Inhibition of Platelet Glycoprotein Ib, Glycoprotein IIb/IIIa, or Both by Monoclonal Antibodies Prevents Arterial Thrombosis in Baboons

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Abstract—The antithrombotic efficacy of the monoclonal antibodies 6B4-Fab and MA-16N7C2 against platelet glycoprotein (GP) Ib and GP IIb/IIIa, respectively, on acute platelet-mediated thrombosis was evaluated in a baboon model of femoral artery stenosis, which is a modification of the original Folts model: platelet thrombi form on the injured stenosed artery, producing cyclic flow reductions (CFRs). A dose of 0.6 mg/kg 6B4-Fab significantly reduced the CFRs by 59±15%, whereas 2 mg/kg 6B4-Fab completely abolished the CFRs without prolongation of the bleeding time. MA-16N7C2 inhibited CFRs by 43±8% at a dose of 0.1 mg/kg and abolished the CFRs at a dose of 0.3 mg/kg but with a significant prolongation of the bleeding time. Finally, the combination of 0.6 mg/kg 6B4-Fab and 0.1 mg/kg MA-16N7C2 fully prevented the CFRs without prolongation of the bleeding time. The present study demonstrates that the inhibition of platelet GP Ib function by 6B4-Fab is a powerful intervention to prevent platelet thrombus formation in injured arteries without prolongation of the bleeding time; the latter is in contrast to the result after the inhibition of GP Ib/IIIa. Moreover, we demonstrate that combining a GP Ib blocker with a GP IIb/IIIa blocker can achieve a strong antithrombotic effect without increasing the bleeding time. This provides new information that will be beneficial in designing clinical therapeutic approaches.

Key Words: platelet glycoprotein Ib ■ platelet glycoprotein IIb/IIIa ■ cyclic flow reductions ■ antithrombotic agents ■ bleeding time

Platelets adhere to the subendothelium of damaged blood vessels through the collagen–von Willebrand factor (vWF)–platelet glycoprotein (GP) Ib axis. vWF forms the bridge between platelets and collagen in the vessel wall (especially under high-shear conditions) and in stenosed arteries and microvessels, where it initiates the formation of platelet aggregates. When vWF binds to GP Ib, platelets are slowed down, allowing direct platelet–collagen receptor interactions via, for example, integrin α6β1 and glycoprotein VI, which activate platelets, finally resulting in a conformational change in the GP IIb/IIIa receptor to enable binding to fibrinogen and vWF, leading to the formation of platelet aggregates.1,2 After extensive vessel injury or rupture of atherosclerotic plaques with subsequent exposure of thrombogenic surfaces, platelet aggregation can progress to result in complete thrombotic occlusion of the injured vessel.3,4 Various clinical studies and studies in experimental animals have clearly shown that inhibition of platelet aggregation, through prevention of the binding of the adhesive proteins to GP IIb/IIIa, is an effective approach to prevent thrombosis.5–8 Unfortunately, this approach increases the risk of bleeding, especially at the doses that are effective in preventing thrombotic episodes.8 Blocking GP Ib9–11 or vWF12 results in an inability of the platelets to attach to the exposed subendothelium. Therefore, the GP Ib–vWF axis is an attractive target on which to focus for the prevention of thrombus formation in stenosed arteries.

In a recent study in baboons,13 we showed that inhibition of platelet adhesion, through prevention of the binding of vWF to GP Ib by monoclonal antibody 6B4-Fab fragments, markedly inhibited thrombosis under relatively low shear conditions, without causing thrombocytopenia or major lengthening of the bleeding time. 6B4 is a potent GP Ib blocker, which, by interacting with GP Ibα (amino acids 201 to 268),14 prevents vWF binding induced by either ristocetin, botrocetin, or shear. In the present study, we investigated the effect of inhibition of platelet adhesion in a modified Folts model,15 in which the cyclic flow reductions are caused by platelet-dependent thrombi that form under high-shear conditions at injured stenosed sites of an artery. The model has been described to represent some of the events that occur in patients with unstable angina and is widely accepted to be effective and clinically relevant for testing potential antithrombotic agents.16,17

The aims of the study were 2-fold. First, we evaluated the antithrombotic efficacy of 6B4-Fab in high-shear conditions...
in baboons and compared it with the efficacy of the GP IIb/IIIa blocking monoclonal antibody MA-16N7C2 to under the same conditions. The second aim was to determine whether the combination of an anti–GP IIb and anti–GP IIb/IIIa treatment could result in a synergistic antithrombotic effect and a lowering of the bleeding time prolongation associated with the anti–GP IIb/IIIa agent.

