Quantifying GPIIb/IIIa Receptor Binding Using 2 Monoclonal Antibodies

Discriminating Abciximab and Small Molecular Weight Antagonists

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Background—Dosing of glycoprotein (GP) IIb/IIIa receptor antagonists is frequently based on the inhibition of platelet aggregation, which may be influenced by the agonist used or concurrent medications. Here we describe a monoclonal antibody-based technique to quantify total and ligand-occupied GPIIb/IIIa receptors.

Methods and Results—In vitro binding of monoclonal antibodies, LYP18 (Mab1) and 4F8 (Mab2), to the GPIIb/IIIa complex, was characterized using purified receptor and to platelets by flow cytometry. Patients undergoing coronary angioplasty received a single 20 mg dose of the oral GPIIb/IIIa antagonist, xemilofiban, or matching placebo, and antibody binding was compared with inhibition of platelet aggregation. Mab1 and Mab2 were bound to purified GPIIb/IIIa and to unoccupied, inactivated receptor on platelets. Mab2 identified the β3 subunit, whereas Mab1 was complex-specific. Neither antibody interfered with the other’s binding, suggesting that they identified distinct sites. Mab1 identified 53 300±5423 GPIIb/IIIa sites per platelet, whereas Mab2 identified 50 120±5066 sites per platelet. Mab1 binding was inhibited by abciximab in a dose dependent manner (IC50, 0.85±0.1 μg/mL), whereas Mab2 binding was unaffected. In contrast, the 2 small molecular weight antagonists, SC-57101A (IC50, 0.22±0.06 μmol/L) and eptifibatide (IC50, 0.35±0.14 μmol/L) inhibited Mab2 but not Mab1 binding. In patients treated with xemilofiban, Mab1 binding was unaltered but Mab2 binding decreased from 37 930±2061 sites per platelet at baseline to 8318±870 sites per platelet 6 hours after dosing (P<0.0001). Platelet aggregation to adenosine diphosphate (20 μmol/L) fell to 3±3% of baseline in line with the inhibition of Mab2 binding (correlation coefficient 0.8, P<0.0001).

Conclusions—Mab1 and Mab2 bind to GPIIb/IIIa and are differentially displaced by abciximab and small molecular weight antagonists. These antibodies may be used to monitor receptor number and occupancy during administration of a GPIIb/IIIa antagonist. (Circulation. 1999;99:2231-2238.)

Key Words: glycoproteins • platelet aggregation inhibitors • abciximab • thrombosis

Glycoprotein (GP) IIb/IIIa is one of a family of integrins that are heterodimers of an α and β subunit and act as receptors for adhesion proteins.1 GPIIb/IIIa is the principle integrin on platelets and is the receptor for fibrinogen, which mediates platelet aggregation and adhesion. Antagonists of GPIIb/IIIa, including the monoclonal antibody abciximab2 and several peptide (eg, eptifibatide)3 and nonpeptide small molecules,4 are potent inhibitors of platelet aggregation; given as short-term infusions, they reduce the complications of coronary angioplasty when combined with aspirin.5-8 Orally active agents have also been developed9 that will hopefully extend the use of GPIIb/IIIa receptor antagonists to long-term therapy for patients with stable coronary disease.

Currently, dosing of GPIIb/IIIa receptor antagonists is based on the inhibition of platelet aggregation. This has several disadvantages. Platelet aggregation may vary depending on the agonist used, the platelet count, ingestion of food or alcohol, smoking, and the use of concurrent medications.10-12 Platelet aggregation is also insensitive to very high or low levels of receptor occupancy. Aggregation reflects cross-linking of GPIIb/IIIa receptors from adjacent platelets and is unaffected at levels of receptor occupancy <30% to 50%.13 Similarly, as receptor occupancy exceeds 80%, aggregation may be completely inhibited despite the presence of unoccupied receptors. The inhibition of residual unoccupied receptors may have functional effects as evidenced by a further increase in bleeding time with levels of occupancy >80%.14

Here, we describe the binding of 2 monoclonal antibodies to the platelet GPIIb/IIIa, LYP18 (Mab1), previously characterized to bind to the ligand recognition site and to be complex specific,15 and 4F8 (Mab2). Binding of these antibodies can be used in a flow cytometric assay to directly quantify occupied and unoccupied GPIIb/IIIa. We also pres-
Materials and Methods

