Anticoagulant Activities of a Monoclonal Antibody That Binds to Exosite II of Thrombin†

Fangru Lian,‡ Li He,‡ Niall S. Colwell,‡,§ Pete Lollar,‖ and Douglas M. Tollefsen*,‡

Division of Hematology, Departments of Internal Medicine and of Biochemistry & Molecular Biophysics, Washington University, St. Louis, Missouri 63110, and Division of Hematology−Oncology, Department of Medicine, Emory University School of Medicine, Atlanta, Georgia 30322

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ABSTRACT: A monoclonal IgG isolated from a patient with multiple myeloma has been shown to bind to exosite II of thrombin, prolong both the thrombin time and the activated partial thromboplastin time (aPTT) when added to normal plasma, and alter the kinetics of hydrolysis of synthetic peptide substrates. Although the IgG does not affect cleavage of fibrinogen by thrombin, it increases the rate of inhibition of thrombin by purified antithrombin ~3-fold. Experiments with plasma immunodepleted of antithrombin or heparin cofactor II confirm that prolongation of the thrombin time requires antithrombin. By contrast, prolongation of the aPTT requires neither antithrombin nor heparin cofactor II. The IgG delays clotting of plasma initiated by purified factor IXa but has much less of an effect on clotting initiated by factor Xa. In a purified system, the IgG decreases the rate of activation of factor VIII by thrombin. These studies indicate that binding of a monoclonal IgG to exosite II prolongs the thrombin time indirectly by accelerating the thrombin−antithrombin reaction and may prolong the aPTT by interfering with activation of factor VIII, thereby diminishing the catalytic activity of the factor IXa/VIIIa complex.

Recently, we reported the first example of a monoclonal antithrombin IgG in a patient with multiple myeloma (*2*). We found that the monoclonal IgG binds to human α-, *β*-, and *γ*-thrombin, as well as to bovine thrombin; by contrast, it does not bind to prothrombin, other vitamin K-dependent coagulation factors, or fibrinogen. Using a panel of 55 surface mutants of recombinant thrombin, we demonstrated that the epitope for the IgG includes Arg-101, Arg-233, and Lys-236[1] in anion-binding exosite II (*3*). This result explained the inability of the IgG to bind to prothrombin, since exosite II is covered by the zymogen's fragment 2 domain. We also found that the IgG at low concentrations alters the kinetics of hydrolysis of various peptide *p*-nitroanilide substrates. Thus, binding of the monoclonal IgG to exosite II induces a conformational change in the catalytic site of thrombin.

On several occasions, when the concentration of the monoclonal IgG reached ~5 g/dL (330 *μ*M), the patient with myeloma experienced life-threatening bleeding and had marked prolongation of the thrombin time and the activated partial thromboplastin time (aPTT)[2] but only slight prolongation of the prothrombin time (PT). The bleeding would subside after the IgG concentration was reduced to <2 g/dL by plasmapheresis. Cessation of bleeding was accompanied by partial correction of the thrombin time without an appreciable change in the aPTT. Further study revealed that the reptilase time was also prolonged in the presence of high concentrations of the IgG, suggesting an effect on fibrin monomer polymerization independent of binding of the IgG to thrombin (*2*). However, the thrombin time was prolonged to a much greater degree than was the reptilase time. When the IgG concentration was decreased to <2 g/dL (130 *μ*M) by dilution with normal plasma in vitro, the reptilase time corrected while the thrombin time remained prolonged. These results suggested that high concentrations of the IgG decrease the rate of fibrin monomer polymerization but that lower concentrations inhibit the coagulant activity thrombin.

In this study, we investigated the mechanism by which low concentrations of the monoclonal IgG prolong the thrombin time and the aPTT of plasma. We were surprised to find that the IgG does not alter the ability of thrombin to clot purified fibrinogen. Rather, binding of the IgG to thrombin accelerates the thrombin−antithrombin reaction and thereby prolongs the thrombin time indirectly. Furthermore, the IgG inhibits activation of factor VIII by thrombin and may prolong the aPTT by interfering with formation of the factor IXa/VIIIa complex.

