Reduction in Thrombus Formation by PG-1 F(ab')$_2$, an Anti-Guinea Pig Platelet Glycoprotein Ib Monoclonal Antibody

Jonathan L. Miller, Mareme Thiam-Cisse, and Ludovic O. Drouet

We have recently demonstrated that intraperitoneal injection into guinea pigs of F(ab')$_2$ fragments of PG-1, a murine monoclonal antibody recognizing the guinea pig homologue of human platelet glycoprotein Ib, produces virtually complete functional block of the platelet von Willebrand factor receptor without inducing a hemorrhagic state. To assess the ability of this antibody to protect against thrombosis resulting from laser-induced injury to mesenteric small arteries, we injected guinea pigs with either PG-1 F(ab')$_2$ (2.3 mg/kg) or with buffer alone 24 hours before study. For each animal, four mesenteric vessels were studied consecutively for 15 minutes each. The number of thrombi, time to first thrombus, and time to embolization of first thrombi were recorded. In the control animals most individual vessels had three to four thrombi, whereas in the antibody-treated animals, vessels most frequently had only a single thrombus or even none at all. The mean number of thrombi per vessel in the antibody-treated animals (1.125) was significantly lower than the mean in the control animals (3.40), with $p<0.001$. However, there was no significant difference between groups with respect to time to first thrombus after laser injury. Detachment of thrombi in the control animals occurred in an easily recordable, discrete fashion, with 75% of first thrombi embolizing in less than 5 minutes. In the antibody-treated animals, gradual dissolution rather than discrete detachment was typically observed. In 31% of the vessels studied in these animals, no embolus occurred; for the remaining vessels only 9% of thrombi were observed to embolize within 5 minutes of their formation. PG-1 F(ab')$_2$ treatment accordingly served to break the repetitive cycle of thrombus production, embolization, and rethrombosis in this model system. (Arteriosclerosis and Thrombosis 1991;11:1231-1236)
dye-pulsed laser is capable of producing an endothelial lesion that, by electron microscopy, is observed to result in a relatively pure platelet thrombus without visible fibrin deposition. We now report the protective effects of PG-1 F(ab')2 on thrombus formation in this model system.

**Methods**

**Antibody Preparation**

PG-1 antibody was purified from mouse ascites and F(ab')2 fragments produced by pepsin digestion, as previously described. Either PG-1 F(ab')2 (683 μg/ml) in phosphate-buffered saline (PBS) or PBS by itself was frozen in coded 1-ml aliquots.

**Animals**

Hartley strain male guinea pigs weighing 300 g were used. Prior to the experiment, 1 ml of either PG-1 F(ab')2 (2.25 mg/kg) or PBS was injected into the guinea pig intraperitoneally. The code for antibody versus PBS treatment was not broken until completion of study of all 12 animals. Twenty-four hours after injection of the antibody or PBS, the animal was anesthetized with sodium thiopental (50 mg/kg in two divided doses, 10 minutes apart) given subcutaneously. This treatment produced surgical anesthesia for more than 2 hours, which exceeded the entire 80-90-minute period of in vivo study.

After attainment of anesthesia, the abdomen was shaved, and a 3-cm incision was made through the abdominal wall and peritoneum to expose the mesentery. Individual mesenteric small arteries of 150-250 μm i.d. were then transilluminated on a thermally controlled stage of an Olympus (Tokyo, Japan) dissecting microscope. The peritoneum was regularly irrigated with saline to prevent dehydration. All animals exhibited stability of rectal temperature and heart rate throughout the experiment and were maintained on natural breathing without the aid of a respirator. At the termination of the experiment, animals were sacrificed by administration of an overdose of sodium thiopental. All animal studies were conducted within the guidelines of INSERM and CNRS institutional policies.

