Collagen-Platelet Interactions: Evidence for a Direct Interaction of Collagen With Platelet GPIa/IIa and an Indirect Interaction With Platelet GPIIb/IIIa Mediated by Adhesive Proteins

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Using intact human platelets as the immunogen and a functional, collagen-coated bead agglutination assay, we have produced a murine monoclonal antibody (6F1) that blocks the interaction between platelets and collagen in the presence of Mg"++. 6F1 affinity-purified the platelet glycoprotein la/IIa complex, and approximately 800 molecules of 6F1 bound per platelet at saturation. 6F1 nearly completely inhibited collagen-induced platelet aggregation and inhibited platelet adhesion to collagen by >95% when plasma proteins were absent. Antibody 10E5, which blocks the binding of adhesive glycoproteins to GPIIb/IIIa, produced only minor inhibition (~25%) of adhesion under the same circumstances. In contrast, when tested in platelet-rich plasma (PRP), 6F1 had only a minor effect on collagen-induced platelet aggregation, prolonging the lag phase but not the slope or maximum aggregation. Similarly, when collagen was precoated with plasma, 6F1 caused less inhibition of platelet adhesion (53%) than without the precoating (>95%). Antibody 10E5 inhibited this adhesion by 32%, and the combination of 6F1 and 10E5 was more effective than either alone, inhibiting it by 90%. Time course studies of platelet agglutination of collagen-coated beads using PRP containing physiologic concentrations of divalent cations showed early inhibition by 6F1, indicating that the GPIa/IIa receptor operates in this environment. With more prolonged incubation, however, 6F1 was less effective; this later agglutination could be partially prevented by adding 10E5 or PGE1 to the 6F1. These data support a model wherein collagen can directly interact with GPIa/IIa and can indirectly interact with GPIb/IIIa via intermediary adhesive proteins. The physiological significance of these interactions, and potential interactions with other receptors, remains to be established.

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MATERIALS AND METHODS

Platelet Preparation

Whole blood was anticoagulated with 0.01 volume 40% Na citrate and platelet-rich plasma (PRP) was prepared by centrifugation at 700 g for 3.5 minutes at 22°C. GPF for the screening assay were prepared by layering the PRP onto a column of Sepharose 2B and eluting with a modified Tyrode's solution containing no added CaCl2 and 2 mmol/L MgCl2 (138 mmol/L NaCl, 2.7 mmol/L KCl, 0.4 mmol/L NaH2PO4, 12 mmol/L NaHCO3, 2 mmol/L MgCl2, 0.2% bovine serum albumin (BSA), 0.1% glucose, 0.01 mol/L HEPES, pH 7.4). Supernatants from the fractions used in the screening assay were found to be devoid of von Willebrand factor, the largest adhesive glycoprotein, as judged by electroimmunoassay. For studies to assess the interaction of platelets in PRP with collagen in the presence of physiologic concentrations of divalent cations, blood was anticoagulated with heparin (4 U/mL), final concentration; Sigma Chemicals, St Louis; Grade I; 166 U/mg), hirudin, (2 to 6 U/mL, final concentration; Calbiochem, San Diego; B grade), or PPACK (10 µmol/L, final concentration; Calbiochem; stock solution in 1 mM/L HCl neutralized immediately before use with NaOH), and PRP was prepared as above.

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Antibodies

Antibodies 10E5, directed against GPIIb and/or GPIIIa22 and 6D1, directed against GPIb,23 have previously been described. A control IgG antibody directed against Hageman factor, was a gift of Drs Sesha Reddigi and Michael Silverberg, State University of New York at Stony Brook. Murine monoclonal antibodies 12F1, directed at platelet glycoprotein Ia (VLA-α), and A-1A5, directed at platelet glycoprotein IIa (VLA-β)24,25 were the gifts of Dr Virgil Woods, Jr, University of California at San Diego. A monoclonal antibody from control ascites (Bethesda Research Laboratories, Rockville, MD) was purified by protein A affinity chromatography as previously described.24

Purification of Type I Rat Skin Collagen

Young female Sprague-Dawley rats (150 to 200 g) were fed a diet containing 0.2% L-aminopropionitrile for 15 days, and then killed. Their skins were removed, cut into small pieces, and incubated for 18 hours at 4°C in 1 L of 4 mmol/L EDTA, 5 mmol/L benzamidine, 10 mmol/L N-ethylmaleimide, 0.5 mmol/L β-aminopropionitrile, 0.9 mmol/L phenylmethylsulfonylfluoride. Type I collagen was then purified by a series of differential salt precipitation steps as described by Miller and Rhodes with only minor modifications.22 The purity of the final, lyophilized preparation was evaluated by electron microscopic analysis of this preparation using negative staining, indicating the absence of significant amounts of cross-linked collagen.

Other collagen preparations used in this study included a suspension of native collagen fibrils from equine tendons (Collagenagent Horm; Hormon-Chemie, Munich, GMBH) and a suspension of collagen fibrils from calf skin (Sigma Diagnostics, St Louis). The equine tendon preparation was diluted 1:9 immediately before use in aggreometry in the acidic buffer supplied by the manufacturer and maintained at 4°C throughout the experiment.

Collagen-Coated Bead Screening Assay

Our previously described assay23 was adapted to identifying clones secreting antibodies to the collagen receptor. Purified type I collagen was dissolved at 2 mg/mL in 3% HAc and dialyzed against 0.03 mol/L Na citrate, pH 6.0, three times and then the dry bead pellet was incubated with the collagen suspension for 18 hours at 4°C with rocking. The beads were then washed in 0.15 mol/L NaCl, 0.01 mol/L Tris/Cl, pH 7.4, 0.05% Na azide, resuspended to the original volume in the same buffer, and kept at 4°C.