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* To whom correspondence should be addressed at the Division of Hematology, Box 8125, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110. Tel: 314-362-8830. Fax: 314-362-8826. E-mail: tollefsen@im.wustl.edu.

‡ Washington University.

§ Present address: Department of Pharmacology and Therapeutics, University College, Cork, Ireland.

‖ Emory University School of Medicine.

[1] Thrombin residues are numbered throughout the text according to their topological equivalence with chymotrypsinogen, as suggested by Bode et al. (*1*).

[2] Abbreviations: aPTT, activated partial thromboplastin time; PT, prothrombin time; HCII, heparin cofactor II; PCPS, phosphatidylcholine/phosphatidylserine vesicles; TSP, Tris/NaCl/poly(ethylene glycol) buffer.

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EXPERIMENTAL PROCEDURES

Materials. The monoclonal human IgG and Fab fragments were prepared as previously described (*3*). Human α-thrombin, factor Xa, and fibrinogen were purchased from Haematologic Technologies (Essex Junction, VT). Purified thrombin mutants were generously provided by Drs. Scott W. Hall and Lawrence L. K. Leung, Stanford University, Stanford, CA (*4*). Published procedures were used to isolate human factor IX (*5*), human factor X (*6*), and human von Willebrand factor (*7*). Human factor IXa was purchased from Haematologic Technologies or isolated as described (*8*). Albumin-free human recombinant factor VIII was a gift from Baxter Biotech. Human antithrombin was purified by heparin−agarose chromatography as described previously (*9*). Heparin cofactor II (HCII) was isolated from human plasma by immunoaffinity chromatography using sheep anti-HCII IgG (Affinity Biologicals, Hamilton, Ontario, Canada) according to the manufacturer's protocol and purified to homogeneity by chromatography on a Mono-Q column. Tosyl-Gly-Pro-Arg-*p*-nitroanilide (Chromozym TH) and MeO-CO-cyclohexylglycyl-Gly-Arg-*p*-nitroanilide (Spectrozyme Xa) were obtained from Boehringer Mannheim (Indianapolis, IN) and American Diagnostica (Greenwich, CT), respectively. Normal human plasma and plasma depleted of antithrombin and/or HCII using antibodies immobilized on agarose beads were purchased from Affinity Biologicals; the depleted plasmas contained <1% antithrombin and/or HCII antigen as measured by ELISA and had normal assays for aPTT, PT, and fibrinogen. Rabbit brain cephalin was obtained from Pel-Freez Biologicals (Rogers, AR). Unilamellar 75% phosphatidylcholine/25% phosphatidylserine (w/w) (PCPS) vesicles were prepared as described (*10*). Hexadimethrine bromide (Polybrene) was purchased from Sigma (St. Louis, MO).

Coagulation Assays. Clotting times were determined with a fibrometer (Becton Dickinson, Sparks, MD) at 37 °C. Reagents were diluted in 0.15 M NaCl, 0.05 M Tris-HCl, 1 mg/mL poly(ethylene glycol) 8000, pH 7.4 (TSP buffer). The thrombin time of normal or inhibitor-depleted plasma was determined by preincubation of 100 μL of plasma with or without 1 μL of IgG or Fab (various concentrations) for 1 min followed by addition of 100 μL of thrombin (10 nM). In other experiments, 75 μL of fibrinogen (4 mg/mL) was preincubated for 1 min with or without 3 μL of IgG (18.7 mg/mL), 30 μL of antithrombin (35 μM), and 3 μL of Polybrene (2.5 mg/mL) in a final volume of 270 μL of TSP buffer. Coagulation was initiated by addition of 30 μL of thrombin (50 nM). Polybrene was used to neutralize a trace amount of heparin present in the antithrombin preparation. The aPTT was determined by preincubation of 100 μL of plasma and 100 μL of APTT-FS reagent (#A2176, Sigma Diagnostics, St. Louis, MO) with or without 1 μL of IgG (18.7 mg/mL) for 3 min, followed by addition of 100 μL of prewarmed $CaCl_2$ (20 mM) to initiate coagulation. To determine the clotting time after addition of factor Xa or IXa, 100 μL of plasma and 60 μL of rabbit brain cephalin (diluted 1:10 from stock with 0.15 M NaCl, 0.01 M Tris-HCl, pH 7.4) were preincubated with or without 1 μL of IgG (18.7 mg/mL) for 1 min. Then 40 μL of factor Xa (0.38−3.2 nM) or factor IXa (9.2−290 nM) was added, followed immediately by 100 μL of $CaCl_2$ (20 mM) to initiate coagulation.

