An Autoantibody Directed Against Human Thrombin Anion-Binding Exosite in a Patient With Arterial Thrombosis: Effects on Platelets, Endothelial Cells, and Protein C Activation

By Emmanuel Arnaud, Monique Lafay, Pascale Gaussem, Véronique Picard, Martine Jandrot-Perrus, Martine Aiach, and Francine Rendu

An autoantibody, developed by a patient with severe and recurrent arterial thrombosis, was characterized to be directed against the anion-binding exosite of thrombin, and inhibited all thrombin interactions requiring this secondary binding site without interfering with the catalytic site. The effect of the antibody was studied on thrombin interactions with platelets and endothelial cells from human umbilical veins (HUVEC). The autoantibody specifically and concentration-dependently inhibited α-thrombin-induced platelet activation and prostacyclin (PGI₂) synthesis from HUVEC. It had no effect when γ-thrombin or the thrombin receptor activation peptide SFLLR were the inducers. The effect of the antibody on protein C activation has been studied. The antibody blocked the thrombin-thrombomodulin activation of protein C. The inhibition of the activation was maximal with a low concentration of thrombomodulin. The fact that the autoantibody inhibited concentration-dependent α-thrombin-induced platelet and endothelial cell functions emphasizes the crucial role of the anion-binding exosite of thrombin to activate its receptor. In regard to the pathology, the antibody inhibited two vascular processes implicated in thrombin-antithrombotic functions, PGI₂ secretion, and protein C activation, which could be implicated in this arterial thrombotic disease.

We previously described an autoantibody specific for human thrombin in a patient presenting with unexplained severe recurrent arterial thrombosis. This antibody inhibits the interaction of thrombin with fibrinogen, fibrin, heparin cofactor II, and thrombomodulin, and has no effect on the interaction of thrombin with synthetic substrates. The study of various recombinant thrombin mutants allowed the demonstration that the antibody recognized a narrow region in the anion-binding exosite.

Antithrombin antibodies are not frequent, and are usually observed with bleeding diseases. The occurrence of such autoantibodies is usually associated with autoimmune disorders or a cross-immunization with bovine thrombin, but never with a thrombotic disease. No other immune and coagulation disorder was detected in the present case. Because abnormalities of platelets and/or the vessel wall are typically involved in arterial thrombosis, the present study was designed to evaluate the antibody effect on thrombin interactions with platelets and endothelial cells, which are both mediated by a thrombin receptor recently identified. The purified autoantibody inhibited α-thrombin-induced platelet aggregation and release, endothelial cell prostacyclin production, and thrombin-thrombomodulin protein C activation in a concentration-dependent manner.

MATERIALS AND METHODS

Case report. The patient R.D. has been described previously. Briefly, she presented with a history of recurrent arterial thrombosis without clearness of atherosclerosis, vasculitis of the arterial wall, or cardiologic disorders. In 1988, an extensive laboratory investigation showed a prolonged thrombin clotting time (TCT) performed with human thrombin with otherwise normal biochemical and immunologic tests. Further studies allowed the description of the presence of an immune inhibitor of thrombin characterized as antithrombin polyclonal IgGs specific for human thrombin, and still present at the time we prepared this report.

Purification of the antibody. Specific IgGs were obtained by a three-step procedure from the patient (IgG D), and from a normal subject (control IgGs). Plasma was diluted with 0.01 mol/L phosphate buffered saline (PBS), pH 7.8 (1:1 vol/vol), and precipitated by the addition of increasing doses of ammonium sulfate. The fractions, which precipitated between 30% and 45% of saturated ammonium sulfate, were solubilized in PBS and dialyzed overnight against 20 mmol/L Tris-HCl, 0.15 mol/L NaCl, pH 7.8. This solution was diluted 1:1 with 20 mmol/L Tris-HCl, pH 7.8, and loaded on a Q-Sepharose column in a flow-pressure liquid chromatography system (Pharmacia, Uppsala, Sweden). Protein was eluted by NaCl gradient (from 0 to 0.5 mol/L) for 40 minutes. The activity of each fraction was determined by the increase in human TCT (H-TCT). The active fractions were dialyzed and injected on Protein G-Sepharose. Non-specific protein was eluted by 20 mmol/L Tris-HCl, 2 mol/L NaCl, pH 7.4. IgGs were eluted by glycine buffer, pH 2.7, and immediately neutralized by addition of 2 mol/L Tris. Protein concentration was measured in a spectrometer Uvikon 930 (Kontron Instruments, Montigny, France) at 280 nm with a coefficient of extinction (E₁₇₅₈) of 13.58.

