Expression of Markers of Platelet Activation and the Interpatient Variation in Response to Abciximab

Claude Bihour, Catherine Durrieu-Jais, Laurent Macchi, Christel Poujol, Pierre Coste, Pierre Besse, Paquita Nurden, Alan T. Nurden

Abstract—Our study concerns the biological effects of abciximab (c7E3 Fab, ReoPro), a powerful new antiplatelet drug that blocks glycoprotein (GP) IIb-IIIa complexes. Samples were examined from 6 patients with coronary artery disease who received a bolus of abciximab followed by a 10-μg/min infusion for at least 18 hours before percutaneous transluminal coronary angioplasty. Inhibition of ADP-induced PA was maximal for 4 patients but partial (79% and 53%) for 2 others during the infusion. Flow cytometry performed with monoclonal antibodies (PAC-1, AP-6, and F26) specific for the “activated” GP IIb-IIIa complex revealed large decreases in the expression of activation markers on platelets during therapy, but these decreases were less marked when inhibition of ADP-induced PA was incomplete. Residual aggregation was seen for all patients during the infusion when TRAP 14-mer peptide or thrombin was the stimulus. Unblocked GP IIb-IIIa complexes were detected on thrombin-stimulated platelets from the patients by immunoelectron microscopy performed using the monoclonal antibody AP-2. Unblocked GP IIb-IIIa complexes were also detected by flow cytometry when platelets preincubated for 1 hour in vitro with abciximab under saturating conditions were (1) incubated with TRAP 14-mer or (2) permeabilized with Triton X-100. In confirming interpatient variation in the platelet response to a standard dose of abciximab, our results also show that an uninhibited internal pool of GP IIb-IIIa complexes may mediate a residual response to strong agonists. (Arterioscler Thromb Vasc Biol. 1999;19:212-219.)

Key Words: platelet aggregation ■ activation markers ■ GP IIb-IIIa complexes ■ abciximab ■ arterial thrombosis

Increased signs of platelet activation have been commonly reported in patients with unstable angina (UA).1-5 Thrombi located in coronary arteries of these patients are white platelet-rich thrombi, therein emphasizing the importance of the platelet contribution to the pathogenesis of UA.6 The occurrence of acute ischemic events during the 24 hours immediately after a high-risk percutaneous revascularization procedure has been related to the platelet activation status measured before the intervention.7 The ex vivo detection of markers localized on the membrane of circulating activated platelets by flow cytometry is a sensitive test designed to indicate the risk of acute cardiac events.7-14 These procedures have been facilitated by the increasing availability of monoclonal antibodies (mAbs) recognizing determinants that are only expressed after platelet activation.15 

mAbs that block the function of GP IIb-IIIa (integrin αIIbβ3), such as 7E3, represent a powerful new generation of antithrombotic agents.16 Administration of abciximab (c7E3 Fab fragments, ReoPro), a chimeric antibody fragment prepared for in vivo use, is a potent inhibitor of platelet thrombus formation in humans.17 The Evaluation of IIb/IIIa Platelet Receptor Antagonist 7E3 in Preventing Ischemic Complications (EPIC) and CAPTURE trials, among others, have shown a beneficial effect of sustained blockage of the glycoprotein (GP) IIb-IIIa receptor in patients undergoing high-risk percutaneous transluminal coronary angioplasty (PTCA), with a reduced rate of acute ischemic complications.18,19

Our study was designed to investigate the effect of abciximab on the expression of conformation-dependent activation markers on GP IIb-IIIa complexes of platelets of patients with coronary disease. We also evaluated the effect of the drug on platelet aggregation (PA) induced by a range of physiological agonists. Our results showed that flow cytometry permitted the detection of persistent signs of platelet activation in patients in whom inhibition of ADP-induced aggregation was incomplete during the infusion. At the same time, evidence was obtained for the continued presence of unblocked internal pools of GP IIb-IIIa complexes during the infusion that could provide a residual platelet response in the presence of a major hemostatic challenge.