Kinetics of Protease Inhibition. Reactions were performed at room temperature in polystyrene cuvettes. Inhibition of thrombin was assayed in incubations containing 10 nM thrombin, 0.5 μM antithrombin or 0.25 μM HCII, and 25 μg/mL Polybrene, with or without 187 μg/mL IgG, in a final volume of 100 μL of TSP buffer. Thrombin was added last to initiate the reaction. At various time points (1−50 min), 500 μL of Chromozym TH (100 μM in TSP buffer) was added, and the rate of change of absorbance at 405 nm was determined. Factor Xa inhibition was assayed in incubations containing 10 nM factor Xa, 1.8 μM antithrombin, and 25 μg/mL Polybrene, with or without 187 μg/mL IgG, in a final volume of 100 μL of TSP buffer. At various times after addition of factor Xa (2−10 min), 500 μL of Spectrozyme FXa (100 μM in TSP buffer) was added, and the rate of change of the absorbance at 405 nm was determined. The pseudo-first-order rate constant for inhibition (k_{obs}) was obtained by fitting the data to the equation: $\ln [E]_t = -k_{obs}t$, in which $[E]_t$ is the protease activity at time t. The second-order rate constant (k) was calculated from the equation: $k = k_{obs}/[I]_0$, in which $[I]_0$ is the initial concentration of the inhibitor.

Factor VIII Activation Assay. Factor VIII (1 nM) was incubated with 0.2 nM thrombin and various concentrations of monoclonal Fab in 0.15 M NaCl, 20 mM Hepes, 5 mM $CaCl_2$, 1 mg/mL poly(ethyleneglycol) 8000, pH 7.4, at room temperature in the presence or absence of 200 nM von Willebrand factor. After 5 min, the mixture was diluted in the same buffer to 0.8 nM factor VIII in 2.5 nM factor IXa, 20 μM PCPS, and 200 nM factor X. The initial rate of factor X activation was measured using a chromogenic substrate for factor Xa, MeO-CO-cyclohexylglycyl-Gly-Arg-*p*-nitroanilide, as described previously (*6*). Under the conditions of the assay, the initial rate of factor X activation is linearly proportional to the concentration of activated factor VIII (factor VIIIa).

RESULTS

Addition of the monoclonal antithrombin IgG to normal human plasma prolonged the clotting time induced by 5 nM thrombin, and Fab fragments derived from the monoclonal IgG had a similar effect (Figure 1). With both antibody preparations, the thrombin time reached a plateau at about twice the control value. The half-maximal effect occurred in the presence of approximately 20 nM IgG or 50 nM Fab. Initially, we considered the possibility that the antibody interferes with the ability of thrombin to cleave fibrinogen. However, the IgG did not prolong the thrombin time of purified human fibrinogen (Table 1), which indicates that neither fibrinogen cleavage nor fibrin monomer polymerization is inhibited significantly at the relatively low concentration of IgG (1.2 μM) used in this experiment. Previously, we found that much higher concentrations of the IgG (≥130 μM) prolong the reptilase time of plasma, apparently by interfering with fibrin monomer polymerization (*2*).

Addition of the monoclonal IgG to a mixture of fibrinogen and antithrombin, each present at approximately its normal plasma concentration, prolonged the thrombin time to an

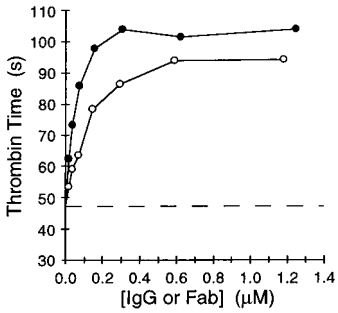

FIGURE 1: Effect of the monoclonal IgG on the thrombin time. Normal human plasma was supplemented with monoclonal IgG or Fab fragments at the concentrations indicated, and clotting was initiated by addition of an equal volume of 10 nM thrombin. The data represent the average of duplicate determinations. The dashed line indicates the control clotting time (without antibody). (●) +IgG; (○) +Fab.