The active fractions were passed on a Fast desalting column (Pharmacia) and lyophilized on a Vacuum station (Virtilis Co, OSI, Elaocourt, France). Lyophilized fractions were stored at 4°C and solubilized in distilled water extemporaneously. Protein concentration was so that 0.15 mg/mL corresponded to 1 µmol/L IgGs.

Purity was tested by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 7.5% of the fractions under nonreducing conditions.

H-TCT. TCTs were performed in purified system on a STA4 automatic (Siago, Asnières, France). One hundred microliters of a solution of purified human fibrinogen (2.5 g/L) (KabiVitrum, Stockholm, Swe...
den) and 50 μL of solution of IgG were incubated for 90 seconds at 37°C. After addition of human thrombin (1 NIH-U/mL) (Stago) in 20 mmol/L Tris HCl, 0.15 mol/L NaCl, 0.5% polyethylene glycol 6,000, pH 7.8, buffer, the clotting time was recorded. The antithrombin activity was determined by the amount of thrombin neutralized, expressed as 1 U corresponding to the neutralization of 1 U-NIH thrombin. A reference curve was established by performing various TCTs in the presence of increasing concentrations of thrombin.

**Thrombin receptor-derived peptide.** The peptide SFLLR was synthesized and purified by Dr Troalen (Institut Gustave Roussy, Villejuif, France). The peptide FSLLR was used as a control.

**Platelet preparation and functions.** Blood was collected from donors who had not taken any drug for 7 days, and anticoagulated with ACD-C (12.4 mmol/L sodium citrate, 13 mmol/L citric acid, 11 mmol/L glucose; 9:1 vol/vol). Platelet-rich plasma (PRP) was obtained by 15 minutes of centrifugation of blood at 100 X g, and incubated for 20 to 30 minutes with [14C] 5-hydroxytryptamine ([14C] 5-HT) (18.5 kBq/10 mL) (Amersham, Les Ulis, France) at room temperature. Platelets were isolated by a 15-minute centrifugation at 1,100 X g and 20°C on a metrizamide gradient (Nycomed Pharma AS, Oslo, Norway). The metrizamide gradient platelets (MGP) thus obtained were adjusted to 3 X 10^8/mL in a buffer, pH 7.4 (10 mmol/L Hepes, 140 mmol/L NaCl, 3 mmol/L KCl, 5 mmol/L NaHCO3, 10 mmol/L glucose, 0.5 mmol/L MgCl2).

Platelet aggregation was recorded on an aggregometer (Chrono Log, Coultronics, Margency, France). The suspension was prewarmed to 37°C for 30 seconds before the addition of 0.1 to 10 μmol/L ADP (Stago), 5 to 12 μg/mL collagen type I (polymerized in 0.05 mol/L Tris, pH 7.8, 2 minutes at 37°C; Stago), and 0.35 to 1 mmol/L human or bovine thrombin (Stago). For platelet release, the reaction was performed in the presence of 1 mmol/L imipramine (Sigma, St Quentin Fallavier, France) and ended 2 or 3 minutes after addition of the agonist by transfer to a solution of 0.1 mol/L EDTA 1:5 (vol/vol) maintained in ice, and immediately centrifuged at 15,000g during 30 seconds. [14C] 5-HT was measured in the supernatant by liquid scintillation in a β-radiometer counter (Kontron, Trappes, France). The results were expressed as percent of [14C] 5-HT secreted to the total amount of labeled [14C] 5-HT contained in unstimulated platelets. All samples were in duplicate.

**Endothelial cell isolation and culture.** Human umbilical veins (HUVEC) were washed with PBS (GIBCO, Pontoise, France) at 37°C, and endothelial cells obtained by incubation with 0.025% collagenase (GIBCO) at 37°C in PBS buffer for 12 minutes. Cell viability was tested by 0.01% Blue trypan coloration (Sigma). Cells were grown to confluence on gelatin-coated flasks, T-25 Primaria (Falcon Dickinson, Lincoln Park, NJ) with culture medium M199/ RPMI 1640 (GIBCO) supplemented with 20% fetal calf serum (Whittaker, Fontenay-sous-Bois, France), 250 IU/mL penicillin, 100 μg/mL streptomycin, and 1 mmol/L glutamine in a 5% CO2/95% air atmosphere at 37°C. The cells were passaged with 0.025% trypsin-EDTA, and grown in six-well culture plates with the same experimental conditions. Cells were used at confluency at the second or third passage.

