CONCISE REPORT

Inhibition of Dog Platelet Function by In Vivo Infusion of F(ab')$_2$ Fragments of a Monoclonal Antibody to the Platelet Glycoprotein IIb/IIIa Receptor

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To assess the potential of monoclonal antibodies that inhibit platelet function in vitro as in vivo therapeutic agents, F(ab')$_2$ fragments (0.17 to 0.81 mg/kg) of a murine monoclonal antibody (7E3) that binds to platelet glycoproteins IIb and/or IIIa and blocks platelet aggregation induced by ADP were infused into three dogs. Soon after infusion, platelets recovered from the dogs showed a decreased aggregation response to adenosine diphosphate, with the highest dose producing nearly total inhibition. These platelets also showed decreased ability to bind ^{125}I-7E3, which was assumed to reflect occupancy of the sites by the unlabeled F(ab')$_2$ fragments. At the highest dose, the binding decreased by 85%, reflecting the binding of ~44,000 molecules of 7E3 F(ab')$_2$ per platelet. Platelet counts decreased after antibody infusion by less than 20%, and none of the dogs showed spontaneous bleeding. Both the aggregation and binding results reverted toward normal within one day. We conclude that it is possible to profoundly inhibit platelet function by in vivo infusion of F(ab')$_2$ fragments of a monoclonal antiplatelet antibody without producing spontaneous hemorrhage or significant thrombocytopenia.

THE SEARCH FOR potent and specific inhibitors of platelet function continues because platelets have been implicated in a variety of serious pathologic processes and none of the currently available agents is ideal.[1] Abundant evidence indicates that binding of fibrinogen, and perhaps other adhesive glycoproteins (von Willebrand factor, thrombospondin, and fibronectin), to or near the glycoprotein IIb/IIIa (GPIIb/IIIa) complex when platelets are activated plays a major role in platelet physiology[2,3]; in fact, all of the agonists that are thought to initiate platelet aggregation in vivo (adenosine diphosphate [ADP], epinephrine, collagen, and thrombin) appear to depend absolutely on the binding of fibrinogen or the other ligands to this receptor.[2,4] We recently showed that, in vitro, monoclonal antibodies directed against specific epitopes on the GPIIb/IIIa complex can completely block platelet aggregation and fibrinogen binding induced by ADP.[4,5] We have now begun to assess the potential of such antibodies as in vivo therapeutic agents by analyzing the effect of injecting F(ab')$_2$ fragments of antibody 7E3 into dogs.[5] These fragments produced a profound decrease in platelet aggregation in response to ADP that correlated with the dose of antibody injected and the number of F(ab')$_2$ molecules bound per platelet.

MATERIALS AND METHODS

The IgG1 murine monoclonal antibody 7E3 has been described in detail previously.[5] The intact antibody was converted to F(ab')$_2$ fragments by digestion with pepsin A (Worthington Biochemical Corp, Freehold, NJ) at 37 °C either by treatment of ascites with 0.1 vol of 1 mol/L sodium citrate, pH 3.75, and 0.025 mg/mL pepsin for 27 hours followed by purification by ion-exchange chromatography (DE-52; Whatman Chemical Separation, Clifton, NJ) with a NaCl gradient[6] or by purifying intact 7E3 from ascites by chromatography on DE-52, adjusting the buffer to 0.2 mol/L NaCl and 0.2 mol/L Na acetate, pH 4.0, digesting with 2% (wt/wt) pepsin for 17 hours, dialyzing against 0.1 mol/L Na phosphate, 0.05% NaN_3, pH 8.0, and collecting the flow-through peak from a column of protein A–Sepharose (Boehringer Mannheim Biochemicals, Indianapolis, Ind). In either case, digestion was shown to be complete by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The final F(ab')$_2$ fragments were dissolved in either 0.15 mol/L NaCl or 0.1 mol/L NaCl, 5 mol/L Tris-Cl, pH 7.5, at concentrations of 0.2 to 0.75 mg/mL. Although a sensitive Limulus amebocyte lysate assay (Pyrogent, Mallinckrodt, St Louis) could detect small amounts of endotoxin in the antibody preparations assayed (0.2 to 3.8 ng/mL), the dogs did not show any evidence of physiologically significant endotoxemia (hyperpyrexia was excluded by serial rectal temperatures in the one dog tested, and significant diffuse intravascular coagulation was excluded by serial plasma fibrinogen determinations in the two dogs tested).

