Occupancy of the Internal and External Pools of Glycoprotein IIb/IIIa following Abciximab Bolus and Infusion

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ABSTRACT

The internal pool of GPIIb/IIIa, which is expressed upon platelet activation, may be inaccessible to inhibition by GPIIb/IIIa antagonists. To determine the occupancy of the internal and external pools of GPIIb/IIIa and platelet function following an abciximab bolus and infusion, 15 patients undergoing elective percutaneous transluminal coronary angioplasty were administered abciximab as a bolus and 36-h infusion. GPIIb/IIIa receptor number and occupancy in resting and TRAP-6 (20 μM)-activated samples (to expose the internal pool of GPIIb/IIIa) was quantified using a monoclonal antibody-based assay. Antibody binding was quantified by flow cytometry and platelet inhibition by light transmittance aggregation and by the rapid platelet function analyzer (Accumetrics, San Diego, CA). The target of >80% receptor occupancy (range 82–99% occupancy) of the external pool of GPIIb/IIIa was achieved in all patients at 3 min. Receptor occupancy of the combined internal and external pools of GPIIb/IIIa was less, ranging from 75 to 93% and again was maximal at 3 min. Platelet aggregation was markedly inhibited to 20 μM ADP (maximal, 11 ± 2% of baseline), but less so to 5 μM TRAP-6 (maximal, 36 ± 25% of baseline). Following discontinuation of the drug, there was a gradual fall in receptor occupancy over 15 days coinciding with the disappearance of abciximab from the platelet surface. Maximum inhibition of platelet function and receptor occupancy of the external pool of GPIIb/IIIa occurs within 3 min of an abciximab bolus and infusion. However, some internal receptors that are expressed by potent agonists are not occupied, which may explain the incomplete inhibition of platelet aggregation.

Glycoprotein (GP) IIb/IIIa is the major integrin on platelets, with 80,000 receptors on the membrane surface and up to an additional 30,000 in internal granules (Wagner et al., 1996). GPIIb/IIIa interacts with fibrinogen to mediate platelet aggregation and adhesion, events that occur early in arterial thrombosis. Several antagonists have been developed against the receptor that prevent fibrinogen binding and inhibit platelet aggregation (Topol et al., 1999), including the chimeric mouse/human monoclonal antibody abciximab. However, even at high levels of receptor occupancy by abciximab, some degree of platelet aggregation persists (Mascelli et al., 1998). One possible explanation is that the drugs fail to gain access to the internal pool of receptors. The internal pool is present on the membranes of granules that fuse with the surface membrane when platelets are stimulated with strong agonists, such as thrombin (Nurden et al., 1999). Thus, the number of GPIIb/IIIa receptors expressed on the platelet surface may increase considerably upon stimulation. Many of the drugs are highly polar and may fail to gain access into the platelet. In addition, there is evidence that the internal pool is complexed with fibrinogen and so may be inaccessible to the drug (Nurden et al., 1996).

Abciximab is a potent antagonist of the platelet GPIIb/IIIa and is effective in preventing coronary thrombosis following coronary interventions (The EPIC Investigators, 1994; The EPILOG Investigators, 1997; The EPISTENT Investigators, 1998). The recommended dosing regime of abciximab is designed to provide greater than 80% receptor occupancy. Abciximab dissociates slowly, with platelet-bound antibody detected up to 15 days following a single bolus and 12-h infusion (Mascelli et al., 1998). Abciximab has also been located in platelet granules bound to internal pools of GPIIb/IIIa, although the extent of occupancy of this pool of GPIIb/IIIa receptors is unknown (Nurden et al., 1999). In this study we have estimated the extent of abciximab binding to the internal and external pools of GPIIb/IIIa over a 15-day period following its administration to patients undergoing percutaneous coronary intervention and related the receptor occupancy to platelet inhibition. The assay used is based on the differential displacement of two anti-GPIIIa monoclonal antibodies mAb1 and mAb2, to directly quantify GPIIb/IIIa receptor number and occupancy (Quinn et al., 1999).