Monoclonal Antibodies
Monoclonal antibody 1 (Mab1, clone LYP18), a murine monoclonal antibody to the GPIIb/IIIa complex; monoclonal antibody 2 (Mab2, clone 4P8), a murine monoclonal antibody to the beta III subunit; isotypic control; fluorescein isothiocyanate (FITC) labeled F(ab’)_2; fragments of human Ig absorbed, sheep anti-mouse IgG antibodies; and calibration beads with 4 different known amounts of antibody per bead were provided by Dr M Canton (Biocytex, Marseille, France). SC-57101A (a HCl salt of the active component of the oral GPIIb/IIIa antagonist, orbofiban) and H’S-52021B, a radiolabeled GPIIb/IIIa ligand, were gifts of Dr Robert Anders (J.D. Searle, Skokie, Ill). Eptifibatide was a gift of Dr David Phillips (Cor Therapeutics, San Francisco, Calif). The monoclonal antibody abciximab was purchased from Eli Lilly and Co, Ind. GPIIb/IIIa was purified according to the protocol of Phillips et al.17 Ligand dilutions were performed in PBS.

Ligand Binding Studies
Nine parts blood from healthy donors, who had not taken aspirin or any other anti-platelet agent in the previous 7 days, was collected to one part sodium citrate (3.8%). This was centrifuged at 150g for 10 minutes and the platelet rich plasma (PRP) was aspirated. PRP was diluted to 1x10^9 platelets/mL with PBS and incubated with different concentrations of ligand for 30 minutes at room temperature. Aliquots of each dilution were incubated with Mab1 or Mab2 (10^-6 M) for 30 minutes at room temperature. FITC-labeled Mab1 or Mab2 was then added and incubated for 10 minutes and analyzed on the flow cytometer.

Platelet Aggregation
For ex vivo platelet aggregation studies, blood was collected in 3.8% sodium citrate at a final dilution of 1:10 and was centrifuged at 850g for 3 minutes to procure PRP. Following PRP removal, the remaining plasma was centrifuged at 2500g for 5 minutes to obtain platelet poor plasma. Platelet aggregation was studied following the addition of adenosine diphosphate (20 μmol/L) to PRP at 37°C by light transmission (Biodata PAP-4, Biodata Corporation). Aggregation was not adjusted for the platelet count of each sample; however, the aggregation at the different time-points was expressed as a percentage of baseline aggregation, before administration of the drug. There was no significant difference in whole blood platelet count at the different time-points with a mean±SEM (x10^12/L) of 213±16 at baseline, 201±10 preprocedure, 215±13 post procedure and 189±9 at the 6 hour time-point.

3HSC-52021B-Binding
3HSC-52021B is a triatiated form of a potent benzamidine GPIIb/IIIa receptor antagonist.16 Earlier experiments (not shown) demonstrated that 3HSC-52021B binds in a dose-dependent saturable manner in whole blood from healthy volunteer donors, with a K_d of 93.4 nmol/L. Ligand binding was determined using a modification of the technique described by Wang et al.19 Whole blood was incubated with a 3HSC-52021B (5 nmol/L) for 30 minutes. Bound ligand was separated from unbound using a cell harvester (Brandel Inc). Each sample was eluted through Whatmann GFb microfiber filters (Whatmann International Ltd) with 3.5 mL of Tris (10 mmol/L) buffer at 4°C. The filters were washed 3 times with 3.5 mL of ice-cold buffer and placed in scintillation fluid (Ecocism A, National Diagnostics). Filters were analyzed using a scintillation counter (Wallac, 1214 RACKBETA). Each sample was analyzed in triplicate with one aliquot containing excess unlabelled compound (500 μmol/L). This was taken to represent nonspecific binding. Results are expressed as the percentage of baseline radioactivity before administration of the GPIIb/IIIa receptor antagonist.

Ex Vivo Studies
The protocol was reviewed and approved by the Irish Medicines Board and the Ethics Committee at St. James’s Hospital and all patients gave written, informed consent.

