CONCISE REPORT

Antithrombin Milano: A New Variant With Monomeric and Dimeric Inactive Antithrombin III

By M. Wolf, C. Boyer, A. Tripodi, D. Meyer, M.J. Larrieu, and P.M. Mannucci

A qualitative defect of antithrombin III (AT III) has been demonstrated over three generations in eight members of an Italian family by the discrepancy between a normal amount of antigen and decreased antithrombin and anti-Xa activity in the presence or in the absence of heparin. By two-dimensional immunoelectrophoresis in the absence of affinity chromatography, the activity in the presence or in the absence of heparin was measured. Two peaks of AT III were present in all patients' plasma. AT III was purified from normal and propositus plasma by sulfate dextran precipitation followed by heparin affinity chromatography. The elution profile of the patient's AT III was abnormal and allowed the separation of two populations of AT III, normal and abnormal. The first fraction (normal AT III) contained AT III activity, migrated as a single peak by two-dimensional immunoelectrophoresis and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), demonstrated a single band with a molecular weight (mol wt) identical to that of normal AT III (60,000). Conversely, the last fraction, devoid of AT III activity, migrated as a single abnormal peak by two-dimensional immunoelectrophoresis in the absence of heparin. By SDS-PAGE, two bands were observed: one with a mol wt of 60,000 and a second one with a mol wt of 120,000. Western blots clearly demonstrated cross-reactivity of the 120,000 and 60,000 mol wt bands with monospecific antisera to human AT III. Reduction of the 120,000 mol wt band converted it to a single 60,000 mol wt band, suggesting the presence of an abnormal dimeric form of AT III. The name AT III Milano is proposed for this new variant.

THE FIRST qualitative defect of antithrombin III (AT III) was described by Sas et al in 1974. Several cases of these genetic variants, referred to as type II AT III deficiency, have now been reported. Variants are characterized by a normal amount of AT III antigen with decreased functional activities (affinity for heparin and/or the ability to inactivate thrombin or factor Xa). In all of these cases except in AT III Milano, various amounts of both normal and abnormal AT III are synthesized and present in plasma. We now report a new abnormal AT III present at the heterozygous state in several members over three generations of an Italian family, AT III Milano. This abnormal AT III is characterized by the presence of two types of inactive molecules, with a normal (60,000) and abnormal (120,000) molecular weight (mol wt). The molecular defect at AT III Milano thus appears to be distinct from those of previously described genetic variants.

MATERIALS AND METHODS

Antithrombin III Assays

Quantitative and qualitative estimation of AT III was achieved as previously described. Three monospecific rabbit anti-human AT III antisera were used, either from commercial sources (Behringwerke, Warburg, FRG, and Diagnostica Stago, France) or prepared in our laboratory.

AT III activity was measured in the absence (progressive antithrombin activity) or presence of heparin (heparin cofactor activity), using the chromogenic substrate S-2238 (Kabi, Sweden) as previously described. Anti-Xa activity was also tested in the absence or presence of heparin, using the chromogenic substrate S-2222 (Kabi).

Results were expressed either as the percentage of control pool plasma or as U/mL. One unit is defined as the amount of AT III present in 1 mL of control plasma.

Purification of AT III

AT III was purified from normal and propositus plasma according to the method of McKay, with the following modifications: after two precipitations of plasma by dextran sulfate, 500,000 mol wt (Pharmacia, Uppsala, Sweden) in the presence of 0.05 mol/L calcium chloride, the supernatant was poured on a heparin-Sepharose 4B (Pharmacia) column equilibrated with 0.05 mol/L Tris, 0.01 mol/L Na citrate, pH 7.4 (Tris citrate buffer), containing 0.15 mol/L NaCl. After extensive washing with the same buffer containing 0.25 mol/L NaCl, the proteins were eluted using a linear salt gradient from 0.25 to 2.00 mol/L NaCl in 0.1 mol/L Tris, 0.01 mol/L Na citrate, pH 7.4. Three-milliliter fractions were collected and AT III antigen was measured. Three to five eluted fractions (10 to 15 mL) containing AT III were pooled, concentrated, dialyzed against Tris citrate buffer, and frozen at −80 °C. AT III antigen and progressive antithrombin activity were estimated on each pooled fraction.