![Fig 1. Purification of the autoantibody on Q-Sepharose. Active ammonium sulfate precipitation fractions were dialyzed and injected on Q-Sepharose. Elution was performed with a gradient of NaCl from 0 to 0.5 mol/L during 40 minutes. Human TCT (•) and absorbance (+) were analyzed on each elution fractions.](image)

![Fig 2. SDS-PAGE in 7.5% polyacrylamide of control IgGs and IgG D (6 μmol/L) under nonreducing conditions.](image)
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Thrombin). The intensity of aggregation was expressed as the percent of light transmission measured 2 minutes after introduction of thrombin relative to that of platelet-poor plasma.

![Platelet aggregation in response to different agonists in the presence of IgG (80 μg/mL). MGP platelets were adjusted to 3 x 10⁷/mL. Control (C) or patient’s (D) IgGs were added before the agonist. Platelets were aggregated by different thrombin forms (bovine α-thrombin, human γ-thrombin, human α-thrombin). The intensity of aggregation was expressed as the percent of light transmission measured 2 minutes after introduction of thrombin relative to that of platelet-poor plasma.](image)

Prostacyclin liberation induced by various agonists. HUVEC were stimulated by various agonists during 5 minutes at room temperature. The reaction was stopped by the addition of 0.5 mL of a mixture of 50 mM acetic acid, 0.1N HCl in methanol. Prostacyclin (PGI₂) was measured in the culture supernatant by the assay of the stable analog of prostacyclin, the 6-keto prostaglandin Fl₂ (PGF₁₂), with a competitive radioimmunologic assay with [¹H] 6-keto PGF₁₂ on a scintillation proximity assay system (Amersham).

Human protein C activation. Protein C activation was determined in presence of IgG D and control IgGs in Tris HCl 20 mMol/L, NaCl 0.1 mol/L, pH 7.4, buffer, containing 0.1% bovine serum albumin (wt/vol) (Sigma). IgGs (1 to 6 μmol/L) were incubated with 3.3 mMol/L human thrombin during 5 minutes at 22°C. Rabbit thrombomodulin (American Diagnostica Inc, Greenwich, CT) was added (15 to 45 mMol/L). After 5 minutes at 37°C, the reaction was started by the addition of protein C (0.75 μmol/L; Stago) at 37°C. After 2, 4, and 8 minutes of activation, aliquots of 25 μL were taken and added to 4 μmol/L antithrombin purified as described¹ and 0.14 IU/mL heparin in a 96-well microplate, according to Zhang and Castellino.² Activated protein C generation was quantified at 22°C using 0.8 mMol/L substrate S 2366 (Chromogenix, Molndal, Sweden) at 405 nm with a kinetic program on a microplate reader MR 9000 (Dynametech Laboratories, Guyancourt, France). The initial velocity (Vi) corresponded to the slope of the linear relation between the variation of absorbance per minute and the time of incubation.

RESULTS

Purification of the autoantibody. The IgGs were first purified by chromatography on Protein G-Sepharose representing a fourfold enrichment of the antithrombin activity. However, because control IgGs isolated with the same procedure induced unspecific effects on any agonist-induced platelet aggregation, a further purification was required. This was performed on a thrombin-Sepharose column as previously described.¹ The enrichment of the immunopurified fraction was poor, and was not improved by blocking the thrombin-active site by the specific inhibitor tosyl-lysine-chloromethylketone (TLCK). Therefore, we used another strategy, taking advantage of the elution of the patient’s IgGs displaying antithrombin activity in only two of the 25 eluted fractions from a Q-Sepharose column (Fig 1). The patient’s and normal IgGs used in this study were purified by the three-step procedure described in the Methods. Ammonium sulfate precipitation, Q-Sepharose, and Protein G-Sepharose columns led to a final 22-fold enrichment. The active fraction of IgGs was included in a band corresponding to migration of antithrombin IgGs, as shown on SDS-PAGE (Fig 2). The specific activity (110 U/mg) of such an active fraction was higher than that obtained after immunopurification on active site–blocked thrombin-Sepharose column.

Effect of the autoantibody on platelet functions. Purified antithrombin IgGs had no aggregating effect by themselves, nor had any unspecific effect on ADP- or collagen-induced aggregation (data not shown). However, when human thrombin was the inducer, an inhibitory effect was observed (Fig 3). Identical inhibitory effect was measured whether the IgGs were preincubated for 10 minutes at 37°C with thrombin or were added just before thrombin. Normal IgGs were used as control, and never had any effect on any platelet function.

