Effects of Monoclonal Antibodies against the Platelet Glycoprotein IIb/IIIa Complex on Thrombosis and Hemostasis in the Baboon

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Abstract

To assess the hemostatic consequences and antithrombotic effectiveness of blocking the platelet glycoprotein (GP) IIb/IIIa receptor for fibrinogen and other adhesive glycoproteins in vivo, well characterized murine monoclonal antibodies against the platelet GP IIb/IIIa complex, AP-2 and LJ-CP8, were infused intravenously into baboons. Four animals each received doses of 0.2, 0.4, and 1.0 mg/kg of purified AP-2 IgG, and three animals were given 1.0 mg/kg of the $F(ab)_2$ fragment of AP-2. Five additional animals were given 10 mg/kg LJ-CP8 IgG. At the highest dose, radiolabeled AP-2 IgG bound to an average of 33,000 sites on the circulating platelets. Serial measurements included platelet count, bleeding time, platelet aggregation (induced by ADP, collagen, and γ-thrombin), and ^{111}In-platelet deposition onto Dacron vascular grafts. Bleeding times were markedly prolonged after injection of 1.0 mg/kg AP-2 IgG (19.2±3.4 min), 1.0 mg/kg AP-2 $F(ab)_2$ (16.5±1.8 min), and 10 mg/kg LJ-CP8 (> 30 min) vs. control studies (4.6±0.2 min), and remained prolonged for 48 h. With each antibody platelet aggregation was initially reduced or absent, with partial recovery over 48 h in a manner that was inversely related to dose. AP-2, both whole IgG and $F(ab)_2$ fragment, but not LJ-CP8, caused a dose-dependent reduction (20–46%) in the circulating platelet count over 24 h. Neither AP-2 nor LJ-CP8 caused a reduction in intraplatelet platelet factor 4, β-thromboglobulin, or [^{14}C]serotonin. Graft-associated platelet thrombus formation was reduced by 73% (1.0 mg/kg AP-2 IgG and 10 mg/kg LJ-CP8) and 53% (1.0 mg/kg AP-2 $F(ab)_2$) relative to control values. In contrast, neither heparin (100 U/kg) nor aspirin (32.5 mg/kg twice a day) showed antithrombotic efficacy in this model. Thus, antibodies that functionally alter the platelet GP IIb/IIIa complex may produce immediate, potent, and transient, antihemostatic, and antithrombotic effects.

Introduction

Platelets bind fibrinogen and other adhesive glycoproteins, such as von Willebrand factor and fibronectin, following stimulation by appropriate physiologic agonists such as thrombin and ADP (1–18). As shown by studies in patients with Glanzmann thrombasthenia, who are deficient in membrane glycoproteins (GP)[1] IIb and IIIa, and by studies in patients with severe afibrinogenemia, the inability of platelets to bind fibrinogen normally may result in significant platelet dysfunction that is characterized by prolonged bleeding and an abnormal aggregation response (19–30). The crucial role of the platelet GP IIb/IIIa receptor has been further documented by a number of recent reports describing monoclonal antibodies that interact specifically with platelet GP IIb and/or GP IIIa, inhibit the binding of fibrinogen and other adhesive glycoproteins, and produce a thrombasthenic-like state in normal platelets in vitro (31–42).

Based on these observations, it has been suggested that such antibodies may be useful as antithrombotic therapeutic agents (43, 44). Preliminary studies in dogs with the $F(ab')_2$ fragment of a murine monoclonal antibody to GP IIb/IIIa have shown dose-dependent inhibition of ADP-induced platelet aggregation ex vivo, and normalization of flow patterns in a platelet-dependent model of coronary artery stenosis. The antibody infusions were not associated with either spontaneous bleeding or marked thrombocytopenia, but relevant hemostatic measurements, such as the bleeding time, were not evaluated in these studies (43, 44).

We have therefore used a baboon animal model to assess the effects of injected antibodies against platelet GP IIb/IIIa with respect to the following variables: platelet count, bleeding time, platelet aggregation, content of platelet storage granules, and platelet deposition onto Dacron vascular grafts. A baboon animal was used in this study since this species is hemostatically similar to man with respect to coagulation factors, platelets and their granular contents, and immunologic characteristics of platelet and plasma proteins as studied using human radioimmunoassays (45–48). The antithrombotic efficacy of injected antibody was assessed using a gamma scintillation camera to measure ^{111}In-platelet deposition onto Dacron vascular grafts incorporated acutely into an externalized femoral arteriovenous shunt. This method was chosen since the measurements of platelet incorporation into forming arterial thrombus are quantitative and reproducible, and since the graft materials used have clinical applications in man (48). These studies indicate that the administration of antibodies that block the platelet GP IIb/IIIa receptor results in altered hemostasis in vivo, as shown by prolongations of the bleeding time. The use of such antibodies may represent a particularly promising strategy for antithrombotic therapy.

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1. *Abbreviations used in this paper:* GP, glycoprotein; PPP, platelet poor plasma; PRP, platelet rich plasma; RCD, Ringer citrate dextrose; β-TG, β-thromboglobulin; T_{max}, maximum transmission; V_{max}, maximum velocity.

Methods

Animals. 39 normal male baboons (*Papio anubis*) were used in these studies. Four animals served as controls to assess the effect of acute graft placement on circulating platelet counts. 29 animals were subsequently given intravenous antibody. No animal received antibody more than once. Four animals were given ^{131}I-labeled AP-2 to assess antibody distribution and kinetics in vivo. Six additional animals were given either the combination of heparin (100 U/kg; Abbott Laboratories, North Chicago, IL) plus oral aspirin (32.5 mg/kg twice daily, Sigma Chemical Co., St. Louis, MO), or aspirin alone at this dose (five animals).

The animals weighed 8–12 kg and had been observed to be disease-free for at least 6 wk before use. All animals had a chronic arteriovenous (A-V) shunt surgically implanted between the femoral artery and vein. Ketamine hydrochloride (10 mg/kg, i.m.) was given as a preanesthetic agent, and the operation was performed under general 1% halothane anesthesia. All procedures were approved by the institutional Animal Care and Use Committee in accordance with federal guidelines (Guide for the Care and Use of Laboratory Animals, 1985). The permanent shunt system consisted of two 25-cm lengths of tubing, 3.0 mm i.d. (Silastic; Dow Corning Corp, Midland, MI). The cannulas were sterilized by autoclaving before surgical placement. Blood flow was established by connecting the two Silastic shunt segments with a 1-cm length of blunt-edge Teflon tubing (2.8 mm i.d.). As described in detail previously, the permanent Teflon-Silastic shunt does not detectably shorten platelet survival or produce measureable platelet activation (47, 48, 62). When used, Dacron vascular grafts were subsequently interposed between the segments of the permanent A-V shunt.

