A Murine Monoclonal Antibody That Blocks Fibrinogen Binding to Normal Platelets Also Inhibits Fibrinogen Interactions With Chymotrypsin-Treated Platelets

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We recently described a monoclonal antibody, 10E5, that completely blocks adenosine diphosphate (ADP) induced fibrinogen binding to platelets and aggregation induced by ADP, epinephrine, and thrombin. Multiple lines of evidence indicate that 10E5 binds to platelet membrane glycoproteins IIb and/or IIIa. Because it has been reported that platelets treated with chymotrypsin aggregate when fibrinogen is added, we tested the effect of 10E5 antibody on chymotrypsin-induced fibrinogen binding and platelet aggregation. Aspirin-treated human platelets were washed in modified Tyrode’s buffer (pH 7.5), incubated for 5 minutes at 22°C with 300 μg/mL chymotrypsin, and washed again. The amount of 10E5 antibody bound to these platelets (37,232 ± 2,928 molecules/platelet; mean ± SEM, N=9) was similar to that bound to unstimulated control platelets (36,910 ± 2,669) and did not differ significantly from the amount of antibody bound to ADP-treated platelets (P < .01, N = 5). The amount of 10E5 bound to chymotrypsin-treated platelets correlated directly with the amount of fibrinogen bound to separate aliquots of the same platelet samples (r = .876, P < .001). The 10E5 antibody caused virtually complete inhibition of both the binding of fibrinogen to chymotrypsin-treated platelets and the aggregation induced by exogenous fibrinogen. Immunoprecipitation studies of 125I-labeled chymotrypsin-treated platelets revealed that the 10E5 antibody bound proteins with molecular weights characteristic of glycoproteins IIb and IIIa. These data suggest that the fibrinogen receptor on chymotrypsin-treated platelets is identical to that on ADP-treated platelets and that this receptor is either near to, or on, the glycoprotein IIb/IIIa complex.

THE BINDING OF FIBRINOGEN to specific receptors on the platelet surface has been shown to mediate platelet aggregation in response to a variety of agonists, including adenosine diphosphate (ADP), thrombin, and epinephrine.1-5 The observation that platelets from patients with Glanzmann's thrombasthenia fail to both aggregate and bind fibrinogen2,5,6 when stimulated with these agonists suggested that the platelet membrane glycoproteins IIb (GPIIb) and IIIa (GPIIIa), which are missing in this disorder,7 may be responsible for the platelet-fibrinogen interactions.

Chymotrypsin treatment also exposes fibrinogen binding sites on normal human platelets,8,9 but not the platelets from patients with thrombasthenia.9 Because chymotrypsin treatment was accompanied by the appearance of a 70,000 molecular weight platelet membrane (glyco)protein that was different from GPIIb and GPIIIa,10,11 it was suggested that this (glyco)protein may act as a fibrinogen receptor. It was not shown, however, that the appearance of the 70,000 protein was necessary for fibrinogen binding.

We recently described a murine monoclonal antibody (10E5) that completely blocks both fibrinogen binding and aggregation of human platelets induced by ADP and creates a thrombathenin-like state.11 Immunoprecipitation and affinity chromatography studies suggest that this antibody is directed against GPIIb and/or GPIIIa,12 and recent evidence showing the EDTA-dependence of the antibody suggests that it is directed at an epitope formed when GPIIb and GPIIIa exist as a complex.13 The present study was designed to use the 10E5 antibody to assess whether the fibrinogen receptor exposed by ADP is the same as that exposed by chymotrypsin. In particular, we tested the effect of 10E5 on fibrinogen binding to, and agglutination of, chymotrypsin-treated platelets and identified the protein(s) on these platelets to which the antibody bound.

MATERIALS AND METHODS

Platelet Preparation

Blood was collected from normal volunteers into 0.10 vol 3.2% sodium citrate and 0.05 vol 1 mmol/L acetylsalicylic acid. Platelet-rich plasma (PRP) was obtained by centrifugation at 280 × g for 12 minutes. This PRP was acidified to pH 6.5 with citric acid and centrifuged at 1,000 × g for 20 minutes. Platelets in the resulting pellet were washed twice in HEPES (N2-hydroxyethylpiperazine-N2-ethane-sulfonic acid) buffered modified Tyrode’s solution, pH 6.5, and resuspended in the same buffer at pH 7.5 (HBMT) (300,000–400,000 platelets/μL).