**Dye-Pulsed Laser**

A pulsed N2 laser (Sopra Bois-Colombes, France) capable of delivering as much as 2.5 mJ of energy and with a pulse duration of 5 nsec was used at a fixed repetition rate of 1.5 Hz as the source to pump a dye laser tuned to 427 nm, the wavelength of the first absorption peak of hemoglobin. Stilbene 420 (Exciton Optilas, Evry, France) dissolved in ethanol/water (75%:25%, vol/vol) at a final concentration of 2.1×10−3 M was used with the laser to achieve this wavelength. Energy delivered by the dye laser was monitored with a pyroelectric energy meter (Laser Precision Inc., Yorkville, N.Y.). The directed laser beam was focused on a 100-μm-diameter section of vessel. Vascular injury was produced by a sequence of 30 laser pulses, each delivering 25 μJ of energy.

**Measurement of Thrombosis and Embolization**

After laser injury each artery was observed for a period of 15 minutes. At the end of this 15-minute period, the mesentry was moved to the region of another loop of bowel, permitting study of a second mesenteric small artery. In sequential manner two additional arteries were studied, so that in a period of 80-90 minutes the results of laser injury to four different arteries from a single animal could be studied.

After each laser injury to an artery, the time was recorded at which thrombus formation in the injured vessel could first be visually detected. In preliminary experiments this measurement was found to be a more reliable indicator of thrombus formation than were attributes of thrombus size due to the limitations of real-time, three-dimensional measurement in this kinetic system. During the 15-minute observation period, thrombi typically detached from the vessel wall, and new thrombi reformed at the site of injury. The total number of thrombi sequentially forming at each injury site was recorded. Additionally, for the first-forming thrombus in each vessel, the time to embolization of the thrombus was recorded.

**Platelet Aggregation**

After completion of the study of all four vessels, blood was drawn from the abdominal aorta into trisodium citrate (1 vol blood:0.1 vol 0.13 mol/l citrate), and platelet-rich plasma (PRP) (5×103-5.5×104 platelets/μl) prepared by centrifugation at 2,100g for 75 seconds at 15°C. Platelet-poor plasma (PPP) was prepared by a second centrifugation at 4,000g for 15 minutes at 15°C. For study of ADP-induced aggregation, ADP (1.2 μmol/l) was added to 400 μl PRP in an aggregometer cuvet, with the blank cuvet containing PPP. For study of ristocetin-induced aggregation, the PRP was diluted with saline (1 vol PRP:3 vol 0.9% NaCl), 400 μl of the diluted PRP was added to an aggregometer cuvet, and ristocetin (2 mg/ml) was then added; the blank cuvet contained PPP similarly diluted with saline.

**Statistical Analysis**

Administration of PG-1 F(ab')2 or PBS to the 12 animals was conducted in a blind, randomized fashion, and the code was not broken until completion of the study of all 12 animals. Comparisons between antibody-treated and control animals were performed by the two-tailed, unpaired t test and by the Mann-Whitney U rank test. Paired t tests were used to detect any differences between the first and fourth vessels studied in the individual animals, as a measure of possible deterioration of the animal preparation over time.
Results

Throughout this investigation, no differences in the appearance, behavior, or tendency to bleed during the experimental procedures were observed that provided any suggestion as to whether the individual animal had received PG-1 F(ab')2, or PBS, permitting the entire study to be completed in a blinded fashion. None of the animals was rendered thrombocytopenic after injection of PG-1 F(ab')2 or PBS. Of the six animals receiving PG-1 F(ab')2, four showed full inhibition of ristocetin-induced aggregation. In the remaining two animals injected with PG-1 F(ab')2, the platelet response to ristocetin remained intact, suggesting failure of effective antibody delivery to the platelets in vivo. Indeed, the results after laser injury in these two animals were indistinguishable from the results in the control animals. Because the aim of the present studies was to assess the effects on experimental thrombosis after successful blockade of platelet GPIb, results from the two animals representing apparent antibody delivery failure were excluded from inclusion in the antibody treatment group. Additionally, for one of the six control animals receiving PBS, aggregation studies were not satisfactorily performed. Although the results for this animal after laser injury were indistinguishable from the other control animals, these results were excluded from inclusion in the control group, as it was impossible to verify that ristocetin-induced platelet aggregation was intact. PRP from all animals included in the study retained responsiveness to ADP, with no significant difference seen in the extent or the rate of ADP-induced aggregation between the PG-1 F(ab')2 treatment or control groups.