Platelet count and hematocrit determinations were performed on whole blood collected in 2 mg/ml disodium EDTA using a J. T. Baker (model 810; Allentown, PA) whole blood analyzer. Before the antibody studies, whole blood platelet counts averaged 370,000±86,000 platelets/μl (±1 SD) and hematocrits averaged 34±5%. Bleeding time measurements were performed on the shaved volar surface of the forearm using the standard template method as previously described for studies in baboons (49). Bleeding time measurements were performed in duplicate and were averaged.

Antibody preparation and characterization. The preparation and characterization of AP-2 has been reported (37). LJ-CP8 was prepared and characterized by methods described previously (50), with the only modification that human washed platelets used as immunogen were treated with chymotrypsin (51) before injection. LJ-CP8 reacted with GP IIb and/or GP IIIa when the two were in complex, as shown by (*a*) its lack of reactivity with platelets from patients with Glanzmann thrombasthenia; (*b*) solid-phase immunoisolation studies (50, 52) demonstrating that the antibody bound to the complex of two platelet membrane proteins having the electrophoretic mobility of GP IIb and GP IIIa (53); (*c*) lack of reactivity with normal platelets incubated with 5 mM EDTA for 15 min at 37°C (54, 55). LJ-CP8 and AP-2 were of the IgG_1 subclass. Purified IgG was prepared from ascitic fluid as previously described (50). Bivalent $F(ab)_2$ fragments were prepared from AP-2 IgG by papain digestion as described (56). Contaminating Fc fragments were removed using protein A Sepharose. The material was homogeneous as determined by SDS-PAGE, exhibiting a single predominant band at 110,000 mol wt.

Radioiodination of antibodies. Labeling of IgG with Na^{131}I or ^{125}I (Amersham Corp., Arlington Heights, IL) was performed with the method of Fraker and Speck (57) using Iodogen (Pierce Chemical Co., Rockford, IL). AP-2 $F(ab)_2$ was labeled with ^{125}I by the chloramine-T procedure (58). Radiolabeled IgG molecules bound to platelets were analyzed by means of PAGE in the presence of SDS, following reduction with dithiothreitol, as described (50). Specific activities of the labeled antibodies were: LJ-CP8 (2.0 μCi/μg); AP-2 IgG (0.05–0.5 μCi/μg); AP-2 $F(ab)_2$ (2.1 μCi/μg).

Binding studies. The number of IgG molecules bound per platelet was calculated on the basis of the specific activity, after separating the platelets from platelet-rich plasma by means of centrifugation through 20% sucrose, as described (12). ^{125}I-labeled LJ-CP8 bound to normal human platelets; as an average, 48,000 molecules bound per cell with a K_d of 9.3×10^{-8} M. The binding of LJ-CP8 to baboon platelets was not evaluated directly. ^{125}I-labeled AP-2 bound to baboon platelets. At saturation, between 49,000 and 52,000 IgG molecules bound per platelet with a K_d of $2.4–2.5 \times 10^{-9}$ M (range of two separate experiments). ^{125}I-labeled AP-2 $F(ab)_2$ binding to baboon platelets was similar, exhibiting 53,000 molecules bound per platelet at saturation with a K_d of 3.3×10^{-9} M (one experiment). In all cases, binding was saturable. Binding isotherms were analyzed by means of Scatchard-type analysis to determine the dissociation constant and number of binding sites using the computer assisted program Ligand (59). Nonsaturable binding, calculated as a computer-fitted parameter defined as the ratio of bound to free ligand at infinite ligand concentration, was effectively zero in each case ($< 1 \times 10^{-15}$).

Both AP-2 IgG and LJ-CP8 inhibited the binding of baboon fibrinogen, purified as described previously (60), to thrombin-stimulated baboon platelets. AP-2 inhibited the binding by 55%, whereas LJ-CP8 inhibited the binding completely. Similar results were obtained with human fibrinogen and platelets. The methods used to measure the binding of antibody and fibrinogen to platelets have been previously reported in detail (12, 50). Both LJ-CP8 and AP-2 IgG, when added at saturating concentrations, completely inhibited aggregation of human or baboon platelet-rich plasma (PRP) induced by ADP, collagen or γ-thrombin. AP-2 $F(ab)_2$ added at saturating concentrations completely abolished collagen-induced platelet aggregation; ADP-induced platelet aggregation was only partially inhibited. For example, the maximal increase in light transmission (see below) in response to 15 μm ADP was reduced 50% (two studies).

Platelet aggregation studies. Platelet aggregation was measured using an aggregometer (Chrono-Log, Havertown, PA) by recording the increase in light transmission through a stirred suspension of PRP maintained at 37°C. Platelet-rich and platelet-poor plasmas were prepared as previously described (49). In all cases, the platelets in PRP were adjusted to a count of 200,000 platelets/μl. Aggregation was induced by the addition of 10 μM and 20 μM ADP (Sigma Chemical Co., St. Louis, MO), 10 μg/ml and 20 μg/ml collagen (Hormon Chemie, Munich, FRG), and 17 μg/ml and 34 μg/ml γ-thrombin (a gift from Dr. J. W. Fenton II, Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY). The response to each agonist was compared with control studies. Aggregometer tracings were quantitatively analyzed to determine T_{max}, the maximum increase in light transmittance, and V_{max} (percent per minute), the aggregation velocity at the maximum slope of the light transmittance curve (61). Typical results are illustrated in Fig. 1. At each dose of injected antibody, platelet aggregation was assessed in at least three different animals acutely (at 2 h) and at daily intervals thereafter.

