Antithrombotic Effect of Platelet Glycoprotein Ib–Blocking Monoclonal Antibody Fab Fragments in Nonhuman Primates

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Abstract—Platelet adhesion in arterial blood flow is mainly supported by the platelet receptor glycoprotein (GP) Ib, which interacts with von Willebrand factor (vWF) that is bound to collagen at the site of vessel wall injury. Antibody 6B4 is a monoclonal antibody (MoAb) raised against purified human GPIb. MoAb 6B4 inhibits both ristocetin- and botrocetin-induced, vWF-dependent human platelet agglutination. MoAb 6B4 furthermore blocks shear-induced adhesion of human platelets to collagen I. We studied the antithrombotic effect of this inhibitory murine MoAb 6B4 in a baboon model of arterial thrombosis. When injected into baboons, intact IgG and its F(ab')2, fragments caused almost immediate thrombocytopenia, whereas injection of the Fab fragments alone did not. Fab fragments were subsequently used to investigate their in vivo effect on platelet deposition onto a thrombogenic device, consisting of collagen-rich, glutaraldehyde-fixed bovine pericardium (0.6 cm²), at a wall shear rate ranging from 700 to 1000 s⁻¹. Baboons were either pretreated with Fabs to study the effect of inhibition on platelet adhesion or treated 6 minutes after placement of the thrombogenic device to investigate the effect on interplatelet cohesion. Pretreatment of the animals with bolus doses ranging from 80 to 640 μg/kg Fab fragments significantly reduced ¹¹¹In-labeled platelet deposition onto the collagen surface by ≈43% to 65%. Only the highest dose caused a significant prolongation (doubling) of the bleeding time. Ex vivo ristocetin-induced platelet agglutination was equally reduced. Treatment with a bolus of 110 μg/kg Fab fragments after a thrombus was allowed to form for 6 minutes had no effect on further platelet deposition. We therefore conclude that Fab fragments or derivatives of inhibitory anti-GPIb antibodies may be useful compounds to prevent thrombosis. (Arterioscler Thromb Vasc Biol. 2000;20:1347-1353.)

Key Words: platelet adhesion ■ platelet aggregation ■ thrombosis ■ glycoprotein Ib ■ monoclonal antibodies
that binds to vWF,\textsuperscript{15} and recombinant vWF fragments like VCL,\textsuperscript{16–18} all of which inhibit vWF-GP\textsubscript{IIb} interaction. All of these studies showed the potential feasibility of antithrombotic therapy based on inhibition of the GP\textsubscript{IIb}-vWF axis.

A number of potent, inhibitory anti-GP\textsubscript{IIb} antibodies have been produced and were extensively tested with respect to their in vitro effect under both static (platelet agglutination, vWF binding) and flow conditions.\textsuperscript{19} We are aware of only 2 successful in vivo studies on guinea pigs, in which F(ab\textsuperscript{9})\textsubscript{2} fragments of PG\textsubscript{1,1}, a monoclonal anti–guinea pig GP\textsubscript{IIb} antibody, were used.\textsuperscript{20,21} These fragments were shown to significantly prolong the time to arterial microvascular graft thrombosis without prolonging the bleeding time.\textsuperscript{21} In another guinea pig model, the fragments could effectively reduce thrombus formation on a laser-induced injury.\textsuperscript{20} However this PG\textsubscript{1,1} antibody is specific for guinea pig platelets and does not cross-react with human platelets. Part of this rather surprising lack of in vivo studies is due to the low cross-reactivity of the anti-human GP\textsubscript{IIb} MoAbs with platelets from commonly used laboratory animals. This situation necessitates the use of nonhuman primates as experimental animals. However, even then, attempts to perform in vivo studies are hampered because injection of the anti-GP\textsubscript{IIb} MoAbs, as well as the snake venom protein echinatin that reacts with GP\textsubscript{IIb}, invariably causes severe thrombocytopenia.\textsuperscript{10,13,22,23}