ABSTRACTIONS: GP, glycoprotein; mAb, monoclonal antibody; PBS, phosphate-buffered saline; TRAP, thrombin receptor-activating peptide; RPFA, rapid platelet function analyzer.
Materials and Methods

Patient Population. This was a single center open label trial in 15 patients undergoing elective percutaneous coronary angioplasty in St. James’s Hospital, Dublin, Ireland. Males and females between the ages of 21 and 70 with clinically significant coronary artery disease suitable for coronary angioplasty were recruited into the study. Exclusion criteria included abciximab administration within the previous 35 days, active internal bleeding, a history of a bleeding diathesis, major trauma, serious bleeding, gastrointestinal or genitourinary bleeding within the previous 6 weeks, a cerebrovascular accident within the previous 2 years or any cerebrovascular accident with a residual neurologocial deficit, a platelet count less than 100,000/µl, the administration of oral anticoagulants within the previous 7 days (unless the international normalized ratio was <1.4), systolic blood pressure >180 mm Hg or diastolic >100 mm Hg, or body weight >120 kg. The protocol was reviewed and approved by the Irish Medicines Board and the Ethics Committee at St. James’s Hospital and all patients provided written informed consent.

Study Protocol. Patients received a bolus (0.25 mg/kg) followed by a 36-h infusion (0.125 µg/kg/min to a maximum of 10 µg/min) of abciximab 18 to 24 h before elective coronary intervention. Coronary angioplasty was performed in the usual manner. Unfractionated heparin was administered as a bolus (50–70 U/kg to a maximum of 7000 U) to achieve an activated clotting time of >200 s. All patients received 300 mg of aspirin 4 h before the procedure. Patients who had a coronary stent inserted received the ADP receptor antagonist (250 mg of ticlopidine b.i.d. or 75 mg of clopidogrel daily) starting immediately following the procedure and continued for 4 weeks.

Blood Samples. Blood samples were collected from a peripheral vein into 3.8% sodium citrate at a final dilution of 1 in 10. Samples were collected at baseline (day 1); before the abciximab bolus; and at 1, 3, 5, 10, 30, and 60 min and 12, 24, and 36 h after the initial bolus of abciximab. Additional samples were taken on days 3, 5, 7, 9, 12, and 15. The baseline and samples obtained during the 1st h were taken from an 18-gauge peripheral venous line after the initial 5 ml of blood was discarded. Subsequent sampling was performed by peripheral venous puncture. Blood samples for platelet counts were collected into EDTA at baseline 2 to 4 and 24 h after the abciximab bolus and on day 3, 7, and 15.

Washing Procedure. The baseline sample and samples obtained during the 1st h after the abciximab bolus obtained for analysis of mAb1, mAb2, and isotype binding were washed immediately at 4°C to prevent any unbound abciximab from binding during performance of the assay. The blood was diluted 1 in 10 (200 µl in 1800 µl) at 4°C in PBS containing prostaglandin E1 (1 µM) and apyrase (25 µg/ml) and centrifuged at 3000g for 2 min. The supernatant was discarded and the whole blood was resuspended in 200 µl of PBS. The washing procedure did not alter antibody binding or the effect of abciximab on mAb1 binding. All later samples were analyzed in whole blood.

GPIIb/IIIa Receptor Number and Occupancy. GPIIb/IIIa receptor number and occupancy were quantified using the GPIIb/IIIa receptor occupancy kit (Becton Dickinson, Oxford, UK), which contains the anti-GPIIbIIIa monoclonal antibodies mAb1 (clone LYP18), mAb2 (clone 4P8), isotypic control antibody, and calibration beads. Analyses were performed immediately after blood collection on resting and TRAP-6- (peptide SFLLRN, provided by Dr. Pat Harriott, Queen’s University, Belfast, Ireland) activated samples. For activation, washed samples obtained in the 1st h and whole blood samples from later time points were activated with TRAP-6 (20 µM) for 5 min before staining with antibody. Samples were stained with secondary antibody and fixed in 2% formaldehyde/PBS and stored at 4°C. Antibody binding was quantified by flow cytometry (FACSCALIBRE; Becton Dickinson, Oxford, UK) within 12 h of staining as described previously (Quinn et al., 1999). Receptor occupancy was calculated using the equation: (baseline mAb1 sites – time point mAb1 sites)/(baseline mAb1 sites) × 100.