The assay consisted of incubating 35 μL of GFP and 35 μL of culture supernatant for 30 minutes at 22°C in the wells of a microtiter plate and then adding 5 μL of the collagen-coated beads. The platelets were rotated at 220 to 270 rpm for eight minutes at 22°C and then the extent of bead agglutination was assessed macroscopically with the aid of a magnifying mirror; inhibition of the agglutination by culture supernatants provided preliminary evidence that the clone was secreting an antibody that interfered with the interaction between the platelets and collagen. This assay was also used for assessing the interaction between platelets in PRP and collagen. For these studies, PRP was prepared from blood anticoagulated with citrate, heparin, PPACK, or hirudin and 70 μL of PRP (150,000/μL) was incubated with buffer, purified 6D1 (10 μg/mL, final concentration), purified 10E5 (10 μg/mL, final concentration), or a combination of 6D1 and 10E5 (each at 10 μg/mL, final concentration), for 30 minutes at 22°C before 5 μL of beads was added. The plates were rotated at 270 rpm and observed at 30 seconds to one-minute intervals until the agglutination was completed.

Immunization and Antibody Production

A BALB/c mouse was injected with six weekly intraperitoneal (IP) injections of ~0.2 mL of buffer containing ~10^7 fresh, human platelets that had been washed two or three times in 0.15 mol/L NaCl, 0.01 mol/L Tris/Cl, 10 mmol/L EDTA, pH 7.4. The platelets in injections no. 2 and 4 were mixed 1:1 with complete Freund's adjuvant before injecting. Approximately 3.5 months after this series of injections, the mouse received a booster injection of ~10^7 platelets IP. Six weeks later the mouse received an intravenous (IV) booster injection of ~10^8 platelets in 0.15 mol/L NaCl, 0.01 mol/L Tris, pH 6.5, and the next day received ~10^7 platelets IP. Three days later, the mouse was killed and the spleen removed. Splenocytes were fused to the nonspecific myeloma cell line X63-Ag 8.653 at a ratio of ~8:1 with 40% polyethylene glycol (vol/vol) as previously described.22 Supernatants of the wells showing growth were tested in the screening assay; two remained consistently positive (6F1 and 8D3) and 6F1 was selected for further study. The cells were subcloned twice by limiting dilution to insure monoclonality,23 and the clone was expanded. Culture supernatants were precipitated with 50% ammonium sulfate, and the antibody purified by protein A affinity chromatography as previously described.22 Antibody was also purified from the ascites fluid of mice injected with 6F1 as previously described.22 F(ab')2 fragments of 6F1 were prepared by 2% (wt/wt) ethanolamine hydrochloride, 0.1% SDS, 0.05% 2-mercaptoethanol, 0.1% N-hydroxysuccinimidegroups were washed in 0.01 mol/L Na citrate, heparin, PPACK, or hirudin and 70 μL of 6F1 was incubated with buffer, purified F(ab')2 fragments of 6F1 were prepared by 2% (wt/wt) ethanolamine hydrochloride, 0.1% SDS, 0.05% 2-mercaptoethanol, 0.1% N-hydroxysuccinimide, detergent. Ouchterlony immunoprecipitation analysis demonstrated 6F1 to be of the IgG1 subclass.

Affinity Purification

Purified 6F1 (1.5 mL; 1.3 mg/mL) was dialyzed against 0.1 mol/L HEPES, pH 7.5, and coupled to 1 mL of Affi-gel 10 (Bio Rad), which had previously been briefly washed with H_2O at 4°C. After rocking overnight at 4°C, the gel was blocked with 1 mol/L ethanolamine, pH 8.2, at 4°C for one hour and then washed with the elution buffers (I = 0.15 mol/L NaCl, 0.01 mol/L Tris/Cl, 1 mol/L phenylmethylsulfonyl fluoride (PMSF), 5 mmol/L N-ethylmaleimide (NEM), 0.05% sodium azide, 0.1% Triton X-100, pH 7.4; II = same as I except 1.0 mol/L NaCl instead of 0.15 mol/L NaCl; III = 0.05 mol/L diethylamine, 5 mmol/L NEM, 0.05% sodium azide, pH 11.5). Platelets obtained from 50 mL whole blood anticoagulated with 10 mmol/L EDTA were washed twice in 0.15 mol/L NaCl, 0.01 mol/L Tris/Cl, 10 mmol/L EDTA, 1 mmol/L PMSF, pH 7.4, and resuspended to 1 mL in the same buffer. The platelets and 2.6 mCi[^1] were then added to a glass vial containing 250 μg of dried iodogen (Pierce Chemical Corp, Rockford, IL; 50 μL of 5 mg/mL iodogen in dichloromethane). After ten minutes at 22°C the platelets were washed five times in the above buffer, and then solubilized in 2 mL of 0.15 mol/L NaCl, 0.01 mol/L Tris/Cl, 1 mol/L EDTA, 1 mmol/L PMSF, pH 0.05% azide, 1% Triton X-100, pH 7.4, at 4°C for 30 minutes. After solubilization, the solution was centrifuged at 12,000 g for 30 minutes at 22°C. The immobilized 6F1 was then incubated with 150 μL of the solubilized, iodinated platelets overnight with rocking at 4°C, after which the gel was packed into a column. The column was then eluted at 12 mL/h at
4°C with 30 bed volumes of buffer I, 30 volumes of buffer II, and 30 volumes of buffer III. The eluted fractions (0.5 mL) were monitored for radioactivity and the peak fractions were analyzed by SDS-PAGE using both staining with silver reagent and radioautography.  