The mean number of thrombi per vessel in the control animals ranged from 2.5 to 4.0 (Table 1). Most individual vessels had three to four thrombi, with an occasional vessel experiencing as few as two or as many as five. In contrast, vessels of the antibody-treated animals never exhibited more than two thrombi and most frequently had only a single thrombus—or even none at all. The mean number of thrombi per vessel in the antibody-treated animals (range of 0.75 to 1.50) was greatly reduced below that of the control group (Figure 1), with a high degree of statistical significance, both by the two-tailed unpaired $t$ test ($p=0.0002$) and by the nonparametric Mann-Whitney $U$ rank test ($p=0.0143$).

There was not a significant difference within either the antibody-treated or the control group between the number of thrombi in the first versus the fourth vessel studied. Despite the significant reduction in the number of thrombi, the actual time elapsed from laser injury to appearance of first thrombus (Table 2) was not significantly different between control animals and those treated with PG-1 F(ab')2.

Detachment of thrombi from injury sites in the control animals occurred as easily recordable, discrete events. Time to embolization of first thrombi in the control animals averaged 239 seconds. Seventy-five percent of these thrombi had embolized in less than 5 minutes after thrombus formation, with 100% of thrombi detaching within 6.5 minutes of their initial formation. In the PG-1 F(ab')2-treated ani-

\[ \text{TABLE 1. Effect of PG-1 F(ab')2 on Thrombus Formation in Mesenteric Small Arteries After Laser Injury} \]

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Thrombi occurring in vessel No.</th>
<th>Mean thrombi/vessel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Antibody</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Antibody</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Antibody</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Antibody</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Control</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

For each animal receiving either 2.25 mg/kg PG-1 F(ab')2 (Antibody) or an equal volume of phosphate-buffered saline (Control), the number of thrombi occurring within 15 minutes is indicated for each of four mesenteric small arteries consecutively subjected to laser injury.

\[ \text{FIGURE 1. Bar graph showing reduction in vascular thrombi by PG-1 F(ab')2. Values are mean±SEM for thrombi occurring in each of four consecutively studied mesenteric small arteries in the four antibody animals receiving 2.25 mg/kg PG-1 F(ab')2, and the five control animals receiving an equal volume of phosphate-buffered saline. Mean of the antibody group (1.125) is significantly different from that of the control group (3.40), with } p=0.0002 \text{ by the two-tailed unpaired } t \text{ test and } p=0.0143 \text{ by the Mann-Whitney } U \text{ rank test.} \]
ever, the inhibitory effects of PG-1 F(ab')$_2$ treatment may be less prominent. For example, guinea pigs whose ristocetin-induced platelet aggregation could be detected. For the remaining 11 vessels, time to embolization of first thrombi could be determined. In contrast to vessels in the control animals, rapid detachment of thrombi was rare, with only 9% of these vessels exhibiting thrombus detachment in less than 5 minutes of initial formation. The average time to embolization was 374 seconds, more than 1.5 times that of the control vessels.