Three different dogs weighing 11 to 17 kg were studied. At the time of, or shortly before, obtaining the first blood sample, each dog was mildly anesthetized with a 10-mg intravenous injection of acepromazine (Aveco Co, Fort Dodge, Ind). Blood samples of 10 mL and 1 mL were collected via a 19-gauge scalp vein infusion set into 0.01 vol 40% trisodium citrate and 0.037 vol 0.27 mol/L Na_2 EDTA, respectively. After obtaining a control sample, the antibody fragments were injected intravenously, and additional samples were obtained at various times thereafter. Platelet-rich plasma (PRP) was prepared from the citrated blood by repetitive one- to two-minute centrifugations at 500 to 700 g at 22 °C and adjusted to 3.0×10^{11} platelets per liter with platelet-poor plasma when the count was greater than this. ADP-induced platelet aggregation was performed immediately after PRP preparation as previously described.[4] Binding of intact ^{125}I-7E3 to platelets in PRP was performed as previously described[5] using a near-saturating concentration of antibody (0.017 mg/mL). Since the off-rates for binding of antibody 7E3[5] and its F(ab')$_2$ fragment are exceedingly slow, we reasoned that binding of injected F(ab')$_2$ fragments to platelets in vivo would prevent the subsequent binding of ^{125}I-7E3 in vitro. The difference between the number of ^{125}I-7E3 molecules bound to platelets obtained before and after injecting the antibody fragments was assumed to reflect the number of F(ab')$_2$ molecules bound. Platelet counts and hematocrits were performed on the EDTA-anticoagulated blood either by electronic resistive particle counting after predilution (Coulter S+ IV, Coulter Electronics, Hialeah, Fla) or by phase-contrast microscopy using the Unopette system (Becton Dickinson, Rutherford, NJ).

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Supported by grant No. 19278 from the National Heart, Lung, and Blood Institute.

Submitted Aug 6, 1985; accepted Sept 4, 1985.

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0006-4971/85/6606-0037$03.00/0

Fibrinogen was assayed on plasma by radial immunodiffusion (Behring Diagnostics, La Jolla, Calif). Bleeding times were attempted by both the nail cuticle method[7] and a modification of the Ivy technique,[8] but reproducible results on control and treated animals could not be obtained.

RESULTS

The effect of 10 mg of acepromazine alone on blood counts and platelet aggregation was determined in a control dog. There were no differences in the initial rates or total extents of platelet aggregation induced by either 2 or 4 μmol/L ADP four hours after the injection. There was, however, a significant decrease in the hematocrit value at four hours (preinjection, 0.48; after four hours, 0.39) and a 15% decrease in the platelet count (preinjection, 3.28×10^{11}/L; after four hours, 2.79×10^{11}/L). Similar results were obtained with another dog injected with 4 mg of acepromazine. The decreases in hematocrit and platelet count presumably reflect atony of the spleen with transient enlargement of this organ and sequestration of blood cells. All three experimental animals had decreases in their hematocrit values during the experiments (dog 1, 0.47 preinjection and 0.39 at one hour postinjection; dog 2, 0.37 preinjection, 0.34 at 1.5 hours and 0.34 at four hours postinjection; dog 3, 0.36 preinjection, 0.32 at one-half hour and 0.33 at four hours postinjection). In all cases, the hematocrit value returned to preinfusion levels by the next day.

Figure 1 shows the results of the platelet aggregation and ^{125}I-7E3 binding assays. Dog 1 (panel A) received a total dose of 0.17 mg/kg of 7E3 F(ab')$_2$ fragments (given in three injections of 1.4, 0.94, and 0.56 mg at hourly intervals) and sustained a 40% decrease in the initial slope and a 61% decrease in the maximal extent of ADP-induced platelet aggregation (4 μmol/L) one hour after the last injection. The binding of ^{125}I-7E3 decreased by 20% from 56,400 molecules per platelet on the preinjection sample to 45,400 on the one-hour postinjection sample, suggesting that 11,000 7E3 F(ab')$_2$ molecules were bound per platelet. Both the initial rate of aggregation and the binding of ^{125}I-7E3 returned to the preinjection values by the next day. With 2 μmol/L ADP, the total extent of platelet aggregation also normalized after one day, but with 4 μmol/L (Fig 1A), there was some residual inhibition. No inhibition was seen when the dog was retested six days later. The platelet count decreased by less than 10% at one hour, from the control value of 2.36×10^{11}/L to 2.2×10^{11}/L. Making the assumptions that the dog had a blood volume of 72 mL/kg, that two thirds of the platelets were in the intravascular space, and that an average of 11,000 7E3 F(ab')$_2$ molecules were bound per platelet, one calculates that 30% of the 7E3 F(ab')$_2$ molecules injected were bound to platelets at the one-hour point.