**Flow Cytometric Analysis**

Whole blood was anticoagulated with 10 mmol/L EDTA and PRP (3.9 x 10^8 platelets/mL) was prepared. Aliquots (0.5 to 0.6 mL) of the PRP were incubated for 30 minutes at 22°C with buffer (0.15 mol/L NaCl, 0.01 mol/L Tris/Cl, pH 7.4), specific antibodies 10E5, 6D1, or 6F1, or the control monoclonal antibody purified from ascites such that the final concentration for all the antibodies was 10 μg/mL. The platelets were then washed three times in the above buffer containing 10 mmol/L EDTA and resuspended to 0.2 mL. A 100 μL sample was then incubated with affinity-purified, FITC-labeled goat anti-mouse F(ab')2, (Tago, Burlingame, CA) such that the final dilution of the antibody was 1/9. After 30 minutes at 22°C, the platelets were washed twice in the above buffer and analyzed. In the study comparing the fluorescence intensity of antibodies 6F1, 12F1, and A-1A5, 0.1 mL of platelets washed once was incubated with the antibodies at final concentrations of 26 to 30 μg/mL.

The cyometric analysis was performed on a FACStar (Becton-Dickinson, Rutherford, NJ) using a 70 μm aperture and an Argon laser at 225 mW (Innova 70; Coherent Laser Products, Palo Alto, CA). Data analysis was performed with the Consort 30 program supplied by the manufacturer and each analysis consisted of 10^4 particles. The platelet peak from the control antibody sample was first analyzed by forward angle light scatter using gain 8; the gates were set to include ~90% of the particles in the monotonous platelet peak and to exclude the larger particles that were thought to represent platelet doublets or contaminating erythrocytes. The fluorescence of this control sample was then analyzed on a log scale with the 256 channels represented on the abscissa encompassing 4 logs (1 log per 64 channels). In the experiment analyzing antibodies 10E5, 6D1, and 6F1, 95% of the platelets reacted with the control antibody had fluorescence intensities less than channel 38, and so a platelet was considered positive if its fluorescence intensity was greater than 38 on this scale.

**Mg²⁺-Dependent Collagen-Coated Plate Adhesion Assay**

The assays previously described by Ill et al²⁸ and Santoro²⁹ for adhesion of platelets to monomeric collagen were modified as follows: (a) purified type I rat skin collagen (2 mg/mL in 3% HAC) was diluted with distilled water to reach a final concentration of 33 μg/mL in 0.05% HAC, (b) 0.1 mL of the collagen solution was used to coat the wells of a polystyrene microtiter plate that had not been pretreated for tissue culture use (Falcon 3915, Becton-Dickinson) for one to two hours at 22°C or 37°C, (c) the wells were aspirated and blocked with 0.1 mL of a 0.5% BSA solution for one hour at 22°C, and then washed three times with 0.5% BSA, 0.15 mol/L NaCl, 0.05 mol/L Tris/Cl, 5 mM mol/L glucose, pH 7.4, (d) 3Cr-labeled gel-filtered platelets were prepared from whole blood anticoagulated with ACD-A (8.5 mL blood:1.5 mL ACD-A) by preparing PRP, concentrating the platelets by adding 0.1 vol of ACD-A and centrifuging for ten minutes at 2,000 g at 22°C, resuspending the platelets in 1/10 the original volume in wash buffer without BSA, incubating with 0.5 mCi Na₃¹⁵CrO₄/mL platelet suspension (ICN Biomedicals Inc, Cleveland) at 22°C or 37°C for 30 to 60 minutes, gel-filtering over Sepharose 2B (Pharmacia, Piscataway, NJ) in the wash buffer indicated above, and adjusting the platelet count to 150,000 to 400,000 μL, (e) platelets were first incubated at 22°C for one hour with buffer, antibody (at the indicated concentrations) or the peptide RGDS (40 μg/mL), (f) 100 μL of the platelet suspension was adjusted to 2 mmol/L MgCl₂ and added to each well, (g) after incubation at 22°C for 30 to 60 minutes, the plates were washed three times with the above buffer, (h) the adherent platelets were solubilized with 100 μL 2% SDS for 30 minutes, and (i) the radioactivity was quantified in a gamma spectrometer. Control wells were prepared by coating either with 0.5% BSA solution or the same collagen solution denatured by heating to 100°C for 20 minutes (gelatin). In some experiments, wells were coated with 10 μg/mL purified fibronectin (Collaborative Research, Bedford, MA) in 0.15 mol/L NaCl, 0.1 mol/L phosphate, 0.05% azide, pH 7.4, for 1.25 hours. In other experiments designed to assess the effects of plasma proteins, collagen-coated wells were blocked with plasma instead of 0.5% albumin.

**Other Techniques**

Platelet aggregation was performed as previously described with PRP or GFP.³² Purified human thrombin for aggregation studies was a kind gift of Dr John Fenton, State of New York Laboratories, Albany. SDS-PAGE was performed as described by Laemmli.²⁶ Radio labeling of 6F1 with 125I using the iodogen technique (~1,500 to 4,000 cpm/μg) and the binding of 125I-6F1 to platelets³³ were performed as described. The number of molecules bound was determined from the net radioactivity in the platelet pellet and the specific activity of the antibody. In the presence of excess unlabeled antibody, the binding of trace amounts of radiolabeled 6F1 was reduced by more than 98%. This indicated that nonspecific binding of 6F1 is minimal, and so it was not considered in the calculations. Results were displayed graphically according to the method of Scatchard²² using the technique described by Trucco and de Petris.³⁳ Radioimmunoelectrophoresis was performed as previously described with the arcs developed with a combination of rabbit antisera to whole, human platelets prepared in our laboratory and 125I-6F1.³⁴ Von Willebrand factor antigen was assayed by electroimmunoassay.³⁵

**RESULTS**

**Characterization of Screening Assay and Identification of Positive Clones**

GFP in the 2 mmol/L Mg²⁺ buffer agglutinated the collagen-coated beads and this agglutination was abolished by EDTA, indicating a requirement for divalent cations. Antibody 10E5, directed at the GPIIb/IIIa receptor, had virtually no effect on the final agglutination, even though it completely inhibits the agglutination of fibrinogen-coated beads by platelets.²² Thus, platelet aggregation, which is inhibited by 10E5, is not required for full agglutination of the collagen-coated beads. The peptide RGDS, even at 1 mg/mL, had only a modest effect. The supernatants from clones 6F1 and 8D3 completely inhibited the agglutination and 6F1 was selected for further study. Purified 6F1 was positive in the screening assay at final concentrations ≥ 0.75 to 3.0 μg/mL; the F(ab')2 fragment of 6F1 was similarly active, indicating that the antibody combining site was responsible for the inhibition.

