Analysis of GPIIb/IIIa Receptor Number by Quantification of 7E3 Binding to Human Platelets

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A large number of glycoprotein (GP) IIb/IIIa receptors are present on the surface of platelets. Studies to define precisely the number of GPIIb/IIIa receptors using specific monoclonal antibodies (MoAbs) or fibrinogen binding have, however, yielded varying estimates of receptor number. To refine the quantitative estimation of GPIIb/IIIa receptors on resting platelets, we have used the MoAb 7E3, which has high affinity for GPIIb/IIIa. Quantitative binding studies were performed using radiolabeled conjugates of 7E3 IgG, as well as fragments and derivatives of 7E3. For platelets obtained from any single individual, the numbers of 7E3 F(ab')2 and IgG molecules bound per platelet were equivalent (−40,000), whereas the number of Fab molecules bound per platelet was consistently approximately twofold higher (−80,000). To investigate the basis of the quantitative disparity in binding of intact 7E3 and 7E3 F(ab')2 versus 7E3 Fab, we studied the binding of a newly constructed, bispecific (Fab')2 molecule containing only a single 7E3 combining site. Because this construct bound to the same extent as the Fab species, the larger size of the intact 7E3 and 7E3 F(ab')2 molecules could not explain the reduced number of molecules that bound per platelet compared to the Fab fragment. Rather, it appears that the valency of the antibody is the critical factor determining the number of antibody molecules bound per platelet. Thus, we conclude that the binding of 7E3 Fab corresponds most closely with surface GPIIb/IIIa number and that the number of GPIIb/IIIa receptors is approximately 80,000 per platelet.

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The platelet surface is densely populated with receptors for adhesive protein ligands.1 Most prominent among these receptors is the glycoprotein IIb/IIIa complex (GPIIb/IIIa). An accurate assessment of the number of surface GPIIb/IIIa receptors is important for understanding the normal and pathological responsiveness of platelets. The GPIIb/IIIa receptor on resting platelets does not bind ligands with high affinity; after activation, the receptor develops a high binding affinity for fibrinogen and other adhesive proteins that enables the receptor to cross-link platelets one to another in stable platelet aggregates.2 Approximately 40,000 fibrinogen molecules have been reported to bind per activated platelet,3,5 but this cannot be directly translated into receptor number because fibrinogen is dimeric and has a total of six regions that may potentially interact with GPIIb/IIIa receptors. Studies of the binding of small monovalent agents such as peptides and snake venom proteins6-9 to GPIIb/IIIa have yielded varying estimates of the number of molecules bound per platelet which may reflect differences in binding affinities or other properties. Monoclonal antibodies (MoAbs), by virtue of their high affinity and specificity for GPIIb/IIIa and the similarity of results using different antibodies, have been considered the most reliable indicators of GPIIb/IIIa copy number. Regardless of the antibody used, a range of 35,000 to 50,000 antibody molecules have been reported to be bound per platelet,6-17 with approximately a twofold variability in the number of receptors present on platelets from different individuals.15 Our previous studies with antibody 7E318 and studies by others using different anti-GPIIb/IIIa MoAbs19-20 indicated that more Fab' molecules bound per platelet than intact IgGs or F(ab')2 fragments of the same antibodies. Various mechanisms can be proposed to explain these observations, among which are: (1) the existence of unique receptor pools that are accessible only to Fab fragments and not to IgGs, based on size-dependent access, (2) steric interference caused by the binding of another antibody molecule to an adjacent GPIIb/IIIa receptor, (3) internalization of Fab molecules into platelet α-granules, with perhaps binding to GPIIb/IIIa receptors in the α-granule membrane, (4) recruitment of GPIIb/IIIa receptors to the platelet surface from the α-granule pool triggered specifically by Fab fragments, or (5) the simultaneous binding of the bivalent antibody species [IgG and F(ab')2] to two different GPIIb/IIIa receptors. To differentiate between these mechanisms, we have studied the binding to platelets of 7E3 IgG, F(ab')2, and Fab, as well as a newly constructed, bispecific (Fab')2 molecule containing only a single 7E3 combining site, but having the same size as 7E3 F(ab')2.

MATERIALS AND METHODS

Chemicals and reagents. All chemicals were purchased from commercial sources and used without purification. Abbreviations. Purified antibody fragments of IgG are defined as follows: Fab denotes the IgG fragment produced by papain digestion, comprised of a light chain linked to the proteolytically cleaved heavy chain, and functionally monovalent for antigen binding; F(ab')2 is a functionally bivalent fragment, a dimer of two Fab' fragments and is produced by papain digestion of IgG; Fab' is a monovalent fragment derived by mild reduction of the F(ab')2 fragment followed by alklylation with iodoacetamide to prevent reoxidation of the sulfhydryl residues10; (Fab')2 designates a bivalent fragment obtained by chemical or oxidative reconstitution of two Fab' fragments; a bispecific (Fab')2 fragment is reconstituted from two different Fab' fragments.

