of the slope of the respective logit-log dose-response curves, as well as determination of the various associated statistical indices were obtained by fitting the raw data to a “four parameter” model as described by Rodbard et al. Statistical analyses of data were conducted by standard techniques.

Affinity Chromatography

For purposes of comparison, antithrombin from normal plasma was chromatographed with the same procedure as the antithrombin of the propositus. Approximately 50 ml of plasma was thawed at room temperature and applied to a heparin-Sepharose 4B column (1.4 x 35 cm) at a flow rate of 10 ml/hr, which had been preequilibrated with 0.01 M Tris-HCl, 0.01 M sodium citrate, 0.02% (w/v) sodium azide, pH 8.3, containing 0.145 M sodium chloride. The column was washed with 1 column volume of the 0.145 M salt buffer, and then 2 column volumes of buffer containing 0.4 M sodium chloride. A linear gradient of sodium chloride from 0.4 M to 1.2 M in the above buffer (150 ml in each chamber) was applied to the column at a flow rate of 10 ml/hr. Fractions of 4 ml were collected, and those containing significant antithrombin immunoreactivity were dialyzed against 0.01 M Tris, 0.145 M NaCl, pH 8.3. Sodium chloride concentrations were determined with the aid of a conductivity bridge (Altex Conductivity Bridge, Beckman Instruments, Inc., Cedar Grove, NJ).

CASE REPORT

The family pedigree is shown in Fig. I. The propositus (V-19) is a 21-yr-old male college student who spontaneously developed thrombophlebitis of the left leg in July 1979. Treatment with warfarin was instituted by his private physician. Laboratory studies revealed low functional antithrombin activity (progressive antithrombin assay), while immunoreactive antithrombin was in the normal range.

A strong family history of recurrent lower extremity thrombosis and pulmonary emboli was obtained. There was no history of consanguinity. The brother (V-20) of the propositus had died at the age of 17 with massive pulmonary emboli following a 4-mo history of painful leg swelling and shortness of breath. He expired in spite of apparently adequate anticoagulation with heparin (activated partial thromboplastin time of 2-2.5 times control). The father (IV-16) had a long history of leg-swelling, but no diagnosis had been made. At age 41, evaluation revealed evidence of deep vein thrombosis and he was placed on warfarin therapy. His paternal grandfather (III-9) died with massive thromboses at age 29. In all, medical histories of 46 family members were obtained. Twelve of these individuals in direct line of descent from the first generation have experienced spontaneous thromboembolic events. In three of the women (III-5, IV-12, IV-13), an episode of deep venous thrombosis occurred during the puerperium. No individual has had recurrence while on warfarin, but at least one subject (IV-15) has had recurrent thromboses after discontinuation of the drug. There was no history of thrombotic disease in the maternal pedigree.

RESULTS

The propositus (V-19) and his father (IV-16) were extensively studied. Both demonstrated prolongations of the prothrombin time, activated partial thromboplastin time, and decreased levels of vitamin-K-dependent coagulation factors consistent with warfarin therapy. Thrombin time, euglobulin lysis time, bleeding time, platelet glass bead retention, platelet factor 3, and platelet aggregation studies were within normal limits. α-2-Macroglobulin and α-1-antitrypsin levels were normal in both subjects.
Plasminogen levels in the propositus were 50% of normal by both immunologic and functional assays (normal ranges, 70%–130% and 66%–134%, respectively). The father and 17 other paternal relatives (both with and without a history of thrombosis) had normal levels of immunoreactive plasminogen. The mother of the propositus (IV-17) and his maternal aunt (outside of the clinical pedigree) had low normal levels of immunoreactive plasminogen (71% and 72%, respectively), but definitely low levels of functional plasminogen (63% and 41%, respectively).

**Evaluation of Antithrombin**

Nineteen members of the pedigree in direct line of descent from the first generation were available for study. Immunoreactive antithrombin levels were within the normal range in all family members studied. Mean antithrombin antigen level (RID) was 96% of normal in 8 members with a history of prior thromboembolic events (Table 1), and 111% of normal in 19 individuals without a history of thrombosis (Table 2). These results were virtually identical with those obtained by radioimmunoassay (data not shown).

Normal antithrombin patterns were obtained when the plasma of either the propositus or his father was run in a two-dimensional crossed immunoelectrophoretic system, with and without heparin incorporation in the first gel dimension.