Table 1: Thrombin Times (s)[a]

	−IgG	+IgG
experiment 1		
fibrinogen	55.3 ± 0.5	53.6 ± 4.2
fibrinogen + antithrombin + Polybrene	60.5 ± 1.6	147.9 ± 12.1
experiment 2		
parent plasma	57.4 ± 7.8	136.5 ± 23.6
antithrombin-depleted plasma	47.0 ± 0.6	52.3 ± 0.4
HCII-depleted plasma	56.1 ± 2.0	131.5 ± 23.8

[a] Clotting times after addition of thrombin are expressed as the mean ± 1 SD of 3−4 determinations. The final concentrations of reagents in experiment 1 were as follows: fibrinogen, 1 mg/mL; antithrombin, 3.5 μM; Polybrene, 25 μg/mL; monoclonal IgG, 1.2 μM; and thrombin, 5 nM. In experiment 2, plasma was supplemented with monoclonal IgG at a concentration of 1.2 μM, and clotting was initiated by addition of an equal volume of 10 nM thrombin. Parent plasma is the starting plasma from which antithrombin or HCII was removed by immunoabsorption. See Experimental Procedures for details.

extent similar to that observed after addition of the IgG to plasma (Table 1). This experiment indicates that the IgG can promote the inhibition of thrombin by antithrombin in a purified system. To determine whether prolongation of the thrombin time by the IgG in plasma requires antithrombin, we conducted experiments with plasma from which the antithrombin had been removed by immunoabsorption. The data in Table 1 show that the IgG had little effect on the thrombin time of antithrombin-depleted plasma but prolonged the thrombin time of both heparin cofactor II (HCII)-depleted plasma and parent plasma (i.e., the starting material for preparation of both inhibitor-depleted plasmas). Therefore, prolongation of the thrombin time in plasma by the monoclonal IgG requires antithrombin but not HCII.

Figure 2 (panel A) shows the effect of the monoclonal IgG on the time course of inhibition of thrombin by purified antithrombin. This experiment was performed under pseudo-first-order conditions (i.e., with a molar excess of antithrombin) in the absence of heparin. The monoclonal IgG increased the second-order rate constant for inhibition of thrombin approximately 2.7-fold, from 2.2×10^5 to 5.9×10^5 M^{-1} min^{-1}. In a parallel experiment (Figure 2, panel B), the IgG had no effect on the rate constant for inhibition of factor Xa (5.3×10^4 M^{-1} min^{-1}), which is consistent with the observation that the monoclonal IgG does not bind to factor Xa (*2*). Figure 2 (panel C) shows that the IgG also increased the rate constant for inhibition of thrombin by purified HCII from 0.6×10^5 to 1.0×10^5 M^{-1} min^{-1} in the absence of a

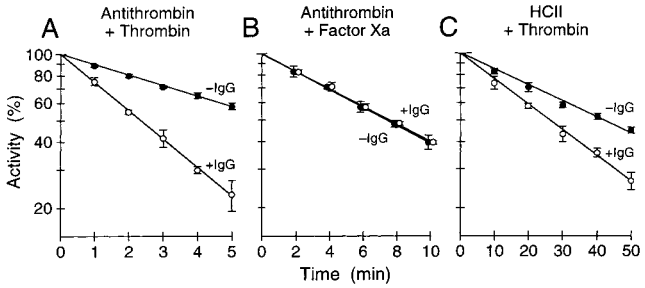

FIGURE 2: Time courses of protease inhibition by antithrombin and HCII. Incubations contained 0.5 μM antithrombin, 25 μg/mL Polybrene, and 10 nM thrombin (panel A); 1.8 μM antithrombin, 25 μg/mL Polybrene, and 10 nM factor Xa (panel B); or 0.25 μM HCII and 10 nM thrombin (panel C). The concentration of IgG in each experiment was 1.2 μM. At various times after addition of thrombin or factor Xa, the amidolytic activity was determined with a chromogenic substrate as described under Experimental Procedures. The data are expressed as percentages of the activity determined in the absence of antithrombin or HCII. The error bars indicate the mean ± 1 SD of 3 determinations. (●) −IgG; (○) +IgG.