We have studied the antithrombic efficacy of a novel murine MoAb, 6B4 (IgG\textsubscript{1}), raised against human platelet GP\textsubscript{IIb}, in in vitro and in vivo studies. In vitro, 6B4 and its F(ab\textsuperscript{9})\textsubscript{2}, and Fab fragments all potently inhibited the binding of vWF to human GP\textsubscript{IIb} under both static and flow conditions, and they also bound to baboon platelets. In the in vivo studies in which 6B4 was injected into baboons, the intact MoAb caused immediate and severe thrombocytopenia; injection of its F(ab\textsuperscript{9})\textsubscript{2}, fragments resulted in a moderate decrease in platelet count, whereas the Fab fragments did not have a major effect on platelet count. Furthermore, the Fab fragments were studied in a baboon model of platelet-dependent arterial thrombosis. Platelet deposition onto collagen-rich bovine pericardium was inhibited when the fragments were injected into the baboons before a thrombus was generated. On the other hand, when the Fab fragments were injected after a thrombus was allowed to form, no inhibition of further thrombosis was observed.

Methods

Preparation and Purification of Intact MoAb 6B4, F(ab\textsuperscript{9})\textsubscript{2}, and Fab Fragments

The antibody used in these studies, 6B4 (subtype IgG\textsubscript{1}), is a murine MoAb raised against purified human GP\textsubscript{IIb}. GP\textsubscript{IIb} was purified from outdated platelet concentrates collected by the Belgian Red Cross in the Free State in accordance with the National Code for Animal Use by guest on October 15, 2017 http://atvb.ahajournals.org/ Downloaded from

MoAb 6B4 was dialyzed overnight against a 0.1 mol/L citrate buffer (pH 3.5). The antibody was digested by incubation with pepsin (Sigma Chemical Co; 1 part pepsin to 200 parts MoAb) for 1 hour at 37°C. Digestion was stopped by adding 1 volume of a 1 mol/L Tris-HCl buffer (pH 9) to 10 volumes of antibody solution.

Monovalent Fab fragments were prepared by pepsin digestion. A 1:10 (vol/vol) solution of 1 mol/L phosphate buffer (pH 7.3) was added to the antibody. Papain (Sigma) was added at a ratio of 1 volume papain to 25 volumes of the phosphate buffer containing the MoAb, 10 mmol/L L-cysteine-HCl (Sigma), and 15 mmol/L EDTA. After incubation for 3 hours at 37°C, digestion was stopped by adding freshly prepared iodoacetamide solution (Sigma) to a final concentration of 30 mmol/L, which was then kept in the dark at room temperature for 30 minutes.

Both F(ab\textsuperscript{9})\textsubscript{2}, and Fab fragments were further purified from contaminating intact IgG and Fc fragments by using protein A-Sepharose. The purified fragments were finally dialyzed against PBS. The purity of the fragments was determined by SDS–polyacrylamide gel electrophoresis, and the protein concentration was measured by using bichinchoninic acid protein assay reagent A (Pierce Chemical Co).

Further Antibody Characterization

MoAb 6B4 binds to a (His\textsubscript{1}–Val\textsubscript{289}) recombinant GP\textsubscript{IIb} (rGP\textsubscript{IIb}) fragment expressed by Chinese hamster ovary cells,\textsuperscript{26} indicating that its epitope is localized within the amino-terminal region of GP\textsubscript{IIb}. MoAb 6B4 Fab was further tested for inhibition of ristocetin- and botrocetin-induced binding of vWF to the rGP\textsubscript{IIb} fragment by ELISA.\textsuperscript{27} Microtiter plates were coated with 5 μg/mL MoAb 2D4 for 48 hours at 4°C. MoAb 2D4, an anti-GP\textsubscript{IIb} MoAb that we produced, binds to the rGP\textsubscript{IIb} fragment but does not block vWF binding. Nonabsorbed sites were blocked with 3% skimmed milk, whereafter the plates were washed with Tris-buffered saline containing 0.1% Tween 20. Purified rGP\textsubscript{IIb} fragments were immobilized on MoAb 2D4 by incubating 2 μg/mL rGP\textsubscript{IIb} for 2 hours at 37°C. After being washed with Tris-buffered saline–Tween 20, increasing concentrations of 6B4 Fab fragments diluted in Tris-buffered saline–Tween 20 were added, followed by 1.25 or 0.6 μg/mL purified human vWF, respectively, when ristocetin (300 μg/mL) or botrocetin (0.5 μg/mL) was used as a modulator. Binding of vWF was determined by incubating the resulting mixture for 1 hour with horseradish peroxidase–conjugated polyclonal anti-vWF antibody (Dako), diluted 1/3000 in Tris-buffered saline–Tween 20. The color reaction, stopped by addition of 4 mol/L H\textsubscript{2}SO\textsubscript{4}, was generated with o-phenylenediamine (Sigma). Ristocetin was supplied by abp, and purified vWF was purchased from the Red Cross, Belgium. The purification of botrocetin from crude Bothrops jararaca venom (Sigma) was performed as previously described.\textsuperscript{28}