MoAbs. The intact 7E3 and the 7E3 fragments bind to platelet GPIIb/IIIa and also to the α,β3 vitronectin receptor. The murine 7E3, the murine/human chimeric 7E3,21 and OC125 (anti-ovarian cancer antigen)22 MoAbs were produced from hybridoma cell lines. The intact IgG molecules were isolated from cell supernatant by...
affinity chromatography on Protein A-Sepharose (Pharmacia, Uppsala, Sweden). The Fab fragments were prepared by papain digestion and the Fab' fragments were prepared by pepsin digestion. The fragments were further purified on Protein A-Sepharose to remove Fc-containing materials, and/or other gel filtration and/or ion exchange chromatography procedures to remove other contaminants. The OC125 Fab' fragment was prepared by mild reduction of the corresponding Fab', fragment followed by alklylation with iodoacetamide to prevent reoxidation of the disulfide bonds. The murine 7E3 Fab and the human/murine chimeric 7E3 Fab (ReoPro, Abczimab; Centocor, Malvern, PA) fragments which are essentially identical in platelet binding and platelet function inhibition properties, were used interchangeably in these experiments, and are collectively identified as 7E3 Fab. The 7E3 IgG and the OC125 and 7E3 Fab') fragments used in these analyses were the native murine molecules.

Preparation of chemically cross-linked (Fab')2 fragments. The method of preparation of the bispecific 7E3×OC125 (Fab')2 fragment was the same used to prepare the bispecific 7E3×P4B6 (Fab')2 fragment. Briefly, parental Fab')2 fragments of an antibody to a cancer antigen not present on platelets, OC125, and 7E3 were reconstituted with an excess of NalI (Amersham Corp, Arlington Heights, IL) in tubes precoated with 5,5'-dithiobis(N,N'-nitrobenzoic acid, then the mixture was treated again with L-cysteine to reduce any free sulfhydryl groups were capped with bis-maleimido methyl ether (BMME). Any remaining free sulphydryl groups were capped with 5,5'-dithiobis(N,N'-nitrobenzoic acid, then the mixture was treated again with L-cysteine to reduce any remaining free sulphydryl groups. The specific species was resolved from the recombined, cross-linked parental molecules using hydrophobic interaction HPLC with a TSK phenyl-5-PW column (Bio-Rad, Hercules, CA). As a control, the BMME linking process was repeated using 7E3 Fab' for both arms of the recombined 7E3 (Fab')2 molecule.

Estimation of antibody concentrations. Antibody concentrations (milligrams per milliliter) were assigned by dividing the absorption at 280 nm by 1.4. Molecular weights were estimated for the 7E3 IgG, OC125 Fab', OC125 Fab', and the bispecific 7E3×OC125 (Fab')2; and assigned based on the amino acid composition for the antibody molecular weight = 150,000; OC125 Fab' = 100,000; OC125 Fab' = 50,000; bispecific 7E3×OC125 (Fab')2 = 100,000; 7E3 Fab (Fab')2 = 97,600; reconstituted 7E3 (Fab')2 = 97,600.

Radioiodination of proteins. Antibodies were iodinated using a modification of the iodogen method29 to a specific activity of 1 to 3 μCi/μg. Briefly, 90 μg of antibody was incubated with 0.5 mCi of Na125I (Amersham Corp, Arlington Heights, IL) in tubes precoated with 5 μg of iodine (Pierce, Rockford, IL) for 5 minutes at room temperature. The reaction was halted by the addition of 5 mmol/L ascorbic acid and removal of the incubation mixture from the reaction vial. Free iodine was removed by gel filtration on a PD-10 column (Pharmacia). Antibodies were diluted with unlabeled antibody to achieve desired specific radioactivities.

Gel filtration chromatography. The monomeric nature of the iodinated antibody molecules and fragments was confirmed using gel filtration chromatography. The 7E3 Fab, Fab', and IgG molecules were subjected to a mock iodination using cold sodium iodide under comparable conditions used to radiolabel these same molecules. The gel filtration retention times of the cold I-labeled antibody and antibody fragments were analyzed using a Bio-Rad Bio-Sil SEC 250 column (300 × 7.8 mm) with a mobile phase of 0.2 mol/L sodium phosphate, pH 6.8 at a flow rate of 1.0 mL per minute. The retention times of the unlabeled 7E3 Fab, 7E3 Fab', and 7E3 IgG molecules were identical to those of the corresponding I-labeled molecules (retention times of 9.64, 8.36, and 7.86 minutes, respectively), indicating that none of these antibody molecules aggregated as a result of radioiodination.