Methods
Preparation of Monoclonal Antibodies 6B4-Fab and MA-16N7C2
Antibodies 6B4, raised against human GP IIb,13,19 and MA-16N7C2, directed against GP IIb/IIIa,7,18 were purified from ascitic fluid from BALB/c mice on protein A Sepharose. 6B4-Fab was prepared from the IgG by papain digestion, as previously described.13

In Vitro Flow Studies
The effects of 6B4-Fab and MA-16N7C2 on platelet adhesion to collagen was studied in a Sakariassen-type parallel-plate flow chamber at a shear rate of 1500 s⁻¹, as previously described.19 Blood was collected from healthy volunteers by using LMW-heparin (25 U/mL, Clexane) as an anticoagulant. Glass coverslips were coated with human collagen type 1 (Sigma Chemical Co), dissolved in 50 mmol/L acetic acid (1 mg/mL), and dialyzed against PBS for 48 hours. Fifteen milliliters of blood, preincubated with vehicle or compound at 37°C for 5 minutes, was perfused in the flow chamber for 5 minutes at 37°C. The platelets were fixed with methanol and stained with May-Grünwald–Giemsa. Platelet surface coverage was analyzed by using an image analyzer. An average of 10 fields per coverslip were analyzed. The results were expressed as percentage of total surface covered with platelets.

Folts Model in Baboons
Nineteen baboons (Papio ursinus) of either sex, weighing 12 to 18 kg, were used. All procedures were approved by the Ethics Committee for Animal Experimentation of the University of the Orange Free State in accordance with the National Code for Animal Use in Research, Education, Diagnosis and Testing of Drug and Related Substances in South Africa.

The experimental procedure was modified from the one described by Folts.15 Baboons were anesthetized with ketamine hydrochloride (10 mg/kg IM), intubated with a cuffed endotracheal tube, and ventilated by a respirator with oxygen supplemented with 0.5% Fluthane to maintain anesthesia. Body temperature was maintained at 37°C with a heating table. A catheter was placed in a femoral vein for drug administration and blood sampling. A segment of another femoral artery was gently dissected free from surrounding tissue, and a perivascular ultrasonic flow probe (Transonic Systems Inc) was placed around the distal dissection site. The mean and phasic blood flow were recorded continuously throughout the experiment. Baboons were allowed to stabilize for 30 minutes. The proximal dissection site of the femoral artery was injured by applying 3 occlusions of the artery for 10 seconds at 2-mm intervals with the use of a spring-loaded forceps. A spring-loaded clamp was placed in the middle of the injured site to produce an external stenosis of 65% to 80%. A gradual decline in blood flow due to platelet adhesion and aggregation was observed. When flow reached zero, blood flow was restored by pushing the spring of the clamp to mechanically dislodge the platelet-rich thrombus. This repetitive pattern of decreasing blood flow after mechanical restoration is referred to as cyclic flow reductions (CFRs). Additional endothelial injury and appropriate external stenosis selections were repeated if needed. Finally, stable CFRs were obtained in 17 of 18 baboons.

After a 60-minute control period, test agents were given via an intravenous bolus injection, and monitoring was continued for up to 60 minutes after drug administration. The antithrombotic effect was quantified by comparing the number of CFRs per hour before and after drug administration. Blood samples for the different laboratory measurements (platelet count, coagulation, platelet aggregation, receptor occupation, and plasma levels) were drawn at −60, 0, 30, 60, 150, and 300 minutes and 24 hours after treatment.