The effect of 6B4 Fab on shear-induced platelet adhesion to collagen was tested in a Sakariassen-type parallel-plate flow chamber at shear rates of 650, 1300, and 2600 s\textsuperscript{–1} as previously described.\textsuperscript{29} Human collagen type I (Sigma) was dissolved in 50 mmol/L acetic acid (1 mg/mL), dialyzed for 48 hours against PBS, subsequently sprayed onto plastic Thermaxx coverslips, and stored at room temperature overnight before use. Twelve milliliters of blood anticoagulated with low-molecular-weight heparin (25 U/mL; Cleaxane, Rhône-Poulenc Rorer) was preincubated with 6B4 Fab fragments at 37°C for 5 minutes. This was then used to perfuse the collagen-coated coverslips. After 5 minutes of perfusion, the platelets were fixed with methanol and the coverslips stained with May–Grünewald/Giemsa. Platelet adhesion (percent of total surface covered with platelets) was evaluated with a light microscope connected to an image analyzer. An average of 30 fields per coverslip were analyzed. Platelet adhesion was expressed as percent maximal platelet adhesion obtained in the absence of inhibitor.

Animal Studies

Normal male baboons (Papio ursinus) were used. The animals weighed between 10 and 15 kg and were disease-free for at least 6 weeks before the experiments. All procedures were approved by the Ethics Committee for Animal Experimentation of the University of the Free State in accordance with the National Code for Animal Use
in Research, Education, Diagnosis and Testing of Drugs and Related Substances in South Africa.

The baboons had permanent polytetrafluoroethylene (Teflon)–silicone rubber (Silastic) arteriovenous shunts implanted in the femoral vessels. Blood flow through the shunts varied between 100 and 120 mL/min, resulting in wall shear rates between 700 and 1000 s⁻¹, which compares well with the shear rates found in medium-size arteries. Handling of the baboons was achieved through anesthesia with ketamine hydrochloride (≈10 mg/kg IM; Anaket-V, Centaur Laboratory).

Study Protocol
To test the effect of the MoAb on platelet count, 6B4, its F(ab)², fragments, or its Fab fragments were administered to 3 different baboons. The plasma volume was calculated by assuming a blood volume of 65 mL/kg body mass and correcting for the hematocrit. Platelet-dependent arterial thrombus formation was induced by using bovine pericardium (0.6 cm² fixed in buffered glutaraldehyde). The pericardium was built into the wall of Silastic tubing (3-mm inside diameter). The method of preparation of the thrombogenic device has been described in detail, except that fixed bovine pericardium was used instead of Dacron vascular graft material. In each experiment, a thrombogenic device prefilled with saline to avoid a blood-air interface was incorporated as an extension segment into the permanent arteriovenous shunt by means of Teflon connectors.

Two approaches were followed to determine the effect of 6B4 Fab fragments on platelet adhesion and interplatelet cohesion onto the collagen of the bovine pericardium.

Dose-Response Effect of 6B4 Fab Fragments on Platelet Adhesion and Deposition
Seven baboons were used in the first studies; in total 13 perfusion experiments were performed. In the first 5 experiments (3 baboons), a thrombogenic device was placed to determine the deposition of platelets (see the section below on graft imaging and quantification of platelet deposition). After 30 minutes, the device was removed and blood flow through the permanent arteriovenous shunt reestablished. Fifteen minutes after removal of the device, each baboon was treated with a bolus of 80 μg/kg 6B4 Fab fragments (in 2 mL saline) and 15 minutes after treatment, a second thrombogenic device was placed for 30 minutes to determine the effect of the Fab fragments on thrombogenesis. The device was again removed and blood flow through the permanent shunt established. This step was followed by a second bolus injection of Fab fragments (80 μg/kg) to attain a cumulative dose of 160 μg/kg. After 15 minutes, a third thrombogenic device was placed for 30 minutes and platelet deposition was measured. In 4 other experiments (2 baboons), the same study protocol was used, except 2 doses of 320 μg/kg were administered. Sham studies were done in 4 other experiments (2 baboons). In these studies, the same protocol of placement of thrombogenic devices was followed, but the baboons were not treated with Fab fragments.