Platelet-Bound Abciximab. Platelet-bound abciximab was quantified using a polyclonal anti-abciximab antibody (Centocor, Malvern, PA). Whole blood was incubated with antibody (40 µg/ml) for 20 min at room temperature. Antibody binding was quantified using fluorescein isothiocyanate-labeled secondary anti-rabbit antibody. Analyses were performed using identical settings on the flow cytometer at the different time points. Results are expressed as the geometric mean fluorescence intensity of 5000 events.

Abciximab Plasma Levels. Abciximab plasma levels were measured using an immunosassay with two monoclonal antibodies specific to abciximab for both capture and detection of free plasma abciximab.

Platelet Aggregation and Rapid Platelet-Function Assay. Platelet aggregation and rapid platelet function assay (RPFA) studies were performed immediately after blood withdrawal. Platelet aggregation was assayed following the addition of ADP (20 µM) or TRAP-6 (5 µM) to platelet-rich plasma at 37°C by light transmission (Biodata PAP-4; Biodata Corporation, Horsham, PA) as previously described (Quinn et al., 1999). The rapid platelet function assay function (Accumetrics, San Diego, CA) is an automated whole blood assay, which quantifies inhibition of GPIIb/IIIa based on the ability of the platelets to agglutinate fibrinogen-coated beads (Coller et al., 1997).

Statistical Analysis. Results are expressed as mean ± S.E.M. Analysis was performed using Friedman’s nonparametric repeated measures analysis of variance and p < 0.05 was considered significant. Dunn’s multiple comparison test was used to compare individual time points when a significant difference was identified by ANOVA.

Results

Patient Characteristics. Fifteen patients were enrolled in the study between May and November 1999. Two patients discontinued the abciximab infusion early; one patient with poor dentition developed significant gingival bleeding after 32 h of the abciximab infusion. The second patient discontinued the infusion after 30 h because of epistaxis. Both patients were continued in the study. One patient did not attend for the day 9 follow-up and another failed to attend for the day 12 visit. Patient characteristics are presented in Table 1. Seven patients underwent coronary stent insertion of which six received 75 mg of clopidogrel daily and one 250 mg of ticlopidine b.i.d. for 4 weeks. Two patients were readmitted with unstable angina during the 30 days of follow-up. One was treated medically and the other underwent angioplasty of a separate coronary vessel.

Receptor Number and Occupancy. There was no significant change in the platelet count throughout the study (data not shown). At baseline, mAb1 identified 68,139 ± 4,177 GPIIb/IIIa (Fig. 1) receptors per platelet in resting samples, whereas mAb2 recognized 58,278 ± 4,461 sites/platelet. TRAP (20 µM) activation was used to expose the internal pool of GPIIb/IIIa. With activation, mAb1 binding

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<th>TABLE 1 Demographic details</th>
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<td>Age ± S.D. (years)</td>
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<tr>
<td>Male gender</td>
</tr>
<tr>
<td>Previous MI</td>
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<tr>
<td>Stable angina</td>
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<td>Diabetes</td>
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Male, myocardial infarction.
increased by 12% to 76,537 ± 5,823 sites/platelet (Fig. 1). The target of >80% receptor occupancy of the resting external pool of GPIIb/IIIa was achieved in all patients within 3 min of the abciximab bolus with mAb1 binding falling to 6052 ± 907 sites/platelet (receptor occupancy range 82–99%, \( p < 0.001 \)). There was a trend toward the recovery of receptor occupancy in the early part of the infusion such that five patients had less than 80% receptor occupancy in resting platelets at 60 min. This trend had reversed by 12 h (presumably reflecting the continuous infusion of the drug) and only one patient had less than 80% receptor occupancy at this time. Receptor occupancy of the combined internal and external pool of GPIIb/IIIa, exposed by TRAP activation, was less marked. mAb1 binding reached a minimum of 11,991 ± 1,209 sites/platelet (receptor occupancy range 75–93%, \( p < 0.001 \)) in activated platelets. As in resting samples, transient recovery of the combined internal and external pool of GPIIb/IIIa was observed at 60 min, receptor occupancy being less than 80% in nine patients.