Baboons were divided into 6 groups, and drug treatment was as follows: (1) vehicle (saline), n = 2; (2) 6B4-Fab at 0.6 mg/kg, n = 5; (3) 6B4-Fab at 2.0 mg/kg, n = 3; (4) MA-16N7C2 at 0.1 mg/kg, n = 3; (5) MA-16N7C2 at 0.3 mg/kg, n = 2; and (6) 6B4 at 0.6 mg/kg plus MA-16N7C2 at 0.1 mg/kg, n = 2. All agents were diluted in saline for injection. In addition, 4 mg/kg 6B4-Fab was injected in 1 baboon to determine the effect of such a high dose on platelet count, receptor occupation, bleeding time, and platelet aggregation. CFRs were not measured in this baboon.

Platelet Count, Coagulation, and Bleeding Time
All blood samples were collected in 0.32% (f.c.) trisodium citrate. The platelet count was determined by using a Technicon H1 blood cell analyzer (Bayer Diagnostics). Prothrombin time and activated partial thromboplastin time were measured at 37°C by use of a coagulometer (Clotex II, Hyland). The template bleeding time was measured at the surface of the forearm by using a Simplate II device (Organon Teknika). The volar surface of the forearm was shaved, and a pressure cuff was applied and inflated to 40 mm Hg. The time elapsed until the visual cessation of blood loss was recorded as the bleeding time. The wound was carefully dabbed every 15 seconds with filter paper.

Platelet Aggregation
Ex vivo platelet aggregation studies were performed on citrated blood with an aggregometer (Elvi 840, Pabisch). Platelet-rich plasma and platelet-poor plasma were prepared by differential centrifugation. The platelet count in platelet-rich plasma was adjusted to 200 000 platelets per microliter with autologous platelet-poor plasma. Platelet aggregation was induced by 1.5 mg/mL ristocetin (ABP), 20 μmol/L ADP (Sigma), or 50 μg/mL collagen (Nycomed Arzneimittel GmbH) and measured by the change in light transmission.

Plasma Concentration
Plasma levels of antibodies were measured by ELISA with the use of plates coated with 5 μg/mL goat anti-mouse IgG (Sigma) and developed with peroxidase-conjugated goat anti-mouse Fab-specific IgG (Sigma). The plasma concentration of the antibodies in each sample was calculated from standard curves constructed by adding known amounts of these antibodies to baboon plasma.

Receptor Occupancy
Ex vivo binding of ¹²⁵I-labeled 6B4-Fab or MA-16N7C2 IgG was used to determine the number of receptors occupied by 6B4-Fab and MA-16N7C2 IgG, respectively. Labeling of the antibodies with sodium ¹²⁵I (Amersham) was performed by using Iodogen (Pierce Chemical Co). Platelet-rich plasma (200 000 platelets per microliter) was incubated with a near saturating dose (2 μg/mL) of ¹²⁵I-MA-16N7C2 or ¹²⁵I-6B4-Fab for 15 minutes at room temperature. A fraction of this mixture was then layered onto 20% sucrose buffer (wt/vol) containing 0.1% BSA in Eppendorf tubes and centrifuged for 4 minutes at 10 000 g. Platelet pellet–associated radioactivity was determined, and the number of radiolabeled molecules bound per platelet was calculated. The results were calculated by subtracting the number of labeled molecules bound to the platelets after treatment from that obtained before treatment and are expressed as the percentage of receptors occupied.