Effect of Anti-GPIb 6B4 Fragments on Interplatelet Cohesion
In a second series of 12 experiments, 6 baboons were used. In all baboons, a thrombogenic device was placed for 24 minutes. In 6 experiments (3 baboons), the baboons received a bolus injection of Fab fragments of 110 μg/kg. The fragments were injected 6 minutes after placement of the thrombogenic device to allow enough platelets to be deposited to cover the collagen surface. In the other 6 experiments (3 baboons), the baboons did not receive any Fab fragments.

In both approaches, blood was collected at different time points to determine platelet count and hematocrit (EDTA), circulating and platelet-associated radioactivity, the ex vivo aggregation of platelets in response to ristocetin, and the plasma concentrations of Fab fragments (see the section on laboratory measurements). The time points at which the blood was collected are given in the figures.

Graft Imaging and Quantification of Platelet Deposition
Autologous blood platelets were labeled with ¹¹¹In-tropolone and imaging and quantification of the deposition of the ¹¹¹In-labeled platelets were done as described in detail. In brief, image acquisition of the grafts, including proximal and distal Silastic segments, was done with a large-field-of-view scintillation camera fitted with a high-resolution collimator. The images were stored on and analyzed with a Medical Data Systems A′ computer (Medtronic) interfaced with the scintillation camera. Dynamic 2-minute image acquisition was started simultaneously with the start of blood flow through the devices. A 2-minute image (128×128-byte mode) of a 3-mL autologous blood sample collected in EDTA was also acquired each time that the grafts were imaged to determine circulating blood radioactivity (blood standard). A region of interest of the graft segment was selected to determine the deposited and circulating radioactivity in each of the dynamic images. Radioactivity in a region of similar size in the proximal segment of the extension tubing was determined and subtracted from the radioactivity in the graft region to calculate deposited radioactivity. Platelet deposition was expressed as the total number of platelets deposited. The method to calculate this has been described in detail.

Laboratory Measurements
Receptor Binding Studies
6B4, its F(ab)², fragments, or Fab fragments were labeled with Na¹²¹I (Amersham) by using the Iodo-Gen method. Iodo-Gen was purchased from Pierce. Baboon PRP, adjusted with autologous plasma to a count of 100 000 platelets/μL, was incubated with different concentrations of iodinated 6B4, F(ab)², fragments, or Fab fragments for 15 minutes at room temperature. The mixture was layered onto 20% sucrose buffer (wt/vol) containing 0.1% (wt/vol) BSA and centrifuged for 4 minutes at 10 000g in Eppendorf tubes. The top fluid, including the plasma, was removed and the pellets were counted in a gamma counter. Binding studies were performed in duplicate on the PRP of 2 baboons.

In Vitro and Ex Vivo Platelet Aggregation
The aggregation of platelets in response to ristocetin (ABP) was done on 10 mL of blood collected in 1 mL of 3.2% trisodium citrate. PRP was prepared by differential centrifugation and the platelet count adjusted to 200 000 platelets/μL with autologous plasma. The aggregation response was measured in a Monitor IV Plus aggregometer (Helena Laboratories) and recorded for 5 minutes. The percent aggregation at 5 minutes was calculated as the difference in light transmission between PRP and platelet-poor plasma.

In vitro studies, the PRP was preincubated for 5 minutes with serial dilutions of intact IgG 6B4, F(ab)², fragments, or Fab fragments before aggregation was initiated. Inhibition of aggregation was calculated from the difference in the aggregation response of platelets without and with antibody or fragments. In the ex vivo determinations, inhibition was calculated from the difference in the aggregation response of platelets before and after treatment of the baboons.

