Monoclonal Antibodies Against Plasma Protease Inhibitors: Production and Characterization of 15 Monoclonal Antibodies Against Human Antithrombin III. Relation Between Antigenic Determinants and Functional Sites of Antithrombin III

By P. Hérion, M. Francotte, D. Siberdt, G. Garduno Soto, J. Urbain, and A. Bollen

Fifteen hybridomas secreting monoclonal antibodies against human antithrombin III, originating from two mouse strains, have been produced by the cell fusion technique. Eight monoclonal antibodies belong to the class IgG1, five to the class IgG2a, and two to the class IgG2b. All light chains belong to the κ group. No cross-reaction of the monoclonal antibodies have been observed with a crude preparation of albumin nor with α1-antitrypsin and α2-antiplasmin. Five of these monoclonal antibodies exhibit a relatively high avidity for antithrombin III. Inhibition experiments showed that the 15 monoclonal antibodies define seven more or less independent antigenic regions on the antithrombin III molecule. Examination of the effects of these antibodies on the inhibitory capacity of antithrombin III toward thrombin activity, either in the presence or in the absence of heparin, showed that several monoclonal antibodies inhibit the antithrombin III activity and allowed to relate some of the antigenic determinants to functional sites on the antithrombin III molecule.

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MATERIALS AND METHODS

Heparin sepharose CL6B and dextran sulfate were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden; Chromozym TH (Tos-gly-pro-arg-pNa) and aprotinin, from Boehringer, Mannheim, FRG; heparin, from Serva, Heidelberg, FRG; and thrombin, from Kabi, Sweden.

Antithrombin III was purified from a pool of citrated human plasma according to the method described by McKay. Antithrombin III was detected using specific goat anti-human antithrombin III antiserum (Immuno, Vienna). Sodium dodecyl sulfate-acrylamide gel electrophoresis showed that the preparation obtained consisted of a major single protein band (the purity was estimated at more than 90%). One hundred nanograms of the purified antithrombin III inhibits 0.1 nKat of thrombin in the amidolytic assay.

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Cells and media were as described previously in Hérion et al.\textsuperscript{26} Immunization of mice, cell fusion and cloning, antibody labeling, immunoglobulin isotyping, isoelectrofocusing, solid phase radioimmunoassay (SPRIA), and indirect enzyme-linked immunosorbent assay (ELISA) were performed as described previously.\textsuperscript{26} Limbro's polyvinylchloride plates (Nunc, Denmark) were coated by incubation with a solution of antithrombin III (6 \(\mu\)g/mL or 0.6 \(\mu\)g/mL) in acetate buffer, pH 5.0.

**Antithrombin III Activity**

The procedure using the chromogenic substrate of thrombin, Tos-gly-pro-ang-pNa, described by Bartl and Lill,\textsuperscript{27} was adapted to a buffer, pH 5.0. Antithrombin III Activity

To prevent adsorption of proteins during the assay, the plates were first incubated with a solution of bovine serum albumin then washed extensively. The reaction buffer contained Tris-HCl (0.1 mol/L, pH 8.2), NaCl (0.15 mol/L), EDTA (0.01 mol/L), polyethylene glycol (M, 6,000, 0.5%), and aprotinin (6.5 IU/mL). Heparin (3 U/mL, final concentration) was added as needed. Antithrombin was incubated with thrombin in this buffer (20 \(\mu\)L final reaction volume) either for five minutes at 20 \(\degree\)C in the presence of heparin or for 90 minutes at 37 \(\degree\)C in the absence of heparin, 20 \(\mu\)L of substrate solution (1 mg/mL in \(\mathrm{H}_2\mathrm{O}\)) was added, and samples were mixed and incubated for five minutes at room temperature. The reaction was then terminated by adding 50 \(\mu\)L of 50% acetic acid. Absorbance was read at 400 nm in a microelisa automatic reader (Dynatech AM 120). Calibration curves were constructed for thrombin and antithrombin III. To assay the effects of the monoclonal antibodies on the antithrombin III activity, antithrombin III was preincubated for four hours at 4 \(\degree\)C with antibody containing serum-free supernatants of hybridoma cells, which had been extensively dialyzed against Tris-HCl 0.1 mol/L, pH 8.2, NaCl 0.15 mol/L, EDTA 0.01 mol/L buffer.

