A Murine Monoclonal Antibody That Completely Blocks the Binding of Fibrinogen to Platelets Produces a Thrombasthenic-like State in Normal Platelets and Binds to Glycoproteins IIb and/or IIIa

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ABSTRACT To define better the role of the fibrinogen receptor in platelet physiology and to characterize it biochemically, a murine monoclonal antibody that completely blocks the binding of fibrinogen to the platelet surface was produced by the hybridoma technique with the aid of a functional screening assay. Purified $F(ab')_2$ fragments and/or intact antibody completely blocked aggregation induced by ADP, thrombin, or epinephrine and the binding of radiolabeled fibrinogen to platelets induced by ADP. The antibody did not block agglutination of formaldehyde-fixed platelets by ristocetin or shape change induced by either ADP or thrombin. ADP- and epinephrine-induced release of ATP was completely inhibited by the antibody, but inhibition of release induced by collagen and thrombin was dose dependent and partial. The antibody also dramatically inhibited platelet retention in glass-bead columns, platelet adhesion to glass, and clot retraction. Thus, the antibody induced a thrombasthenic-like state. Immunofluorescent studies confirmed the specificity of the antibody for normal platelets and megakaryocytes and suggested that there is a marked decrease in detectable antigen in thrombasthenic platelets. Radiolabeled antibody bound to an average of ~40,000 sites on normal platelets but it bound to <2,000 sites on the platelets of a patient with thrombasthenia. The antibody immunoprecipitated both glycoproteins IIb and IIIa, and both glycoproteins bound to an affinity column of the antibody. These studies indicate that there is probably a single anatomic site that is crucial to the binding of all fibrinogen molecules and that this site is most likely on the glycoprotein IIb/IIIa complex. It also suggests that the thrombasthenic phenotype can be completely accounted for on the basis of the inhibition of fibrinogen binding to platelets.

INTRODUCTION

The interaction of fibrinogen with its platelet receptor occupies a central role in platelet physiology. Current evidence indicates that it is required to achieve normal values for the skin bleeding time, the adhesion of platelets to glass surfaces, and the aggregation of platelets induced by ADP, epinephrine, thrombin, or arachidonic acid metabolites (1–11). There is general agreement that patients with Glanzmann's thrombasthenia have a functional defect of their platelet fibrinogen receptors, as evidenced by the inability of their platelets to bind fibrinogen (12–15). As these patients' platelets are deficient in the membrane glycoproteins GPIIb and GPIIIa (16, 17) and as there is evidence that these glycoproteins complex together in association with fibrinogen (18), the GPIIb/IIIa complex has been tentatively identified as the fibrinogen receptor. Controversy persists, however, since it has been reported that thrombasthenic platelets have abnormalities in other surface glycoproteins (19) and one group has proposed that thrombasthenic platelets do indeed have receptors for fibrinogen, but are unable to expose them in response to agonist activation (20).

To define better the role of the fibrinogen receptor in platelet physiology and to characterize it biochemically, we have employed the hybridoma technique

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(21) to produce murine monoclonal antibodies that block the receptor. By using a screening assay based on inhibition of the interaction of platelets with fibrinogen-coated beads (14), it was possible to select for those antibodies that best blocked the binding site for fibrinogen. This report characterizes the most potent monoclonal antibody found among 59 clones producing such antibodies.