Platelet preparation. Blood donors were selected from normal healthy adult volunteers who had not taken aspirin or any antimitic for a minimum of 10 days. Whole blood was drawn by venipuncture into 1:100 final volume of 40% trisodium citrate, and centrifuged at 600g for 6 minutes (or equivalent) at 22°C for preparation of platelet-rich plasma (PRP). For some experiments, platelets were isolated from the plasma by gel-filtration27 of PRP on Sephacryl 2B-CI (Pharmacia) pre-equilibrated in modified Tyrode's buffer containing 20 mmol/L HEPES, pH 7.5, 187 mmol/L NaCl, 4 mmol/L KCl, 50 mmol/L Na2HPO4, 1 mmol/L MgCl2, 5.5 mmol/L glucose, and 2% bovine serum albumin. PGE (final concentration 1 mmol/L) was added to the gel-filtered platelet suspensions and the platelets were allowed to incubate at room temperature for at least 30 minutes before the initiation of binding studies. Platelet counts of the PRP and gel-filtered platelets were determined using a resistance particle counter (Coulter ZM, Hialeah, FL). Platelet counts of the PRP were adjusted to 300,000 ± 50,000 platelets/μL with autologous platelet-poor plasma if necessary.

Direct platelet binding assay. A direct binding method1 was used for measuring the number of antibody and antibody fragment molecules able to bind to platelets in both PRP and gel-filtered platelet suspensions. PRP was incubated with 125I-labeled antibody or fragment for 30 to 60 minutes at room temperature. Duplicate (3) 100-μL aliquots of the PRP were then centrifuged through 200 μL of 30% sucrose to separate free from platelet-bound antibody. The amount of molecules of antibody bound per platelet was calculated as: the fraction of the total radioactivity that was platelet bound × the total antibody content (μg) × the appropriate conversion factor to obtain molecules per μg [Avogadro's number divided by (the antibody molecular weight × 10^12 μg/g)], divided by the number of platelets in the sample.

Rate of dissociation of radiolabeled 7E3 Fab and (Fab')2 from platelets. Studies were performed by first incubating a subsaturating amount of 125I-7E3 Fab (0.2 μg/mL) or 125I-7E3 Fab (2 μg/mL) with PRP for 30 minutes at 37°C and determining the amount of radiolabeled antibody bound. Antibody dissociation was initiated by the addition of a 100-fold excess (20 μg/mL) of unlabeled 7E3 Fab or Fab')2 to the incubations containing 125I-7E3 Fab or Fab')2, respectively. The amount of bound radioactivity was determined following separation of the platelet-bound fraction from the unbound antibody fraction by centrifugation through 30% sucrose (described above). Platelet-bound radioactive antibody was determined at various time points up to 5 hours after the addition of unlabeled antibody and expressed as a percentage of the initial amount bound.

Sequential saturation platelet binding assay. A two-step assay was developed to assess the effect of prior exposure of platelets to various antibodies on the ability of 125I-7E3 Fab to bind in a subsequent incubation step. PRP was first incubated with unlabeled antibodies at 10 μg/mL for 60 minutes at 22°C. In the second step, 125I-7E3 Fab was added to a final concentration of 15 μg/mL, and incubated for an additional 30 minutes at 22°C. Quantification of bound radiolabeled antibody was performed as described above for the direct platelet binding assay.

Binding of antibody to thrombin-stimulated platelets. The binding of 125I-7E3 IgG or 125I-7E3 Fab to gel-filtered platelets was determined after stimulation with α-thrombin (Enzyme Research Laboratories, South Bend, IN), according to the procedure of Lombardo et al.24 Aliquots of gel-filtered platelets were incubated with either 0.1 U/mL of thrombin or an equivalent volume of Tyrode's buffer for 10 minutes at 22 to 25°C. Hirudin (Sigma, St Louis, MO) was then added at a 16-fold excess (U/V) and allowed to incubate
for 5 minutes at room temperature to neutralize the thrombin. Binding studies were then performed as described above.

**Purification of platelet GPIIb/IIIa.** GPIIb/IIIa was purified from outdated human platelets (50 to 100 U) obtained from the American Red Cross (Penn-Jersey Region, Philadelphia, PA). The platelets were pelleted, washed in 20 mmol/L Tris-Cl, 150 mmol/L NaCl, 0.4% sodium citrate, pH 7.0, buffer, and pelleted again. The GPIIb/IIIa was extracted by stirring overnight at room temperature in 1% Triton X-100, 20 mmol/L Tris-Cl, 150 mmol/L NaCl, 1 mmol/L CaCl₂, pH 7.4. The platelet fragments were separated from the soluble GPIIb/IIIa by centrifugation and filtration. The filtered supernatant was applied to an affinity column containing 100 g anti-GPIIb/IIIa MoAb IgG. The bound GPIIb/IIIa was eluted with 50 mmol/L diethanolamine, 0.1% Triton X-100, 1 mmol/L CaCl₂, pH 11.5. The eluted GPIIb/IIIa was immediately neutralized with 2 mol/L Tris-Cl, 10 mmol/L CaCl₂, pH 7.0. The GPIIb/IIIa was concentrated in a stirred cell ultrafiltration device and stored with 1% sodium azide at 4°C. The GPIIb/IIIa purity and activity was assessed using nonreducing and reducing gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), high-performance liquid chromatography gel filtration analysis, immunoblot, and binding of 7E3 and 10E5 antibodies in an enzyme-linked immunosorbent assay (ELISA). The isolated GPIIb/IIIa receptor exhibited approximately 98% purity by the gel filtration analysis, with some minor contaminants observed by SDS-PAGE and immunoblot analyses.