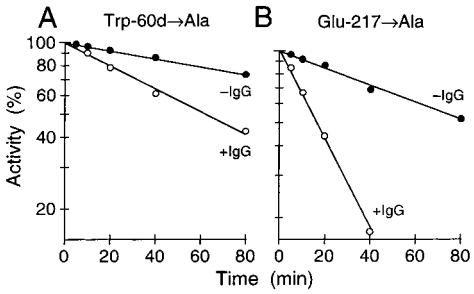

FIGURE 3: Effect of mutations flanking the catalytic site of thrombin. Incubations were performed as described in the legend to Figure 2 (panel A). (●) −IgG; (○) +IgG.

glycosaminoglycan. In control experiments (not shown), polyclonal human IgG had no effect on the rate of inhibition of thrombin by antithrombin or HCII.

Substitutions of alanine for Trp-60d and Glu-217, which flank the catalytic site of thrombin, have been reported to decrease the rate of inhibition by antithrombin in the absence of heparin by approximately 1 order of magnitude (*11*, *12*). Figure 3 shows the kinetics of inhibition of these thrombin variants by antithrombin in the absence or presence of the monoclonal IgG. The IgG increased the rate constant from 0.8×10^4 to 2.2×10^4 M^{-1} min^{-1} for the Trp-60d variant and from 1.7×10^4 to 8.3×10^4 M^{-1} min^{-1} for the Glu-217 variant. Thus, these mutations do not affect the ability of the IgG to stimulate the thrombin−antithrombin reaction, even though the basal rate of inhibition is much lower than that of wild-type thrombin (2.2×10^5 M^{-1} min^{-1}; see Figure 2, panel A).

The monoclonal IgG and Fab fragments derived from it prolonged the aPTT of normal human plasma as shown in Figure 4. The effect appeared to saturate over the range of IgG and Fab concentrations used in this experiment (0−1.2 μM). At a concentration of 1.2 μM, the IgG caused a similar degree of prolongation of the aPTT in normal plasma as compared with plasma lacking either antithrombin or HCII (Table 2). Thus, in contrast to the thrombin time, prolongation of the aPTT requires neither antithrombin nor HCII.

To identify steps in the intrinsic and common coagulation pathways that are inhibited by the monoclonal IgG, we

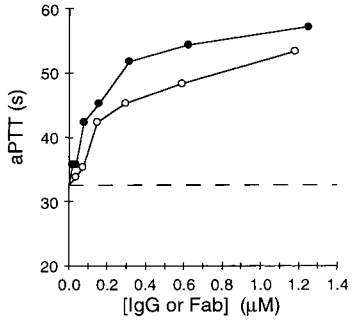

FIGURE 4: Effect of the monoclonal IgG on the aPTT. Normal human plasma was supplemented with monoclonal IgG or Fab fragments at the concentrations indicated, and aPTT assays were performed as described under Experimental Procedures. The data represent the average of duplicate determinations. The dashed line indicates the control clotting time (without antibody). (●) +IgG; (○) +Fab.

Table 2: Activated Partial Thromboplastin Times (aPTT) (s)[a]

	−IgG	+IgG
parent plasma	34.3 ± 0.4	51.0 ± 0.8
antithrombin-depleted plasma	33.5 ± 1.3	51.0 ± 0.3
HCII-depleted plasma	34.9 ± 2.0	54.4 ± 1.1

[a] Clotting times are expressed as the mean ± 1 SD of 4 determinations. The concentration of monoclonal IgG in plasma, prior to the addition of aPTT reagent and $CaCl_2$, was 1.2 μM.