### Discussion

The formation of vascular thrombi is complex, involving vessels, cellular elements of the blood, and plasmatic factors. In the present study we have focused on one aspect of this process, the role of the platelet membrane GPIb/IX vWF receptor. In guinea pigs whose ristocetin-induced platelet aggregation was blocked by the in vivo administration of PG-1 F(ab')$_2$, there was a highly significant reduction of thrombus production after dye-pulsed laser injury to mesenteric small arteries. As we have previously demonstrated, the PG-1 F(ab')$_2$ treatment does not result either in the production of a hemorrhagic state in these animals or a fall of the platelet count to levels producing a bleeding tendency. Thus, in this model system a MoAb directed against GPIb appears to have afforded a degree of protection from thrombosis without the concomitant production of a bleeding diathesis. After more severe tissue injury, however, the inhibitory effects of PG-1 F(ab')$_2$ treatment may be less prominent. For example, guinea pigs receiving PG-1 F(ab')$_2$ have not been found to show any abnormal prolongation of the skin template bleeding time.

The continued ability of ADP to induce aggregation of PRP from the PG-1 F(ab')$_2$-treated animals suggests that the reduction of thrombus formation in these animals is not mediated by inhibition of fibrinogen binding to platelet GPIIb/IIIa. In the case of human platelets, Weiss and coworkers have recently demonstrated that other adhesive ligands such as vWF, vitronectin, or fibronectin may be involved in platelet adhesion and thrombus formation on subendothelium when studied at high shear rates. Whether such ligands may contribute to hemostasis in the guinea pig by binding to guinea pig platelet GPIIb/IIIa is currently unknown.

The clinical observation that patients with Bernard-Soulier disease, in whom there is a deficiency of platelet GPIb/IX, have a bleeding tendency together with decreased platelet adhesiveness provides evidence for the importance of intact vWF binding sites in primary hemostasis. However, because there may be other abnormalities of these platelets in addition to a deficiency of GPIIb/IX, Bernard-Soulier disease does not provide an ideal model to assess the role of GPIb/IX itself. In fact, Sakariassen and colleagues, studying the adherence of human platelets to de-endothelialized human umbilical arteries in an annular perfusion chamber, found that whereas a MoAb directed against human GPIb inhibited platelet adhesion at shear rates above 500 sec$^{-1}$ but not at 300 sec$^{-1}$, Bernard-Soulier platelets showed decreased adhesion at all shear rates.

The present studies overcome this limitation, providing the first in vivo evidence that specific inhibition of platelet GPIb/IX impairs thrombus formation at sites of vascular injury. This observation is additionally consistent with the in vitro studies by Peterson and coworkers, in which a MoAb directed against

### Table 2. Effect of PG-1 F(ab')$_2$ on Time to First Thrombus in Mesenteric Small Arteries After Laser Injury

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Thrombi occurring in vessel No.</th>
<th>Mean time (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody</td>
<td>1</td>
<td>None</td>
</tr>
<tr>
<td>Antibody</td>
<td>2</td>
<td>None</td>
</tr>
<tr>
<td>Antibody</td>
<td>3</td>
<td>40</td>
</tr>
<tr>
<td>Antibody</td>
<td>4</td>
<td>50</td>
</tr>
<tr>
<td>Mean</td>
<td>270</td>
<td>45</td>
</tr>
<tr>
<td>Control</td>
<td>40</td>
<td>45</td>
</tr>
<tr>
<td>Control</td>
<td>45</td>
<td>40</td>
</tr>
<tr>
<td>Control</td>
<td>40</td>
<td>45</td>
</tr>
<tr>
<td>Control</td>
<td>55</td>
<td>45</td>
</tr>
<tr>
<td>Mean</td>
<td>58.5</td>
<td></td>
</tr>
</tbody>
</table>