Statistical Analysis
Data are expressed as mean ± SEM. The Student t test (2-tailed) or 1-factor ANOVA followed by the Fisher test was used for statistical evaluation. A value of P < 0.05 was considered to be statistically significant.
Results

In Vitro Flow Studies in the Flow Chamber

6B4-Fab completely inhibits platelet adhesion to collagen in flow at 5 μg/mL at 1300 s⁻¹ and 2.5 μg/mL at 2600 s⁻¹. In the present study, we analyzed the combined effects of partially inhibitory concentrations of 6B4-Fab and MA-16N7C2 on human platelet adhesion to human collagen type I in vitro after 5 minutes of perfusion at 1500 s⁻¹ in a flow chamber. Concentrations of 1.5 and 2.25 μg/mL 6B4-Fab inhibited platelet adhesion by 38% and 53%, respectively, whereas 0.5 and 0.75 μg/mL MA-16N7C2 inhibited the surface coverage by platelets by 7% and 44%, respectively, mainly by reducing platelet aggregate formation. A combination of 1.5 μg/mL 6B4-Fab and 0.5 μg/mL MA-16N7C2 inhibited surface coverage by 76%, whereas 88% inhibition was achieved when 2.25 μg/mL 6B4-Fab and 0.75 μg/mL MA-16N7C2 were given in combination (Figure 1).

Platelet Count, Coagulation, and Bleeding Time

The platelet count was not significantly affected by injection of up to 4 mg/kg 6B4-Fab or MA-16N7C2 (in agreement with previous observations) or the combination of 6B4-Fab and MA-16N7C2. No significant changes of prothrombin time and activated partial thromboplastin time were observed in any of the groups.

The bleeding time (baseline 2.04±0.59 minutes, interanimal coefficient of variation 29%, n = 13) was prolonged only after the injection of 0.3 mg/kg MA-16N7C2, but it returned to normal levels 24 hours later (Figure 2).

Antithrombotic Effect

The frequency of the CFRs (Figure 3) was not significantly different between the different groups before the treatments were started, nor was it changed by the injection of saline (107±7%). A dose of 0.6 mg/kg 6B4-Fab resulted in a significant reduction of the CFRs by 59±15%. A dose of 2 mg/kg abolished the CFRs, which could not be restored by increasing the intimal damage or by increasing the stenosis. MA-16N7C2 significantly inhibited CFRs by 43±8% at a dose of 0.1 mg/kg and abolished the CFRs at 0.3 mg/kg. The combination of 0.6 mg/kg 6B4-Fab and 0.1 mg/kg MA-16N7C2 completely blocked the CFRs.

Platelet Aggregation

The effects of 6B4-Fab and MA-16N7C2 on ex vivo platelet aggregation are shown in Figure 4. 6B4-Fab inhibited the ex vivo ristocetin-induced platelet aggregation in a dose- and time-dependent manner. Aggregation was totally absent 30 minutes after injection of 0.6 mg/kg 6B4 Fab or injection of the combination of 6B4-Fab and MA-16N7C2. Significant inhibitory effects at these doses persisted for 150 minutes and returned to normal values within 24 hours.

ADP- and collagen-induced platelet aggregation was significantly inhibited after the administration of 0.1 or 0.3 mg/kg MA-16N7C2 and also when 0.6 mg/kg 6B4-Fab and 0.1 mg/kg MA-16N7C2 were combined. It is of interest that the combination of 0.6 mg/kg 6B4-Fab fragments and 0.1 mg/kg MA-16N7C2 had a more pronounced effect on the inhibition of ADP-induced platelet aggregation than did a single dose of 0.1 mg/kg MA-16N7C2, the reason for which is unclear at present.

Figure 1. Inhibition by 6B4-Fab and MA-16N7C2 of platelet-dependent surface coverage of collagen-coated coverslips as determined after 5-minute perfusion of whole blood at 1500 s⁻¹. A and E, PBS control. B, 6B4-Fab (1.5 μg/mL). C, MA-16N7C2 (0.5 μg/mL). D, 6B4-Fab (1.5 μg/mL) plus MA-16N7C2 (0.5 μg/mL). F, 6B4-Fab (2.25 μg/mL). G, MA-16N7C2 (0.75 μg/mL). H, 6B4 Fab (2.25 μg/mL) plus MA-16N7C2 (0.75 μg/mL). Data are given as mean±SEM (n=4). *P<0.05 vs PBS control.