Blood samples were drawn, into 3.8% sodium citrate, from patients receiving a single dose of the oral GPIIb/IIIa receptor antagonist, xemilofiban 20 mg, or a matching placebo administered in a randomized, blinded fashion 30 to 90 minutes before coronary balloon angioplasty or stent placement. All patients received aspirin (150 to 300 mg) before the procedure and heparin at the time of intervention to achieve an ACT of >300 s. Blood sampling was performed from a peripheral vein at baseline before administration of the drug. The intervention was performed no sooner than 30 minutes but within 90 minutes of randomized drug administration. Blood samples were obtained again from the femoral venous sheath after insertion and immediately postangioplasty, discarding the initial 5 mL of blood in each case. Peripheral venous blood was also obtained just before the next dose of study medication, 4 to 7 hours following the initial dose. Samples were collected into 3.8% sodium citrate for platelet studies. Platelet aggregation studies and monoclonal antibody binding by flow cytometry were performed within 2 hours of blood sampling. 3HSC-52021B binding studies were performed within 24 hours. Previous experiments had confirmed stability of the 3HSC-52021B binding assay for up to 7 days, when samples were stored at 4°C (data not shown). For Mab1 and Mab2 binding, whole blood was diluted 1 in 4 with platelet poor plasma from the corresponding aggregation studies at each time-point. This was performed in order to ensure that Mab1 and Mab2 (5 μg/mL) were at saturating concentrations. Platelet poor plasma is used to prevent dilution of the ligand. Diluted samples were incubated with antibody for 20 minutes at room temperature, stained with secondary antibody, and analyzed by flow cytometry.
**Statistical Analysis**

Continuous data are presented as mean±SEM. Clinical data from xemilofiban- and placebo-treated patients was compared by ANOVA. Within the groups of treated and untreated patients, data from the different time-points was compared by an initial ANOVA with subsequent Dunnett’s t test (when $P<0.05$) to compare each time-point to baseline. Percent of baseline Mab2 binding and percent of baseline aggregation were correlated using Pearson’s correlation coefficient.

**Results**

**Binding of Mab1 and Mab2 to GPIIb/IIIa**

Mab1 and Mab2 were bound to purified GPIIb/IIIa in a dot blot assay, where no binding was detected with an isotypic antibody. Following protein gel electrophoresis of the purified receptor (performed under reducing conditions), Mab2 identified GPIIb/IIIa, binding to the $\beta_3$ subunit (Figure 1). As Mab1 binding is complex specific, it did not identify the receptor under these reducing conditions.

**Binding of Mab1 and Mab2 to Platelets In Vitro**

In undiluted PRP and in PRP diluted to $1 \times 10^7$ platelets/mL from healthy donors, Mab1 and Mab2 bound in a concentration-dependent and saturable manner. Mab1 had a slightly lower affinity than Mab2 with $K_d$ of $5.3 \pm 1.3 \mu g/mL$ and $3.8 \pm 1.1 \mu g/mL$ ($n=3$), respectively, in PRP and $1.52 \pm 1.4 \mu g/mL$ and $1.4 \pm 1.5 \mu g/mL$ ($n=3$), respectively, in PRP diluted to $1 \times 10^7$/mL (Figure 2). Maximum binding occurred at 6 $\mu g/mL$ in dilute PRP and at 15 $\mu g/mL$ in undiluted PRP. Mab1 identified a mean of $53,300 \pm 5,423$ GPIIb/IIIa receptors sites per platelet at maximum binding. The number of sites determined by Mab2 was slightly lower at $50,120 \pm 5,066$ sites. Mab1 binding was reduced to background fluorescence in platelet preparations incubated with EDTA (10 mmol/L at 37°C for 30 minutes), whereas Mab2 binding was reduced but was not completely abolished and the antibody still recognized $13,041 \pm 577$ sites per platelet. In competition studies using FITC-labeled Mab1 and Mab2, Mab1 did not interfere with Mab2 binding and Mab2 did not inhibit Mab1 binding, suggesting that they bind to separate sites (Figure 3).