Chymotrypsin treatment routinely consisted of incubating washed platelets at 22°C with 300 μg/mL of the enzyme dissolved in 0.15 mol/L NaCl. The platelets were treated similarly, except that they were incubated in the presence of 5 mmol/L EDTA and resuspended in HBMT. Control platelets were treated similarly, except that they were incubated with 0.15 mol/L NaCl instead of chymotrypsin. In some experiments, platelets were incubated with chymotrypsin in the presence of...
either 0.1 µmol/L prostaglandin E1 (PGE1) (dissolved in ethanol as a 2.8 mmol/L stock solution) or 1 mg/mL potato apyrase (grade I, Sigma) for various times up to 2 hours at either 22°C or 37°C.

**Monoclonal Antibody and Fibrinogen Preparation**

The murine monoclonal antibody (10E5) and another monoclonal antibody directed at GPIb were obtained as previously described \(^{1,2}\) and were iodinated using lactoperoxidase and Na\(^{125}\)I (New England Nuclear Corp, Boston, Mass). \(^{1,2}\)

Fibrinogen was purified from fresh frozen plasma and iodinated with \(^{125}\)I (New England Nuclear) using chloramine T, also as described. \(^{3}\)

**Binding of 10E5 Antibody**

Control platelets, platelets stimulated with 10 µmol/L ADP, and chymotrypsin-treated platelets were incubated at 22°C with 0.5–12 µg/mL \(^{125}\)I-labeled 10E5 antibody (specific activity 20–40 cpm/ng) or another monoclonal antibody (6D1) to GPIb. \(^{4}\) As previous studies indicated that equilibrium binding was achieved after 5 minutes, \(^{13,14}\) platelet-bound antibody was separated from free antibody after 5 minutes by centrifugation (12,000 g) through silicone oil in a microfuge (Beckman Instruments, Palo Alto, Calif). To quantify bound antibody, the tips of the microcentrifuge tubes containing platelet pellets were amputated using a dog nail cutter and counted in a gamma counter (Micromedic, Havertown, Pa). Because nonspecific binding was previously found to be negligible, \(^{13,14}\) total binding did not require correction.

**Fibrinogen Binding**

Fibrinogen binding was performed as described previously. \(^{2}\) Chymotrypsin-treated platelets were incubated with a saturating concentration of labeled fibrinogen (1 mg/mL, specific activity 1,000 cpm/µg) in the absence or presence of 5 mmol/L EDTA, the latter to assess nonspecific binding.

To determine the effect of 10E5 antibody on fibrinogen binding to chymotrypsin-treated platelets, platelets were first incubated with 10 µg/mL unlabeled antibody (a near saturating concentration), followed after 5 minutes by a 1-minute incubation with 125I-fibrinogen, with or without EDTA.

**Identification of 10E5 Antibody Binding Site(s) on Chymotrypsin-Treated Platelets**

Control and chymotrypsin-treated platelets were iodinated with iodine 125 using lactoperoxidase and H\(_2\)O\(_2\). \(^{13}\) Labeled platelets were washed three times with 0.15 mol/L NaCl in the presence of 5 mmol/L EDTA and were resuspended in one tenth the original volume using HBMT. The incorporation of iodine into platelet membrane proteins varied between 1% and 3% in five separate experiments. No differences in iodine incorporation were noted between control and chymotrypsin-treated platelets.