Vascular grafts. Uncrimped knitted Dacron grafts (Sauvage exter-

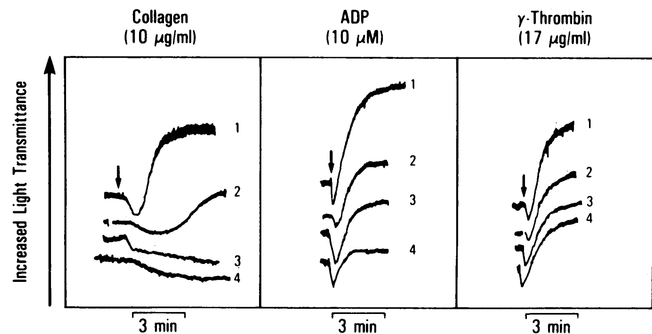

Figure 1. Typical aggregation tracings were from animals given 1.0 mg/kg AP-2 IgG, and are representative, for each agonist used, of measurements taken pre-IgG (curve *1*), and at 2 h (curve *4*), 24 h (curve *3*), and 48 h (curve *2*) after administration.

nal velour, mean porosity 2,000–2,200 ml/H_2O per min at 120 mmHg) were a gift from U. S. Catheter, Incorporated, Billerica, MA. All grafts were 10 cm in length, 4.0 mm i.d. Before evaluation in the arteriovenous shunt system, the grafts were rendered impervious to blood leakage by an external wrapping of Parafilm (American Can Co., New York) and placement inside a 10-cm length of 5.2 mm i.d. "heat shrink" Teflon tubing (Small Parts Inc., Miami, FL). Silicone rubber tubing, 10 cm × 4.0 mm i.d., was connected to both ends of the graft segment with Silastic medical adhesive (type A, Dow Corning). This procedure produced impervious grafts rigidly constrained to a linear geometry, and having an inner diameter of precise dimensions (4.0 mm). The resulting flow channel was smooth in its transition from the Silastic to the graft without imperfection due to the coupling procedure. The avoidance of a hemodynamic "step" at the graft junctions has been found essential to insure reproducible and uniform platelet deposition following blood exposure (48).

The 4.0-mm i.d. Silastic tubing segments proximal and distal to the Dacron graft were subsequently connected to the 3.0 mm i.d. Silastic tubing comprising the chronic arteriovenous shunt with 2 cm long tapered Teflon connectors (Small Parts, Inc.). Mean blood flow rates through vascular graft segments incorporated into the A-V shunt system were measured continuously using a Doppler ultrasonic flowmeter (L and M Electronics, Daly City, CA) and "C" shell cuff type transducer probe that fitted snugly around the Silastic tubing comprising the chronic shunt. In all studies initial blood flow rates ranged from 160 to 240 ml/min.

Grafts were initially placed and imaged 1 h after the injection of autologous ^{111}In-labeled platelets. Antibodies were injected on the following day. Subsequent grafts were placed 10 min after the antibody was infused. When heparin and aspirin were given, grafts were placed 10 min after intravenous heparin administration. In studies with aspirin, grafts were placed 1 h after the first dose given on the second day of administration. In all studies the grafts were placed for 1 h and then removed.

Platelet labeling with [^{111}In]oxine and [^{14}C]serotonin. Autologous baboon blood platelets were labeled with [^{111}In]oxine as previously described (48). In brief, whole blood (100 ml) was collected directly into plastic bags (TA-3; Fenwal Laboratories, Deerfield, IL) containing 20 ml acid-citrate-dextrose anticogaulant (NIH formula A). The blood was centrifuged in the bag at 300 *g* for 10 min. The supernatant PRP was transferred to a second bag and pH adjusted to 6.5 by the addition of 0.15 M citric acid (0.1 ml/10 ml PRP). The red blood cell fraction was returned to the donor animal. The platelets were formed into a pellet by centrifugation of the PRP at 1,300 *g* for 15 min. The supernatant platelet-poor plasma (PPP) was completely decanted and discarded. To remove residual plasma proteins, the bag containing the platelet pellet was carefully washed once by overlaying with 30 ml of Ringer citrate dextrose (RCD; pH 6.5) which was decanted and discarded. The pellet was then gently resuspended in 5.0 ml RCD, and incubated for 30 min with 800–1,000 μCi [^{111}In]oxine (Amersham Corp.). Contaminating red cells were removed by a final slow centrifugation at 200 *g* for 5 min. Labeling efficiencies averaged > 90%. Previous studies with this method have shown that the labeled platelet population is functionally normal as demonstrated by equivalent reductions in circulating platelet count and circulating platelet radioactivity in response to infused collagen suspensions or blood exposure to vascular grafts (48, 62).

In five additional studies, platelets harvested according to the same method were simultaneously labeled with 500 μCi of [^{111}In]oxine and 20 μCi of [^{14}C]serotonin as described previously (63). These studies were included to examine the possibility that platelet granular serotonin might be preferentially lost as compared to the cytoplasmic ^{111}In-platelet label. To determine the platelet radioactivities, serial 3.0-ml blood samples were harvested twice by the addition of 5.0 ml RCD and centrifugation at 250 *g* for 5 min. The pooled supernatant PRP was then centrifuged at 1,300 *g* for 20 min and the supernatant PPP decanted. The platelet pellets were solubilized with 1 ml distilled water and mixed with 18 ml Riafluor (New England Nuclear, Boston, MA) for liquid scintillation counting. 1-ml samples of supernatant PPP were also counted for ^{14}C-activity in the same manner. After injection of the labeled cells, total PPP ^{14}C-activities averaged 2.1±1.2% of the platelet pellet activities, indicating negligible in vitro release of [^{14}C]serotonin for platelets harvested by this method. At the end of each experiment, 1-ml samples of the scintillation cocktail containing the solubilized pellets were counted for ^{111}In-platelet activity using a gamma counter. After allowing at least 30 d for the ^{111}In to decay (half-life: 2.8 d), the remaining mixture was counted for [^{14}C]serotonin activity. This approach insured that the same platelet preparation was counted for both radioisotopes.

Serotonin release assay. A serotonin release assay was used to assess the capacity of monoclonal antibodies to induce platelet release in vitro. Baboon blood was anticoagulated with sodium citrate. The platelet concentration in PRP was adjusted to 300,000/μl, and the citrate concentration was held constant at 0.012 M. The PRP was incubated with 50 nCi/ml [^{14}C]serotonin (57 mCi/mmol) for 30 min at 37°C. Imipramine (10 μM) was added to inhibit the uptake of released serotonin (if added prior to the addition of [^{14}C]serotonin, total uptake was reduced by ~ 65%). Separate 1-ml aliquots of PRP were then incubated with 16 μl of normal saline or AP-2 IgG stock solution (2.5 mg/ml AP-2 in 0.17 M phosphate buffered saline, pH 7.2) to give a final AP-2 concentration of 40 μg/ml PRP. Samples were incubated for up to 60 min at 37°C under both stirred (aggregometer cuvette) and unstirred conditions. Cell-free supernatant activity was counted at 15, 30, and 60 min after centrifugation of the PRP at 12,000 *g* for 2 min. Apparent release of newly absorbed [^{14}C]serotonin varied from 2 to 5% over 60 min in both stirred and unstirred control samples. At all times, apparent release in AP-2 treated samples ranged from 0 to 2% and was equivalent to control values indicating negligible antibody-induced release. In contrast, in positive control studies with added collagen (40 μg/ml), release of platelet serotonin averaged 66% after 60 min.