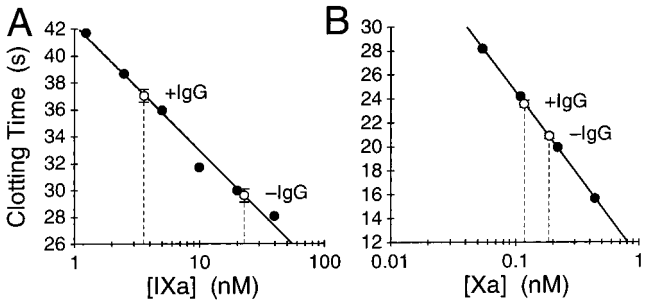

FIGURE 5: Factor IXa and factor Xa clotting times. Plasma depleted of both antithrombin and HCII was supplemented with monoclonal IgG at a concentration of 1.2 μM, and the clotting time was determined after addition of rabbit brain cephalin, $CaCl_2$, and factor IXa (panel A) or factor Xa (panel B) at the final concentrations indicated. See Experimental Procedures for details. The closed circles show standard curves determined with various concentrations of each protease in the absence of the monoclonal IgG. The open circles and error bars indicate the mean ± 1 SD of 3 determinations made after addition of 23 nM factor IXa (panel A) or 0.19 nM factor Xa (panel B) to plasma in the presence or absence of the IgG. As indicated by the dashed lines, the apparent activity of factor IXa was reduced to 3.6 nM (84% decrease) in the presence of the IgG, while the apparent activity of factor Xa was reduced to 0.12 nM (37% decrease).

determined the clotting times initiated by either purified factor IXa or factor Xa in the presence and absence of the IgG. The clotting time after addition of factor IXa to antithrombin/HCII-depleted plasma increased from 29.6 ± 0.5 s (mean ± 1 SD) in the absence of the IgG to 37.1 ± 0.5 s in the presence of the IgG. The mean values are plotted in Figure 5 (panel A, open circles) along with a standard curve generated with various known concentrations of factor IXa (closed circles). The IgG had the net effect of decreasing the apparent factor IXa clotting activity by 84%. In similar experiments with factor Xa, the clotting time increased from 20.9 ± 0.2 s in the absence of the IgG to 23.6 ± 0.3 s in the presence of the IgG. From the graph in Figure 5 (panel B), the IgG appeared to decrease the factor Xa clotting activity by 37%. The predominant effect of the monoclonal IgG on the aPTT, therefore, appears to occur at the level of factor IXa.

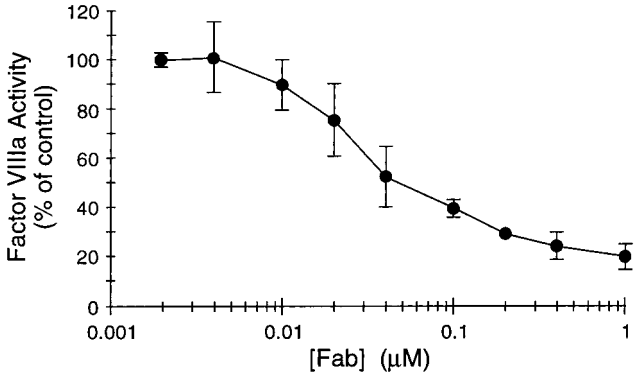

FIGURE 6: Activation of factor VIII by thrombin. Factor VIII (1 nM) was activated with 0.2 nM thrombin in the presence of 200 nM von Willebrand factor and various concentrations of monoclonal Fab fragments as described under Experimental Procedures. The data are expressed as percentages of the control value obtained in the absence of Fab. The mean value and range of duplicate determinations are shown.

Factor VIII is converted to VIIIa by thrombin during the aPTT reaction, and factor VIIIa is required for efficient activation of factor X by factor IXa. Diminished activation of factor VIII, therefore, can prolong the factor IXa clotting time. Figure 6 shows that monoclonal antithrombin Fab fragments inhibited thrombin-induced activation of factor VIII by ~80% when assayed in the presence of von Willebrand factor, which binds and stabilizes factor VIII. The half-maximal effect occurred at ~30 nM Fab. Qualitatively similar results were obtained in experiments performed in the absence of von Willebrand factor (not shown), although the degree of inhibition appeared to be somewhat less.