Iodinated platelet suspensions (1 ml) were lysed with 5 ml of 1% Triton X-100, 0.15 mol/L NaCl, 0.02 mol/L Tris/C1, 6 mmol/L N-ethylmaleimide (NEM), and 1 mmol/L leupeptin, pH 7.4. The suspension was sonicated (three times for 15–20 seconds, power reading 30–40, using a model 9100 Lab Line instrument with probe Model MT1; Lab Line Instruments, Melrose, Ill) and left at 4°C overnight. It was recentrifuged at 4°C for 15 minutes (5,000 g) and again at 4°C for 60 minutes (40,000 g). The supernatant was divided into three aliquots: one was incubated either with 10E5 antibody (150 µL, 420 µg/mL); a second was incubated with the same concentration of a control murine monoclonal antibody; and the last was retained as a control for electrophoresis. A solid-phase immunoprecipitin was produced by washing a 10% suspension of protein A-containing staphylococci three times (Enzyme Center, Boston, Mass) in phosphate-buffered saline (PBS) containing 1% Triton X-100, 0.02% sodium azide, 0.1% sodium deoxycholate (SDS), 0.1% bovine serum albumin (BSA), pH 8.0 and adding an equal volume of this suspension to the platelet lysates containing antibodies. The suspension was incubated overnight and washed seven times in the above PBS solution. The staphylococci were air dried and subsequently heated to 100°C in a solution of one part 3.3% SDS, 6 mmol/L NEM, and one part 2% SDS, 80 mmol/L imidazole/C1, 0.05% bromophenol blue, pH 6.8, to release bound antigen and antibody. The suspensions were then centrifuged at 12,000 g for 5 minutes and the supernatant applied nonreduced, or after reduction with 0.1% 2-mercaptoethanol, to 5%–15% gradient polyacrylamide gels and electrophoresed. \(^{12,14}\) The gels were stained with Coomassie brilliant blue, dried, and autoradiograms prepared. \(^{12,14}\)

**RESULTS**

**Chymotrypsin Treatment**

Incubation of platelets with chymotrypsin for 5 minutes at 22°C resulted in removal of approximately 93% of the surface glycoprotein Ib, as assessed by the binding of another monoclonal antibody (6D1) directed against this glycoprotein. \(^{14}\) These chymotrypsin-treated platelets aggregated in response to adding 0.3–0.6 mg/mL purified fibrinogen (Fig 1). The aggregation was virtually completely inhibited by 10 µg/mL of 10E5 antibody (Fig 1). When the time of incubation with chymotrypsin was increased, the aggregation induced by fibrinogen decreased (Fig 2).

**Binding of 10E5 Antibody and Fibrinogen to Chymotrypsin-Treated Platelets**

The amount of 10E5 antibody bound to chymotrypsin-treated platelets was similar to that bound to unstimulated control platelets and did not differ significantly from the amount of antibody bound to ADP-treated platelets (\(P < 0.1, N = 5\)). At a near-saturating concentration of antibody (10 µg/mL), 37,232 ± 2,928 (mean ± SEM, N = 9) molecules of 10E5 bound per chymotrypsin-treated platelet, compared to 36,910 ± 2,669 molecules of antibody bound per

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**Fig 1.** Aggregometer tracings of washed chymotrypsin-treated platelets aggregated with 0.3 mg/mL fibrinogen with and without 10 µg/mL 10E5 antibody, as indicated. The aggregometer was set so that a baseline was established at 10% light transmission with PRP and 100% transmission with a buffer blank.
with chymotrypsin for longer than 5 minutes at 37°C, and this correlated with the previously noted decrease in aggregation. The results of a typical experiment are illustrated in Fig 2. Control platelets incubated at 37°C for 90 minutes bound fibrinogen (36,112 ± 2,106 molecules per platelet; N = 3) and 10E5 (37,047 ± 1,368 molecules per platelet) normally, after 5-minute incubation with chymotrypsin. The platelets from different individuals varied in the amount of fibrinogen and 10E5 antibody that they bound. However, the correlation between the amount of 10E5 antibody and fibrinogen bound by a single individual’s platelets was very good (r = .876, P < .001).

Identification of 10E5 Binding Sites on Chymotrypsin-treated Platelets

Figure 4 depicts an autoradiogram of a 5%–15% gradient SDS-polyacrylamide gel of whole chymotrypsin-treated platelets (lane A), the nonreduced immunoprecipitate of chymotrypsin-treated platelet lysates incubated with 10E5 antibody (lane B), and a nonreduced immunoprecipitate using a control antibody (lane C). Radioactive bands with molecular weights characteristic of GPIIb and GPIIIa are seen in lane B. No radioactive bands were observed in samples immuno-
precipitated with the control antibody (lane C). A minor radioactive band of mol wt 59,000 was sometimes seen after electrophoresis of immunoprecipitates of chymotrypsin-treated platelets, but only after prolonged exposure of the autoradiogram (1 week, compared to 2–3 days for GPIIb and GPIIIa). Degradation of GPIIIb was noted, however, with longer chymotrypsin treatment. Results of similar studies using platelets that were not treated with chymotrypsin are shown in lanes D–F. Figure 5 illustrates the appearance and increase in intensity of a 68,000 mol wt radioactive band on an autoradiogram of a 7.5% SDS gel of nonreduced whole platelets incubated for 5, 30, 60, and 90 minutes with chymotrypsin. After 90 minutes, another radioactive band (mol wt 55,000) appeared. Concomitant losses of radioactivity in the GPIIIa region are seen as well.