On day 3, that is, 12 h following discontinuation of the infusion, mAb1 binding in resting platelets increased, indicating dissociation of abciximab from GPIIb/IIIa. However, 51% of the GPIIb/IIIa surface receptors were still occupied on day 7, 5 days after discontinuation of the infusion (mAb1 binding 36,978 ± 2,760 sites/platelet) and 28% were still occupied (mAb1 binding 48,825 ± 2,840 sites/platelet) on day 15, 13 days after discontinuation of the infusion. The recovery of the entire pool of GPIIb/IIIa was more rapid. By day 15, 13 days after discontinuation of the infusion, only 12% of receptors in the activated samples failed to bind mAb1 (mAb1 binding 66,973 ± 4,488 sites/platelet). There was no change in mAb2 binding during the course of the study, indicating that abciximab had no effect on the total number of platelet GPIIb/IIIa receptors \( (p = 0.08) \).

**Platelet Aggregation.** Maximum inhibition of ADP- (20 \( \mu \)M) and TRAP (5 \( \mu \)M)-induced platelet aggregation was observed within 3 min of the bolus administration (Fig. 2). ADP-induced platelet aggregation reached a nadir of 8 ± 2% aggregation \( (11 ± 2% \) of baseline, \( p < 0.001 \)) at 3 min, whereas TRAP-induced aggregation fell to 23 ± 1% \( (36 ± 5% \) of baseline, \( p < 0.001 \)) at the same time point. Some recovery of ADP and TRAP-induced platelet aggregation occurred during the abciximab infusion at the 12-h time point. However, this trend was reversed during the continued infusion. Platelet aggregation had recovered to 36 ± 5% for ADP and 54 ± 4% for TRAP on day 3, 12 h after discontinuation of the infusion and had fully recovered by day 7, 5 days after the end of the infusion. In the seven patients receiving the ADP receptor antagonists clopidogrel and ticlopidine, there was a trend toward greater inhibition of ADP-induced platelet aggregation at day 15 (79 ± 10% of baseline aggregation).

**Rapid Platelet Function Assay.** Inhibition of the RPFA was similar to the inhibition of platelet aggregation, although more pronounced (Fig. 3). Maximum inhibition occurred within 3 min of the abciximab bolus to 6 ± 1% of baseline \( (p < 0.001) \). Recovery of RPFA began 12 h after stopping abciximab but was not complete until day 12, 9 days after discontinuation of the drug. The ADP receptor antagonists clopidogrel and ticlopidine did not alter the recovery of the RPFA.

**Platelet-Bound and Plasma Abciximab.** Platelet-bound abciximab was maximal at 1 min after the bolus (Fig. 4). Consistent with the platelet aggregation, RPFA, and

![Fig. 1. Receptor occupancy of the internal and external pools of GPIIb/IIIa detected by mAb1 in resting (●) and TRAP-6-activated samples (□). Abciximab administered as a bolus and infusion for 36 h (shaded area) markedly suppressed mAb1 binding, which recovered slowly following discontinuation of the drug: ***\( p < 0.001 \), **\( p < 0.01 \).](image1)

![Fig. 2. Inhibition of ADP- (●, 20 \( \mu \)M) and TRAP-6 (□, 5 \( \mu \)M)-induced platelet aggregation, presented as a percentage of baseline aggregation. The shaded area represents the period of the infusion. ***\( p < 0.001 \), **\( p < 0.01 \), *\( p < 0.05 \).](image2)

![Fig. 3. Inhibition of the RPFA. Note that the assay detects the aggregation of fibrinogen-coated beads mixed with platelets following stimulation with iso-TRAP. The shaded area represents the period of the infusion. ***\( p < 0.001 \), **\( p < 0.01 \), *\( p < 0.05 \).](image3)
mAb1 binding data, there was a gradual decline in abciximab bound to platelets that paralleled a fall in plasma abciximab despite the continued infusion of the drug (Fig. 5). Curiously, there was an abrupt rise in platelet-bound abciximab along with further inhibition of mAb1 binding and platelet aggregation at 24 to 36 h, without a change in plasma drug levels. Dissociation of platelet-bound abciximab was observed 12 h after discontinuation of the infusion although platelet-associated abciximab was detected even at day 15. Abciximab plasma levels reached a maximum (2428 ± 272 ng/ml) within 1 min of the bolus administration. This had fallen to 393 ± 80 ng/ml at 1 h. Plasma abciximab was undetectable in all patients at 7 days after the initial bolus (Fig. 5).