**GPIIb/IIIa binding ELISA.** The binding of the native murine 7E3 Fab' was tested with a coated-plate ELISA. The assay compared half-maximal binding concentrations of twofold dilutions of the 7E3 and OC125 fragments (evaluated from 1 to 1,000 ng/mL) to purified GPIIb/IIIa, which was detected using a peroxidase-conjugated, murine Fab' fragment-specific, goat antibody.

**OC125 activity assay.** The 7E3 (Fab'), OC125 Fab', OC125 Fab' (Fab'2), and 7E3×OC125 (Fab') molecules were analyzed for the ability to inhibit binding of OC125 IgG (anti-ovarian cancer antigen) in a CA 125 radioimmunoassay (RIA) (Centocor). The CA 125 RIA was a simultaneous, sandwich immunometric assay that used a single antibody, OC125, as both capture and signal antibody using anti-body-coated beads and 125I-labeled antibody. Over the concentration range evaluated (0.08 to 20 μmol/L), OC125 Fab' inhibited the binding of 125I-labeled OC125. The 7E3 Fab' fragment had no inhibitory activity in the CA 125 RIA over the same concentration range. The bispecific 7E3×OC125 (Fab'), and the OC125 Fab' molecules attained complete inhibition of binding of the 125I-labeled OC125 antibody at concentrations of 1.3 and 5 μmol/L, respectively.

**RESULTS**

Comparative binding of 7E3 IgG, 7E3 Fab' (Fab'2), and 7E3 Fab. The results of an experiment in which the binding of 125I-7E3 IgG and 125I-7E3 Fab to unstimulated platelets in PRP measured after 1 hour of incubation at 22°C are shown in Fig 1. One hour exceeds the amount of time required to achieve equilibrium binding of all species of the 7E3 antibody. These results are representative of a number of similar experiments, and indicate that saturation binding was achieved for both forms of the 7E3 antibody after 1 hour at concentrations greater than or equal to 5 μg/mL. Approximately twice as many 7E3 Fab fragments were bound per platelet compared to 7E3 IgG (~80,000 vs ~40,000, respectively), which is consistent with our previous observations. Attempts to further refine the estimates of maximum binding by curve fitting, Scatchard analysis, or other linearizations of binding data did not significantly alter the estimates of maximum binding obtained by direct examination of the full binding curve data. Because saturation binding of both forms of 7E3 required a minimum concentration of 5 μg/mL, in subsequent studies antibody concentrations of ±15 μg/mL were routinely used.

Studies comparing the binding of 7E3 IgG and 7E3 Fab at a saturating concentration were performed in experiments with platelets obtained from 12 normal human donors (Fig 2). The inter-subject range of 7E3 IgG binding was 50,200 to 50,400 molecules per platelet and the range of 7E3 Fab binding was calculated.

**Fig 1.** Saturable binding of 7E3 IgG and 7E3 Fab to human platelets. Platelet-rich plasma (300,000 platelets/μL) was incubated with varying concentrations of 7E3 IgG or OC125 IgG, determined at 30 minutes at 22°C. The lines connect the Fab and IgG binding values for each individual to highlight the consistent twofold difference in the extent of binding. For this population, the mean ± SD number of 7E3 binding sites per platelet was 87,200 ± 9,600 for 7E3 Fab and 41,300 ± 4,100 for 7E3 IgG.
binding was 71,700 to 102,000 molecules per platelet. In this analysis, an average of 41,300 (±4,100) molecules of 7E3 IgG bound per platelet, and 87,200 (±9,800) 7E3 Fab molecules bound per platelet. Despite the variability in binding between individuals, the results for each patient showed a consistent near 2 to 1 relationship between Fab and IgG binding (mean ± SD, 2.1 ± 6.1; range, 1.9 to 2.3).