Methods

Study Subjects

Studies on 6 adult male patients who underwent PTCA for UA and who received abciximab form the basis of this report. Patients were

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hospitaledized in the coronary care unit of the Hôpital Cardiologique (Pessac, France), each of them exhibiting clinical signs of angina at rest with associated electrical changes. A qualifying coronary angio-
gram within 48 hours of their most recent episode of myocardial ischemia showed a culprit lesion in a single native coronary vessel suitable for PTCA. Further information on the patients is given in Table 1.

Patients received abciximab as a bolus injection (0.25 mg/kg) followed by a continuous infusion (10 μg/min) for at least 18 hours (Table 1). The infusion continued until 1 hour after the completion of the PTCA, as described for the CAPTURE trial.19 Vascular sheaths were maintained for at least 24 hours after the end of the infusion. Each patient received intravenous heparin adjusted to give an aPTT between 2.0 and 2.5 times normal values. Heparin was continued for 24 hours (patient 1), 48 hours (patients 4, 5, and 6), or 72 hours (patients 2 and 3) after the completion of PTCA. Each patient then received subcutaneous calciparin, which continued over the period of our study. Aspirin (250 mg oral daily) was also initiated before the abciximab bolus was given. Nitrates, calcium antagonists, and/or β-blockers were maintained throughout the infusion and for varying periods after PTCA. Patients 1, 2, 5, and 6 were enrolled in the CAPTURE trial; the 2 remaining patients were studied after the trial had ended but were treated similarly. Approval was obtained from the National Ethics Committee before the onset of the study.

Murine mAbs Used in This Study

PAC-1, a murine IgM-κ mAb, binds to a conformation-dependent determinant on activated GP IIb-IIIa complexes unoccupied with adhesive protein.20,21 It was purchased from the University of Pennsylvania (Philadelphia) through the courtesy of Prof S. Shattil. AP-6, an IgM, obtained by immunization of mice with the β204–227 peptide, was a gift from Dr T. Kunicki (La Jolla, Calif). The epitope for AP-6 is a ligand-induced binding site (LIBS) exposed on GP IIb-IIIa complexes after agonist-induced fibrinogen binding.22 The anti–receptor-induced binding site (RIBS) IgG murine mAb, F26, recognizes receptor-bound fibrinogen on platelets23 and was provided by Dr H. Gralnick (Bethesda, Md). AP-2 (from Dr Kunicki) is an IgG mAb that reacts with a complex-dependent determinant common to unactivated and activated GP IIb-IIIa.24 Platelet secretion was assessed using an anti–P-selectin mAb, VH10, prepared by us.10

Studies on Platelets From Patients

Blood Sampling

Blood was collected on at least 4 occasions from each patient (see Figure 1) and 2 anticoagulant systems were used (see below). The initial samples were always taken before the bolus injection of abciximab but after the onset of heparin and aspirin. For all patients, samples were taken near the end of the infusion and before PTCA. Sampling was continued at intervals for up to 5 days after the infusion. For patients 3 and 5, blood was also collected 3 hours into the infusion. Initially, blood was withdrawn from the femoral artery via the vascular sheath used for cardiac catheterization. After removal of the catheter, peripheral blood was obtained by clean venipuncture. The initial 3 mL of blood was always discarded.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age, y</th>
<th>Clinical Syndrome</th>
<th>Mono- or Tri- (1), Bi- (2) vessel IHD</th>
<th>Culprit Vessel Segment</th>
<th>Global Ejection Fraction, %</th>
<th>Intracoronary Thrombus</th>
<th>Time (h) From First Ischemic Pain to Abciximab Bolus</th>
<th>Time (h) From Abciximab Bolus to PTCA</th>
<th>Duration (h) of Infusion</th>
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<td>Cx proximal</td>
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<td>Present</td>
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</tbody>
</table>

*Mono- (1), Bi- (2) or Tri- (3) vessel IHD indicates ischemic heart disease; MI, myocardial infarction; Cx, circumflex coronary artery; and LAD, left anterior descending coronary artery.