DISCUSSION

Anion-binding exosites I and II are two patches of positively charged amino acid residues that lie on the surface of thrombin, each separated from the catalytic site by ~20 Å. The exosites facilitate interactions of thrombin with other macromolecules. For example, exosite I binds fibrinogen, the thrombin receptor, thrombomodulin, hirudin, and heparin cofactor II, whereas exosite II binds glycosaminoglycans, prothrombin fragment 2, factor V, and factor VIII (*13, 14*). Binding of thrombomodulin or other ligands to exosite I alters the catalytic activity of thrombin (*15*−*17*). Ligands that bind to exosite II also produce allosteric changes that can be detected with active site directed fluorescence probes (*8, 18*) or functional assays (*19*−*22*). The monoclonal IgG used in this study binds to Arg-233 and nearby residues in exosite II and alters the kinetics of hydrolysis of various peptide *p*-nitroanilide substrates (*3*). The effects on catalytic activity produced by the IgG differ qualitatively from those produced by heparin or prothrombin fragment 2 (see ref *3* for details). Thus, various ligands that bind to exosite II can have different effects on the conformation of the catalytic site.

The allosteric effect induced by binding of the monoclonal IgG to thrombin appears to be responsible for increasing the

rate of inhibition by antithrombin. The control experiment with factor Xa, to which the IgG does not bind, argues against a direct stimulatory effect of the IgG on antithrombin (Figure 2, panel B). Thrombin is inhibited when it attacks the reactive site loop of antithrombin, resulting in cleavage of the P1−P1′ peptide bond and formation of an acyl ester between the P1 residue and the active site serine of thrombin. Kinetically, the thrombin−antithrombin reaction involves the initial formation of an encounter complex with a high equilibrium binding constant (K_d = 1.4 mM) followed by rapid conversion to the stable complex (k_2 = 10 s^{-1}) (*23*). Heparin accelerates the reaction by lowering the K_d approximately 1000-fold. We previously showed that the IgG increases the selectivity of thrombin for the substrate MeO-CO-cyclohexylglycyl-Gly-Arg-*p*-nitroanilide as indicated by a 2.6-fold increase in the k_{cat}/K_m, the primary effect being a 1.8-fold increase in the k_{cat} value (*3*). This substrate mimicks the reactive site of antithrombin, which contains the sequence Gly-Arg-Ser (P2-P1-P1′). By analogy, the IgG might increase the k_2 of the thrombin−antithrombin reaction, although additional experiments would be necessary to rule out an effect on the K_d. The absence of an effect of the IgG on fibrinogen clotting could be related to the amino acid sequences of thrombin cleavage sites in the fibrinogen Aα and Bβ chains (Val-Arg-Gly and Ala-Arg-Gly, P2-P1-P1′), which might be insensitive to the allosteric change induced by the IgG. Alternatively, binding of fibrinogen to exosite I could negate the allosteric effect produced by the IgG.

Trp-60d and Glu-217 lie on opposite sides of the active site cleft of thrombin and are essential for normal inhibition by antithrombin. Thus, these residues could potentially mediate the allosteric effect of the IgG on the thrombin−antithrombin reaction. Replacement of Trp-60d by alanine increases the initial affinity of thrombin for antithrombin about 100-fold (K_d = 13.6 μM) but dramatically lowers the rate of stable complex formation (k_2 = 0.007 s^{-1}) (*11*). The net effect is a 13-fold reduction in the second-order rate constant for inhibition (k_2/K_d). Similarly, substitution of alanine for Glu-217 decreases the apparent second-order rate constant about 30-fold (*12*). The experiment in Figure 3 indicates that facilitation of the thrombin−antithrombin reaction by the IgG is independent of Trp-60d and Glu-217. The result with the Trp-60d mutant is consistent with our previous finding that deletion of Pro-60b, Pro-60c, and Trp-60d in thrombin (desPPW) does not affect the ability of the monoclonal antibody to increase the rate of hydrolysis of MeO-CO-cyclohexylglycyl-Gly-Arg-*p*-nitroanilide (*3*). Therefore, other amino acids near the catalytic site appear to be responsible for mediating the allosteric effect of the IgG.