In accord with the data obtained by Shadle and Barondes³⁵ and Santoro,³⁹ agglutination was much less apparent or entirely absent when Ca²⁺ was substituted for Mg²⁺, and the agglutination in the presence of the Mg²⁺ was inhibited by adding increasing concentrations of Ca²⁺. Agglutination still occurred when the MgCl₂ concentration was 1 mmol/L and the CaCl₂ concentration was 2 mmol/L, simulating the
physiologic concentrations of these divalent cations. The agglutination did, however, take somewhat longer to occur, with nearly full agglutination taking ~4.5 minutes compared with ~2 minutes for GFP in the presence of 2 mmol/L MgCl₂ alone. 6F1 also inhibited the agglutination in the presence of 1 mmol/L MgCl₂/2 mmol/L CaCl₂, but it was not as complete as with the 2 mmol/L Mg⁺⁺ buffer.

**Affinity Purification**

Figure 1 shows the radioautography results of the affinity purification procedure in which iodinated solubilized platelet proteins were reacted with immobilized 6F1 and then the bound proteins were eluted. The solubilized platelet preparation (PIts) shows the typical pattern of iodinated platelet proteins when analyzed nonreduced and reduced. The majority of radioactivity eluted with buffer I, and only a small peak eluted with the high salt buffer (II). A sharp radioactivity peak eluted immediately after beginning the high pH buffer (III) and this eluate (E) is shown in Fig 1. Two proteins were contained in the eluate; their migrations in the gels indicated that one was mol wt ~154 Kd nonreduced and ~166 Kd reduced, and the other was mol wt ~128 Kd nonreduced and ~150 Kd reduced. These same two proteins were specifically precipitated by 6F1 from radiolabeled platelets and shown to differ from GPIIb, GPIIb, and GPIIa, which were immunoprecipitated in the same experiment with antibodies 6D1 and 10E5 (data not shown).

The iodination patterns, molecular weights, and characteristic changes with reduction identify these bands as being GPIa and GPIIa. Whereas previous data indicate that GPIa and GPIIa exist as a noncovalent complex in the platelet membrane, an antibody directed against either component might precipitate both proteins. GPIIa, however, has also been shown to complex with two different proteins that migrate in the region occupied by what was previously identified as GPIIc. and so an antibody directed at GPIIa might also precipitate these proteins. On nonreduced gels, the two proteins in the GPIc region are mol wt ~150 Kd, which is slightly lower than the mol wt of GPIa. On reduction the heavy chains of these proteins migrate as mol wt 134 Kd. No proteins with this pattern could be identified.

**Radioimmunoelectrophoresis**

When Triton X-100–solubilized platelet proteins were first separated by electrophoresis and then reacted with a combination of a rabbit anti-whole platelet serum and 125I-6F1, a single major radioactive precipitin arc was observed. When compared with the arcs produced by antibodies 10E5 and 6D1, the 6F1 arc was found to be slightly less anodal than the 10E5 arc and considerably less anodal than the 6D1 arc. The mobility of the 6F1 arc is consistent with that previously identified for the GPIa/IIa arc in crossed immunoelectrophoresis.

**125I-6F1 Binding Assay**

125I-6F1 bound to platelets in both citrated PRP and GFP in a specific and saturable manner. Maximal binding with near-saturating doses of 6F1 was achieved after 30 minutes at 22°C. Both intact 6F1 and 6F1-F(ab')₂ inhibited the binding of 125I-6F1 by >90% when added in excess. In contrast, excess 10E5 inhibited 6F1 binding to platelets in PRP by only 2% and 6D1 inhibited binding by only 23% when tested on two separate occasions. Antibody 8D3, which was also positive in the collagen-bead screening assay, inhibited 125I-6F1 binding by 71%, suggesting that it binds to a site near to the 6F1 epitope. In three binding experiments analyzed according to the method of Scatchard, the antibody’s mean dissociation constant (kd) was ~0.52 nmol/L. The maximum number of antibody molecules that could bind per platelet was estimated by the binding of near-saturating concentrations of 6F1 (39 to 134 nmol/L) to PRP and GFP. Similar results were obtained with both platelet preparations (809 ± 302 [mean ± SD] for PRP [n = 10] and 764 ± 264 for GFP [n = 5]).

**Flow Cytometry**

The fluorescence intensity produced by reacting fresh platelets first with near-saturating concentrations of the different monoclonal antibodies and then with FITC-labeled goat anti-mouse IgG F(ab')₂ is shown in Fig 2A. More than
83% of the particles in the platelet peak gave a positive reaction with 6F1. Since only 86% of the particles were positive with 6D1 (anti-GPIb) and 82% were positive with 10E5 (anti-GP Ib and/or IIa), it is likely that the unreactive 14% to 18% of the particles were not platelets. This was supported by fluorescence microscopy showing that all identifiable platelets adherent to a glass slide were positive with 10E5 and that some particles the size of platelets, but which appeared to be nonadherent erythrocyte fragments and debris, did not fluoresce. Our data are, however, inadequate to completely exclude the possibility that there is a small subpopulation of unreactive platelets. The fluorescence intensity for 6F1 (mean channel, 72) was considerably less than that for 6D1 (mean, 106) and 10E5 (mean, 131), which is consistent with the greater density of receptors reactive with these antibodies (~25,000 GPIb, and ~45,000 GPIb/IIa molecules per platelet). The slight tailing of fluorescence intensity with 6F1 at the highest intensity values may represent either minimal platelet agglutination (doublets) of a small percentage of the platelets, or perhaps a subpopulation of platelets with a higher density of 6F1 receptors.