In some experiments, normal or propositus citrated plasma was directly applied on small columns containing 5 mL of heparin.
Antithrombin Levels in Three of the Affected Family Members With AT II Milano

<table>
<thead>
<tr>
<th>Case No.</th>
<th>AT III Antigen (Laurell) (%)</th>
<th>Heparin Cofactor (%)</th>
<th>Progressive (%)</th>
<th>AT III Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>118</td>
<td>51</td>
<td>58</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
<td>110</td>
<td>53</td>
<td>50</td>
<td>70</td>
</tr>
<tr>
<td>3</td>
<td>115</td>
<td>52</td>
<td>56</td>
<td>69</td>
</tr>
</tbody>
</table>

Normal range 80–120

Sepharose 4B. AT III was eluted, and the fractions were pooled and treated as described above.

Anti-AT III IgG Preparation

IgG was purified from the rabbit anti-AT III antiserum previously described. Monospecific IgG was obtained by immunoadsorption on purified AT III bound to cyanogen bromide (CNBr)-activated Sepharose 4B (0.3 mg per milliliter of beads) and elution with 0.1 mol/L glycine, pH 2.4. IgG was radiolabeled by the method of Fraker and Speck,11 using 150 μg IgG and 1 mCi of 125I, with a specific activity of 3 μCi/mg.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS (0.01%)-PAGE (10%) was performed according to Laemmli’s method.12 Samples were reduced with 5% β-mercaptoethanol. Molecular weights were estimated using markers in the range of 14,000 to 200,000 (Pharmacia). Proteins were either stained by Coomassie brilliant blue or were transferred onto nitrocellulose by Western blotting.12 AT III being revealed by 125I-labeled anti-AT III IgG and autoradiography.

Case Report

The propositus was a 25-year-old male who developed thrombo-phlebitis of the right leg following surgery for a tibial fracture of the same leg. Laboratory studies revealed a decreased heparin cofactor activity, contrasting with a normal amount of AT III antigen in eight of 14 tested members. Family analysis demonstrated an autosomal dominant mode of inheritance. Only two of these members had a history of deep vein thrombosis, the propositus following surgery and his mother following parturition.

RESULTS

In three members of the family, the propositus (case 2), his sister (case 3), and his grandmother (case 1), both heparin cofactor (mean, 52%) and progressive antithrombin (mean, 62%) activities were decreased to the same extent (Table 1). Similar results were obtained for anti-Xa activity. The levels of AT III antigen were consistently in the normal range (Table 1).

The qualitative abnormality of AT III was confirmed by two-dimensional immunoelectrophoresis in the eight affected members (Fig 1). In absence of heparin, two peaks of AT III with complete immunologic identity were observed, the first peak at the same position as in control plasma, the second with a more anodal mobility. In the presence of heparin, only one peak similar to that of the control was demonstrated.

The results of heparin-Sepharose chromatography of AT III from normal and propositus plasma are illustrated in Fig 2. Normal AT III (Fig 2A) eluted from 0.9 to 1.5 mol/L NaCl, and AT III-progressive activity and antigen levels were similar in all tested fractions, with a mean ratio of activity to antigen of 0.94. In all fractions, AT III migrated as one peak by two-dimensional immunoelectrophoresis and demonstrated whether unreduced or reduced a single band of 60,000 mol wt by SDS-PAGE.

In contrast, propositus AT III (Fig 2B) eluted in a broader peak from 0.9 to 1.8 mol/L NaCl. The ratio of AT III activity (progressive antithrombin) to antigen progressively decreased from 1.04 in the first pooled

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Fig 1. Two-dimensional immunoelectrophoresis of AT III: control plasma without (A) and with (C) heparin; propositus plasma without (B) and with (D) heparin.
fraction (21 to 24) to less than 0.02 in the last one (40 to 44). In the first pooled fraction, a single peak with a normal migration was observed by two-dimensional immunoelectrophoresis in the absence of heparin. Similarly, a single band with a molecular weight identical to that of normal AT III (60,000) was obtained by PAGE (Fig 3). In the intermediate pooled fractions (25 to 40), two peaks of AT III were demonstrated by two-dimensional immunoelectrophoresis, and a single peak with a more anodal migration than normal AT III was identified in the last pooled fraction (40 to 44), in which no antithrombin activity was detected. In those fractions (Fig 3B), two bands of 60,000 and 120,000 mol wt were consistently observed in unreduced gels by PAGE. Following reduction with β-mercaptoethanol, a single band of 60,000 mol wt was detected. In both unreduced and reduced gels, the bands were revealed following Western blotting by specific immunoadsorbed 125I-labeled anti-AT III IgG.