Prolongation of the thrombin time of plasma containing the monoclonal IgG is primarily due to stimulation of the thrombin−antithrombin reaction. Although the IgG also increases the rate constant for inhibition of thrombin by HCII, the contribution of this effect is relatively minor, because HCII is present in plasma at a lower concentration and inhibits thrombin at a lower rate in comparison to antithrombin (*24*). Based on the rate constants derived from the data in Figure 2, the half-time for inhibition of thrombin by antithrombin at a plasma concentration of 2.5 μM decreases from ∼75 to ∼30 s in the presence of the IgG, whereas the half-time for inhibition by HCII at a plasma concentration of 1.2 μM decreases from ∼580 to ∼350 s. Thus, stimulation of the thrombin−HCII reaction by the IgG is insufficient to prolong the thrombin time of antithrombin-depleted plasma (Table 1). Furthermore, the data in Table 1 indicate that the thrombin time of antithrombin-depleted plasma is shorter than that of parent plasma and suggest that, even in the absence of the IgG, some of the thrombin added to parent plasma is inhibited by antithrombin before clotting occurs.

In addition to stimulating the thrombin−antithrombin reaction, the monoclonal IgG interferes with the ability of thrombin to activate factor VIII (Figure 6). Although a conformational change in the active site of thrombin may contribute to this effect, the IgG probably competes directly with factor VIII for binding to thrombin. Consistent with this view, replacement of Arg-93, Arg-97, and Arg-101 in exosite II by alanine markedly reduces the ability of thrombin to activate factor VIII (*14*). Arg-101 appears to be part of the epitope for the IgG (*3*), and nearby residues including Arg-93 and Arg-97 are also likely to be blocked by the antibody. Prolongation of the aPTT of plasma containing the IgG does not depend on the presence of antithrombin or HCII (Table 2) and could result from impaired feedback activation of factor VIII by thrombin. Factor VIIIa is required for efficient activation of factor X by factor IXa, while factor Va serves as the cofactor for activation of prothrombin by factor Xa. The hypothesis that the IgG interferes primarily with the ability of thrombin to activate factor VIII in the aPTT assay is supported by the observation that the IgG inhibits clotting induced by factor IXa to a greater degree than that induced by factor Xa (Figure 5). The observation that the PT is only slightly prolonged by the IgG is also consistent with this hypothesis. The relatively minor inhibitory effect of the IgG on clotting induced by factor Xa could potentially be due to inhibition of factor V activation by thrombin, although exosite II does not appear to be involved in the rate-limiting proteolytic step of this reaction (*14*).

In summary, we have shown that a monoclonal IgG that binds to exosite II of thrombin increases the rate of inhibition of thrombin by antithrombin and decreases the rate at which thrombin activates factor VIII. The IgG increases the thrombin time and the aPTT of normal human plasma to approximately twice their baseline values in a dose-dependent manner, and these effects saturate at concentrations of IgG in the range of 0.1−1.0 μM. At much higher concentrations (≥130 μM), the IgG also prolongs the reptilase time by interfering with fibrin monomer polymerization, which has been reported to be relatively common in patients with multiple myeloma (*25*). When first identified, the IgG was present in the patient's plasma at a concentration of ∼0.4 g/dL (27 μM), and the aPTT was prolonged to 63.9 s (normal 24.1−32.3 s), but the patient did not have spontaneous bleeding and underwent coronary artery bypass surgery without excessive blood loss. At this concentration of the monoclonal IgG, the aPTT was within the range usually considered to be therapeutic for a patient receiving intravenous heparin. Only when the IgG concentration increased to ∼5 g/dL (330 μM) did life-threatening bleeding occur, presumably due to the effects of the IgG on the thrombin time and aPTT combined with the effect on fibrin monomer polymerization. Thus, a novel anticoagulant agent could be developed that selectively targets exosite II of thrombin and thereby prolongs the thrombin time and aPTT in a manner similar to that of the monoclonal IgG. Whether the effects

produced by the IgG on the thrombin time and the aPTT will translate into antithrombotic activity in vivo remains to be tested in animal models.

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