**RESULTS**

**Immunization of Mice**

Mice belonging to five strains were hyperimmunized by three injections of purified antithrombin III at three-week intervals. The tertiary antibody response was tested by Ouchterlony assay and SPRIA (results not shown). On this basis, one Balb/c mouse and one A/J mouse were selected for fusion. After a rest period of four months, they were given a booster of 200 \(\mu\)g antigen in saline administered intraperitoneally and intravenously on day 4 before fusion. The spleen of the A/J mouse yielded 140 \(\times\) \(10^6\) nucleated cells and that of the Balb/c yielded 92 \(\times\) \(10^6\) cells.

**Cell Fusion, Cloning, and Antibody Secretion**

For each mouse, five fusion experiments were performed involving, respectively, 27 \(\times\) \(10^6\) or 18 \(\times\) \(10^6\) spleen cells. The ratio of spleen cells to myeloma cells was 3:1 and 2:1, respectively. After fusion, the cells were plated in 96-microwell plates at two cell densities (\(\pm\) \(10^6\) and \(10^7\) fused spleen cells per well) on a feeder layer consisting of 5 \(\times\) \(10^6\) thymocytes and 5 \(\times\) \(10^6\) macrophages per well, in HAT-containing medium (hypoxanthine, 13.6 \(\mu\)g/mL; aminopterin, 1.76 \(\mu\)g/mL; and thymidine, 3.8 \(\mu\)g/mL). Hybridoma growth was recorded by microscopic examination of the wells by day 8 after fusion, and detection of antibodies in the supernatant by the indirect ELISA was performed by days 9 through 12. The fusion efficiencies (mean number of clones containing more than 10\(^2\) cells by day 8 after fusion) were around 40 per 10\(^6\) fused spleen cells for the A/J mouse and the Balb/c mouse, while the rate of clones secreting antibodies against \(\alpha\)-antiplasmin were, respectively, 8.8\% and 1.5\%.\textsuperscript{28}

Twenty clones from the A/J fusion and six from the Balb/c fusion were recloned in soft agar. By immunoprecipitation using rabbit anti-mouse immunoglobulin serum,\textsuperscript{28} we detected immunoglobulin-secreting subclones in the case of twenty-three original clones. Examination of the clones picked up from the agar plates for specific antibody secretion resulted in the selection of seventeen distinct clones producing antibodies directed against antithrombin III and one clone producing an antibody that seemed to react with human albumin. These clones were expanded in large cultures, frozen, injected into pristane (2,6,10,14-tetramethylpentadecane)-"primed" mice (Balb/c or Fl [Balb/c × A/J]), and eventually subcloned to examine antibody secretion stability. Because of their instability, two clones were discarded.

**Immunoglobulin Class and Subclass of the Monoclonal Antibodies**

The immunoglobulin class was determined by the Ouchterlony test on concentrated culture supernatants. The results presented in Table 1 indicate that eight monoclonal antibodies belong to the class IgG1, five to the IgG2a, and two to the IgG2b class. All light chains belong to the \(\kappa\) group.

**Specificity and Avidity of the Monoclonal Antibodies**

The relative avidities of the monoclonal antibodies and their specificity were examined by the indirect ELISA, using solid phases coated with different dilutions of antigen or solid phases coated with possible cross-reacting antigens (crude human albumin, \(\alpha\)-antiplasmin, and \(\alpha\)-antitrypsin). Some typical curves are shown in Fig 1, and the characteristic figures relating to the 15 monoclonal antibodies are reported in Table 1. The antibodies display a heterogeneous pattern of binding level and avidities. Only monoclonal antibodies BATR1, AATR2, AATR5, AATR6, AATR9, and AATR10 have a binding level corresponding to an OD \(\simeq 0.250\) on plates coated with 30 ng of antithrombin per well. No significant binding occurred on crude human albumin, \(\alpha\)-antitrypsin, and \(\alpha\)-antiplasmin.
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Table 1. Class and Binding Characteristics of the Monoclonal Antibodies