Aggregation Response in Platelet-Rich Plasma

Blood was anticoagulated with 3.8% (wt/vol) sodium citrate (1 vol:9 vol) and platelet-rich plasma (PRP) prepared by centrifugation at 120g for 10 minutes at room temperature.25 Platelets were stimulated with 0.5 to 8 μmol/L ADP, 0.72 mg/mL arachidonic acid, 25 μmol/L TRAP 14-mer (SFLLRNPNDKYEPE) purchased from Neosystem, or 1.5 mg/mL ristocetin. Results were expressed as percentage light transmission at maximum aggregation. Inhibition was calculated relative to the response of the sample taken before the onset of abciximab therapy. On occasion, activated platelets (see below) were also evaluated using citrated PRP that had been incubated with 10 μmol/L ADP without stirring.

Aggregation of Washed Platelets by Thrombin and Detection of Unblocked GP IIb-IIIa Complexes by Immunoelectron Microscopy

Blood samples were collected into acid-citrate-dextrose NIH formula A (ACD-A) (1 vol anticoagulant: 6 vol blood). Washed platelets were prepared according to our previously published procedures.10 Aggregation was tested for each patient at each time point using 0.5

Figure 1. PA, measured in citrated PRP in response to 3 doses of ADP, as indicated, for each of 6 patients who received an abciximab bolus of 0.25 mg/kg followed by a continuous infusion of 10 μg/min. Shaded areas represent the duration of the abciximab infusion. The bolus was administered at time zero. Light transmission changes were recorded as described in Methods.
Platelet Activation and Abciximab Therapy

U/mL human α-thrombin (Ortho Diagnostics). Immunogold staining was performed on platelets obtained from patients 3 and 4 near the end of the abciximab infusion to investigate the surface expression of unblocked pools of GP IIb-IIIa at the time of maximum inhibition of ADP-induced PA. Here, unstirred suspensions of washed platelets were incubated with or without 0.5 U/mL α-thrombin for 10 minutes before being fixed and processed for immunogold staining with the mAb AP-2, using ultrathin cryosections according to the procedures previously detailed by us.8 Bound AP-2 was located using goat anti-mouse IgG adsorbed onto 5-nm gold particles (1:100 dilution, Auroprobe EM GAM G5; Amershams). Sections were examined in a Jeol JEM-1010 electron microscope.22

Ex Vivo Detection of Activated Platelets

The procedures for ex vivo detection of activated platelets have been described previously by us.10 Here, ACD-A was used as anticoagulant because prior studies showed that it minimizes platelet activation during blood collection and subsequent experimentation. PRP was prepared at 120g for 10 minutes. Aliquots (10 μL) were immediately added to polystyrene tubes containing 100 μL 137 mmol/L NaCl, 2 mmol/L KCl, 12 mmol/L NaHCO3, 0.3 mmol/L NaH2PO4, 1 mmol/L MgCl2, 5.5 mmol/L glucose, 5 mmol/L HEPES, and 0.1% (wt/vol) BSA, pH 7.4 (HEPES-buffered modified Tyrode’s, HBMT) and a predetermined concentration of 1 of the following mAbs: PAC-1 (IgM, 6.25 μg/mL), AP-6 (ascites, 1:1000 vol/vol), F26 (IgG, 5.0 μg/mL), VH10 (IgG, 5.0 μg/mL). Controls were effected in the absence of primary antibody or using the same amount of myeloma mouse IgG (or IgM) (Sigma) whose subclass matched that of the test mAb. Samples were incubated for 15 minutes at room temperature without stirring and bound antibody was measured using DTAF-conjugated, affinity-purified, F(ab′)2 fragments of donkey anti-mouse IgM (Jackson ImmunoResearch) or FITC-conjugated affinity-purified F(ab′)2 fragments of goat antibody to mouse IgG (Fcγ fragment specific, Jackson ImmunoResearch) as described.10 In vitro experiments performed by incubating washed platelets (prepared as above) from normal volunteers with 10 μg/mL abciximab (Lilly-France) confirmed that the secondary antibodies did not cross-react with platelet-bound abciximab (c7E3 Fab fragments) in flow cytometry. Furthermore, the same antibodies did not detect abciximab adsorbed onto nitrocellulose in a dot-blot assay performed using a chemiluminescence revelation procedure (data not shown).