Whereas the maximum number of 6F1 molecules that could bind to platelets (~800) was less than the ~1,800 molecules per platelet reported by Pischel et al for antibody...
12F1, which is directed at GPIa and reacts with the GPIa/IIa complex, we used flow cytometry in an attempt to ascertain whether the discrepancy was due to 6F1 recognizing only a subpopulation of GPIa/IIa receptors. Platelets were incubated with a control antibody, 6F1, 12F1, or A-1A5. The last antibody, which is directed against GPIIa and thus presumably reacts with GPIa/IIa and the two complexes migrating in the GPIc/IIa region, has a maximal binding of ~4,900 molecules per platelet. As seen in Fig 2B, 6F1 and 12F1 gave virtually identical patterns, whereas A-1A5 gave a stronger fluorescence signal.

Further Assessment of the Effects of 6F1 on Platelet-Collagen Interactions in the Absence of Plasma

Platelet aggregation. Antibody 6F1 at 10 μg/mL did not inhibit platelet aggregation of GFP induced by ADP (5 and 11 μmol/L), thrombin (0.12 and 0.25 U/mL), arachidonic acid (1.8 mmol/L), or calcium ionophore A23187 (4.6 μmol/L). Similarly, no inhibition of aggregation was noted in citrated PRP when the agonists were ADP (5 μmol/L), ristocetin (1.2 mg/mL), arachidonic acid (1.9 mmol/L), platelet activating factor (20 μmol/L), or A23187 (6.9 μmol/L). In sharp contrast, antibody 6F1 and 6F1-F(ab')2 fragments dramatically inhibited collagen-induced aggregation of gel-filtered platelets in a dose-dependent manner that correlated with the percentage of 6F1 molecules bound per platelet (Fig 3A). Not only was the lag phase, total extent of aggregation, and the slope of aggregation inhibited, but so also was the platelet shape change (indicated by the initial increase in optical density and the thinning of the tracing), which presumably reflects the initial reaction that follows adhesion of platelets to the collagen. Despite this profound inhibition, if stirring was continued for eight to ten minutes, some shape change and aggregation occurred. Moreover, the inhibitory effect of 6F1 could be overcome by increasing the concentration of collagen used in the assay.

![Figure 3](http://www.bloodjournal.org) Effect of antibody 6F1 on platelet aggregation using GFP and PRP. (A) GFP in the modified Tyrode's buffer containing 2 mmol/L Mg++ (0.4 mL; ~3.2 x 10^6 platelets/μL) were incubated with 6F1 (17 μL) at the indicated final concentrations for 30 minutes at 22°C. Aggregation was initiated with 6 μL of equine tendon collagen suspension (0.1 mg/mL). The depicted experiment is typical of more than 20 other experiments. The binding of 6F1-6F1 for 30 minutes at 22°C was simultaneously performed on other aliquots of platelets using the same final concentrations of 6F1 as were used for the aggregation study. The percentage of maximum 6F1 binding, which was 870 molecules of 6F1 per platelet in this experiment, are also indicated. (B) Citrated PRP (3.2 x 10^6 platelets/μL; 0.4 mL) was incubated with buffer or 6F1 (100 μL; 85 μg/mL, final concentration) at 22°C for ~35 minutes and then aggregation was initiated with 30 μL of calf skin collagen (2 mg/mL; 113 μg/mL, final concentration). The lag times, that is the time from adding the collagen until the aggregation curve descends below the baseline, are indicated.
Mg$^{2+}$-dependent collagen-coated plate adhesion assay. Control $^{51}$Cr-labeled platelets in the presence of 2 mmol/L Mg$^{2+}$ adhered to the monomeric, type I collagen-coated microtiter wells, forming a dense monolayer that could be readily observed with the aid of an inverted microscope. In contrast, platelets pretreated with 6F1 (20 μg/mL) adhered very poorly to the collagen-coated wells, with results comparable with that seen with platelets adhering to the control, albumin-coated wells. These data were quantified by solubilizing the adherent platelets in SDS and counting the released radioactivity (Table 1). The density of platelets adhering under control conditions (-6.5 x 10⁸/mm²) is similar to that indicated by Santoro’s data (~7.8 x 10⁸/mm²).10 6F1 treatment reduced platelet binding to collagen by ~96%, reducing it to the level observed with control platelets binding to albumin-coated wells and wells coated with denatured collagen (gelatin). In dose-response studies, 6F1 inhibited adhesion by 50% at ~0.1 μg/mL. 6F1 F(ab')₂ fragments were as effective as intact 6F1, thus excluding an effect by the Fc region of the antibody. The specificity of the effect of 6F1 was established by studies showing that 10E5 inhibited adhesion by 50% at ~0.1 μg/mL. 6F1 treatment reduced platelet binding to collagen by ~96%, reducing it to the level observed with control platelets binding to albumin-coated wells and wells coated with denatured collagen (gelatin). In dose-response studies, 6F1 inhibited adhesion by 50% at ~0.1 μg/mL. 6F1 F(ab')₂ fragments were as effective as intact 6F1, thus excluding an effect by the Fc region of the antibody. The specificity of the effect of 6F1 was established by studies showing that 10E5 (3 to 20 μg/mL) reduced the binding to collagen by only a modest amount (~25%), and that 6D1 actually increased the adhesion by a small amount (~17% in the experiment in Table 1 and 20% ± 12% in a total of four experiments). The peptide RGDS (40 μg/mL) reduced the adhesion by ~44% in the experiment depicted and by 34% in another experiment. This last observation appears to be consistent with the partial effect of RGDS observed in the screening assay and the ~20% decrease in adhesion to collagen-coated wells observed by Santoro.10