Repeated analysis of a sample from a single donor drawn at the same time revealed a coefficient of variation of 0.55% for Mab1 and 1.3% for Mab2 in the number of sites identified. Analysis of samples from 4 different healthy donors revealed a coefficient of variation of 5.4% for Mab1 and 12.6% for Mab2 in the number of sites identified.
Figure 3. Histogram of mean fluorescence intensities of FITC-labeled Mab1 and Mab2 binding in flow cytometric competition assays. A, Incubation with isotypic control before addition of FITC-Mab1 (solid histogram) or FITC-Mab2 (open histogram) did not effect binding of FITC-Mab1 or FITC-Mab2. B, Initial incubation with unlabeled Mab2 reduced FITC-Mab2 binding but did not effect FITC-Mab1 binding. C, Initial incubation with unlabelled Mab1 reduced FITC-Mab1 binding but did not effect FITC-Mab2 binding.
Increasing concentrations of eptifibatide reduced Mab2 binding progressively increased after 2 hours, indicating displacement of the abciximab from its binding site, whereas Mab2 binding remained constant. In dilute unfixed PRP incubated with eptifibatide (12.5×10^{-6} M) or SC-57101A (6.3×10^{-6} M), Mab2 binding increased after 2 hours indicating the displacement of ligand. Fixing samples in 1% formaldehyde, after incubation with Mab1 and Mab2 prevented this displacement, and binding remained constant for up to 15 days (data not shown).

**Clinical Studies**

Figure 4 shows the effect of xemilofiban (20 mg) or placebo on platelet aggregation. 

！HSC-52021B binding and number of sites detected by Mab1 and Mab2. In patients receiving placebo (n=7), Mab2 binding remained stable with 44 652±4189 sites, 38 013±2681 sites, and 40 047±1986 sites detected preprocedure, postprocedure, and at 6 hours, respectively (P=0.41). The corresponding number of sites identified by Mab1 were 59 230±4892, 56 679±5332, and 56 955±4537 at each time-point (P=0.98). Platelet aggregation was largely unchanged at 94±2%, 87±6%, and 97±2%, at the corresponding time points (P=0.15). Similarly, radioligand binding was uninhibited at 123±24% preprocedure, 162±24% postprocedure, and 115±8% at 6 hours (P=0.12).

In patients receiving xemilofiban (n=9), there was significant inhibition of platelet aggregation, radiolabeled ligand binding, and Mab2 binding (P<0.002). This occurred progressively over the period of observation, which is consistent with a peak plasma concentration of 2 to 6 hours.20 At baseline, Mab1 and 2 identified 59 910±1932 and 37 930±2061 sites per platelet, respectively. Mab1 binding was unchanged following drug at 50 795±2808 sites just before the procedure, 50 260±2737 immediately postprocedure, and 48 485±3228 sites at the 6-hour time-point (P=0.92). In contrast, Mab2 binding decreased to 19 540±4819 (51% of baseline) sites just before the procedure, 13 373±4866 (32% of baseline) immediately postprocedure (at an average of 92 minutes after the initial drug dose), and reached a minimum at the 6-hour time-point of 8318±870 sites per platelet before the next dose of drug (P<0.0001). Aggregation was reduced to 61±8%, 30±15%, and 3±3% of baseline at the corresponding time-points (P<0.0001). Radioligand binding was also inhibited to 79±20% before the procedure, 77±16% immediately postprocedure, and 57±10% at 6 hours (P<0.05 for postprocedure and 6-hour sample compared with baseline). Mab2 expressed as a percent of baseline correlated with platelet aggregation, also expressed as a percent of baseline (r=0.8; P<0.0001, Figure 6).

**Discussion**

**Binding of Mab1 and Mab2 to GPIIb/IIIa**

Previous studies have shown that Mab1 binds to GPIIb/IIIa on platelets and the vitronectin receptor, α,β3, on endothelial and melanoma cells.21–23 Its binding to GPIIb/IIIa requires an intact heterodimer because it fails to bind to either subunit under reducing conditions or in crossed immunoelectrophoresis.15 The binding site is at or close to the ligand recognition sites of GPIIb/IIIa, as Mab1 inhibits fibrinogen binding and
platelet aggregation. Mab2 binds to the β3 subunit of the platelet GPIIb/IIIa, but further characterization is required to define the precise epitope. Mab2 recognizes the receptor under reducing conditions, suggesting that it recognizes a linear epitope. This may explain the continued recognition of the epitope when the receptor complex is disrupted by chelating Ca²⁺. Mab2 also binds to endothelial cells. This is consistent with its epitope also being present on the β3 subunit of the vitronectin receptor αvβ3 (data not shown).