Flow Cytometry

After the incubation of the patients’ platelets with antibodies, samples were diluted with 1 mL HBMT and analyzed using a Becton-Dickinson FACScan flow cytometer as described.10 Fluorescence histograms were obtained for 10,000 cells, data being analyzed using Lyssys II software (Becton-Dickinson). Histograms were composed from fluorescence data obtained using a logarithmic mode of amplification. Antibody binding was expressed as the percentage of platelets positive for antibody. Mean fluorescence intensity (MFI, arbitrary units converted to a linear scale10) was a measure of the extent of antibody binding to individual platelets. The gate for activated platelets was set so as to include <1% of the events seen when identical platelet samples were incubated with the control murine immunoglobulin (IgM or IgG) used at the same concentration as the murine mAb. Percentages of activated platelets in PRP prepared from ACD-A–anticoagulated blood taken from 10 age-matched healthy and medication-free control subjects, given as mean±SD (range), were as follows: F26, 2.2±0.7 (0.8 to 3.4); PAC-1, 4.7±3 (1.3 to 9.3); AP-6, 1.1±0.3 (0.1 to 2); and VH10, 3.8±2.4 (1.7 to 9.2).

Confirmation that Unblocked Internal Pools of GP IIb-IIIa Can Be Detected by AP-2 in the Presence of Abciximab

Here, we made use of our previous observation that platelets saturated with abciximab fail to bind the mAb AP-2.14,24,26 Citrated whole blood from control donors was incubated for 1 hour at 37°C in the presence of 5 or 10 μg/mL abciximab. PRP was prepared and aliquots were incubated with or without 25 μmol/L TRAP 14-mer peptide or 10 μmol/L ADP in the presence of AP-2 (ascites 1:200 vol/vol) or VH10 (as above). After 15 minutes, FITC-conjugated affinity-purified F(ab′)2 fragments of goat antibody to mouse IgG (Fcγ fragment specific) were added and the samples processed for flow cytometry as described in the previous section. Second, selected samples were fixed with 1% (wt/vol) paraformaldehyde (PFA), permeabilized or not with 1% wt/vol Triton X-100, and the accessibility of the internal pools of GP IIb-IIIa to AP-2 was determined by flow cytometry as in an earlier study.27

Statistics

Data for PA testing were expressed as mean±SD. The mean values were compared by paired Student’s t test. The mean percentages of activated platelets were analyzed by ANOVA using the Kruskal-Wallis nonparametric test. Wilcoxon’s test was used when appropriate to compare the variation of values as a function of time.

Results

Clinically, each PTCA was successful. None of the patients presented ischemic chest pain after the procedure. Creatinine kinase (CK-MB) isozyme levels were not seen to rise after PTCA. No undue bleeding was observed.

Platelet Aggregation

Before the bolus injection of abciximab, PA with ADP was as expected for subjects receiving aspirin, often reversible (particularly for 2 and 4 μmol/L ADP). Testing of samples taken after the bolus and 3 hours into the infusion (patients 3 and 5) confirmed that near-maximal inhibition of PA had already been achieved (Figure 1). ADP-induced PA at all doses tested was virtually totally inhibited (>98%) for patients 1, 2, and 3 at time points of 12 hours or more into the abciximab infusion. However, patient 6 showed a significant residual and irreversible aggregation to 4 μmol/L ADP. Even at the lowest dose of ADP, complete inhibition was not observed for this patient. Patients 4 and 5 showed a somewhat intermediate profile. Considerable intersubject variation was also seen during the recovery of ADP-induced PA after abciximab infusion had ended. No correlations were found between the extent of the inhibition of PA and the platelet count.