Plasma levels of 6B4 Fab fragments were measured with a sandwich ELISA. In brief, microtiter plates were coated overnight at 4°C with 5 μg/mL polyclonal goat anti-mouse IgG (Sigma). After unoccupied binding sites were blocked with BSA, serial dilutions of baboon plasma were added to the wells and incubated for 2 hours. Bound 6B4 Fab fragments were detected by using goat anti-mouse IgG (Fab-specific) conjugated to peroxidase (Sigma) and developed by using o-phenylenediamine as described above. Standard curves were constructed by adding known amounts of 6B4 Fab fragments to baboon plasma.

Bleeding Time
The bleeding time was determined by using the Simplate II device (Organon Teknika) according to the instructions of the manufacturer. The volar surface of the forearm of the baboons was shaved, and a pressure cuff was applied and inflated to 40 mm Hg.

Statistical Analysis
Student’s t test for paired data was used to test for statistically significant differences. Data given in the text are mean±SE. Probability values <0.05 were considered statistically significant.
Results

In Vitro Effect of 6B4 Fab Fragments on Human Platelets

MoAb 6B4 Fab fragments blocked the ristocetin-(1 mg/mL) and botrocetin-(0.5 μg/mL) induced human platelet agglutination, with an IC$_{50}$ of 1.2 ± 0.3 μg/mL (24 ± 6 nmol/L) and 2.0 ± 0.5 μg/mL (40 ± 10 nmol/L), respectively. For the intact 6B4 IgG, these values were 0.3 μg/mL (2.0 nmol/L) and 0.8 μg/mL (5.3 nmol/L), respectively. 6B4 binds to an epitope localized on the amino-terminal part (His1–Val289) of GPIbα. The inability of 6B4 to bind to denatured GPIb during Western blotting (not shown) strongly suggests that this antibody binds to a conformation-sensitive epitope on GPIbα.

The 6B4 Fab fragments dose-dependently inhibited both the ristocetin-(300 μg/mL) and botrocetin-(0.5 μg/mL) induced binding of vWF to the (1–289) rGPIbα fragment (Figure 1), with an IC$_{50}$ of 1.8 μg/mL (36 nmol/L) and 2.5 μg/mL (50 nmol/L), respectively.

In Vitro Effect of MoAb 6B4 and Its F(ab$'$)$_2$ and Fab Fragments on Baboon Platelets

Binding of the antibody and its fragments to baboon platelets was dose dependent and saturable. Half saturation (K$_{D50}$) was obtained with 4.7 nmol/L for 6B4 IgG, 6.4 nmol/L for F(ab$'$)$_2$, and 49.2 nmol/L for Fab (Figure 3). At saturating concentrations, ristocetin-induced aggregation was completely abolished (Figure 4). The IC$_{50}$ of platelet aggregation was obtained at 4.5 nmol/L, 7.7 nmol/L, and 40 nmol/L for 6B4 IgG, F(ab$'$)$_2$, and Fab fragments, respectively.

Effect of Injection of MoAb 6B4, F(ab$'$)$_2$, and Fab Fragments on Peripheral Platelet Count in Baboons

In 1 baboon, 100 μg/kg intact antibody caused a profound decrease in the blood platelet count ($<30 \times 10^9$/L) within 10 minutes after injection. After 48 hours, the platelet count was still <100 $\times 10^9$/L. When 100 μg/kg 6B4 F(ab$'$)$_2$ fragments was injected into 2 baboons, the platelet count decreased rapidly to between 120 and 150 $\times 10^9$/L, ie, by ~60%, but then reached preinfusion values within 24 hours. Finally, when 80 to 320 μg/kg of the monovalent 6B4 Fab fragments was injected, the platelet count (45 minutes after injection) decreased by only ~10% to 20% and by only 26% when 640 μg/kg was used with an IC$_{50}$ of 1.8 μg/mL (36 nmol/L) and 2.5 μg/mL (50 nmol/L), respectively.