The comparative binding of 7E3 IgG, 7E3 F(ab')2, and 7E3 Fab to platelets from a panel of normal donors (n = 7) is presented in Fig 3. The mean ± SD binding of 7E3 Fab was 81,900 ± 9,200 molecules per platelet. The numbers of 7E3 IgG and 7E3 Fab (±2); molecules bound per platelet were similar (40,600 ± 4,100 and 43,400 ± 5,900, respectively), and both were approximately 50% of the number of 7E3 Fab molecules bound per platelet. The similarity of 7E3 IgG and 7E3 F(ab')2 binding suggests that the loss of the Fc domain of intact 7E3 IgG does not affect maximal binding, even though it reduces the molecular weight by approximately one third.

To further investigate the difference in binding of 7E3 F(ab')2 and 7E3 Fab to platelets, the rates of dissociation of each fragment were compared. Dissociation of platelet-bound, 125I-7E3 F(ab')2 and 125I-7E3 Fab was measured after addition of a 100-fold excess of the respective unlabeled antibody as shown in Fig 4. The 125I-7E3 F(ab')2 dissociated from platelets much slower than did 125I-7E3 Fab under these conditions. After 5 hours of incubation with the competing unlabeled 7E3 F(ab')2, 96% of the 125I-7E3 F(ab')2 remained bound to platelets. Only 42% of the 125I-7E3 Fab remained bound after the same interval. The significantly slower dissociation of 7E3 F(ab')2 is consistent with it binding to two different GPIIb/IIIa receptors simultaneously.

Studies to assess whether 7E3 Fab can bind to a subpopulation of GPIIb/IIIa receptors inaccessible to 7E3 IgG and 7E3 F(ab')2. To assess whether all forms of 7E3 have equal accessibility to the same population(s) of GPIIb/IIIa receptors, a two-step binding experiment was designed. If the larger, bivalent 7E3 molecules could not access certain GPIIb/IIIa receptors that the smaller 7E3 Fab fragments could engage, then radiolabeled 7E3 Fab should be able to bind to platelets pretreated with 7E3 IgG. In the first step, aliquots of PRP were preincubated for 1 hour with 10 µg/mL of 7E3 Fab, 7E3 F(ab')2, 7E3 IgG, or a non–platelet-specific control antibody, OC125 F(ab')2. In the second step, 125I-7E3 Fab (15 µg/mL) was added, and the incubation was continued for an additional 30 minutes. The rates of dissociation of 7E3 IgG and 7E3 F(ab')2 (Fig 4) from the platelet surface are slow, ensuring that antibody that bound to the platelets in the preincubation step would not dissociate significantly in the subsequent 30-minute incubation step.

The results shown in Fig 5 indicate that preincubating platelets with 7E3 IgG or 7E3 F(ab')2 reduced the subsequent binding of 7E3 Fab by 88% and 95%, respectively. Preincubation with unlabeled 7E3 Fab itself produced a similar reduction (94%) in radiolabeled 7E3 Fab binding. The control antibody, OC125 F(ab')2, did not reduce 7E3 Fab binding. If the twofold increase in 7E3 Fab binding compared with 7E3 IgG or 7E3 F(ab')2 binding were caused by differences in access to a subpopulation of GPIIb/IIIa receptors, one would have expected the 7E3 IgG and 7E3 F(ab')2 preincubation to only decrease 7E3 Fab binding by approximately 50%.

Internal pools of GPIIb/IIIa receptors. Platelets contain an internal reservoir of GPIIb/IIIa receptors that can join the outer platelet membrane surface when the platelet undergoes the release reaction and α-granule membranes fuse with the open canalicular system and ultimately the plasma membrane. A sub saturating amount of 125I-7E3 Fab (0.2 µg/mL) or 125I-7E3 F(ab')2 (0.2 µg/mL) was added to PRP and incubated for 30 minutes at 37°C in a 5% CO2 incubator. The dissociation of 125I-7E3 Fab or 125I-7E3 F(ab')2 was evaluated after addition of a 100-fold excess (20 µg/mL) of the respective unlabeled antibody fragment to the platelet suspension and removal of aliquots at the indicated time. The amount of residual bound radioactivity was determined after separation of the platelet-bound fraction from the unbound antibody by centrifugation through 30% sucrose, and expressed as a percentage of the initial amount bound.
PLATELET GP\textsubscript{IIb/IIIa} RECEPTOR QUANTIFICATION

Fig 5. Effect of prior exposure of platelets to saturating concentrations (10 \(\mu\)g/mL) of unlabelled 7E3 IgG, 7E3 Fab, or the control antibody OC125 F(ab\textsuperscript{'})\textsubscript{2} on the subsequent binding of \(^{125}\text{I}-7E3 Fab\) (15 \(\mu\)g/mL). Initial incubations were performed for 60 minutes at 22°C and the secondary incubation for 30 minutes at 22°C. After separation of platelets from the plasma, the number of platelet bound \(^{125}\text{I}-7E3 molecules were quantified. The number of \(^{125}\text{I}-7E3 Fab\) molecules is expressed as a percent of the binding that was measured in the absence of antibody in the initial incubation. The height of bars represents the average of the determinations from four normal donors and the error bars correspond to 1 SD.