<table>
<thead>
<tr>
<th>Monoclonal Antibody</th>
<th>Class</th>
<th>Supernatant Titer (x 10^-3</th>
<th>Max A4920/300</th>
<th>Max A4920/30</th>
<th>Relative Avidity</th>
</tr>
</thead>
<tbody>
<tr>
<td>BATR1</td>
<td>IgG1</td>
<td>2.0</td>
<td>1.15</td>
<td>0.40</td>
<td>0.079</td>
</tr>
<tr>
<td>BATR3</td>
<td>IgG2a</td>
<td>1.6</td>
<td>0.67</td>
<td>0.08</td>
<td>0.079</td>
</tr>
<tr>
<td>BATR7</td>
<td>IgG1</td>
<td>1.1</td>
<td>0.29</td>
<td>0.04</td>
<td>NK</td>
</tr>
<tr>
<td>AATR1</td>
<td>IgG1</td>
<td>2.5</td>
<td>0.57</td>
<td>0.09</td>
<td>0.063</td>
</tr>
<tr>
<td>AATR2</td>
<td>IgG1</td>
<td>0.50</td>
<td>0.62</td>
<td>0.52</td>
<td>0.40</td>
</tr>
<tr>
<td>AATR5</td>
<td>IgG1</td>
<td>0.50</td>
<td>0.73</td>
<td>0.63</td>
<td>0.36</td>
</tr>
<tr>
<td>AATR6</td>
<td>IgG2a</td>
<td>0.020</td>
<td>0.38</td>
<td>0.25</td>
<td>0.45</td>
</tr>
<tr>
<td>AATR7</td>
<td>IgG2b</td>
<td>2.5</td>
<td>0.57</td>
<td>0.11</td>
<td>0.40</td>
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<tr>
<td>AATR9</td>
<td>IgG1</td>
<td>0.22</td>
<td>0.75</td>
<td>0.55</td>
<td>0.36</td>
</tr>
<tr>
<td>AATR10</td>
<td>IgG2b</td>
<td>0.056</td>
<td>0.50</td>
<td>0.26</td>
<td>0.25</td>
</tr>
<tr>
<td>AATR11</td>
<td>IgG2a</td>
<td>10.00*</td>
<td>0.17</td>
<td>0.02</td>
<td>NK</td>
</tr>
<tr>
<td>AATR12</td>
<td>IgG1</td>
<td>10.00</td>
<td>0.98</td>
<td>0.10</td>
<td>NK</td>
</tr>
<tr>
<td>AATR17</td>
<td>IgG2a</td>
<td>0.79</td>
<td>0.65</td>
<td>0.07</td>
<td>0.020</td>
</tr>
<tr>
<td>AATR20</td>
<td>IgG1</td>
<td>0.22</td>
<td>0.70</td>
<td>0.09†</td>
<td>0.0032</td>
</tr>
<tr>
<td>AATR24</td>
<td>IgG2a</td>
<td>0.35</td>
<td>0.63</td>
<td>0.10</td>
<td>0.022</td>
</tr>
</tbody>
</table>

The antibodies originating from the Balb/c strain and the A/J strain are respectively designated by BATR and AATR. The titer is expressed as the dilution of supernatant giving an A4920 = 0.3 in the indirect ELISA. The Max A4920/300 and Max A4920/30 are the optical density values measured at 490 nm, at the plateau of the binding curves obtained with increasing concentration of antibodies, on plates respectively coated with 300 ng or 30 ng of antithrombin III per well (see Fig 1). The relative avidity is expressed as the ratio Ab30/Ab300, where Ab30 and Ab30 represent the dilutions of culture supernatants giving a same A4 value, in binding curves generated using increasing concentration of antibodies on plates respectively coated with 300 ng of and 30 ng antithrombin III per well. NK, not known.

*An A4920 value of 0.3 is not attained. The indicated dilution gives an OD = 0.1.
†Plateau not attained with undiluted supernatant.

Epitope Specificity

The epitope specificity of the monoclonal antibodies was examined by inhibition experiments, where the binding of 35S-internally labeled antibodies to the antigen (immobilized on a solid phase) was tested in the presence of the other monoclonal antibodies. The results of these inhibition experiments are summarized in Table 2 and distinguish several groups of monoclonal antibodies.

A first group comprises BATR1 and BATR3, another one, BATR7 and AATR11. Two antigenic sites are defined by unique monoclonal antibodies: AATR1 and AATR10. Three other epitopes are defined by the following three groups: AATR2, AATR5, AATR6, and AATR9; AATR12, AATR17, and AATR20; and AATR7 and AATR24. Moreover, BATR1 and BATR3 seem to produce conformational changes, as preincubation of antithrombin III with either of them inhibits the binding of AATR12, AATR17, and AATR20, but the reverse is not true. The definition of these groups of completely cross-reacting antibodies draws the tentative antigenic picture of antithrombin III shown in Fig 2. Partial inhibitions were also observed between antibodies belonging to different subgroups, for example, between antibodies AATR2, 5, 6, 9, and 10.