Platelet survival studies. After platelet labeling with ^{111}In, 3-ml blood samples were taken at daily intervals for 4 d, and then counted using a gamma spectrometer. Mean platelet life span was determined by computer least-squares fitting the disappearance data to gamma functions as described previously (47, 48).

Measurement of platelet factor 4 (PF-4) and β-thromboglobulin (β-TG). The platelet contents of PF-4 and β-TG were measured by radioimmunoassay of Triton X-100 platelet lysates as previously described (48). Plasma levels of PF-4 and β-thromboglobulin (β-TG) were also determined by radioimmunoassay on blood samples collected and processed as described previously (48).

Graft imaging. Images of the Dacron grafts, including proximal and distal Silastic segments, were acquired with a scintillation camera (Picker DC 4/11 Dyna; Picker Corp., Northford, CT) and stored on and analyzed by a computer (Medical Data Systems A^3; Medtronic, Ann Arbor, MI) interfaced with the camera. Immediately before imaging the vascular grafts, images were also acquired of 4.0 mm i.d. Silastic tubing filled with autologous blood and having the same luminal volume as the graft segment (blood standard). The activities of the standard and 10-cm graft segments were counted in the same 3.1 × 12.5-cm region of interest (10 × 40 pixels) as defined by image analysis software routines. Images were acquired at 5-min intervals. Deposited ^{111}In-platelet activity, calculated by subtracting the blood standard activity from all dynamic study images, increased monotonically over the exposure period. The total number of platelets deposited after 1 h (labeled plus unlabeled cells) was calculated by dividing the deposited platelet activity by the blood standard platelet activity, and multiplying by the volume of the blood standard (1.26 ml) and the circulating platelet count (platelets/ml) (48).

Statistics. All statistical analyses and curve fittings were done using the PROPHET system of the Division of Research Resources, National Institutes of Health. Statistical comparisons were made using Student *t* test (two-tailed) for paired and unpaired sample groups when the data were normally distributed. Remaining results were compared using the Wilcoxon sign rank test (64). All data in Results are given as the mean±1 SE.

Results

Effect of graft placement and injected antibodies on circulating platelet counts. In a number of studies Dacron vascular grafts were placed daily for 1 h on four sequential days to assess the time course and effectiveness of injected antibody. The effect on the circulating platelet count of repeated graft placement in the absence of injected antibody was also measured (Table I). Placement of control grafts for 1 h in four animals caused a reduction in platelet count from 419,000±43,000 platelets/μl to 378,000±55,000 platelets/μl as measured on the following day (Table I). 2 h after graft placement on the second day the counts were further reduced to 348,000±45,000 indicating that, in untreated animals, an acute reduction in platelet count of ~ 8% was produced by each graft placement per se. Over the following 2 d the platelet counts in the untreated group measured prior to graft placement were unchanged and averaged 380,000 platelets/μl.

Platelet counts were also evaluated sequentially in three of the four animals in each treatment group given AP-2 IgG, in three animals given AP-2 $F(ab)_2$, and in five animals given LJ-CP8. The initial platelet count in each group as well as the reduction in platelet count due to control graft studies (Pre-Ab values, Table I) were quite comparable. However, following AP-2 administration further reductions in platelet count were observed. These changes appeared acutely within 2 h of AP-2 administration and were largely complete by 24 h, although a modest additional reduction in count (from 285,000 platelets/μl to 227,000 platelets/μl) was observed between 24 and 48 h in animals given 0.4 mg/kg IgG. By 24 h, animals given 1.0 mg/kg AP-2 IgG had counts averaging 204,000±43,000 platelets/μl (vs. 380,000 platelets/μl in the control studies). Reductions in platelet count observed at 2 h and 24 h after administration of 1.0 mg/kg AP-2 $F(ab)_2$ were comparable to those measured following injection of the same amount of whole IgG (Table I).

Animals given 10.0 mg/kg LJ-CP8 showed only modest fluctuations in platelet count consistent with graft-mediated platelet removal only. Since the platelet counts in the untreated and LJ-CP8 treated animals remained relatively constant, and because the daily blood loss due to sampling (as calculated from hematocrit determinations) averaged only 5±2%/d, the reduced platelet counts in the AP-2 treated groups reflected a direct effect on platelets.

Thus, while the untreated and LJ-CP8 treated groups maintained normal counts, animals given 0.2 mg/kg and 0.4 mg/kg AP-2 IgG showed acute reductions in platelet numbers ranging from 22 to 33%. In animals given 1.0 mg/kg AP-2 (IgG or $F(ab)_2$), the loss of platelets averaged 45–46%, and these reductions appeared largely irreversible. It is important, however, to emphasize that injection of AP-2 induced maximal inhibition of platelet function within minutes, and that at the later time points the decrease in platelet count per se was never sufficient in any animal to prolong bleeding time measurements.

Effect of infused antibodies on platelet aggregation. Representative aggregometer tracings are given in Fig. 1. Measurements of the extent of platelet aggregation as determined from the maximum increase in light transmission (T_{max}) are presented in Table II. In animals given 0.2 mg/kg AP-2 IgG, values of T_{max} after aggregation induced by collagen, ADP, and γ-thrombin were modestly reduced over 24 h. Increased amounts of infused AP-2 IgG (0.4 and 1.0 mg/kg) caused dose-dependent inhibition of platelet aggregation with only partial recovery of platelet function by 48 h. At the highest dose, T_{max} measurements initially averaged 0–45% of control values, increasing to 17–60% by 48 h. In general, aggregation in response to collagen was suppressed the most, and aggregation in response to γ-thrombin was suppressed the least. AP-2 $F(ab)_2$ (1.0 mg/kg) was less effective than the equivalent amount of whole IgG. T_{max} was initially reduced in response to collagen (36%) and ADP (49%), and to a lesser extent γ-thrombin (84%), returning to essentially normal values by 48 h (Table II).