Effect of 6F1 on Platelet-Collagen Interactions in the Presence of Plasma

Collagen-coated bead agglutination assay. PRPs obtained from blood anticoagulated with heparin, PPACK, and hirudin, designed to maintain near-physiological concentrations of divalent cations, all fully agglutinated the collagen-coated beads within one to three minutes and 6F1 caused complete or nearly complete inhibition of this agglutination (Fig 4). 10E5, in contrast inhibited agglutination only at the earliest time points. If the plates were mixed for a longer period of time, however, the 6F1-treated samples began to agglutinate. This delayed agglutination in the presence of 6F1 could be prevented by treating the platelets with PGE1, suggesting that it was due to platelet activation and/or release. Moreover, the delayed agglutination was partially inhibited by the presence of both 6F1 and 10E5, suggesting that it was mediated, at least in part, by GPIIb/IIIa. Ultimately, however, even the combination of 6F1 and 10E5 failed to completely inhibit the agglutination, indicating that other receptors may also mediate the interactions. The agglutination caused by citrated PRP followed the same pattern as that described above, but agglutination was usually weaker and more delayed, presumably as a result of the chelation of Mg$^{2+}$.

Platelet aggregation. 6F1 had much less effect on inhibiting collagen induced platelet aggregation of citrated PRP than it did on GFP (Fig 3B). The only significant difference was a small increase in the lag phase and this was consistently observed. The inhibition by 6F1 could be slightly enhanced by reducing the collagen concentration, but it

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Fig 4. Time course of agglutination of collagen-coated beads by heparinized PRP. PRP (1.6 x 10⁸ platelets/μL; 70μL) obtained from whole blood anticoagulated with 4 U/mL heparin, was pretreated with buffer (cont), antibody 6FI (10 μg/mL), or antibody 10E5 (10 μg/mL) and then added to microtiter plate wells. The experiment was begun by adding 5 μL of the collagen-coated beads and rotating the plate at 270 rpm for the indicated times. The results depicted are from one of five similar experiments.

Table 1. Effect of Monoclonal Antibodies and an RGD Peptide on Platelet Adhesion to Immobilized Collagen, Gelatin, and Albumin

<table>
<thead>
<tr>
<th>Coating Protein</th>
<th>Albumin (μd)</th>
<th>Gelatin (μd)</th>
<th>Collagen (μd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer control</td>
<td>8,900*</td>
<td>10,600</td>
<td>62,700</td>
</tr>
<tr>
<td>Antibody 6F1</td>
<td>10,600</td>
<td>12,400</td>
<td>1,800</td>
</tr>
<tr>
<td>Antibody 10E5</td>
<td>18,500</td>
<td>15,200</td>
<td>56,100</td>
</tr>
<tr>
<td>Antibody 6D1</td>
<td>10,600</td>
<td>4,500</td>
<td>70,300</td>
</tr>
<tr>
<td>RGDS</td>
<td>11,200</td>
<td>900</td>
<td>35,200</td>
</tr>
</tbody>
</table>

Polystyrene microtiter wells were precoated with 0.1 mL of purified type I collagen (33 μg/mL), albumin (0.5%), or gelatin (33 μg/mL). After blocking with an albumin-containing solution and washing, 0.1 mL of $^{51}$Cr-labeled platelets (~4 x 10⁶/mL) that were pretreated with buffer, antibodies (20 μg/mL), or RGDS peptide (40 μg/mL) were added and allowed to adhere for 30 minutes at 22°C. The plates were then washed, the adherent platelets solubilized in SDS, and the radioactivity determined.

*Adherent platelets/mm²; mean of duplicate values from one of five similar experiments.
never approached that observed in the gel-filtered platelet system. Similar results were obtained with heparinized PRP, hirudin-PRP, and PPACK-PRP.

The inhibitory effect of 6F1 on collagen-induced platelet aggregation of GFP could be diminished significantly by adding back as little as 0.1 mL of citrated plasma to 0.3 mL. GFP (data not shown). Similar results were obtained with heparinized PRP, hirudin-PRP, and PPACK-PRP.

Platelet adhesion assay. The effect of plasma on the adhesion of GFP to collagen-coated microtiter plates was tested by substituting citrated plasma for the bovine serum albumin as the blocking agent added immediately after coating the plates with collagen. The unadsorbed plasma was then washed away so that the platelet adhesion step could be performed in the absence of free plasma, thus avoiding platelet aggregate formation. As demonstrated in Table 2, blocking with plasma slightly increased the number of platelets bound to the collagen-coated plate. It had a profound effect, however, on the ability of 6F1 to inhibit the adhesion, decreasing it from 96% to 53% ($P < .001$). Even at PPP dilutions of 1/100, blocking with PPP decreased the inhibition produced by 6F1. The effect of PPP did not appear to depend on the divalent cation concentration of the PPP since EDTA-PPP and hirudin-chemed PPP were also effective in reducing the inhibitory effect of 6F1. The inhibition produced by 10E5 alone increased from 25% to 32% but this difference was not statistically significant. The combination of 6F1 and 10E5 was much more effective than either antibody alone, achieving 90% inhibition. This augmented inhibition, however, was still significantly less than that produced by 6F1 in the absence of plasma ($P < .05$), raising the possibility that other receptors may be involved.