The intensity of platelet agglutination with ristocetin was little changed in patients receiving abciximab (Table 2). Notwithstanding, it was sometimes reversible during abciximab infusion (data not illustrated). PA with arachidonic acid was minimal, as expected for patients receiving aspirin. PA was induced by way of the thrombin receptor, using 25 μmol/L TRAP 14-mer peptide, the minimum dose that regularly gives full-scale aggregation with citrated PRP in our laboratory. Residual platelet responses to TRAP were seen during the infusion for all patients and ranged from 28% to 71% of pretreatment levels (Table 2). When the aggregation of washed platelets was examined with 0.1 U/mL thrombin, residual PA was again seen for samples taken from all patients during the infusion (Table 2).

Activation-Induced Expression of Unoccupied GP IIb-IIIa Receptors From the Internal Pool

Washed platelets prepared from blood collected from patients 3 and 4 near the end of the abciximab infusion were incubated with thrombin to give maximum secretion. After fixation, ultrathin cryosections were incubated with AP-2 and bound mAb was visualized by immunogold labeling in electron microscopy. Results are shown for patient 3 in Figure 2. Whereas labeling with AP-2 was abundant on the surface of
platelets taken before the onset of abciximab (A), it was much reduced by the end of the infusion (B). Yet, after platelets in the same sample had been stimulated with thrombin, gold beads representing bound antibody were again readily detected at the surface (C). The platelet illustrated in C has a long pseudopod extending back to the surface with considerable labeling. We conclude that unblocked GP IIb-IIIa complexes are made available at the platelet surface after thrombin stimulation. Similar results were obtained for patient 4.

Experiments were continued using normal subjects’ citrated PRP preincubated with abciximab in vitro. As shown in Figure 3, flow cytometry confirmed that abciximab blocked the binding of AP-2 to the surface of unstimulated platelets (compare A and C). In contrast, TRAP 14-mer induced the

<table>
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<th>During Treatment</th>
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<th>Day 2*</th>
<th>Day 3*</th>
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<td>9±12§</td>
<td>26±18‡</td>
<td>32±23†</td>
<td>54±27</td>
</tr>
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<td>Ristocetin (1.5 mg/mL)</td>
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<td>84±11</td>
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<td>TRAP 14-mer (25 μmol/L)</td>
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<td>48±14‡</td>
<td>55±20‡</td>
<td>61±11‡</td>
<td>70±41‡</td>
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<td>Thrombin (0.1 U/mL)</td>
<td>75±6</td>
<td>38±10†</td>
<td>ND</td>
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<td>63±7</td>
</tr>
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</table>

Results are the maximal light transmission (%) and are mean±SD for the 6 patients as a group. During treatment represents post–bolus injection and during continuous infusion. The results for thrombin were obtained using washed platelets. AA indicates arachidonic acid; ND, not done.

*Samples taken on days 1 to 3 after the end of continuous infusion.

†P < 0.05; ‡P < 0.01; §P < 0.001 compared with pretreatment.

Figure 2. Immunogold labeling with the mAb AP-2 of ultrathin cryosections of platelets from patient 3. Samples were taken before (A) and 19 hours after the onset of abciximab therapy (B and C). In A and B, the platelets were unstimulated; in C, the platelets were incubated for 10 minutes with 0.5 U/mL thrombin before being processed. Ultrathin sections were incubated sequentially with AP-2 and goat antibody to murine IgG coupled to 5-nm gold beads before examination by electron microscopy. Note the reappearance of surface labeling for AP-2 on the thrombin-stimulated platelets taken 19 hours after the onset of abciximab. Bar=0.5 μm.