Injection of 10 mg/kg LJ-CP8 totally abolished platelet aggregation acutely, with little recovery of T_{max} by 48 h in

Table I. Effect of Graft Placement and Injected Antibodies on Circulating Platelet Count

		Platelets/μl (×10⁻³)				
Dose	*n*	Control	Pre-Ab	2 h	24 h	48 h
mg/kg						
0	4	419±43	378±55	348±45	380±42	380±44
AP-2 IgG						
0.2	3	424±120	345±87	236±67	259±58	258±52
0.4	3	429±30	347±34	279±36	285±33	227±55
1.0	3	438±29	384±53	199±63	204±43	187±45
AP-2 $F(ab)_2$						
1.0	3	377±33	356±51	198±34	213±44	
LJ-CP8 IgG						
10.0	5	388±26	333±46	322±36	318±41	349±41

In four control baboons, and in animals receiving intravenous antibody (Ab), circulating platelet counts were measured before the placement of 10 cm Dacron vascular grafts (control), and on the following day before the injection of antibody (Pre-Ab). A second series of grafts were placed 10 min following the injections of Ab. Platelet counts were subsequently measured 2 h after antibody administration and prior to grafts evaluated 24 h and 48 h following Ab infusion. All grafts were incorporated into an arteriovenous shunt for 1 h, and then removed. All values are mean±1 SEM.

Table II. Extent of Platelet Aggregation after Antibody Infusion

Dose (*mg/kg*)	Percent of maximum light transmission (T_{max})											
	ADP (*10 μm*)				Collagen (*10 μg/ml*)				γ-Thrombin (*17 μg/ml*)			
	Pre	2 h	24 h	48 h	Pre	2 h	24 h	48 h	Pre	2 h	24 h	48 h
AP-2 IgG:												
0.2	100	70±10	100*		100	51±27	83*		100	85±15	72*	
0.4	100	23±8	58±12	97‡	100	0	32±16	61±23	100	50±25	61±10	81‡
1.0	100	17±4	24±8	52±5	100	0	0	17±17	100	45±24	38±20	60±12
AP-2 F(ab)$_2$:												
1.0	100	49±9	58±11	87‡	100	36±18	25±25	102‡	100	84±19	91±16	110‡
LJ-CP8 IgG:												
10.0	100	0	20±9	66±10	100	0	0	18±17				

Platelet aggregation in response to ADP, collagen, and γ-thrombin was assessed in normal baboons before (Pre) and at 2, 24, and 48 h after the injection of monoclonal antibodies. The maximum extent of light transmission was normalized with respect to preinfusion values. Before antibody infusion, the absolute increases in light transmittance relative to that observed with platelet free plasma (100%), were, at the indicated concentrations of agonists: ADP (41±3%, n = 17), collagen (53±4%, n = 17), and γ-thrombin (59±4%, n = 12). All values are mean±1 SEM of observations in three animals (AP-2 IgG, AP-2 F(ab)$_2$) or five animals (LJ-CP8) unless otherwise indicated. * Single measurement only. ‡ Mean of two observations.

response to collagen (18%), and partial recovery in response to ADP (66%). In several additional studies (data not shown) platelet aggregation was assessed using blood drawn as early as 10 min after the injection of antibody. Results were equivalent to those obtained with samples drawn at 2 h, implying that the maximal effect of the injected IgG was virtually immediate.

Aggregometer recordings were also analyzed quantitatively to determine maximum slope, or V_{max} (percent per minute), in order to assess the effects of infused antibodies on rates of platelet aggregation in vitro (Table III). In general, both V_{max} and T_{max} exhibited similar trends with respect to agonist, antibody, dose, and time following antibody infusion. Thus for each antibody, the largest reductions in V_{max} were observed when the agonist was collagen. Increasing doses of AP-2 IgG caused progressive reductions in V_{max}. AP-2 F(ab)$_2$ was somewhat less effective than the whole IgG at the same dose, and maximum inhibition of aggregation velocity was seen in animals given LJ-CP8 IgG. Similarly, for each agonist, V_{max} was reduced significantly at 48 h after injection LJ-CP8 (10 mg/kg) and AP-2 IgG (1.0 mg/kg), but was reduced to a lesser extent in animals given AP-2 F(ab)$_2$ (1.0 mg/kg).

Effect of injected antibodies on the bleeding time. Measurements of the standard template bleeding time are shown in Fig. 2. In 17 control studies the bleeding time averaged 4.6±0.1 min and was not prolonged significantly by the infusion of 0.2 mg/kg AP-2 IgG (5.2±0.4 min; $P > 0.4$, paired t test). AP-2 IgG at 0.4 mg/kg caused a transient prolongation in bleeding time (9.8±1.5 min; $P = 0.05$) that was normal by 24 h (4.6±0.3 min). The highest dose of AP-2 IgG, 1.0 mg/kg, initially prolonged the bleeding time to 19.2±3.4 min ($P = 0.01$), and values were still abnormal at 24 h (15.8±3.9 min, $P < 0.05$).

Table III. Rates of Platelet Aggregation after Antibody Infusion

Dose (*mg/kg*)	Maximum rate of platelet aggregation (*% per min*)											
	ADP (*10 μm*)				Collagen (*10 μg/ml*)				γ-Thrombin (*17 μg/ml*)			
	Pre	2 h	24 h	48 h	Pre	2 h	24 h	48 h	Pre	2 h	24 h	48 h
AP-2 IgG:												
0.2	102±20	64±23	69*		68±8	19±19	51*		113±20	75±28	63*	
0.4	86±29	18±5	31±3	47‡	38±12	0	9±6	29±15	82±5	28±10	33±13	54‡
1.0	97±2	39±4	33±3	48±9	59±22	0	0	5±5	89±2	40±13	33±11	49±3
AP-2 F(ab)$_2$:												
1.0	84±8	44±5	52±7	62‡	56±14	15±8	16±16	57‡	73±7	45±4	47±5	58‡
LJ-CP8:												
10.0	68±5	4±4	21±7	39±7	30±7	0	0	10±10				

Rates of platelet aggregation (%/min) are expressed as the maximum rate of increase in light transmission, i.e., the slope of each aggregation curve was measured at its inflection point. Total light transmittance varied from 0% (PRP) to 100% (PFP). Aggregation in response to ADP, collagen, and γ-thrombin was measured before (Pre) and 2, 24, and 48 h after single bolus infusions of antibody. Unless otherwise noted, all values are mean±1 SEM of observations in three animals (AP-2 IgG, AP-2 F(ab)$_2$) or five animals (LJ-CP8 IgG). * Observations in one animal only. ‡ Average of measurements in two animals.