**Discussion**

The bolus and 12-h infusion regimen of abciximab that has proven to be effective in a number of large randomized controlled trials (The EPIC Investigators, 1994; The EPILOG Investigators, 1997; The EPISTENT Investigators, 1998) is designed to provide >80% GPIIb/IIIa receptor blockade for the duration of therapy. This level of receptor occupancy is required to prevent thrombotic complications at the time of coronary intervention (The EPIC Investigators, 1994). However, even at this level of receptor occupancy inhibition of platelet aggregation, particularly to potent agonists, may be incomplete. This may be due in part to the inaccessibility of an internal pool of receptors. To address this issue, we have used a monoclonal antibody-based assay to directly quantify abciximab receptor occupancy of the internal and external pool of GPIIb/IIIa (Quinn et al., 1999). The dose of abciximab used differed from the standard regimen used for coronary interventions in that following the standard bolus, the infusion was continued for 36 h to see whether the internal pool became occupied, as previously suggested (Nurden et al., 1999). This regimen has been shown to reduce ischemic events in patients with unstable coronary syndromes before percutaneous intervention (The CAPTURE Investigators, 1997).

Maximum platelet inhibition and receptor occupancy of the internal pool of GPIIb/IIIa occurred within 3 min of the bolus dose. This is similar to previous reports of maximum platelet inhibition and receptor occupancy within 10 min of the bolus dose (Hezard et al., 1999). In addition, the target receptor occupancy of greater than 80% in the external pool of GPIIb/IIIa was achieved in all patients. Platelet-bound abciximab, detected by a polyclonal antibody to abciximab, mirrored the reduction in mAb1 binding indicating that the inhibition of mAb1 binding was a result of abciximab binding to GPIIb/IIIa. This level of receptor occupancy was accompanied by marked suppression of platelet aggregation induced by ADP and suppression of the rapid platelet function assay. However, there was still a substantial degree of platelet aggregation in response to TRAP, a potent agonist that induces platelet adhesion. Moreover, when platelets were stimulated, a pool of unoccupied receptors was detected using the mAb1 assay. Therefore, the persistent platelet aggregation may reflect failure of the drug to inhibit the internal pool of receptors.

Nurden et al. (1999) have shown trafficking of abciximab-bound receptor between the internal and external pools. Therefore, it was possible that greater inhibition of platelet aggregation may occur with a longer period of infusion as the internal pool is occupied. We could show no further suppression of the total pool of GPIIb/IIIa measured as mAb1 binding in TRAP-stimulated platelets. Indeed, there is evidence that the internal pool is occupied by the ligand fibrinogen and therefore may be unavailable to the antagonist (Nurden et al., 1996). Thus, although there is trafficking of the receptor from the surface to the internal pool, this may be insufficient over the time course of the infusion to occupy all of the receptors.

Curiously, there was a transient fall and rise in platelet inhibition, receptor occupancy, and platelet-bound abciximab during the course of the infusion without a change in plasma abciximab concentration. The unexpected rise in binding coincided with the administration of a 300-mg dose of aspirin at the time of the percutaneous intervention. The difference between occupancy of the external and the total pool of receptors persisted, suggesting that this was not a result of abciximab redistributing to the internal pool. Interestingly, there is evidence that aspirin enhances the platelet inhibitory effects of abciximab and increases the binding of abciximab to platelets (Schneider et al., 1999).

Strong agonists such as TRAP-6 induce secretion of GPIIb/IIIa from platelet α-granules and increase the platelet sur-

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**Fig. 4.** Platelet-bound abciximab expressed as the geometric mean fluorescence intensities. The shaded area represents the period of the infusion. ***p < 0.001, **p < 0.01, *p < 0.05.

**Fig. 5.** Free plasma abciximab (limit of detection, 10 ng/ml). The shaded area represents the period of the infusion.
face receptor number by 10 to 30% (Tsao et al., 1995; Gawaz et al., 2000). The internal pool of GPIIb/IIIa is accessible to abciximab (Gawaz et al., 2000) and some labeling of the internal pool occurs within 1 min following the bolus (Nurden et al., 1999). However, our study demonstrates that this process is incomplete as the number of unoccupied sites detected by mAb1 increased upon stimulation. The results suggest that a proportion of the internal pool is inaccessible to abciximab inhibition. It remains to be clarified whether this explains the incomplete inhibition of platelet aggregation to TRAP. In addition to free receptor, TRAP may induce secretion of fibrinogen-bound receptor that may not be inhibited by abciximab (Nurden et al., 1996).

References


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