Figure 3. Flow cytometric analysis showing the presence of unblocked GP IIb-IIIa complexes in platelets. First, citrated PRP from a control subject was incubated for 1 hour at 37°C in the absence (A and B) or presence (C and D) of 10 μg/mL abciximab. Samples were then incubated for a further 15 minutes in the presence or absence of 25 μmol/L TRAP 14-mer peptide, as indicated. The murine mAbs AP-2 or VH10 were also present as indicated. Bound mAb was assessed using FITC-labeled secondary antibody specific for the Fc fragment of murine IgG. In A, the histograms obtained for AP-2 in the presence of TRAP masked that given for unstimulated platelets within the chosen settings of the flow cytometer (see text). In E and F, platelets were fixed with PFA after incubation for 1 hour with abciximab. Platelets were then permeabilized or not with 1% Triton X-100 before being incubated with AP-2 or VH10 as indicated. The results confirmed that labeling of the internal pool with AP-2 was seen after permeabilization. The findings are typical of those obtained for 5 donors.
appearance of a subpopulation of GP IIb-IIIa complexes able to bind AP-2 (Figure 3). The normal appearance of P-selectin (B and D) confirmed that abciximab had little effect on secretion at this concentration of TRAP. Little increase in AP-2 binding was seen after the addition of 10 μmol/L ADP (data not shown). Finally, we looked at the binding of AP-2 to PFA-fixed, Triton X-100–permeabilized platelets. Samples were again volumes of citrated PRP preincubated for 1 hour with abciximab. Typical results are shown in Figure 3 (E and F). As can be seen, a pool of AP-2 binding sites is exposed after permeabilization, which renders accessible the internal receptor pools of platelets (see Reference 27). We point out that in the absence of abciximab, the flow cytometer calibration did not permit a separation of the histograms, representing the high-density surface pool of unblocked GP IIb-IIIa recognized by AP-2 (MFI=549 in the experiment shown in A) and the increased surface expression seen after TRAP stimulation (MFI=605).

Detection of Circulating Activated Platelets

Initial experiments confirmed that abciximab brought about a dose-dependent inhibition of AP-6 and PAC-1 binding when control platelets were stimulated with ADP in vitro. Inhibition was complete at 10 μg/mL, showing that c7E3 Fab either masked the activation-dependent epitopes on GP IIb-IIIa or prevented their formation (data not shown). The binding of abciximab to GP IIb-IIIa did not itself induce the expression of the LIBS epitope recognized by AP-6. Abciximab did not interfere with VH10 binding to P-selectin (data not shown).

Assessment of Platelet Activation Markers During and After Abciximab Infusion

As shown in Figure 4, the percentage of platelets positive for 2 or more of the mAbs directed against activation-dependent markers on GP IIb-IIIa was elevated for 4 patients before the addition of abciximab, whereas for patients 2 and 4 the results were at the upper limit of the range observed for age-matched healthy controls. As shown in Figure 4, abciximab therapy had a marked effect on these levels and the trend was toward reduction. The highest number of activated platelets detected before the bolus injection was from patient 6. For this patient, even 15 hours after the onset of the abciximab infusion, 47% and 13% of platelets remained positive with PAC-1 and AP-6, respectively. Interestingly, this was the patient with the highest residual aggregation response to ADP. Results were not always concordant for all 3 antibodies (see patients 1, 2, and 5). The reason for this variability is unknown but confirms the value of testing a series of mAbs in studies such as these. Taking the patients as a group, the decrease in levels of activated platelets in the samples taken shortly before the PTCA reached statistical significance with respect to those present before abciximab for F26 (P<0.001) and AP-6 (P<0.05). Results for PAC-1 (P<0.06) did not, owing to the fact that platelets of 3 patients failed to give elevated values for this antibody before the onset of abciximab.

Platelet P-selectin expression as measured using VH10 was low in patients 1 through 4 (<10%) both before and during abciximab infusion (data not illustrated). In contrast, the percentage of platelets that had undergone secretion and that were recognized by VH10 were elevated in patient 6 both before abciximab administration (23%) and at 15 hours into therapy (41%). For patient 5, 14% of platelets were recognized by VH10 at 21 hours into the infusion. Nevertheless, mean changes in P-selectin levels did not reach statistical significance within the infusion period.