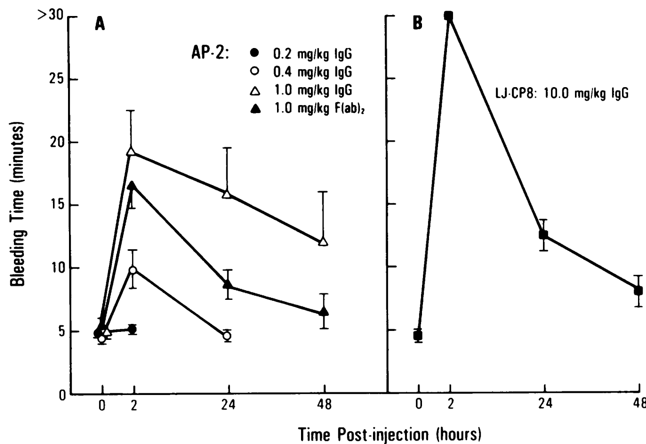

Figure 2. Effects of AP-2 and LJ-CP8 on bleeding time. (*A*) Doses of AP-2 IgG were: 0.2 mg/kg (●), 0.4 mg/kg (○), and 1.0 mg/kg (△). AP-2 F(ab)$_2$ was given at 1.0 mg/kg (▲). (*B*) LJ-CP8 was given at 10.0 mg/kg (■). Whereas the lowest dose of AP-2 did not prolong bleeding acutely, values obtained in animals given 1.0 mg/kg were markedly prolonged acutely and remained abnormal for at least 24 h. LJ-CP8 treated animals were abnormal for at least 48 h. All measurements were performed in duplicate and averaged, and represent the mean±1 SEM of determinations made in three animals (●, ▲), four animals (○), or five animals (△, ■).

Differences at 48 h (12.0±4.0 min) were not statistically significant ($P = 0.14$). AP-2 F(ab)$_2$ (1.0 mg/kg) increased the bleeding time initially (16.5±1.8 min, $P < 0.05$), but values at 24 h (8.7±1.1 min) and 48 h (6.7±1.2 min) were statistically normal ($P > 0.1$).

Infusion of LJ-CP8 (10.0 mg/kg) initially prolonged the bleeding time to > 30 min in five animals (Fig. 2 *B*). Values were significantly prolonged at 24 h (12.6±0.9 min; $P < 0.001$), and modestly prolonged at 48 h (8.8±1.8; $P = 0.07$).

In vivo distribution of ^{131}I-labeled AP-2 IgG. The time course of platelet and plasma radioactivity after the injection of ^{131}I-labeled AP-2 IgG is shown in Fig. 3. At the highest dose of ^{131}I-labeled antibody, 1.0 mg/kg, ~ 33,000 molecules per platelet were initially bound (Fig. 3 *A*). With 0.2 and 0.4 mg/kg IgG, 10,000–15,000 sites per platelet were initially occupied. In all cases, platelet associated IgG decreased progressively to < 2,000 molecules per platelet after 4 d. Thus, it is apparent that platelets bearing anti-GP IIb/IIIa antibodies circulated for hours. Analysis by polyacrylamide gel electrophoresis revealed that the platelet-bound radioactivity was indeed associated with intact IgG molecules (Fig. 3).

Plasma AP-2 levels ranged from ~ 0.5 to 10 µg/ml after the injection of 0.2 and 1.0 mg/kg, respectively. Most of the plasma IgG was cleared within the first 24 h, with a more gradual reduction to < 0.5 µg/ml by 96 h.

Studies with Dacron vascular grafts. Measurements of platelet deposition onto Dacron grafts inserted 10 min after the injection of AP-2 antibodies, and exposed for 1 h, are given in Fig. 4. Control studies were performed in 17 animals subsequently given either AP-2 IgG (nine animals), AP-2 F(ab)$_2$ (three animals), or LJ-CP8 (five animals). Since we have previously shown that graft platelet deposition depends in an approximately linear fashion on the circulating platelet count (65), the experimental groups were chosen to have comparable mean platelet counts in the range 307,000 to 389,000 platelets/µl (Fig. 4). After 60 min of blood exposure, AP-2 IgG reduced graft platelet deposition in a dose-dependent fashion. Thus deposition was reduced by an average of 41% by 0.2 mg/kg AP-2 ($P < 0.1$ vs. controls), 51% by 0.4 mg/kg AP-2 ($P < 0.01$), and 73% by 1.0 mg/kg AP-2 ($P < 0.001$). Platelet

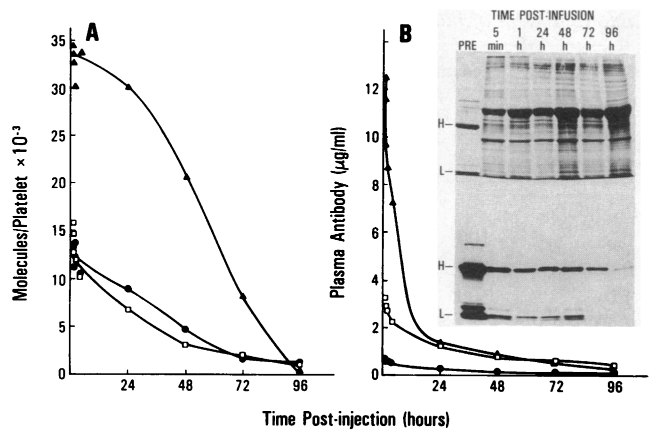

Figure 3. In vivo distribution of ^{131}I-labeled AP-2 IgG. Platelet binding (*A*) and plasma levels (*B*) of ^{131}I-IgG were determined following injection of 0.2 mg/kg (●) and 0.4 mg/kg (□) (one animal each), or 1.0 mg/kg (▲) (two animals). Platelet counts at the time of injection averaged 417,000/µl (●), 475,000/µl (□), and 342,000/µl (▲). At the highest dose, approximately 33,000 IgG molecules were initially bound to platelets. Both platelet and plasma IgG were largely cleared by 96 h. The insert to panel B shows the results of SDS-PAGE of the injected antibody (PRE, lane *1*) and platelet-bound ^{131}I-labeled AP-2 IgG. All samples were analyzed under reducing conditions. Platelets were separated from PRP, obtained at various time intervals following infusion of the antibody, by means of centrifugation through 20% sucrose (12). The platelet pellet was then lysed in SDS-containing buffer and reduced with 50 mM dithiothreitol. The top gel shows Coomassie Brilliant Blue staining, the lower gel the corresponding autoradiography. Intact heavy (*H*) and light (*L*) chains of IgG are bound to platelets over the period of observation. The prominent band seen in the platelet samples stained with Coomassie Brilliant Blue represents albumin present in the sucrose solution used to separate the platelets.