The 6B4 Fab fragments inhibited platelet adhesion to human collagen type I in a concentration-dependent manner at shear rates of 650, 1300, and 2600 s$^{-1}$ (Figure 2), with a 50% reduction of surface coverage obtained at a concentration of 3.5 μg/mL (70 nmol/L), 1.1 μg/mL (22 nmol/L), and 0.5 μg/mL (10 nmol/L), respectively.
Platelet Counts, Plasma Levels of 6B4 Fab Fragments, Ex Vivo Ristocetin-Induced Platelet Agglutination, and Bleeding Times After 6B4 Fab Administration

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<th>Dose, µg/kg</th>
<th>Time, min</th>
<th>Platelet Counts ×10³/µL</th>
<th>% Decrease</th>
<th>Plasma Levels, µg/mL</th>
<th>Ristocetin-Induced (1.5 mg/mL) Platelet Agglutination</th>
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ND indicates not determined. Administration of 80–640 µg/kg 6B4 Fab fragments to baboons was performed. Values are given as mean±SE. Statistical comparisons were made by Student’s t test for paired sample groups: *P<0.05.

μg/kg was injected (the Table). On the basis of this result, the 6B4 Fab fragments were used for further studies.

Effect of Different Doses of MoAb 6B4 Fab Fragments on Platelet Deposition

Platelet adhesion and deposition onto thrombogenic devices sequentially placed 30 minutes apart are summarized in Figure 5. In the sham studies (Figure 5A), placement of the previous graft had no significant effect on platelet deposition that formed on subsequent grafts.

In the treatment studies (Figure 5B), dosages of 80 and 160 µg/kg (2×80 µg/kg 6B4 Fab) significantly inhibited platelet deposition in comparison with control by ~43% and 53%, respectively. Doses of 320 and 640 µg/kg (2×320 µg/kg 6B4 Fab) significantly reduced platelet deposition by 56% and 65%, respectively.

Plasma levels of 6B4 Fab fragments and inhibition of ex vivo agglutination determined on samples obtained 45 minutes or 2 hours after administration changed in both a dose- and time-dependent manner (the Table). Ex vivo ristocetin-induced platelet aggregation was significantly inhibited at doses of 160 and 640 µg/kg. Both ristocetin-induced platelet aggregation and plasma values returned to baseline within 3 hours after antibody injection.

Bleeding times determined in the treatment studies before and 45 minutes after injection of 80 to 320 µg/kg 6B4 Fab fragments were not significantly prolonged. Only a dose of 640 µg/kg significantly prolonged the bleeding time.

Effect of 6B4 Fab Fragments on Interplatelet Cohesion

Treatment of the baboons with 110 µg/kg 6B4 Fab did not affect platelet deposition when the animals were injected after a thrombus was allowed to form for an initial 6 minutes (Figure 6).

Discussion

The initial step in platelet adhesion consists of vWF binding to GPIb. We investigated the effect of inhibiting this interaction on platelet function both in vitro and in vivo. A murine MoAb, 6B4, that binds to a conformational epitope in the amino-terminal part of GPIbα was used. The antibody and its fragments potently inhibited the binding of vWF to an rGPIbα fragment (His1–Val289) and dose-dependently inhibited vWF-dependent human platelet agglutination. The intact antibody and its fragments also dose-dependently inhibited human platelet adhesion to type I collagen in a flow chamber at wall shear rates of 650, 1300, and 2600 s⁻¹. This inhibition was shear dependent, ie, more pronounced at higher shear.

Of interest was the finding that 6B4 did not react with platelets from dogs, hamsters, pigs, or guinea pigs but did...
Figure 6. Influence of late treatment of baboons with 6B4 Fab fragments on platelet deposition. The thrombogenic device was placed at time 0 and platelet deposition determined for 24 minutes (top shaded bar). After 6 minutes (arrow), animals were either left untreated (□) or treated with a bolus of 110 (□) μg/kg 6B4 Fab fragments (n = 6). Values are given as mean ± SE.

bind to baboon platelets with much the same characteristics as to human platelets. As a result, baboons were used for in vivo and ex vivo studies. Both the intact antibody as well as its F(ab')2 fragments caused immediate thrombocytopenia, similar to what was seen when other anti-GPIb MoAbs were injected into different experimental animals.11,12,22,23 On the other hand, the Fab fractions had only a moderate effect on the blood platelet count, and we therefore decided to use the Fab fractions to assess the antithrombotic effect of 6B4 in a baboon model of arterial thrombosis.30 The glutaraldehyde-fixed bovine pericardium was highly thrombogenic: after 30 minutes of exposure to native flowing blood, ≈3×10⁹ platelets deposited on the area of 0.6 cm². In similar studies, only ≈0.7×10⁹ platelets accumulated in 30 minutes on Dacron vascular graft material (0.9 cm²).31 It is therefore also not surprising that a number of control thrombogenic devices became occluded before 30 minutes of exposure to flowing blood.