**DISCUSSION**

These studies indicate that a monoclonal antibody (6F1) prepared against intact platelets, which recognizes an epitope on the GPIa/IIa receptor, can dramatically inhibit the interaction between platelets and type I collagen when tested in the presence of 2 mmol/L Mg$^{2+}$ and the absence of plasma proteins. Similar results were reported by Kunicki et al$^{18}$ while these studies were in progress, but the monoclonal antibody they used was prepared by Wayner and Carter to the human fibroblast class II extracellular matrix receptor.$^{41}$ Moreover, Santoro et al have provided convincing evidence that the GPIa/IIa receptor can mediate the interaction between platelets and collagen in the presence of Mg$^{2+}$ and the absence of plasma proteins.$^{19,21}$ These previous studies indicated that the GPIa/IIa platelet receptor is either identical to or very similar to the VLA-2 antigen found on a variety of cells$^{22,24,25,40,42}$ and our flow cytometry experiments comparing 6F1 and the VLA-2-specific monoclonal antibody 12F1 provide additional support for this observation.

Whereas the studies conducted by Santoro et al$^{10,31}$ and Kunicki et al$^{18}$ used GFP in which plasma proteins were excluded, we designed the present studies to assess the functional role of the GPIa/IIa receptor in a plasma environment. To our surprise, 6F1 had only a modest effect on platelet aggregation using citrated PRP, producing a small but consistent increase in the lag phase. This was not a result of the altered divalent cation concentration produced by the citrate since similar results were obtained with PRP prepared from blood anticoagulated with heparin, PPACK, and hirudin. We excluded the possibility that plasma interferes with 6F1 binding by showing equivalent binding of $^{125}$I-6F1 to gel-filtered platelets and platelets in PRP. Moreover, adhesion studies in which plasma proteins were allowed to adsorb to immobilized collagen before the antibody-treated platelets were added, confirmed the reduced ability of 6F1 to inhibit adhesion under these circumstances. These data suggested that one or more non-GPIa/IIa mechanism can mediate platelet-collagen interactions in the presence of plasma.

To investigate these non-GPIa/IIa mechanism(s) we used our monoclonal antibody (10E5) that blocks the interaction of platelet GPIIIa/IIIa with exogenous fibrinogen, von Willebrand factor, fibronectin, and thrombospondin.$^{22,42}$ Using GFP and 2 mmol/L Mg$^{2+}$, 10E5 had virtually no effect on the collagen-coated bead agglutination assay, and had only a minor inhibitory effect on the adhesion assay. When plasma proteins were allowed to adsorb to the immobilized collagen in the adhesion assay, 6F1 alone lost nearly half of its inhibitory ability, 10E5 alone increased somewhat in its inhibitory ability, but the combination of 6F1 and 10E5 produced more dramatic inhibition than either alone. This suggested that platelets were adhering via both receptors. We would emphasize, however, that even the combination of 6F1 and 10E5 failed to produce complete inhibition, raising the possibility that other receptors are also involved.

To further explore the roles of these two receptors in mediating platelet-collagen interactions, we performed time-course experiments with our collagen bead assay, using PRP prepared from blood anticoagulated with agents that do not alter the divalent cation concentrations. We observed that agglutination occurred rapidly, and at the early time points

| Table 2. Effect of Blocking Agents on the Inhibition of Platelet Adhesion to Collagen by Monoclonal Antibodies 6F1 and 10E5 |
|---|---|---|---|
| | | Adherent Platelets/mm² | Percent Inhibition |
| | | | | Adherent Platelets/mm² | Percent Inhibition |
| **Blocking Agent** | | | | | |
| | | Buffer control | 67,500 ± 11,300* | 6F1† | 2,900 ± 800 | 10E5† | 50,400 ± 6,600 | 6F1† and 10E5† | 2,700 ± 300 |
| | | 71,300 ± 5,100 | 33,800 ± 3,500 | 48,300 ± 6,600 | 7,500 ± 1,400 |
| | | 96 | 25 | 96 |
| | | 3,500 | 53 | 32 | 90 |
| *Mean ± SEM; n = 6. |
| †10 μg/mL. |
6F1 produced nearly complete inhibition, indicating that the GPIa/IIa receptor can mediate platelet-collagen interactions in a plasma milieu at physiological divalent cation concentrations. In contrast, 10E5 had only a modest effect on this early phase. With more prolonged mixing, however, agglutination occurred despite the presence of 6F1. At these later time points, 10E5 alone produced no significant inhibition, but the combination of 6F1 and 10E5 produced greater, but incomplete inhibition, just as in the adhesion assay. Moreover, when the platelets were pretreated with PGE1, 6F1 alone produced marked inhibition even at the later time points, indicating that the GPIIb/IIa-dependent mechanism required platelet activation and/or release.

One possible model that might be drawn from these studies is that the initial interaction between platelets and collagen in the presence of plasma proteins is mediated primarily via GPIa/IIa, with subsequent activation of the GPIIb/IIa receptor, and further platelet-collagen interactions mediated through both of these receptors. This model can account for the slight prolongation of the lag phase of collagen-induced platelet aggregation caused by 6F1, if the fibrillar collagen, by virtue of its multimeric reactive structure, can achieve an affinity for the GPIa/IIa receptor that exceeds that of antibody 6F1. Then, even a small number of occupied receptors may be able to trigger the exposure of GPIIb/IIa, which in turn can lead to both increased platelet-collagen adhesion and aggregation mediated via fibrinogen binding. An alternative explanation is that GPIIb/IIa is capable of a low level of adherence to collagen in the presence of plasma even without platelet activation and that such interaction is itself capable of initiating platelet aggregation mediated by GPIIb/IIa.

It is likely that the augmented role for GPIIb/IIa-mediated adhesion in the presence of plasma results from the adsorption onto the insoluble collagen matrix of adhesive glycoproteins that are known to bind to GPIIb/IIa, fibronectin3, and von Willebrand factor,4 have been shown to bind to both collagen and GPIIb/IIa; thrombospondin binds to collagen6 and may bind to GPIIb/IIa,4 and fibrinogen binds to GPIIb/IIa2 and may bind to collagen.47 Moreover, these adhesive glycoproteins can bind to each other (for example, fibrinogen and fibronectin can bind to thrombospondin)48 making it possible for indirect immobilization of these proteins. Thus, the relative roles of the GPIa/IIa complex and GPIIb/IIa in mediating platelet-collagen adhesion is likely to be determined by the extent of adhesive glycoprotein adsorption, the specific molecular architecture of the adsorbed proteins, and the state of activation of the GPIIb/IIa receptor.