METHODS

Culture medium RPMI 1640 was obtained from Gibco Laboratories (Grand Island, NY); medium 109 (National Collection of Type Cultures) from M. A. Bioproducts (Walkersville, MD); and fetal calf serum from either Flow Laboratories, Inc. (McLean, VA) or Gibco Laboratories. Polyethylene glycol (mol wt 1,000) was obtained from Koch-Light Laboratories Ltd. (Colnbrook, Buckinghamshire, England); Sephadex G-25 and protein A-Sepharose CL-4B from Pharmacia Fine Chemicals (Piscataway, NJ); protein A-containing staphylococci from The Enzyme Center (Boston, MA); *N*-ethylmaleimide and 2,6,10,14-tetramethylpentadecane (Pristane) from Aldrich Chemical Co. (Metuchen, NJ); ADP from Eastman Kodak Co. (Rochester, NY); epinephrine and bovine thrombin from Parke Davis and Co. (Detroit, MI); ristocetin from Lenau (Denmark); Affigel-10 from Bio-Rad Laboratories, Inc. (Richmond, CA); fluorescent slide mounting medium (Aqua-Mount) from Lerner Laboratories (New Haven, CT); control ascites containing a monoclonal antibody from Bethesda Research Laboratories (Rockville, MD); and Hepes buffer and complete Freund's adjuvant from Calbiochem-Behring Corp. (La Jolla, CA). Purified human thrombin was a gift from Dr. John Fenton II, New York State Department of Health, Albany. Rabbit anti-mouse immunoglobulin sera directed against IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgA, and IgM were obtained from Litton Bionetics (Kensington, MD), goat fluorescein isothiocyanate (FITC)[1] antimouse IgG plus IgM from Tago Inc. (Burlingame, CA), and goat FITC- and unlabeled anti-mouse IgG from E-Y Laboratories (San Mateo, CA). Sodium metaperiodate, collagen (insoluble type I from bovine Achilles' tendon), bovine fibrinogen (a crude source of bovine von Willebrand factor; fraction 1, type 1, 84% clottable), leupeptin, diisopropylfluorophosphate, ATP, dimethyl sulfoxide (DMSO), bovine serum albumin, and lactoperoxidase were obtained from Sigma Chemical Co. (St. Louis, MO). Pepsin A (2,500 U/mg) and soluble skin collagen (6.75 mg/ml) were obtained from Worthington Biochemical Corp. (Freehold, NJ), Triton X-100 from Packard Instrument Co., Inc. (Downers Grove, IL) and [^{3}H]$NaBH_4$ (52 Ci/mmol) and ^{125}I from New England Nuclear (Boston, MA). A fresh suspension of particulate collagen was prepared by adding 4 g of the insoluble collagen to 100 ml of ice-cold 0.15 M NaCl, mixing the suspension in a blender (Oster Div., Sunbeam Corp., Milwaukee, WI; mix setting) for 5 min at 4°C and centrifuging the supernatant twice at 2,000 *g* for 5 min. Plasminogen-free fibrinogen (lot number PR 2548), purified according to the method of Mosesson (22) was obtained from Cutter Laboratories Inc. (Berkeley, CA). It was previously shown to be negative for von Willebrand factor antigen by electroimmunoassay when assayed at 10 mg/ml (14).

[1] *Abbreviations used in this paper:* DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; PBS, 0.15 M NaCl, 10 mM sodium phosphate, pH 7.4; PRP, platelet-rich plasma; T-S, 0.15 M NaCl, 10 mM Tris/Cl, pH 7.4; TS-E, 0.15 M NaCl, 10 mM Tris/Cl, 10 mM EDTA, pH 7.4.

Platelet-rich plasma (PRP) preparation, platelet-poor plasma preparation, fixation of platelets with formaldehyde, and platelet aggregation were performed as previously described (23). Aggregation studies were performed in a dual-channel Chrono-Log aggregometer (Havertown, PA) with matched Teflon-coated stir bars (24). PRP samples (0.40–0.45 ml, 3×10^{11} platelets/liter) were pH controlled at 7.70±0.05 with the aid of the device previously described (23). In some experiments, release of platelet ATP and platelet aggregation were measured simultaneously using 50 μl of a Mg^{++}, luciferin-luciferase reagent (Chronolume, Chrono-Log), and an aggregometer adapted to measure light emission (Lumi-aggregometer, Chrono-Log), as previously described (25). Immobilization of fibrinogen on solid polymer beads (Matrex 102, Amicon Corp., Lexington, MA) was performed as previously described (14); after coupling was completed the beads were washed extensively with 0.15 M NaCl, 10 mM Tris/Cl, pH 7.4 (T-S) containing 0.05% sodium azide and kept at 4°C.