brane.\textsuperscript{31} We considered the possibility that the 7E3 Fab fragment could uniquely bind to internal GP\textsubscript{IIb/IIIa} receptors under the conditions used in these binding studies, thus leading to a larger number of bound 7E3 Fab molecules per platelet. This could occur if 7E3 Fab caused externalization of these receptors or if the 7E3 Fab fragment was itself rapidly internalized and bound to internal receptors. To test the first possibility, we measured the release of the contents of both dense bodies (serotonin, adenosine triphosphate) and \(\alpha\)-granules (\(\beta\)-thromboglobulin) when platelets were treated with 7E3 Fab, and it did not produce granule release (data not shown).

If 7E3 Fab, but not 7E3 IgG, was able to gain access to the internal store of receptors on unactivated platelets under the conditions of these binding experiments, then aliquots of activated and unactivated platelets incubated with \(^{125}\text{I}-7E3 Fab\) should show little or no difference in the number of molecules of 7E3 Fab bound. In contrast, if the lower number of 7E3 IgG molecules bound per platelet were caused by an inability to access internal GP\textsubscript{IIb/IIIa} receptor stores, then the activated platelet sample would be expected to show an increased binding of the \(^{125}\text{I}-7E3 IgG\) relative to the unactivated platelet aliquot. To avoid platelet aggregation and fibrin formation by thrombin, studies were performed with gel-filtered platelets. Activation of the gel-filtered platelets with \(\alpha\)-thrombin before incubation with radiolabeled 7E3 Fab or IgG significantly increased the number of both 7E3 IgG and 7E3 Fab molecules bound per platelet (1.4-fold and 1.3-fold, respectively) relative to the unactivated samples (Table 1). Thus, these data do not support either of the hypotheses that the twofold increase in 7E3 Fab binding compared to 7E3 IgG is due to unique activation of platelets by 7E3 Fab or to unique access of 7E3 Fab to the internal pool of GP\textsubscript{IIb/IIIa} present in unactivated platelets.

Studies of the effect of molecular size and valency using the bispecific 7E3xOC125 (Fab\textsuperscript{'})\textsubscript{2} molecule. A bispecific antibody with the size of an F(ab\textsuperscript{'})\textsubscript{2} fragment but with only a single binding site for GP\textsubscript{IIb/IIIa} was synthesized. To ensure that the activity of the chemically cross-linked bispecific (Fab\textsuperscript{'})\textsubscript{2} molecule was comparable to the native 7E3 F(ab\textsuperscript{'})\textsubscript{2}, it was evaluated in a GPIIb/IIIa binding ELISA. The native 7E3 F(ab\textsuperscript{'})\textsubscript{2}, the recombined 7E3 (Fab\textsuperscript{'})\textsubscript{2}, and the bispecific 7E3xOC125 (Fab\textsuperscript{'})\textsubscript{2}, had comparable half-maximal GP\textsubscript{IIb/IIIa} binding concentrations, whereas the native OC125 F(ab\textsuperscript{'})\textsubscript{2} had no activity over the concentration range tested (data not shown). If antibody size is the major factor defining the number of 7E3 molecules bound, then the binding of the 7E3xOC125 (Fab\textsuperscript{'})\textsubscript{2} should parallel that of the 7E3 F(ab\textsuperscript{'})\textsubscript{2}. If valency is the determining factor, then the bispecific (Fab\textsuperscript{'})\textsubscript{2} molecule should bind to the same extent as 7E3 Fab, since the OC125 combining site does not recognize GP\textsubscript{IIb/IIIa}.

The binding of the bispecific 7E3xOC125 (Fab\textsuperscript{'})\textsubscript{2} antibody and a reagent, reconstituted bivalent 7E3 (Fab\textsuperscript{'})\textsubscript{2} fragment were tested against the platelets obtained from the same 7 donors used for the experiment described in Fig 3. As shown in Fig 6, the binding of the bispecific (Fab\textsuperscript{'})\textsubscript{2} (7E3,800 ± 6,300 molecules per platelet) was similar to the binding of the 7E3 Fab (81,000 ± 9,200 molecules per platelet) (Fig 3), and both were significantly greater than the binding of native 7E3 F(ab\textsuperscript{'})\textsubscript{2} (43,400 ± 5,900 molecules per platelet) (Fig 3) or the reconstituted 7E3 (Fab\textsuperscript{'})\textsubscript{2} (38,500 ± 4,200) (Fig 6). The similarity in binding properties of the native 7E3 F(ab\textsuperscript{'})\textsubscript{2} and the reconstituted 7E3 (Fab\textsuperscript{'})\textsubscript{2} indicates that