Dog 2 was given a single injection of 0.57 mg/kg of 7E3 F(ab')$_2$ (Fig 1B). The inhibition of ADP-induced platelet aggregation (4 μmol/L) was more pronounced than with the lower dose of antibody, with a 75% decrease in the initial slope and an 84% decrease in the maximal extent of aggregation four hours after the infusion. The average number of molecules of ^{125}I-7E3 that could bind per platelet decreased from 62,500 to 22,500. Using the aforementioned assumptions, 33% of the injected 7E3 F(ab')$_2$ molecules were bound to platelets at four hours. Despite this, the platelet count only decreased from the preinjection value of 2.65×10^{11}/L to

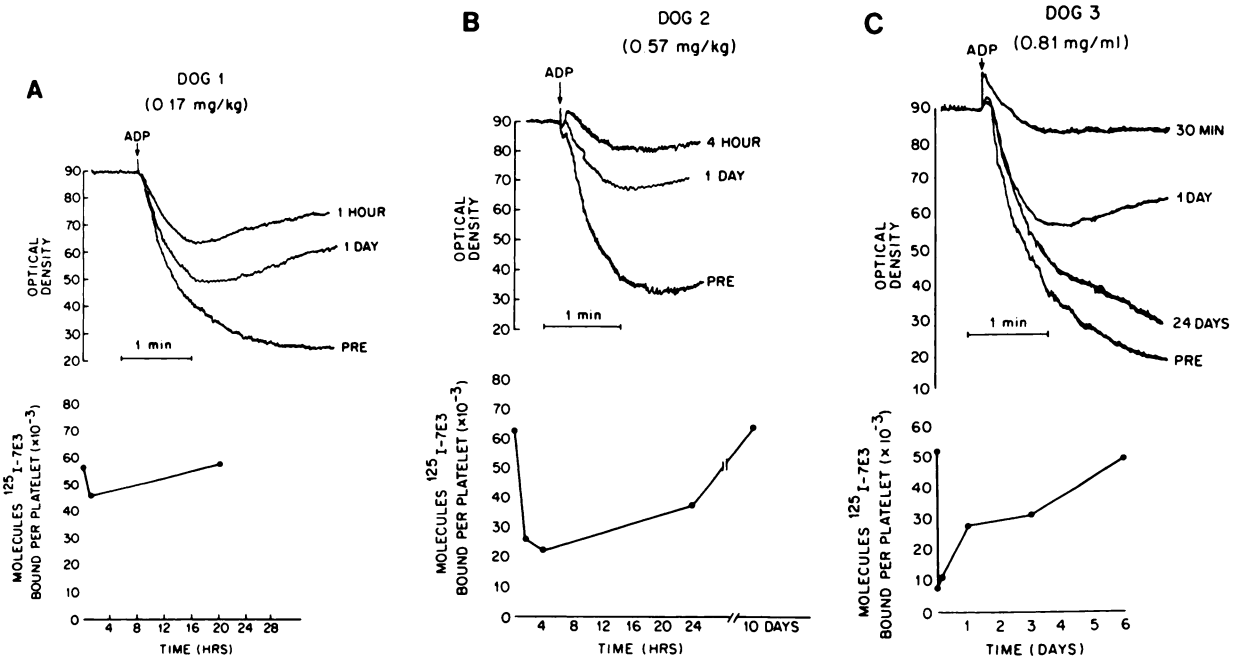

Fig 1. (A through C) Effect of infusion of antibody 7E3 F(ab')$_2$ fragments on platelet aggregation in response to ADP and the in vitro binding of intact ^{125}I-7E3 antibody. After obtaining a control sample (PRE), three different dogs received the indicated doses of 7E3 fragments intravenously. Additional blood samples were then obtained at the indicated times. Platelet-rich plasma (2.35 to 3.00 × 10^{11}/L) was prepared as described and then used in studies of platelet aggregation and ^{125}I-7E3 antibody binding.

2.13×10^{11}/L, a percentage decrease (19.7%) that only modestly exceeded the 15% decrease in the acepromazine control study. The average number of ^{125}I-7E3 molecules that could bind per platelet increased to 38,000 after one day and returned to the preinfusion level within one week. The inhibition of platelet aggregation was still evident at one day, and even after one week a modest decrease (~30%) in aggregation response was noted. Interestingly, the platelet count 1 week after the infusion rose to 3.77×10^{11}/L, a value considerably above the preinfusion count.