Figure 2. Effect of 6B4-Fab and MA-16N7C2 administration on template bleeding time in baboons. Open square indicates 0.6 mg/kg 6B4; solid square, 2.0 mg/kg 6B4; solid circle, 0.1 mg/kg MA-16N7C2; open circle, 0.3 mg/kg MA-16N7C2; and open triangle, 0.6 mg/kg 6B4+0.1 mg/kg MA-16N7C2. Data represent mean±SEM. *P<0.05 vs pretreatment value.

Figure 3. Effects of different doses of 6B4-Fab, MA-16N7C2, or their combination on CFRs in the baboon femoral artery. Saline injection appears as 0.0 mg/kg. The results are expressed as percentage of the pretreatment value. Data represent the mean±SEM. *P<0.05, **P<0.01, and ***P<0.001.
Plasma Concentration and Receptor Occupation

The plasma concentrations of 6B4-Fab and MA-16N7C2 are shown in Figure 5. The plasma concentration of both antibodies peaked at 30 minutes after the bolus injection and declined rapidly thereafter. The occupancy of GP Ib and GP IIb/IIIa receptors by 6B4-Fab and MA-16N7C2 was maximal at 30 minutes; however, it remained elevated for an extended period. GP Ib receptor occupancy after bolus injections of 0.6 mg/kg 6B4-Fab, 2.0 mg/kg 6B4-Fab, and 0.1 mg/kg of MA-16N7C2, respectively, was 26%, 69%, and 84%; and after the combination of 0.6 mg/kg 6B4-Fab and 0.1 mg/kg of MA-16N7C2, respectively, was 26%. The occupation remained almost at the same level 24 hours after treatment.

The relationship between GP Ib receptor occupancy, ristocetin-induced agglutination, CFRs, and bleeding time suggests that occupancy of 20% and of 35% of receptors with 6B4-Fab results in 50% reduction of platelet agglutination and CFRs, respectively, whereas up to 80% occupancy does not result in a significantly prolonged bleeding time (Figure 6).

Discussion

The present study evaluated the antithrombotic effects of the Fab fragments of monoclonal anti–GP Ib antibody (6B4) and of the monoclonal anti–GP IIb/IIIa antibody (MA-16N7C2) in injured and stenosed baboon femoral arteries.

Previous studies have shown that 6B4-Fab inhibited ristocetin- and botrocetin-induced vWF-dependent human platelet agglutination. 6B4-Fab inhibited human platelet adhesion to human collagen type I in a perfusion flow chamber with IC50 values of 3.5, 1.1, and 0.5 μg/mL at shear rates of 650, 1300, and 2600 s-1, respectively, indicating that this compound is more effective under high-shear conditions. In a baboon model with permanent arteriovenous shunt (700 to 1000 s-1), 6B4-Fab significantly reduced platelet deposition onto collagen. The present study evaluated the antithrombotic efficacy of 6B4-Fab under high-shear conditions by using a modified Folts model in baboons: 6B4-Fab significantly reduced CFRs at a dose of 0.6 mg/kg and completely abolished CFRs at 2.0 mg/kg without causing a marked prolongation of the bleeding time, even when a dose of 4 mg/kg 6B4-Fab was given, suggesting that 6B4-Fab is a powerful antithrombotic agent with, possibly, a low bleeding risk.

Although administration of inhibitory antibodies against vWF resulted in bleeding times that were not prolonged or were only minimally prolonged or in bleeding times that resulted in a nearly 7-fold prolongation, the minimal effect of blocking GP Ib on the bleeding time as seen in the present study is correlated with previous findings obtained with antibodies or with a recombinant vWF-A1 domain, which resulted in a prolongation of the bleeding time of, at most, 3-fold. Most likely, the rather exceptional combination of an antiplatelet effect without prolongation of the bleeding time, as observed in the present study and in other settings, is due to the shear dependence of the vWF–GP Ib interaction, which would therefore play a lesser role in the lower shear systems and might be more relevant for the bleeding time measurements. The bleeding problems observed in patients with Bernard-Soulier syndrome may be due to either the complete absence of GP Ib, in contrast to the maximum 80% receptor occupancy that was obtained in the present study, or the
combination of GP Ib deficiency, thrombocytopenia, and giant platelets, which may be less readily arrested at the site of injury.