Table 1. 7E3 Binding to Unstimulated and Thrombin-Stimulated Platelets

<table>
<thead>
<tr>
<th>Molecules Bound per Platelet</th>
<th>PRP</th>
<th>Unstimulated GF Platelets</th>
<th>Stimulated GF Platelets + Thrombin</th>
<th>Ratio of No. of Molecules Bound for Stimulated Platelets/Unstimulated Platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td>7E3 Fab</td>
<td>84,300 ± 10,700</td>
<td>92,900 ± 13,100</td>
<td>126,000 ± 20,600</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>7E3 IgG</td>
<td>37,400 ± 4,500</td>
<td>42,700 ± 3,300</td>
<td>54,300 ± 3,400</td>
<td>1.3 ± 0.1</td>
</tr>
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The number of radiolabeled 7E3 IgG or 7E3 Fab molecules bound per platelet was quantified using platelets from normal donors in both PRP and after gel filtration to remove fibrinogen. The binding of \(^{125}\text{I}-7E3 IgG\) or \(^{125}\text{I}-7E3 Fab\) was also measured using gel-filtered (GF) platelets after the platelets were stimulated for 10 minutes with thrombin (0.1 U/mL). All platelet samples were then incubated with the radiiodinated forms of 7E3 for 30 minutes at room temperature. After the separation of platelets from the unbound 7E3 molecules by centrifugation through 30% sucrose, the number of platelet-bound \(^{125}\text{I}-7E3 molecules were quantified. The numbers represent the average of 5 determinations ± 1 SD.
Fig 6. Binding of radiolabeled conjugates of the recombined 7E3 (Fab')², the bispecific 7E3×OC125 (Fab')², and OC125 F(ab')² to platelets. Platelets were obtained from the same seven normal donors shown in Fig 3. PRP was prepared from citrate-anticoagulated blood, and incubated with 15 µg/mL of radiolabeled antibody for 60 minutes at 22°C. After separation of platelets from the plasma by centrifugation through 30% sucrose, the number of bound antibody molecules was calculated. The height of the bars indicate the average number of molecules of each antibody species bound per platelet and the error bars correspond to 1 SD.

The BMME linkage procedure does not significantly alter the binding properties of 7E3. The nonspecific binding of radiolabeled OC125 F(ab')² control antibody fragment was 400 ± 200 molecules per platelet. Thus, the results with the bispecific 7E3×OC125 (Fab')² fragment indicate that the valency rather than the size of the 7E3 species determines the extent of binding to platelets.

**DISCUSSION**

Investigations designed to establish the number of cellular antigens or receptors routinely rely on quantitative determinations of ligand or specific MoAb binding. Quantitative evaluation of the binding of the ligand fibrinogen to the platelet as a method of estimating the number of GPIIb/IIIa receptors on the surface of unactivated platelets has serious limitations because (1) activation stimuli may not result in all GPIIb/IIIa receptors undergoing the transition to the high affinity ligand binding state, (2) the binding of one fibrinogen molecule may result in steric hindrance that limits the binding of other fibrinogen molecules, (3) internal pools of GPIIb/IIIa may join the surface, and (4) the stoichiometry of fibrinogen binding to GPIIb/IIIa may not be 1:1.

MoAbs and fragments of MoAbs that bind to the unactivated form of GPIIb/IIIa should, in theory, provide a more direct means for assessing receptor number on unactivated platelets, but issues of steric hindrance, access to internal stores, and the stoichiometry of binding may also affect the results. The consistent observation by us⁵⁸ and others⁵⁹,⁶⁰ of a larger number of Fab or Fab' fragments binding per platelet that intact IgG molecules require a rigorous examination if one is to obtain an accurate estimate of the number of platelet surface GPIIb/IIIa, and that is why the current studies were undertaken. We exploited the high-affinity binding properties of the 7E3 MoAb to GPIIb/IIIa receptors on both unactivated and activated platelets, and the ability to make a variety of forms of the 7E3 antibody differing in size and valency. Although the rate of 7E3 F(ab')² and 7E3 Fab fragment binding to unactivated platelets is more rapid than observed for the larger IgG molecule,¹⁰ maximal platelet binding for all 7E3 molecules is obtained rapidly at concentrations of 5 µg/mL. For the present studies, binding incubations were performed for 30 to 60 minutes to ensure that equilibrium binding was attained. Previous studies established that the equilibrium dissociation constants for the interaction of 7E3 IgG, F(ab')², and Fab to resting platelets are similar at 4 nmol/L, 2 nmol/L, and 5 nmol/L, respectively (data not shown). Except for the experiments that required gel-filtered platelets, all other binding studies were conducted in PRP that was prepared from freshly obtained, citrate-anticoagulated blood from donors who had not taken antiplatelet agents.