A clotting assay for progressive antithrombin activity was used to screen family members for functional deficiency of the inhibitor. Progressive antithrombin activity was low in the 8 subjects with a history of

**Table 1. Antithrombin (AT) Levels in Family Members With a History of Thrombotic Disease**

<table>
<thead>
<tr>
<th>Pedigree Number</th>
<th>Age of Patient</th>
<th>AT-Antigen (RID)</th>
<th>Heparin Cofactor (Amidolytic)</th>
<th>Prog. AT (Amidolytic)</th>
<th>Prog. AT (Clotting)</th>
</tr>
</thead>
<tbody>
<tr>
<td>III-5</td>
<td>71</td>
<td>103</td>
<td>42</td>
<td>42</td>
<td>54</td>
</tr>
<tr>
<td>IV-12</td>
<td>41</td>
<td>93</td>
<td>41</td>
<td>57</td>
<td>47</td>
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<tr>
<td>*IV-13</td>
<td>43</td>
<td>100</td>
<td>40</td>
<td>44</td>
<td>63</td>
</tr>
<tr>
<td>*IV-15</td>
<td>40</td>
<td>111</td>
<td>48</td>
<td>48</td>
<td>57</td>
</tr>
<tr>
<td>IV-16</td>
<td>48</td>
<td>80</td>
<td>42</td>
<td>61</td>
<td>69</td>
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<tr>
<td>V-15</td>
<td>26</td>
<td>89</td>
<td>45</td>
<td>57</td>
<td>63</td>
</tr>
<tr>
<td>*V-17</td>
<td>21</td>
<td>96</td>
<td>37</td>
<td>37</td>
<td>56</td>
</tr>
<tr>
<td>*V-19</td>
<td>21</td>
<td>96</td>
<td>42</td>
<td>50</td>
<td>58</td>
</tr>
<tr>
<td>Mean</td>
<td>96</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal range (% of normal pool)</td>
<td>85–115</td>
<td>86–114</td>
<td>87–125</td>
<td>82–119</td>
<td></td>
</tr>
</tbody>
</table>

*On warfarin.
thrombosis (mean 58%, range 47%-69%). No difference was noted in activity between those taking warfarin \((n = 4, \text{mean } 57\%)\) as compared to those not taking warfarin \((n = 4, \text{mean } 59\%)\). Two individuals (IV-13, IV-16) who were investigated while on warfarin therapy were subsequently studied when the drug was discontinued; the two determinations of antithrombin activity were not substantially different (data not shown).

Seven of the eight individuals with low functional antithrombin activity by clotting assay were studied further with assays employing chromogenic substrates. The mean progressive antithrombin activity level was 50% of normal, and the heparin cofactor level was 42% of normal (Table 1). The likely explanation for the higher levels obtained for progressive antithrombin activity employing clotting methodology is the contribution of secondary plasma inhibitors such as \(a_2\)-macroglobulin to thrombin neutralization as compared to those measured with chromogenic substrates.43

The plasma antifactor X activity in the presence of heparin was 41% of normal in the propositus (V-19) and 48% of normal in his father (IV-16). These reductions were similar in magnitude to those obtained with the heparin cofactor assay.

It has been suggested that stanazolol, an anabolic steroid, might increase the synthesis of antithrombin in individuals with congenital deficiency of this inhibitor.42,43 For this reason, both the propositus and his father underwent a 2-mo trial of stanazolol, 2 mg orally t.i.d., without any significant alteration in their antithrombin levels as measured by immunologic and functional assays. It is of interest to note that the plasma level of plasminogen did rise in the propositus from its initially low concentration during stanazolol therapy.

**Table 2. Antithrombin (AT) Levels in Family Members Without a History of Thromboembolic Disease**

<table>
<thead>
<tr>
<th>Pedigree Number</th>
<th>Age of Patient</th>
<th>AT Antigen (RID)</th>
<th>AT Activity (Clotting)</th>
</tr>
</thead>
<tbody>
<tr>
<td>III-12</td>
<td>55</td>
<td>81</td>
<td>85</td>
</tr>
<tr>
<td>IV-4</td>
<td>43</td>
<td>89</td>
<td>92</td>
</tr>
<tr>
<td>IV-6</td>
<td>53</td>
<td>119</td>
<td>113</td>
</tr>
<tr>
<td>IV-9</td>
<td>42</td>
<td>133</td>
<td>81</td>
</tr>
<tr>
<td>V-9</td>
<td>12</td>
<td>85</td>
<td>115</td>
</tr>
<tr>
<td>V-10</td>
<td>8</td>
<td>144</td>
<td>115</td>
</tr>
<tr>
<td>V-11</td>
<td>3</td>
<td>104</td>
<td>94</td>
</tr>
<tr>
<td>V-12</td>
<td>14</td>
<td>115</td>
<td>118</td>
</tr>
<tr>
<td>V-14</td>
<td>28</td>
<td>104</td>
<td>99</td>
</tr>
<tr>
<td>V-16</td>
<td>24</td>
<td>137</td>
<td>120</td>
</tr>
<tr>
<td>V-18</td>
<td>19</td>
<td>110</td>
<td>95</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>111</td>
<td>103</td>
</tr>
</tbody>
</table>