**DISCUSSION**

Our data indicate that a murine monoclonal antibody (10E5) that inhibits ADP-induced platelet aggregation and fibrinogen binding to normal platelets and recognizes an epitope that exists when GPIIb and GPIIIa are complexed also blocks both fibrinogen binding to, and fibrinogen-induced agglutination of chymotrypsin-treated platelets. This strongly suggests that the fibrinogen binding site of chymotrypsin-treated platelets is similar to the fibrinogen binding site of ADP-treated platelets. Indeed, immunoprecipitation studies with platelets incubated with chymotrypsin for 5 minutes show that the 10E5 antibody binds to two platelet membrane glycoproteins with molecular weights similar to GPIIb and GPIIIa (the same glycoproteins involved in 10E5 binding to control platelets). The ratio of GPIIb and GPIIIa in 10E5 immunoprecipitates, particularly of chymotrypsin-treated platelets, however, was notably different from that observed in whole platelets. Because current evidence points to the 10E5 antibody recognizing the GPIIb/GPIIIa complex, the reason for these results is not apparent. Two possible explanations may be offered: (1) GPIIb and GPIIIa may not be present in stoichiometric amounts in the intact platelet, and/or (2) cleavage of GPIIb by chymotrypsin or endogenous platelet proteases may generate GPIIb degradation products that are still able to form the 10E5 epitope when complexed with GPIIIa, but which migrate in a polyacrylamide gel at a location(s) near to GPIIIa.

Recent studies by Kornecki et al demonstrated that polyclonal and monoclonal antibodies that inhibit the aggregation of chymotrypsin-treated platelets in response to fibrinogen, precipitate a 70,000 molecular weight platelet membrane protein. The authors suggested that this protein acted as another receptor for fibrinogen, and, as they also found it in thrombasthenic platelets, they concluded that it was not related to the GPIIb/IIIa complex. Other evidence, however, indicates that it is more likely to be a chymotrypsin-induced proteolytic cleavage product of glycoprotein IIIa. Such an interpretation leaves unresolved the question as to why such a fragment should be present in the platelets of patients with thrombasthenia who lack glycoprotein IIIa. In the present study, a 68,000 mol wt peptide was only noted after 15 minutes of incubation with chymotrypsin.

Additional studies by Kornecki et al, comparing the effects of various concentrations of chymotrypsin on platelet aggregation and fibrinogen binding, noted that fibrinogen binding correlated with the appearance of a 66,000 mol wt GPIIIa fragment. Although experiments performed in the present study are not technically comparable to those performed by Kornecki et al, we found the appearance of a molecular weight 68,000 fragment thought to be derived from GPIIIa to correlate negatively with platelet aggregation and the binding of both fibrinogen and 10E5 antibody.

The degradation of GPIIIa by chymotrypsin and its relationship to fibrinogen binding was also examined by McGregor et al using a monoclonal antibody (P2) directed against GPI, GPIIb, and GPIIIa. These investigators noted that incubating platelets for 30 minutes with chymotrypsin (0.2 mg/mL) led to loss of the P2 epitope, but not fibrinogen binding, and thus to failure...
of the P2 antibody to inhibit fibrinogen-induced platelet aggregation. Our results indicate that chymotrypsin-treatment reduces both fibrinogen and 10E5 binding concordantly, suggesting that the 10E5 antibody may be closer to the fibrinogen binding epitope on the glycoprotein IIb/IIIa complex than P2.

In summary, our data suggest that both ADP-induced platelet aggregation and fibrinogen-induced aggregation of chymotrypsin-treated platelets are mediated by the same platelet receptor, which is either near to, or on, the glycoprotein IIb/IIIa complex.

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