On the other hand, we could not really demonstrate the anticipated higher efficiency of 6B4-Fab in the present high-shear setting compared with the lower shear setting in a previous experiment. This, to a large extent, may be due to the different thrombogenic stimulus that was presented: a collagen-rich glutaraldehyde-fixed bovine pericardium in an extracorporeal arteriovenous shunt in the medium-shear experiments versus a physically damaged and stenosed femoral artery. Part of the explanation may be that in the CFR experiments, ultrahigh-shear levels might occur, which can result in direct GP Ib-vWF–dependent platelet aggregation and may be more resistant to inhibition.

6B4-Fab caused a dose- and time-dependent inhibition of ex vivo ristocetin-induced platelet aggregation. A lower receptor occupancy was needed to inhibit ristocetin-induced platelet agglutination than was needed to prevent CFRs, which is in accordance with the findings of van Zanten et al, who reported that lower available GP Ib numbers were needed to prevent platelet adhesion to collagen under flow-induced compared with ristocetin-induced aggregation. However, the receptor occupancy needed to obtain an effect as observed in the present study may be somewhat underestimated in view of the relative low affinity of 6B4-Fab.

For purposes of comparison, we also used the Folts model to investigate the effect of inhibition of GP IIb/IIIa with MA-16N7C2. A dose of 0.1 mg/kg MA-16N7C2 inhibited CFRs by \( \approx 43\% \), and CFRs were abolished at a dose of 0.3 mg/kg MA-16N7C2. Thus, inhibition of platelet adhesion and inhibition of platelet aggregation are both viable avenues for preventing arterial thrombosis. However, with the high dose of MA-16N7C2, the bleeding time was significantly prolonged. Therefore, inhibition of aggregation may increase the risk of bleeding, in agreement with studies using other antiplatelet agents.

The roles of GP Ib and of GP IIb/IIIa in the formation of a platelet-dependent thrombus are complementary. Indeed, we found that combined inhibition has a more pronounced effect on in vitro platelet accumulation onto collagen in a flow chamber and on the prevention of CFRs in the baboon. With this combination, in addition, a full in vivo antithrombotic effect was obtained without prolongation of the bleeding time.

In conclusion, the present study demonstrates that the inhibition of platelet GP Ib is a powerful intervention in the prevention of platelet thrombus formation in the injured and stenosed baboon femoral artery without prolongation of the bleeding time. The effect on bleeding time is definitely less during inhibition of GP Ib than during inhibition of GP IIb/IIIa. Accordingly, a GP Ib blocker, such as 6B4-Fab, seems to be promising for further development as a compound for the prevention of acute arterial thrombotic syndromes. Moreover, we demonstrated that combining a low

![Figure 5. Plasma levels of 6B4-Fab (A) and MA-16N7C2 (C) and of GP Ib (B) and GP IIb/IIIa (D) receptor occupancy by 6B4-Fab and MA-16N7C2, respectively, as function of the time after their administration to baboons. Open square indicates 0.6 mg/kg 6B4; solid square, 2.0 mg/kg 6B4; solid circle, 0.1 mg/kg MA-16N7C2; open circle, 0.3 mg/kg MA-16N7C2; and, open triangle, 0.6 mg/kg 6B4 + 0.1 mg/kg MA-16N7C2. Data represent the mean±SEM.](http://atvb.ahajournals.org/)

![Figure 6. Relationship between percent GP Ib receptor occupancy by 6B4-Fab and percent ristocetin-induced platelet aggregation, percent CFRs, and bleeding time (BT). To construct the CFR curve, receptor occupancy 30 minutes after administration was used.](http://atvb.ahajournals.org/)
dose of a GP Ib inhibitor with a low dose of a GP Ib/IIa inhibitor has a potent antithrombotic action, again with minimal effects on the bleeding time. This provides new information that will be beneficial in designing clinical therapeutic approaches.

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References
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