| Normal range    |               | 85-115          | 82-119                |

**Affinity Chromatography**

On heparin-Sepharose chromatography, antithrombin of a normal individual emerged as a sharp peak between 0.6 \(M\) and 0.8 \(M\) sodium chloride concentration (Fig. 2A). The antithrombin of the propositus eluted over a wide range of salt concentration (0.6-1.0 \(M\) sodium chloride), and two overlapping peaks of immunoreactivity were apparent (Fig. 2B). Fractions eluted from the affinity matrix containing the inhibitor as determined by radioimmunoassay were assayed for heparin cofactor activity as well as progressive antithrombin activity. From these measurements, the heparin cofactor activity/\(\mu M\) of antithrombin antigen and progressive antithrombin activity/\(\mu M\) of antithrombin antigen could be estimated. These estimates of specific biologic activity are approximately 1.0 for normal antithrombin. These values of the two parameters are apparent across the entire peak of antithrombin immunoreactivity in the chromatogram of the plasma of a normal individual. For the propositus, however, this is only true for the ascending limb of the first peak of immunoreactivity. After this early portion of the chromatogram, the functional activity of the inhibitor/\(\mu M\) of immunoreactive antithrombin steadily decreases until fraction 55. The progressive antithrombin activity is approximately equal to the heparin cofactor level.
at all salt concentrations. The data indicate that the functionally defective molecules (antithrombin "Chicago") of the propositus, which is the predominant species in the second peak of immunoreactivity, can be separated from normal inhibitor. The abnormal antithrombin thus possesses an enhanced capacity to bind to the mucopolysaccharide.

The plateau that is reached in the ratio of functional antithrombin activity to immunoreactive antigen after fraction 55 in the propositus suggests that the abnormal molecules possess a markedly reduced ability to neutralize thrombin in the presence or absence of heparin (approximately 10%–20% of normal). However, the possibility that this low level of activity is due to contaminating normal antithrombin cannot be completely excluded.

DISCUSSION

Patients with antithrombin deficiency have a strikingly high risk of thrombotic disease. If one includes all of the reported patients with congenital reduction of this inhibitor, then about 55% of the people have had at least one thrombotic event. The first thrombotic episode is spontaneous in about 42% of patients, but appears to be related to pregnancy, delivery, contraceptive pill ingestion, surgery, or trauma in the remaining 58% of these individuals. The most common sites of disease are deep leg and iliac veins. About 60% of patients develop recurrent thrombotic episodes, and clinical signs of pulmonary embolism are present in 40% of all cases.

Two major subtypes of inherited antithrombin deficiency with the same clinical picture and autosomal dominant inheritance patterns have been delineated. The "classical" type is caused by a reduced synthesis of biologically normal inhibitor molecules. In these cases, the immunologic and biologic activity of antithrombin within the blood is reduced to the same extent (Fig. 3). The rarer subtype is produced by a discrete molecular defect within the protease inhibitor. For this reason, the plasma levels of antithrombin, as judged by biologic activity measurements, are greatly reduced, while the immunologic determinations of this inhibitor are essentially normal. At least seven families with this subtype of antithrombin deficiency have been reported in the literature. In four of these cases, the mutant antithrombin molecules have been separated from the normal inhibitor, allowing for more detailed characterization of the nature of the biologic defect. However, no detailed structural information is available for any of the abnormal antithrombins at the present time.