**Differential Displacement of Mab1 and Mab2 Binding to Platelets by Antagonists**

Both antibodies bound to platelets in a concentration dependent and saturable manner with equivalent affinities. The number of sites identified by the antibodies is consistent with previously reported numbers of GPIIb/IIIa receptors per platelet identified by bivalent monoclonal antibodies and labeled fibrinogen binding. However, it is possible that our antibodies underestimate the true number of receptors per platelet; Wagner et al have demonstrated discrepancy between bivalent and monovalent antibody binding. This will not, though, affect the ability of the assay to estimate the percentage occupancy. On whole platelets, Mab1 binding was displaced entirely by disruption of the complex using EDTA and by the monoclonal antibody, abciximab. Abciximab is directed at the fibrinogen binding site and prevents fibrinogen binding, platelet aggregation, and platelet adhesion. In contrast, abciximab had no effect on Mab2 binding, even at very high concentrations. This suggests that the Mab1 and Mab2 recognize different epitopes. Indeed, using prelabeled Mab1 and Mab2, we have shown that the antibodies did not compete with each other for binding, again suggesting that they identified different epitopes.

In contrast to abciximab, 2 small molecule antagonists, the peptide eptifibatide and the nonpeptide SC-57101A, displaced Mab2 but not Mab1. It is not clear why these compounds failed to displace Mab1. As with abciximab, SC-57101A and eptifibatide prevent platelet aggregation and fibrinogen binding, suggesting that they bind at or close to the ligand recognition site. However, there may be differences in the regions recognized by antagonists despite their similar effects on fibrinogen binding. Cross-linking studies using iodinated GPIIb/IIIa antagonists KYGRGDS and the cyclic compound, KYGC(s-s)HarGDPWPC(s-s) show that these
compounds cross-link to different sites on the β3 subunit. Thus, antagonists may differ in where they bind within the active pocket and this may explain the differential displacement of Mab1 binding.

Similarly, it is unclear why some compounds and not others displace Mab2. Mab2 binding does not interfere with the binding of the radiolabeled GPIIb/IIIa antagonist, 3HSC-52021B, in contrast with Mab1, suggesting that Mab2 identifies a site remote from the active site and remote from where antagonists bind. Therefore, reduction of Mab2 binding may not reflect a direct interaction between antibody and antagonist. The displacement of Mab2 may be the result of a conformational change in the receptor with a loss or attenuation of the epitope recognized by the antibody. Ligand binding is known to induce conformational changes in the GPIIb/IIIa, usually detected as the expression of neo-epitopes. Ligand attenuation of a binding site or epitope has also been described in the fibronectin receptor (α5β1), where the epitope is present on unbound receptors but disappears from ligand bound receptors.

Assay of Total and Occupied GPIIb/IIIa

The differential displacement of the 2 monoclonal antibodies to the GPIIb/IIIa receptor, Mab1 and Mab2, by abciximab and the synthetic GPIIb/IIIa antagonists provides a means of calculating total receptor number and receptor occupancy by GPIIb/IIIa ligand. This may be useful in following the receptor-ligand interaction, as demonstrated in our patient population. The differential effects of the compounds may also be applied to monitor drug receptor interactions following oral GPIIb/IIIa antagonists in patients previously treated with abciximab. Currently available methods to assess the biological activity of GPIIb/IIIa receptor antagonists are limited by their inability to differentiate between receptor inhibition by abciximab and small molecular weight antagonists. This is important as abciximab can remain bound to platelets for days following a single administration, enhancing the effects of oral GPIIb/IIIa antagonists. Finally, by estimating total as well as bound receptors, this assay provides a means to follow receptor kinetics during long-term drug administration. Thus, changes in receptor number as a result of ligand occupancy or following drug withdrawal may be detected.

In conclusion, Mab1 and Mab2 are 2 monoclonal antibodies that bind to different epitopes of the platelet GPIIb/IIIa and are differentially displaced by abciximab and the small molecule antagonists, eptifibatide, xemilofiban, and SC-57101a. The displacement of Mab1 and Mab2 may be used to monitor drug receptor interactions in vivo and potentially to discriminate between the effects of abciximab and other antagonists in man.

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References


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