Isoelectric Focusing

Autoradiogram obtained after isoelectric focusing of several 35S-internally labeled antibodies confirmed the monoclonality of the antibodies. The results, together with the epitope specificity and binding characteristics, strongly suggest a molecular identity between AATR2 and AATR5 (results not shown).
Effect of the Monoclonal Antibodies on Antithrombin III Activity

The effect of the monoclonal antibodies on the neutralizing activity of antithrombin III toward the enzymatic activity of thrombin was examined using a chromogenic assay of thrombin amidolytic activity, either in the presence or in the absence of heparin. For these assays we used antibodies produced in serum-free medium. As seen in Fig 3, preincubation of antithrombin III with monoclonal antibodies BATRI, AATR7, AATR12, AATR17, or AATR24 significantly reduces the antithrombin III activity in the absence of heparin. Monoclonal antibodies BATRI and AATR17 prevent the neutralization of thrombin in the presence of heparin. Other monoclonal antibodies, like AATR2, AATR12, AATR17, or AATR20, also reduce, although to a lesser extent, the antithrombin III activity in the presence of heparin.

Similar experiments were performed with various amounts of purified monoclonal antibodies. The results of these assays are shown in Figs 4 and 5.

In the absence of heparin (Fig 4), monoclonal antibodies BATRI abolish almost completely the antithrombin III activity. Preincubation of antithrombin III with antibodies AATR24 or AATR17 also reduces the antithrombin III activity. A small effect of the monoclonal AATR20 is observed, as compared with the control (anti-urokinase monoclonal antibodies). As seen in Fig 5A, monoclonal BATRI completely inhibits the activity of the enzyme in the presence of heparin. Monoclonal antibodies AATR20, AATR17, and AATR24 also significantly reduce the extent of neutralization of thrombin in the presence of heparin. Preincubation of these purified monoclonal antibodies with antithrombin III and heparin before assaying thrombin activity gave similar results (Fig 5B). Simi-
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Fig 4. Effect of purified monoclonal antibodies on the antithrombin III activity in the absence of heparin. Various amounts of purified antibodies were preincubated with antithrombin III before assaying thrombin activity. *: BATR 1; Δ, AATR 24; ■, AATR 17; △, AATR 20; ●, unrelated monoclonal antibody (anti-urokinase monoclonal antibody).

lar assays were carried out with various amounts of purified antibodies AATR7 and AATR12. Furthermore, monoclonal antibodies AATR7 and BATR1 behave in a similar manner; this is also true for the AATR17 and AATR12 antibodies.

DISCUSSION

Fusion of the spleen cells of two mice belonging to two strains (A/J and Balb/c), hyperimmunized with human antithrombin III, with a subclone of the nonsecreting SP2/0-Ag14 myeloma line, has led to the isolation of 15 hybridoma clones secreting monoclonal antibodies against antithrombin III. Differences in hybridoma yields between the two individual mice best exemplifies the role of the immune state of the animal in the yield of a fusion experiment. The immune state is subjected to strain and individual variations on a same immunization schedule. Thus, for a greater chance of success, fusion experiments should always involve spleens coming from at least two individual mice. It is worthwhile to note that the Balb/c mouse yielded three times less specific hybridoma (in the first screening) than the A/J mouse, whereas the antibody titer in the Balb/c mouse was five times higher than in the A/J mouse in the tertiary response.

These 15 hybridoma lines are easily clonable in soft agar (without feeder cells) and grow well in vitro (generation time 11 to 16 hours) as well as in vivo (inoculation dose = 10⁶ cells).

The antibodies produced belong to the classes IgG1, IgG2a, and IgG2b. They appear to be specific for antithrombin III and do not cross-react with other protease inhibitors, such as α₁-antitrypsin and α₂-antiplasmin, with which antithrombin III has been shown or is expected to share sequence homologies.

The antibodies display a heterogeneous pattern of relative avidity and binding to antithrombin III, as determined in the solid phase assay.