Extent of the Observed Activation

The heterogeneity in the extent to which individual platelets within the total platelet population bind the activation-dependent mAbs can be considerable. This is illustrated in histograms shown for patient 3 in Figure 5. Levels of bound AP-6 and F26 differed widely within the activated subpopulation observed before abciximab therapy was started; however, the MFI values had both fallen to baseline levels just before the PTCA. Also shown are histograms obtained after PRP from blood collected into citrate was activated in vitro with ADP. Now the expression of the activation-dependent epitopes was complete. This selected result emphasizes how the levels of platelet activation seen in vivo are often partial. As a general rule in our study, a fall in the number of activated platelets was accompanied by a decrease in the MFI (data not given). This finding suggests that measuring numbers of activated platelets indeed reflects the extent of the hemostatic challenge.
of PA after a single bolus injection of this drug. A marked heterogeneity in the extent and duration of inhibition previously demonstrated in patients with UA. Some platelets by the anti-RIBS and anti-LIBS antibodies.

This hypothesis would fit with a continued recognition of the abciximab bolus was administered platelet activation was measured platelet activation levels before and during therapy. The initial results highlight interpatient variability in biological tests performed during and after the administration of abciximab to patients with coronary artery disease and selected for PTCA.

Our results show that abciximab administration according to the CAPTURE guidelines. Platelet activation is known to occur episodically in coronary syndromes, and the pre-abciximab levels seen here confirm this trend and ongoing platelet reactivity before the onset of therapy. The pretherapy values obtained for both the PA response and the numbers of activated platelets served as a reference for subsequent measurements on the same patient. The initial levels of activated platelets were globally much greater for patients with healthy age-matched control donors not taking aspirin. Although aspirin prevents thromboxane production in platelets, our results confirm that aspirin treatment clearly does not stop platelet activation in patients with coronary artery disease. Thus, at the time that the abciximab bolus was administered platelet activation was occurring in at least 4 of the patients. Not least, our study highlights that by performing platelet function testing and/or measuring platelet activation levels before and during therapy, it is possible to identify cases in which the inhibitory effect of abciximab is less than total.

It is interesting to speculate as to why patient 6 appeared to be more resistant to the effects of abciximab, although it cannot be excluded that inhibition was maximal early in the therapy and that aggregation to ADP had begun to recover by 15 hours (despite the continued infusion and the fact that for patient 3 and 5 the inhibition seen at 3 hours was maintained at 14 hours and 21 hours, respectively). One possibility is that a significant number of activated GP IIb-IIIa complexes are occupied by adhesive protein and unable to bind abciximab. This hypothesis would fit with a continued recognition of some platelets by the anti-RIBS and anti-LIBS antibodies. Previous studies on patients with UA have demonstrated marked heterogeneity in the extent and duration of inhibition of PA after a single bolus injection of this drug.

A preliminary report has described how residual platelet function in the presence of a GP IIb-IIIa inhibitor varies with respect to the coronary disease state and the level of platelet activation. Patient 6 confirms that this is so and points to the personalization of the abciximab regimen for the benefit of individual patients. It has previously been described that thrombocytosis can lead to a decreased efficiency of abciximab. However, patient 6 had a platelet count between 139,000/μL pretreatment and 142,000/μL 12 hours postinfusion, whereas binding studies with AP-2 performed during a post-PTCA checkup showed a normal GP IIb-IIIa density on his platelets (data not given). A recent investigation demonstrated an association between the PI42 polymorphism of the GP IIIa gene and the risk for acute coronary thrombosis. We considered that patient 6 may possess the PI42 allele. However, analysis of his PI3 genotype in our laboratory by molecular biology procedures revealed that he is homozygous for PI41 (A.T.N., unpublished data, 1998), so this is not the explanation. Notwithstanding, it is plausible that occasional patients may possess GP IIb-IIIa complexes in which structural differences mean that abciximab binds with an altered affinity. A much larger series of patients will now have to be studied to assess the frequency of situations in which, as for patient 6, significant levels of platelet activation and a residual PA response to ADP also continued during the abciximab infusion.