Dog 3 received 0.81 mg/kg in a single infusion. Within 30 minutes, the maximal extent of aggregation in response to 2 μmol/L ADP decreased by 91%; the decrease in initial slope was more difficult to assess because the platelet shape change remained intact and it was difficult to differentiate between a decay in the shape change response and authentic platelet aggregation (Fig 1C). At 30 minutes there was also an 85% decrease in the number of ^{125}I-7E3 antibody molecules that could bind to platelets (Fig 1C). Thus, an average of 44,000 $F(ab')_2$ 7E3 molecules were bound per platelet, representing 18% of the injected dose. Very similar data were obtained at three hours. During the course of the next week there was recovery of the initial slope of platelet aggregation and the number of molecules of ^{125}I-7E3 that could bind per platelet. Even after six days, however, the response to 2 μmol/L ADP continued to show the aggregation-disaggregation type of response seen at one day (Fig 1C); the original type of response was obtained again when blood was collected 24 days later (Fig 1C). The original platelet count (1.80×10^{11}/L) decreased by 10% at three hours but was above the preinfusion level (2.25×10^{11}/L) when measured the next day. In a separate experiment conducted 8 weeks later, this dog's PRP was incubated in vitro with increasing concentrations of 7E3 $F(ab')_2$ fragments and the aggregation and binding experiments repeated. In vitro incubation with 10 μg/mL of the fragments most closely simulated the maximum inhibition of aggregation and ^{125}I-7E3 binding produced by injecting the 0.81 mg/kg in vivo.

DISCUSSION

These studies indicate that the $F(ab')_2$ fragment of a murine monoclonal antibody that blocks the binding of fibrinogen to platelets can be given in sufficient quantities in vivo to profoundly inhibit platelet aggregation induced by ADP and that this inhibition correlates with the number of $F(ab')_2$ molecules bound per platelet. Most importantly, the inhibition was achieved without inducing spontaneous hemorrhage or significant thrombocytopenia, supporting the potential usefulness of this approach for human therapy. We chose to use $F(ab')_2$ fragments that are devoid of the Fc portion of the immunoglobulin molecule rather than intact antibody so as to avoid premature clearance of antibody-coated platelets by splenic macrophages containing Fc receptors. Since we did not directly measure platelet survival, it is possible that the $F(ab')_2$-coated platelets did suffer some shortening of their life span. Nonetheless, there were only minor decreases in the platelet count despite the evidence that many $F(ab')_2$ fragment molecules were bound to each platelet.

The functional defect produced in vitro by 7E3, like that of antibody 10E5, is virtually identical to that found in patients with Glanzmann's thrombasthenia whose platelets have a deficiency or defect in GPIIb and GPIIIa.[2,4,5] It was reasonable to suspect, therefore, that infusion in vivo of these antibodies would produce functional impairments that simulate the laboratory data and clinical symptoms of these patients. The platelet aggregation data are, in fact, similar to those found in patients with thrombasthenia. However, there was some impairment of aggregation when approximately 80% of the GPIIb/IIIa sites were available in dog 1 and approximately 50% of the GPIIb/IIIa sites were available after one day in dogs 2 and 3. This contrasts with the reported absence of aggregation defects in heterozygous patients with thrombasthenia[9] and raises the possibility that the antibody interferes with platelet function by another mechanism or perhaps that the use of mean values of blocked sites obscures a functionally significant heterogeneity. Alternatively, since the aggregation defects produced by the antibodies under these circumstances were relatively subtle, similar defects may not be appreciated in heterozygous patients with thrombasthenia in whom no internal control response is available for comparison.

Clinically, it appears that the vast majority of bleeding episodes in thrombasthenic patients follow identifiable trauma, with relatively little spontaneous hemorrhage other than that associated with diseased gums.[2,9] The most likely explanation for this observation is that despite the paralysis of the aggregation response the platelets from these patients can still adhere to subendothelial surfaces by interaction with von Willebrand factor via GPIb and collagen via a still-unknown receptor(s). In addition, these platelets retain at least some of their ability to be activated, release the contents of their granules, and facilitate the generation of thrombin. Thus, it is conceivable that these antibodies could be very effective in blocking the accumulation of multiple layers of platelets without producing an unacceptable risk of hemorrhage. Such an effect may be useful in avoiding total occlusion of the blood vessel, as in myocardial infarction and stroke, and in avoiding the accumulation of friable platelet-fibrin material that can embolize, as in transient ischemic attacks, pulmonary emboli and thromboemboli from vascular prostheses and artificial organs.

ACKNOWLEDGMENT

We thank Lisa Lih and Ann Schwartz for outstanding technical assistance and Shirley Murray for outstanding secretarial assistance.

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