Examination of the epitope specificities of these monoclonal antibodies defined the antigenic picture of antithrombin III presented in Fig 2. The 15 monoclonal antibodies define seven more or less independent antigenic regions on the antithrombin III molecule. Moreover, one-way inhibitions observed with antibodies binding to sites g and e suggest that binding of a monoclonal antibody to site g could block the antithrombin III in a conformational state that prevents binding to site e. The meaning of partial inhibitions observed between some antibodies is unclear. They could be explained by weak steric hindrance between labeled and inhibitor antibodies, inducing an apparent decrease in the affinity of the labeled antibody for the antigen. Another factor could be some heterogeneity in the antigen bound to the plate, resulting from a natural microheterogeneity or from partial denaturation during its isolation or its binding to the microtest plate.

Several monoclonal antibodies were shown to pro-
produce neutralizing activity on the inhibiting activity of antithrombin III toward thrombin either in the presence or in the absence of heparin. On the basis of the results detailed earlier, our panel of monoclonal antibodies can be derived into several subgroups. The first subgroup (e.g., BATR 1, AATR 7), which strongly prevents the neutralization of thrombin by antithrombin III in the presence or absence of heparin, is made up of antibodies that recognize directly the binding site or induce conformational changes that prevent the interactions between thrombin and antithrombin. The second class (AATR 12, BATR 17, AATR 24) has the same effects but to a lower extent. The third subgroup contains monoclonal antibodies recognizing epitopes unrelated to the binding of thrombin to antithrombin. They have no effect on heparin binding. The effects of monoclonal antibodies of these different classes on the antithrombin III activity in the presence or in the absence of heparin are summarized in Table 3.

The monoclonal antibody AATR 20 deserves a special mention because the effects are different whether heparin is present or not.* AATR 20 slightly alters the extent of neutralization of thrombin by antithrombin in the absence of heparin. However, in the presence of heparin, the effect of AATR 20 is comparable to what is observed with class II antibodies. The peculiar behavior of AATR 20 can be understood if one assumes that antithrombin III undergoes several conformational changes. In this hypothesis, substantiated by published molecular models, heparin would favor a conformational change that leads to the increased effect of AATR 20.

Relating our results with the antigenic picture shown in Fig 2, it appears that only monoclonal antibodies recognizing the epitopes d, e, or g can produce significant effects on the biological activities of antithrombin III. These observations, together with the fact that one-way inhibitions associated with conformational changes are observed for the binding to these epitopes, suggest some structural molecular identity or overlapping between epitopes d, e, and g and class I, II, and III determinants. Kinetics studies of the effects produced by the monoclonal antibodies, as well as competition between antibody and heparin or thrombin for binding to antithrombin III, could be helpful to evaluate this model further. The contribution of the carbohydrate chains of antithrombin III in the defined antigenic determinants, as well as the binding of the antibodies to the modified form of antithrombin III released from the complex with protease, also remain to be investigated.

Besides these structural immunochemical studies, the monoclonal antibodies produced and characterized in this report could provide valuable tools for dosage and purification of antithrombin III, allowing larger clinical trials for the use of antithrombin III as a therapeutic agent.

ACKNOWLEDGMENT

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REFERENCES


Table 3. Effect of Monoclonal Antibodies on the Antithrombin III Activity

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Effect on AT III Activity</th>
<th>Heparin Absent</th>
<th>Heparin Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(BATR 1, AATR 7)</td>
<td>Strong</td>
<td>Strong</td>
<td></td>
</tr>
<tr>
<td>Class II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(AATR 12, BATR 17, AATR 24)</td>
<td>Moderated</td>
<td>Moderated</td>
<td></td>
</tr>
<tr>
<td>Class III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AATR 20</td>
<td>No effect</td>
<td>No effect</td>
<td>Strong</td>
</tr>
</tbody>
</table>

*Although we cannot completely exclude the possibility that some monoclonal antibodies bind directly to heparin, we believe that this is rather unlikely. Indeed, we performed binding studies of monoclonal antibody AATR 20 on antithrombin III using an ELISA system on PVC Linbro plates, either in the presence or in the absence of heparin. Similar assays were carried out also, with monoclonal antibodies AATR 12 and BATR 1 as controls. The results of these assays showed that the presence of heparin did not affect the binding of monoclonal antibody AATR 20 on antithrombin III.
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Monoclonal antibodies against plasma protease inhibitors: production and characterization of 15 monoclonal antibodies against human antithrombin III. Relation between antigenic determinants and functional sites of antithrombin III

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