Similar results were obtained by staining or Western blotting of AT III, when heparin-Sepharose affinity chromatography was performed directly on small amounts of plasma without prior precipitation by dextran sulfate.

**DISCUSSION**

We report here a new variant of AT III present at the heterozygous state in eight members over three generations of an Italian family. This molecular defect is characterized in plasma by a functionally defective AT III, both in the presence or absence of heparin, as well as by the existence of two populations of AT III by two-dimensional immunoelectrophoresis, the latter being only demonstrated in the absence of heparin. The abnormal AT III was found to elute from heparin-Sepharose at a higher ionic strength than normal AT III. This abnormal pattern of elution allowed the separation of normal and abnormal populations of AT III.
III. The first fraction of patients' AT III was functionally, immunologically, and biochemically indistinguishable from normal AT III. Conversely, in the last fraction, progressive antithrombin activity was lacking, affinity for heparin was increased, AT III moved as a single peak with a more anodal electrophoretic mobility in the absence of heparin, and by SDS-PAGE, two bands were detected, one with a molecular weight similar to that of normal AT III (60,000) and the second with a molecular weight of 120,000. Cross-reactivity of the two bands with monospecific antisera to AT III was unequivocally demonstrated.

The description of this new variant, AT III Milano, confirms the heterogeneity of congenital AT III deficiency. Qualitative defects of AT III have been reported in full in at least eight families since the first description of a mutant AT III by Sas et al in 1974. In three of these eight families, the abnormal AT III has been separated from the normal protein, allowing further identification.

Normal AT III contains distinct binding sites for thrombin and for heparin. Data from the measurement of biological activities, two-dimensional immunoelectrophoresis and/or affinity chromatography on heparin-Sepharose strongly suggest that there are various molecular defects in the previously reported variants. AT III Basel, Paris, Padua, and Toyama are defective only in their heparin binding sites, while AT III Aalborg and Vicenza appear to be defective only in their thrombin binding sites. Conversely, AT III Budapest and Chicago demonstrate a combined defect affecting mainly the thrombin binding site but also the heparin binding site. AT III Milano appears to be similar in that regard. By affinity chromatography on heparin agarose, an abnormal electroelution profile similar to that of our patient was reported in AT III Chicago, but the latter showed normal migration in plasma in the absence of heparin. In AT III Trento, the pattern of two-dimensional immunoelectrophoresis was similar to that of AT III Milano, but no further characterization of this variant was reported. In AT III Budapest, a higher molecular weight (100,000 to 120,000) of the functionally abnormal protein was suggested by gel filtration of plasma onto Sephacryl S-200. These results, however, were not confirmed by using purified AT III. Furthermore, the molecular abnormality of AT III Budapest appears different, since—contrary to AT III Milano—affinity for heparin is decreased.

The most important finding in AT III Milano is the presence of two functionally defective but distinct molecular forms of AT III, with molecular weights of 60,000 and 120,000, respectively. The high-molecular weight band is converted to a single band of 60,000 mol wt in the presence of β-mercaptoethanol, suggesting the existence of a two-chain form linked by one or more disulfide bonds. This observation leads us to propose a tentative model to explain the high-molecular weight form of this new variant. Normal AT III consists of a single chain containing three disulfide bonds. A single mutation involving a cysteine residue would result in the presence of a free thiol group, allowing intermolecular disulfide bridges to form, thus giving rise to a dimer. Such a possibility has been demonstrated for A-I Milano apoprotein. Alternatively, the abnormal AT III may associate to a distinct plasma protein having the same molecular weight, but this would appear unlikely. Preliminary results suggest that association of the abnormal AT III with the same or with a distinct molecule was not related to purification, since similar results were obtained by a single step of purification from plasma.

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