The first such kindred with a mutant antithrombin in association with a profound thrombotic diathesis was described by Sas et al. in 1974. Affected members of this family exhibited a marked diminution of

![Fig. 3. Classification of familial antithrombin deficiency states. In the normal situation, the binding of heparin to inhibitor results in the allosteric activation of the antithrombin molecule (arrow), allowing thrombin to be rapidly inactivated.](image-url)
both progressive antithrombin as well as heparin cofactor activity in the face of normal immunologic levels of the inhibitor. The abnormal protein has been termed antithrombin “Budapest.” This component was found to migrate more slowly than normal antithrombin in a crossed immunoelectrophoretic system containing heparin. It has recently been demonstrated that heparin binds to this abnormal antithrombin with reduced affinity. Based on the above data, one may assume that a major defect is present at the thrombin binding site of antithrombin “Budapest,” with an associated minor abnormality within the heparin binding region.

Tran et al. have studied the plasma of an individual with recurrent superficial thrombophlebitis. The purified inhibitor obtained from this patient is termed antithrombin “Basel” and exhibits normal immunologic and normal progressive antithrombin levels with depressed heparin cofactor activity as compared to normal protease inhibitor. These investigators demonstrated that antithrombin “Basel” eluted at a lower ionic strength from heparin-Sepharose, as compared to normal inhibitor. This implies a reduced affinity of antithrombin “Basel” for the heparin molecule. Therefore, it can be inferred that a defect exists at the heparin binding site of this abnormal protein.

A family with functional deficiency of antithrombin associated with thrombophilia has also been reported by Sorensen et al. The abnormal protein (antithrombin “Aalborg”) has reduced progressive antithrombin activity as well as diminished heparin cofactor activity, but retains normal heparin affinity. Thus, this defect appears to affect the thrombin binding site of the inhibitor.

Wolf et al. have studied another such kindred and have termed the defective protease inhibitor antithrombin “Paris.” This abnormal protein appears to be similar to antithrombin “Basel” in terms of its progressive antithrombin activity, heparin cofactor activity, and heparin affinity. The abnormal protein in this article has been termed antithrombin “Chicago.” The levels of this inhibitor were normal as judged by immunologic measurements. The progressive antithrombin activity and the heparin cofactor activity of the abnormal molecule were markedly reduced as compared to normal inhibitor (approximately 10%–20% of normal). However, in contrast to antithrombin “Budapest,” antithrombin “Chicago” eluted from heparin-Sepharose at a higher ionic strength than normal antithrombin. Thus, we can infer that the molecular defect affects both the thrombin interaction site and the heparin binding domain of the protease inhibitor.

Laboratory analyses revealed that the propositus also exhibited a deficiency of immunoreactive and functional plasminogen (about 50% of normal). This was inherited from the maternal pedigree; all paternal relatives studied had normal levels of this zymogen. A structural abnormality of plasminogen has been reported in association with thrombosis. However, the contribution of this combined abnormality of functional antithrombin and plasminogen to an enhanced thrombotic diathesis in the propositus over that associated with inhibitor deficiency alone cannot be ascertained.

The common feature in all instances of familial antithrombin deficiency associated with thrombotic disease is a reduced level of heparin cofactor activity. This implies that the above mechanism may be important in the prevention of thrombosis in vivo. As the various anomalies of the antithrombin-heparin cofactor system are uncovered and characterized in greater detail, we shall obtain a clearer picture of the relevance of this mechanism to the prevention of thrombotic disease.

Damus et al. have suggested that the nonthrombogenic properties of blood vessels may be due to the presence of heparin species with anticoagulant activity on the vascular endothelium. Several investigators have provided evidence to support this hypothesis. Murphy et al. have noted that dogs undergoing extracorporeal circulation without anticoagulant protection do not experience thrombosis and have attributed this phenomenon to the presence of endogenous heparin-like substances. Lollar and Owen have injected labeled enzyme with antithrombin. However, the latter investigators have suggested that this phenomena is due to the binding of thrombin to the endothelium, with resultant acceleration of enzyme-antithrombin interactions. More recently, Marcum et al. have isolated anticoagulantly active heparin species from the microvasculature and have demonstrated that the mucopolysaccharide is able to enhance the rate of neutralization of thrombin by antithrombin.

The availability of heparin-like components within the vascular tree would permit antithrombin to be selectively activated by blood-surface interfaces where enzymes of the hemostatic mechanism are generated. Thus, the plasma protease inhibitor may be critically placed to neutralize the above enzymes and thereby protect natural surfaces against thrombus formation. It is tempting to speculate that antithrombin “Chicago,” with increased heparin avidity as compared to normal inhibitor, might preferentially interact with these mucopolysaccharides. This defect in the normal inhibitory mechanism might, in part, explain the frequent and severe thromboembolic phenomena seen in all affected members of this kindred who were available for study.
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