In addition to platelet GPIIb/IIIa, 7E3 also binds to another integrin receptor, α,β₃, that also contains the GPIIb (β3) subunit. Although α,β₃ is present on platelets, the number of α,β₃ receptors is very small (~50 to 200 per platelet) representing less than 0.5% of the number of GPIIb/IIIa receptors.³¹ Thus, the potential contribution of platelet α,β₃ to total 7E3 binding was disregarded for these quantitative binding studies.

The results of this study indicate that the most plausible explanation for the twofold higher binding of 7E3 Fab compared with 7E3 IgG is that each of the two antibody-combining sites of the 7E3 IgG engages a separate and adjacent GPIIb/IIIa molecule. Several lines of evidence are consistent with this explanation. No evidence was found to support the existence of discrete surface GPIIb/IIIa receptor populations that are accessible only to the Fab fragment and not to F(ab')² or IgG. Thus, exposure of platelets to saturating concentrations of unlabeled 7E3 IgG or F(ab')² blocked the access of 7E3 Fab to the platelet surface nearly as well as did unlabeled 7E3 Fab. No evidence was obtained to suggest that the 7E3 Fab fragment produced release of α-granule contents or increased surface GPIIb/IIIa expression. Moreover, it is unlikely that selective internalization of 7E3 Fab accounts for the differences in binding because the number of molecules of 7E3 Fab that bound to platelets increased when platelets were preactivated with thrombin, to approximately the same extent as did platelets reacted with 7E3 IgG. It is also unlikely that the larger molecular size of the 7E3 IgG or F(ab')² fragment is responsible for limiting the extent of binding to one half the level of the 7E3 Fab fragment because the bispecific 7E3×OC125 (Fab')² fragment, containing only one 7E3 combining site, bound to platelets to the same extent as 7E3 Fab, ie, twice the level of 7E3 (Fab')². This result establishes that size alone is not a major determinant of the extent of binding and points, instead, to a bivalent mode of binding of IgG and F(ab')² fragments to surface GPIIb/IIIa as explaining the twofold disparity in binding. The results are most consistent with a bivalent binding of 7E3 IgG or F(ab')² to two adjacent receptors on the same platelet, because IgGs are ineffective at bridging between platelets³¹ and no evidence of agglutination was
ever observed after the addition of 7E3 IgG or 7E3 F(ab')2 to platelets. The extraordinary density of GPIIb/IIIa on the surface of platelets, with molecules estimated to be spaced approximately 160 Å apart,* adds plausibility to the hypothesis that the bivalent antibody molecules can actually engage two different receptors, even without having to postulate extensive movement of receptors in the membrane, because the two combining sites of a murine IgG, MoAbs have been estimated to be 117 to 134 Å apart.**

Several laboratories have used MoAb-based techniques to provide estimates of approximately 40,000 GPIIb/IIIa receptors per platelet. This number is widely accepted, in part, because of its concordance with the number of fibrinogen molecules reported to bind to activated platelets.** Studies with another of our antibodies, 7H2, which is directed at GPIIIa, also showed a twofold increase in binding of 7H2 Fab compared to 7H2 IgG, with approximately 80,000 Fab molecules binding per platelet. If the actual number of GPIIb/IIIa receptors is ~80,000, or twice the number of fibrinogen molecules that bind at saturation, this raises the interesting possibility that a single fibrinogen molecule may also bind to two different GPIIb/IIIa receptors on a single platelet. Each intact fibrinogen molecule contains two γ-chain dodecapeptide sequences that have been implicated in binding to GPIIb/IIIa and promoting platelet aggregation. Additionally, fibrinogen contains four arginine-glycine-aspartic acid (RGD) sequences that may contribute alternative sites for GPIIb/IIIa binding, although current data would suggest that these RGD sites are less important than the dodecapeptide sequences.** Also uncertain is the degree to which fibrinogen is structurally constrained to minimize "cis" interactions with GPIIb/IIIa receptors on the surface of the same platelet as opposed to "trans" interactions with GPIIb/IIIa receptors on different platelets. Nevertheless, the multivalent nature of the fibrinogen molecule suggests that at least bivalent binding to a single platelet is possible and may at least partly explain why ~40,000 fibrinogen molecules bind per platelet, even if there are twice as many GPIIb/IIIa receptors.

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*The surface area of a platelet is estimated to be approximately 20 square microns (equivalent to 2 x 10^6 Å²). If the surface area of the platelet is represented as a grid containing 80,000 squares each containing one GPIIb/IIIa receptor, then the area of each square would equal 2.5 x 10^6 Å². The distance between the midpoints of two adjacent squares (equivalent to the length of the side of each square) would then be estimated as 158 Å (≤ 2.5 x 10^4 Å²).

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Analysis of GPIIb/IIIa receptor number by quantification of 7E3 binding to human platelets

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