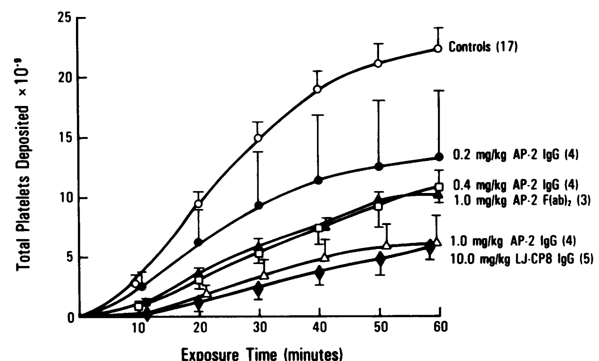

Figure 4. Effects of AP-2 and LJ-CP8 on platelet deposition onto acutely placed Dacron vascular grafts. The time course (0–60 min) of platelet accumulation was reduced in a dose-dependent fashion by AP-2 IgG. Significant effects were also observed in animals given AP-2 F(ab)$_2$ and LJ-CP8 IgG. Antibodies were administered 10 min before graft placement. Mean circulating platelet counts in the study groups were: 389,000±25,000/µl (○), 307,000±76,000/µl (●), 330,000±29,000/µl (□), 343,000±31,000/µl (▲), 384,000±37,000/µl (△), and 355,000±34,000/µl (♦).

accumulation was also reduced by AP-2 $F(ab)_2$ (1.0 mg/kg); at 60 min platelet accumulation was reduced 55% vs. controls ($P < 0.02$). In five animals given 10.0 mg/kg LJ-CP8, graft platelet deposition was reduced 73% vs. the control studies ($P < 0.001$), a value equivalent to the result observed in animals treated with 1.0 mg/kg AP-2 IgG.

While the results obtained with Dacron grafts evaluated on subsequent days following AP-2 infusion were ambiguous due to the observed reductions in ciruclating platelet count (Table I), platelet counts were relatively stable after LJ-CP8 administration. Thus 24 h after LJ-CP8 injection, platelet accumulation onto freshly inserted grafts in four animals averaged $7.5 \pm 2.22 \times 10^9$ platelets per graft at 1 h, and was reduced by 66% vs. control studies ($P < 0.01$, paired t test). In two animals studied at 48 h, graft platelet deposition was still reduced by an average of 46% after the 1-h exposure period.

Three additional groups of animals were also studied following administration of heparin (100 U/kg), oral aspirin (32.5 mg/kg twice daily) or the combination of heparin and aspirin at three doses. These study groups had comparable mean circulating platelet counts, ranging from 365,000 to 450,000 platelets/μl. Neither heparin, aspirin, nor the combination was effective in reducing platelet deposition over the 60-min period following graft placement ($P > 0.2$ vs. control values at all imaging times).

Effect on platelet granular release. To determine whether the antithrombotic effects of AP-2 and LJ-CP8 were mediated through a depletion of platelet granular contents, platelet PF-4, β-TG, and [^{14}C]serotonin were measured before and after antibody administration. No reductions in platelet PF-4 or β-TG were observed over the 24-h period after the injection of either 1.0 mg/kg AP-2 IgG or 10.0 mg/kg LJ-CP8 (Table IV). Similarly, platelet [^{14}C]serotonin radioactivity, expressed as a percentage of pre-IgG values, was not preferentially reduced relative to platelet cytoplasmic ^{111}In radioactivity. Also, 2 h after the injection of 1.0 mg/kg AP-2 IgG into three animals, plasma levels of PF-4 and β-TG averaged 4.1 ± 1.8 ng/ml and 1.8 ± 0.4 ng/ml, respectively, and were equivalent to control values obtained in untreated animals as reported previously (48). These results suggest that the circulating platelets had not undergone either α-granule or dense granule release, and are also in accord with the observed inability of AP-2 IgG to induce platelet release in vitro (see Methods).

Table IV. Effect of Injected Antibodies on Platelet Granular Release

Measurement	Pre-IgG	2 h	24 h
AP-2 IgG (1.0 mg/kg):			
Platelet count (per μl $\times 10^{-3}$)	454±18	214±82	277±52
Platelet PF-4 (μg per 10^9 platelets)	10.6±1.5	8.2±1.5	—
Platelet β-TG (μg per 10^9 platelets)	11.2±0.8	17.1±3.4	—
^{111}In-platelets (% baseline)	100	35.5±20.3	36.6±10.6
[^{14}C]serotonin (% baseline)	100	41.0±18.7	47.3±10.7
LJ-CP8 IgG (10.0 mg/kg):			
Platelet count (per μl $\times 10^{-3}$)	305±46	297±34	290±39
Platelet PF-4 (μg per 10^9 platelets)	7.6±0.6	7.2±0.7	7.2±0.8*
Platelet β-TG (μg per 10^9 platelets)	8.8±0.3	9.2±0.3	10.4±0.6*
^{111}In-platelets (% baseline)	100	0.96±0.07	0.68±0.04
[^{14}C]serotonin (% baseline)	100	0.95±0.05	0.72±0.04

The platelet contents of PF-4, β-TG, ^{111}In-platelet activity and [^{14}C]serotonin-platelet activity were measured prior to (Pre-IgG) and at 2 and 24 h after antibody injection in three aimals (AP-2 IgG) or four animals (LJ-CP8 IgG) unless otherwise indicated. All values are mean±1 SEM.

* Measurements in three animals.

Platelet survival studies. Determination of platelet survival was not appropriate in animals given AP-2 IgG or $F(ab)_2$ since these antibodies caused marked fluctuations in the circulating platelet count. Although platelet counts were largely unchanged following administration of LJ-CP8, this result did not preclude the possibility of some increase in platelet turnover. Therefore, in two animals platelet survival was measured over 4 d after injection of 10 mg/kg LJ-CP8. To maintain a normal platelet count over the study interval, no blood was drawn for other assays, nor were graft thrombosis studies conducted. The mean platelet lifespans averaged 5.24 and 5.18 d, values that were normal for baboons as previously reported (48).