Forty micrometers of the patient’s IgGs inhibited 0.75 mMol/L and 1 mMol/L α-thrombin by 47% and 50% for aggregation and 70% and 60% for secretion, respectively. By contrast, it had no effect when γ-thrombin was the agonist. The inhibitory effect on α-thrombin was increased with increasing IgGs concentrations (Fig 4). Using 120 μg/mL of IgGs (0.8 μmol/L) resulted in an almost complete inhibition of aggregation and secretion of 5-hydroxytryptamine. By contrast, whatever the concentration of control IgGs used, no inhibition was ever observed on γ-thrombin–induced aggregation and release (Fig 5).

The peptide SFLLR described as the receptor amino acid sequence responsible for activation, was also used as an aggregation agonist to further characterize the effect of the patient’s IgGs on the interaction of thrombin with its platelet receptor. Using a 20-μmol/L concentration of the peptide SFLLR, aggregation and [¹⁴C] 5-HT release reached similar
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10 nmol/L α-thrombin. Such a PGI₂ production was observed in the presence of histamine, a potent stimulator used as a control agonist. γ-Thrombin also stimulated HUVEC to produce PGI₂, but to a lower level because 50 nmol/L γ-thrombin produced twice less PGI₂ than did 10 nmol/L α-thrombin.

Fig 4. Dose effect of IgG D on α-thrombin-induced platelet responses. MGP platelets (3.10⁸/µL) were stimulated by 1 nmol/L human α-thrombin after the addition of increasing doses of control or patient’s IgGs. The platelet responses were measured 2 minutes after the addition of α-thrombin. Results are the mean (±SD) of seven experiments. (A) Platelet aggregation. (B) [¹⁴C]5-HT platelet release.

Fig 5. Dose effect of IgG D on γ-thrombin-induced platelet aggregation and release. MGP platelets (3.10⁸/µL) were stimulated by 1 nmol/L human γ-thrombin after the addition of increasing doses of control or patient’s IgGs. The platelet responses were measured 3 minutes after the addition of γ-thrombin. Results are the mean (±SD) of seven experiments. (A) Platelet aggregation. (B) [¹⁴C]5-HT platelet release.

Effect of the autoantibody on endothelial cells. Basal PGI₂ production by HUVEC, measured as the 6-keto PGF₁α, was increased between five to eight times in the presence of

extents as in the presence of 1 nmol/L α-thrombin (Fig 6). No inhibitory effect of patient’s IgGs (80 µg/mL) was observed, not even with higher IgG concentrations (120 or 160 µg/mL) (data not shown). The peptide FSLLR, used as a control, induced no aggregation (data not shown).
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**Fig 6.** Dose effect of IgG D on TRAP-induced platelet responses. MGP platelets \(3 \times 10^8\) plaq/μL were stimulated by 20 μmol/L of thrombin receptor agonist peptide after the addition of 80 μg/mL of control or patient IgGs. The platelet responses were measured 3 minutes after the addition of TRAP. Results are the mean (±SD) of three experiments. (A) Platelet aggregation. (B) [14C] 5-HT platelet release.

In the presence of 2 mg/mL (13 μmol/L) of the patient's IgGs, only the α-thrombin–induced PGI2 was completely inhibited. When γ-thrombin (or histamine) was the inducer, the PGI2 production was unaffected by the presence of the autoantibody (Fig 7).

**Effect of the autoantibody on thrombin-thrombomodulin activation of protein C.** The effect of purified IgGs on protein C activation was first determined in the presence of thrombomodulin 15 nmol/L (Fig 8). The patient's IgGs inhibited protein C activation in a concentration-dependent manner. Almost complete inhibition was observed with 0.9 mg/mL of the patient's IgGs (6 μmol/L), corresponding approximately to the circulating concentration previously evaluated as 5% of the total IgGs.

The effect of different concentrations of thrombomodulin on the inhibition of protein C activation by the patient's IgGs (6 μmol/L) is shown in Fig 9. The patient's IgGs inhibited protein C activation up to 45 nmol/L of thrombomodulin, a concentration above which the antibody no longer had an inhibitory effect. These results show that the antibody-dependent inhibition of protein C activation depends on the number of available thrombomodulin molecules.