Time of appearance (seconds [sec]) of the first thrombus after laser injury to four consecutively studied mesenteric small arteries, as well as the mean for all vessels, is presented for animals receiving either 2.25 mg/kg PG-1 F(ab')$_2$ (Antibody) or animals receiving phosphate-buffered saline (Control). Animals are presented in the same order as in Table 1.
human platelet GPIb was shown to inhibit shear-induced platelet aggregation in citrated PRP. Ikeda et al., using hirudin-anticoagulated human PRP of normal divalent ion concentration, have recently extended this observation, demonstrating marked inhibition of shear-induced platelet aggregation by anti-GPIb or by anti-GPIIb/IIIa MoAbs under conditions of high shear, such as may occur in partially obstructed small arteries or arterioles. Based on the size of the small mesenteric arteries studied in the current investigation, it appears likely that shear rates in excess of 1,000 sec⁻¹ would be obtained. In the absence of direct measurement of individual vessels, however, true shear rates are difficult to assess, and there may be considerable spatial differences in wall shear rate within a short segment of a vessel. Additionally, as shown by direct measurement of platelet shear rate profiles in normal rabbit mesenteric arterioles by Tangelder and colleagues, the actual shear experienced by platelets at the vessel wall may be as much as 1.5 to four times higher than that expected on the basis of a parabolic velocity distribution.

Despite the overall antithrombotic effect of PG-1 F(ab’① ①) administration, the time from laser-induced injury to the first appearance of thrombus was not appreciably affected by the antibody. This observation is of interest since PG-1 F(ab’①), binding to platelet GPIb/IX might well be anticipated to block vWF-mediated adhesion to the injured vessel. However, the established ability of PG-1 F(ab’①), to inhibit a vWF-dependent platelet-platelet association does not necessarily equate to the ability of this antibody to block even GPIb/IX-dependent adhesion of platelets to vascular surfaces. For example, the binding of PG-1 F(ab’①) to platelet GPIb/IX might produce a relatively greater steric hindrance to platelet-platelet rather than to platelet-vessel wall interactions mediated by vWF. It is also possible that the initial platelet adhesion is normally mediated by other platelet membrane components such as guinea pig analogues of human GPIa or GPIIb/IIIa, or that such a pathway may be alternatively used after the binding of PG-1 F(ab’①), to GPIb/IX.

The PG-1 F(ab’①) treatment consistently served to break the repetitive cycle of thrombus production, embolization, and rethrombosis. This cycle normally proceeds with the build-up of a platelet thrombus at the site of injury, followed by detachment of the formed thrombus and the subsequent recruitment of newly arriving platelets to form another thrombus. From the in vitro observations that PG-1 F(ab’①), inhibits vWF binding and vWF-mediated aggregation of guinea pig platelets together with the demonstrated ability of anti-GPIb MoAbs to inhibit vWF-dependent shear-induced aggregation of human platelets, the observed impairment of thrombus formation at sites of vessel injury in the antibody-treated animals may well be explained by interference with a platelet-platelet association mediated by vWF binding to GPIb/IX. Additionally, instability of platelet-platelet associations might lead to a more gradual dissolution of a thrombus, which might be less likely to break off as a single unit than would a more cohesive thrombus developing in the untreated animals. It is interesting that Bellinger and colleagues were able to demonstrate similar inhibition of a platelet-platelet association when a murine MoAb directed against porcine vWF was injected into normal pigs. Although the anti-vWF MoAb did not interfere with the initial adherence of platelets to subendothelium, it strongly inhibited the development of platelet thrombi in coronary arteries, as well as a blockade of ristocetin-induced platelet aggregation in vitro.

The greatly decreased likelihood for a new thrombus to form after eventual embolization of a first thrombus suggests that any exposed surface after embolization in antibody-treated animals is relatively nonthrombogenic. The precise mechanism underlying these phenomena remains unknown. Further development of this model system, permitting three-dimensional morphometric measurement of thrombi in real time, may eventually allow more precise discrimination of the effects of antibody on thrombus build-up in comparison with thrombus dissolution.

Whether selective inhibition of the GPIb/IX complex in humans would be able to reduce the tendency for platelets to participate in thrombus formation without the concomitant production of a hemorrhagic tendency is presently unknown. The present studies in an animal model system do, however, suggest that functional inhibition of the platelet GPIb/IX receptor, either by antibodies or potentially by smaller peptides, might have possible therapeutic potential.

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