Discussion

The results of the present study in baboons demonstrate that infusion of monoclonal antibodies against the platelet glycoprotein IIb/IIIa complex may significantly impair both platelet hemostatic function and the ability of platelets to participate in thrombus formation on arterial Dacron vascular grafts. In general, the time course and dose dependence of the effects of infused antibodies were similar for measurements of platelet aggregation, bleeding time, and graft platelet deposition. Interestingly, the lowest dose of AP-2 IgG (0.2 mg/kg) caused a transient impairment of platelet aggregation but had no detectable effect on bleeding time (Fig. 2). Similarly, in animals given 0.4 mg/kg AP-2 IgG, bleeding times at 24 h were normal, but aggregation in response to all three agonists was still reduced. These results suggest that, at least under some conditions, measurements of abnormal platelet aggregation ex vivo do not necessarily predict platelet hemostatic function in vivo.

The prolonged bleeding times observed in the present study are in agreement with the bleeding tendency consistently observed in patients with Glanzmann thrombasthenia who lack platelet GP IIb and IIIa, and whose platelets are unable to normally bind fibrinogen and other adhesive glycoproteins such as von Willebrand factor and fibronectin (1, 19–27). Previous investigators using a dog model have observed that infusion of an antibody against platelet GP IIb/IIIa did not cause spontaneous bleeding (43, 44). However, since spontaneous bleeding is usually associated only with severe and prolonged thrombocytopenia or profound inhibition of platelet function, hemostatic effects may have been inadequately evaluated in these studies.

In this report we observed comparable dose-dependent reductions in platelet count following injections of both AP-2 IgG and AP-2 $F(ab)_2$ fragments. Thus, the mechanism underlying the fall in platelet count was independent of the Fc portion of the molecule. Previous studies performed in a limited number of dogs given $F(ab')_2$ antibody fragments (43) showed

no significant effect on platelet count. Since different animals and different antibodies were used in these studies it is unwarranted to draw firm conclusions regarding mechanisms on the basis of this comparison, particularly since injection of whole IgG molecules of antibody LJ-CP8 had no effect on platelet count or platelet lifespan, even when given at doses 10 times greater than AP-2. Therefore, the relative merits of using whole IgG or Fab fragments may depend in each case upon the effects of individual antibodies on platelet viability, and on the feasibility of preparing Fab fragments.

Both AP-2 IgG and LJ-CP8 inhibited fibrinogen binding to baboon platelets, the former partially and the latter completely. Maximal effect was obtained with saturating amounts of antibody, and the results of in vitro inhibition studies were used to calculate the amount of antibody to be injected for in vivo experiments. The fact that AP-2 causes only partial inhibition of fibrinogen binding to baboon platelets in vitro is noteworthy, since it demonstrates that even partial blockade of the GP IIb/IIIa receptor may cause significant inhibition of platelet function in vivo. At the highest dose of AP-2 used in this study, 1.0 mg/kg, ~ 33,000 sites per baboon platelet were occupied, in reasonable agreement with the number of GP IIb/IIIa molecules per human platelet (3, 7, 32, 37, 66, 67). Also, AP-2 concentrations that effectively inhibit fibrinogen binding in vitro, > 2 μg/ml, are in good agreement with the effective plasma levels achieved in vivo, i.e., 2-10 μg/ml (Fig. 3) (37). Based on a baboon blood volume of 70 ml/kg and a molecular weight of 160,000 for the monoclonal IgG, it can be calculated from the data shown in Fig. 3 *A* that following the injection of 0.2, 0.4, and 1.0 mg/kg, respectively, ~ 59, 34, and 25% of injected IgG molecules were initially bound to platelets. We also found that intact heavy and light chain constituents of IgG molecules were associated with the platelet for up to 96 h. This lack of processing clearly suggests that the IgG remained bound to the platelet surface and was not internalized.

The acute thrombosis model used in the present studies, involving the acute interposition of Dacron vascular grafts into arteriovenous shunts, has been previously described (48). This model was chosen for several reasons. First, this system permits control of relevant physical variables such as blood flow rate and graft geometry. Second, measurements by external gamma camera imaging are quantitative and reproducible without attenuation of emissions due to intervening tissues. Third, anticoagulants are not required so that forming thrombi are subject to normal filtration, dilution, and inactivation mechanisms in the animal.

Using this model, marked reductions in graft platelet deposition were demonstrated for both antibodies studied. Interestingly, intermediate doses of AP-2, i.e., < 0.4 mg/kg, showed significant effects on platelet deposition without prolonging the bleeding time to a degree likely to be of clinical consequence (e.g., > 10 min). In contrast, neither heparin (100 U/kg) nor aspirin (32.5 mg/kg twice daily) showed antithrombotic efficacy. At these doses, heparin has been shown to prevent the accumulation of morphologically identifiable amounts of fibrin onto vascular grafts (68), and aspirin has been shown to reduce the capacity of platelets to synthesize thromboxane A_2 (TxA_2) to < 1% of control values (47). Thus, in this setting of acute arterial thrombosis associated with prosthetic vascular grafts, blocking the platelet GP IIb/IIIa receptor was substantially more effective than conventional therapies directed against coagulation- or thromboxane-mediated pathways of platelet recruitment. Interestingly, ticlopidine, a potent antiplatelet agent that induces a thrombasthenic-like state in platelets by mechanisms not clearly defined (69), has also been shown to be efficacious in this model (65). However, ticlopidine requires several days of pretreatment to reach full effectiveness, and these effects persist for several days following discontinuance of therapy.

Finally, a number of monoclonal antibodies against platelet glycoproteins have been described which differ markedly with respect to their effects on platelet function in vitro (31-33, 36, 37, 41, 42, 50, 70). Given the possibility that such antibodies could exhibit differential effects on thrombosis vs. hemostasis, or may differ in their capacity to induce immune-mediated platelet removal, further studies are warranted. Such antibodies might be particularly attractive as antithrombotic agents in situations requiring immediate, potent, yet transient inhibition of platelet function in vivo, such as small caliber vascular grafts, percutaneous arterial angioplasty, or post-reperfusion arterial occlusion.

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