Treatment of baboons with 6B4 Fab fragments inhibited platelet deposition on the thrombogenic devices by 43% to 65%. The observed effect must be ascribed to the effect of the antibody, because sequential placement of thrombogenic devices in untreated baboons caused no decreased deposition. No complete inhibition of platelet deposition was observed, even at high doses.

It is possible that this incomplete inhibition was due to the medium shear rates (700 to 1000 s⁻¹) used in this study. In general, in flow chambers a more effective inhibition with vWF-GPIb inhibitors is obtained at higher shear rates,11,12,26 which we also demonstrated here by finding a more pronounced effect of 6B4 on platelet adhesion to collagen when a higher shear was applied in a flow chamber; also, in vivo an arterial thrombus was more readily prevented than a venous one.11 This implies that inhibition of GPIIb would result especially in arterial effects, which in addition could result in less bleeding risk.

The doses used indeed caused a significant reduction in thrombus size without apparently increasing the risk of bleeding since bleeding time was only mildly prolonged at the highest dose. This finding supports data obtained with other GPIIb-αvWF–blocking agents (VCL, anti-αvWF MoAb AJvW-2) that lengthened the bleeding time only moderately.11,12,17,18 This finding is important and might provide a major advantage in the development of antithrombotic agents compared with GPIIb-IIIa antagonists, like ReoPro.37 On the other hand, the GPIIIa–αvWF interaction in contrast to the GPIIIa–fibrinogen interaction is the ultimate first step in platelet adhesion under fast blood flow. Because binding of αvWF to GPIIIa is a time-consuming process, it is reasonable to assume that by inhibiting αvWF-GPIIIa binding, fewer platelets will be activated. The smaller thrombus that finally forms may therefore be a consequence of both fewer platelets that adhere to collagen and less platelet aggregation. Thus, where GPIIIa blockers mainly prevent platelet aggregation, interruption at an earlier stage by a GPIb blocker is expected not only to limit the platelet plug that is formed but also to reduce additional platelet-dependent effects, such as granule release, thought to play a role in the development of arteriosclerosis and restenosis.40,41

There are also indications that the GPIIIa–αvWF complex is involved in platelet-platelet interactions. Ruggeri et al.42 recently reported that blocking the GPIIIa–αvWF interaction, after platelets from PPACK-anticoagulated blood had adhered to bovine collagen in vitro for 100 seconds at 1500 s⁻¹, prevented further thrombus growth measured after another 740 seconds, even at low shear rates that do not normally initiate vWF-dependent platelet adhesion. To test this concept in vivo, we performed a second series of studies to investigate the role of GPIIb in platelet-platelet interactions at intermediate shear rates. A thrombogenic device was placed as an extension segment in the permanent arteriovenous shunt and exposed to native flowing blood. After 6 minutes the baboons were treated with the Fab fractions of 6B4. We postulated that a 6-minute exposure (number of platelets deposited was ≈0.6×10⁹) was sufficient to allow ample coverage of the pericardium with adhering platelets. Inhibition of platelet deposition due to treatment, when compared with sham studies, would therefore reflect inhibition of platelet-platelet interactions. Because no such effect was seen, it strongly suggested that GPIIb does not play a major role in in vivo platelet-platelet interactions under the conditions used in this study.

In conclusion, we have reported on the first anti-human GPIb antibody that can be used successfully to prevent platelet adhesion and thrombus formation in vivo, thereby confirming the predominant role of GPIb in platelet adhesion in vivo. Our studies, however, do not support the hypothesis that GPIIb also plays a part in platelet-platelet interactions in vivo. On the basis of our results on bleeding times and the inhibition of thrombogenesis, we propose that the Fab fragments or derivatives of the anti-GPIIb MoAb 6B4 may be useful compounds in preventing arterial thrombosis in those patients in whom thrombosis is expected, ie, after vascular engraftment, endarterectomy, or balloon catheterization.

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