**DISCUSSION**

The autoantibody studied in the present report was found in a patient suffering from severe arterial thrombosis, and was characterized to be directed specifically against human thrombin. The effects of the autoantibody on thrombin-plasma proteins interactions indicated that it was directed against the anion-binding exosite, and a further study using thrombin mutants allowed the demonstration that the domain of thrombin recognized by the autoantibody clearly overlaps the anion-binding exosite.

Thrombin is a serine protease, generated at the end of a series of enzymatic reactions caused by a vascular lesion, known to play a crucial role in hemostasis. It transforms fibrinogen in fibrin, aggregates platelets, and favors its own production by activating several coagulation factors. At the same time, it is able to downregulate its own production by binding to thrombomodulin on the endothelium surface to activate the coagulation inhibitor protein C, and to activate endothelial cell PGI2 production. Thrombin thus interacts both with cells and circulating proteins, all of which are interactions that require the integrity of the thrombin molecule, ie, a recognition site or anion-binding exosite, and a catalytic domain.

Cellular responses to thrombin are consecutive to the cleavage of the thrombin receptor. Indeed, the thrombin receptor is now well described with its seven transmembrane domains, and a sequence in the N-terminal part homologous to the C-terminal end of hirudin. Thrombin recognition of its receptor occurs through the anion-binding exosite, which bears the specificity, whereas the information for activation is given by the cleavage of the receptor by the catalytic domain of thrombin. The interaction of thrombin with the receptor therefore removes the anion-binding site, which becomes the agonist.

In the case presented here, the autoantibody inhibited specifically α-thrombin–induced platelet aggregation, but had no effect when the synthetic peptide SFLLR or γ-thrombin, the autoproteolytically cleaved thrombin that has lost the anion-binding site, were the agonists. Such a reduction in α-thrombin–induced platelet aggregation was previously shown in conditions in which the anion-binding site was blocked by the presence of the C-terminal sequence of hirudin. Similarly, in the presence of the patient's IgGs, the
PG12 production by in vitro–cultured endothelial cells stimulated by α-thrombin was strongly and concentration-dependently reduced, whereas the patient’s IgGs had no effect when γ-thrombin was the inducer. It is of note that in our conditions, and in the absence of IgGs, a significant amount of PG12 was produced by 50 nmol/L γ-thrombin, a production which has been reported only in response to much higher γ-thrombin concentrations.17

Thus, the antibody rendered thrombin less effective for the activation of its receptor, both on platelets and on endothelial cells, and a similar thrombin/antibody ratio was necessary (1:1,000) to get complete inhibition. The epitope recognized by the antibody involves His 66, Arg 68, and Arg 70, and the sequence Pro 23–Glu 24–Glu 25 of the B-chain of thrombin, which corresponds to a narrow region in the anion-binding exosite.2 Such a specificity renders the antibody pertinent for studying the role of the anion exosite of thrombin in the activation of its receptor. Moreover, the fact that the inhibition was concentration-dependent implies that the graded blocking of the anion-binding exosite leads to the graded response of the receptor to thrombin, as recently demonstrated.18,19

In vitro, the autoantibody delayed all procoagulant actions of thrombin, such as fibrin formation and platelet aggregation, but also the PG12 production from endothelial cells. Although PG12 is the most powerful antiaggregant, the inhibition of its production cannot by itself explain thrombotic events,7,20 as severe as those observed in our patient. Thrombin interaction with other molecules of the endothelial cell surface, such as thrombomodulin, may also be involved in the pathology.

Indeed, the autoantibody dose-dependently prevented the
.activation of protein C by thrombin in the presence of thrombomodulin. Moreover, the antibody competed with thrombomodulin for binding on thrombin anion exosite, increasing thrombomodulin concentration overcame the effect of the antibody. The amount of thrombomodulin expressed on the endothelial cell surface decreases on various stimulation, such as tumor necrosis factor, interleukin-1β, and endotoxin. In such conditions, the antibody could compete with thrombomodulin for protein C activation, and consequently render the vascular antithrombotic response to procoagulant stimuli less effective. In addition, differences in thrombomodulin expression among various endothelia in the body may also explain the localization of the patient’s thrombosis to large arteries.

In conclusion, this antithrombin autoantibody, first detected with a prolonged TCT in the presence of human thrombin, inhibited PG(12) secretion and protein C activation subsequent to thrombin formation on the vascular surface, two major processes, the inhibition of which represents a risk factor for the patient to develop thrombosis.

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E Arnaud, M Lafay, P Gaussem, V Picard, M Jandrot-Perrus, M Aiach and F Rendu