Others have previously identified a potential role for GPIIb and/or GPIIa in platelet adhesion to collagen.44 The most directly relevant studies are those of Shadle et al49 who reported that antibody PM1-1, which is directed at an epitope on GPIIb that is exposed either by chelation of divalent cations or ligand occupancy of GPIIb/IIa,49,50 and which presumably interferes with GPIIb/IIa-mediated phenomena, could inhibit by ~80% the adhesion of GFP to immobilized collagen. They noted, however, that purified GPIIb/IIa could not completely neutralize the inhibitory effect of their polyclonal antibody to platelets, suggesting that an additional receptor(s) could also mediate the adhesion, a conclusion that was also in accord with their affinity chromatography data.51 One possible explanation for the discrepancy between their results and ours is that in the assay used by Shadle and Barondes52 the adherent platelets undergo release of ~50% of their dense granule contents (and presumably a like amount of α granule contents), whereas in Santoro’s assay,53 which formed the basis of the assay used for these studies, release is only ~5%. The release of α granule adhesive glycoproteins and the activation of the GPIIb/IIa receptor during the assay, are both likely to favor the GPIIb/IIa-mediated mechanism.

The complexity of these in vitro data makes it very difficult to predict the likely consequences of isolated GPIa/IIa deficiency in vivo. Two female patients with hemorrhagic diatheses have been reported in association with GPIa deficiency and isolated defects in platelet aggregation induced by collagen, but a simple picture does not emerge from analyzing their abnormalities. The first suffered from easy bruising, menorrhagia, and posttraumatic hemorrhage, but did not bleed excessively after an appendectomy.7,21 Her bleeding time was abnormally prolonged and her platelets in citrated PRP failed to aggregate to a variety of collagens, even at extremely high doses. In addition, her platelets failed to adhere to collagen fibers when tested in the presence of EDTA, suggesting impairment of both cation-dependent and cation-independent mechanisms. Her platelets in citrated PRP also had markedly reduced adherence to immobilized type III collagen in an annular perfusion chamber. A similar defect was found in adhesion to the subendothelial surface of human umbilical arteries. The patient’s platelets demonstrated an ~75% to 85% decrease in surface GPIa and perhaps a partial reduction in GPIIa.7 In preliminary studies of this patient’s platelets, we were able to show that her platelets bound only ~16% of the normal amount of 6F1,52 indicating the identity of the patient’s missing glycoprotein(s) and the glycoprotein(s) identified by 6F1. The second patient had a long history of severe menorrhagia, petechiae, and postoperative hemorrhage.20 Her platelets contained no identifiable GPIa or intact thrombospondin; degraded forms of thrombospondin were detected immunologically. Her platelets in citrated PRP showed an isolated, partial defect in response to collagen, which could be overcome by increasing the collagen concentration by ten-fold or by adding purified thrombospondin (10 μmol/mL). In contrast to the other patient, this patient’s platelets adhered normally to collagen fibers in the presence of EDTA.

Despite the profound inhibition of platelet-collagen interactions produced by 6F1 in buffer systems, the addition of 6F1 to PRP did not simulate the platelet function defects found in either patient. This discrepancy might be accounted for if the patients’ platelets are totally devoid of functional GPIa/IIa receptors, whereas the inhibition by 6F1 is incomplete as a result of the extraordinary affinity of the fibrillar forms of collagen. Thus, only minimal collagen-GPIa/IIa interactions may be needed to initiate the steps that result in platelet aggregation. Alternatively, it is possible that the platelets of these patients have other abnormalities in addi-
tion to the GPIa deficiency that account for their functional defects. For example, the first patient’s platelets failed to interact normally with collagen in the presence of EDTA even though the GPIa/IIa mechanism has been reported to be inoperative in the presence of EDTA. Moreover, the second patient’s platelets, which were reported to be totally deficient in GPIa, interacted normally with collagen in the presence of EDTA. Similarly, the thrombospondin abnormality found in the second patient’s platelets may have contributed to the platelet aggregation abnormality. Since these patients came to medical attention because of their hemorrhagic diatheses it may be that defects in both GPIa/IIa and a non-GPIa/IIa mechanism for interacting with collagen are required to produce clinical symptoms.

A large number of different platelet glycoproteins have been proposed as platelet receptors for collagen over the past two decades and ongoing studies have continued to provide both supportive and negative data on their respective roles. Additional studies will clearly be required to unequivocally assign physiological significance to them. It should, perhaps, come as no surprise that platelet-collagen interactions are so complex, since collagen can exist in a wide variety of polymeric forms that may differ in their affinity for platelet components, and as suggested in the current studies, these polymeric forms may also interact indirectly with platelets by acting as insoluble matrices for adhesive glycoproteins that can then bind to their own platelet receptors.

NOTE ADDED IN PROOF

After these studies were completed, Sonnenberg et al (Laminin receptor on platelets is the integrin VLA-6; Nature 336:487, 1988) and Gralnick et al (A murine monoclonal antibody that identifies a 157/130 kD platelet-collagen receptor; Circulation 78:II-308, 1988), reported on new monoclonal antibodies that specifically inhibited platelet adhesion to collagen and recognized the GPIa/IIa complex.

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Collagen-platelet interactions: evidence for a direct interaction of collagen with platelet GPIa/IIa and an indirect interaction with platelet GPIIb/IIIa mediated by adhesive proteins

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