Kleiman et al reported that PA in response to TRAP was significantly less inhibited after a bolus injection of 0.25 mg/kg abciximab than PA induced by ADP. Our data substantiate this report and show that this was also the case for the patients that we have studied despite the additional 10 μg/mL post-bolus infusion. Using platelets coated with another anti–GP IIb-IIIa, Niiya et al showed that subsequent stimulation with 0.1 U/mL thrombin permitted the binding of 39,000 fibrinogen molecules per platelet as a result of the activation-dependent expression of GP IIb-IIIa complexes from within the platelet. We therefore considered it highly probable that the residual PA to TRAP observed during abciximab infusion was mediated by GP IIb-IIIa receptors coming from an internal platelet compartment. To prove this, we made use of the fact that abciximab inhibits the binding to GP IIb-IIIa of another mAb, AP-2. Three lines of evidence supported our theory. First, a regular surface labeling by AP-2 was located by immunoelectron microscopy after thrombin stimulation of platelets isolated from patients near the end of the abciximab infusion. Then, using flow cytometry and platelets preincubated with abciximab in vitro under saturating conditions, AP-2 was shown to detect (1) unblocked GP IIb-IIIa newly exposed after TRAP 14-mer stimulation and (2) an internal pool of unblocked GP IIb-IIIa complexes in platelets permeabilized with Triton X-100. Whether these complexes can be expressed on the surface occupied with fibrinogen from the α-granules has to be determined. Other experiments confirmed that under these conditions, aspirin had little effect on thrombin-induced secretion from platelets (data not shown).

Bhattacharya et al reported that patients receiving a single dose of 0.25 mg/kg of abciximab showed a partial recovery (50% of baseline) of ADP-induced PA 4 hours postinjection. Tcheng et al emphasized donor variability in the recovery of ADP-PA after treatment in studies performed...
on 11 patients who received a bolus combined with abciximab infusion during 12 hours. Significantly, posttreatment heterogeneity was less when abciximab was given as a bolus combined with a 36-hour infusion. In our study, in which the infusion was for up to 24 hours, the recovery of ADP-induced PA was delayed beyond 24 hours for patients 1 and 2 but was more rapid for patients 3 through 6. Among the factors that may influence the recovery rate are (1) variations in plasma levels of free abciximab, (2) the rate at which surface-bound abciximab dissociates from circulating platelets,36 and (3) the extent to which GP IIb-IIIa receptors exchange between surface and internal pools.37 As a separate part of our study, we examined the distribution of abciximab within the different platelet membrane systems at each time point by immunogold staining using a rabbit antibody specific for c7E3 (provided by Drs R. Jordan and C. Wagner, Centocor, Malvern, Pa). The results indicated that abciximab gains access to the internal membrane pools of GP IIb-IIIa both through thin channels of the surface-connected canalicular system and by endocytosis but that there is not a continued recycling through dissociation and reassociation. Whether this limits the efficacy of abciximab or provides a protective backup to limit the tendency for hemorrhage is another important question to resolve.

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Logic implies that inhibition of GP IIb-IIIa should be optimal before PTCA-induced vessel injury and extend throughout the period when the injured vessel wall is at its most thrombogenic. Limiting the initial platelet-to-platelet interactions through abciximab may also reduce the expression of the internal pool of GP IIb-IIIa complexes on the surface of platelets, a process that also occurs within the aggregate and can help bring fibrinogen onto the surface and thereby promote platelet thrombogenicity.22,41 The finding by Reverter et al42 that abciximab has a dampening effect on platelet-mediated thrombin generation may also help explain the reduced signs of platelet activation during therapy. Finally, our studies highlight a major difference in the functional response between platelets from patients receiving abciximab and those from patients with Glanzmann’s thrombasthenia. In the latter, an inherited GP IIb-IIIa deficiency extends to all membrane systems,43 whereas here we have provided evidence for a pool of functional GP IIb-IIIa complexes that can be expressed after platelet stimulation with TRAP 14-mer or thrombin. Quite simply, it appears that despite the infusion, free abciximab levels are insufficient to inhibit the functioning of internal pools in the face of a strong hemostatic challenge. Whether this limits the efficacy of abciximab or provides a protective backup to limit the tendency for hemorrhage is another important question to resolve.


Expression of Markers of Platelet Activation and the Interpatient Variation in Response to Abciximab

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