Platelet retention in glass-bead columns was measured as previously described (26), with blood being drawn through a 19-gauge butterfly infusion set into a series of syringes containing heparin (40 μl of 1,000 U/ml) and one of the following solutions: 6 μg/ml control monoclonal antibody $F(ab')_2$ fragments, 6 μg/ml 10E5 antibody $F(ab')_2$ fragments, or T-S. The syringes were twirled 10 times between the palms in both the horizontal and vertical axes immediately before being placed in the infusion pump. Platelet adhesion to glass slides was measured by a modification of the technique of Zucker and Vroman (7), as previously described (27). In brief, the slides are precoated with platelet-poor plasma for 5 s, washed, and then covered with PRP for 30 min. After washing again, the slides are fixed and the adherent platelets assessed by phase-contrast microscopy. ADP-induced binding of ^{125}I-fibrinogen (1 mg/ml) to gel-filtered platelets (3×10^{11} platelets/liter) in the presence of control and 10E5 antibody was assessed as previously described (15); nonspecific binding was defined as that amount of fibrinogen that bound to platelets in the absence of ADP. In experiments to assess the ability of 10E5 antibody to displace fibrinogen from its platelet receptor, the antibody was added to gel-filtered platelets after ADP had induced the binding of ^{125}I-fibrinogen and the amount of fibrinogen remaining bound was measured as a function of time. Immunoelectrophoresis was performed on 3-μl samples of ascites or purified antibody in 1.5% agarose employing a 0.065 M barbital/acetate buffer, pH 8.6 (Immunoelectrofilm Kit, Kallestad Laboratories, Inc., Chaska, MN). Electrophoresis was carried out at 100 V for 50 min and the precipitin arcs were developed by overnight incubation with goat anti-mouse IgG. Thin-layer gel electrofocusing of purified antibody was performed on polyacrylamide gels (5% T, 3% C) containing 2.4% (wt/vol) ampholines with a pH range of 3.5 to 9.5, according to the manufacturer's instructions (Ampholine PAG plate, LKB Instruments, Bromma, Sweden). The pH gradient was determined by cutting strips of the gel, eluting the ampholines in water, and measuring the pH. Immunofluorescent studies were performed on washed platelets as previously described (28). Supernatants from the growing cells were used neat or at a 1/2 dilution for the first incubation and FITC-labeled goat anti-mouse IgG was used at a dilution of either 1/120 or 1/240 for the second. The platelets from two related patients with Bernard-Soulier syndrome (A.J. [29] and T.H. [29]) and two unrelated patients with thrombasthenia (M.M. [30] and N.L. [20]) were also tested. PRP was obtained from these patients (and appropriate controls) by gravity sedimentation. Clot retraction of diluted PRP was assayed according to a modification of the method of Wid-

mer and Moake (31) by incubating 0.3 ml PRP ($\sim 2 \times 10^{11}$ platelets/liter), 1.4 ml of 53 mM Na_2HPO_4, 12 mM KH_2PO_4 (pH 7.4) buffer, and 0.14 ml of purified test and control antibody (10 μg/ml final concentration) for 10 min at 37°C. 5 μl of packed erythrocytes (to facilitate visual observations) and 100 μl of bovine thrombin (50 U/ml) were then added. The samples were stirred immediately with a small glass rod and the rod was left in place while the tubes incubated at 37°C; visual inspections of the clots that formed were made every 10–15 min for the first 2 h and at less frequent intervals thereafter.

Antibody production. A BALB/c mouse (Jackson Laboratories, Bar Harbor, ME) was injected intraperitoneally with six weekly 0.2-ml injections of 3×10^8 washed platelets (citrated PRP washed twice in 0.15 M NaCl, 10 mM Tris/Cl, 10 mM EDTA, pH 7.4 [TS-E]), resuspended in $^1/_{10}$ to $^1/_{20}$ of their original volume in TS-E, and mixed 1:1 with complete Freund's adjuvant. The seventh weekly injection was given intravenously into the tail vein and consisted of 0.3 ml containing $\sim 5 \times 10^8$ washed platelets resuspended in T-S without EDTA. Each of the seven platelet suspensions was obtained from a different donor. 3 d after the last injection, the mouse was killed by cervical dislocation and the spleen removed. A suspension of spleen cells in RPMI 1640 was prepared by teasing the spleen apart. After erythrocytes were lysed with ammonium chloride, the spleen cells were fused with a nonsecretory BALB/c mouse myeloma cell line (×63-Ag 8.653 [32]) that had been kept frozen in 10% DMSO, 90% fetal calf serum until 1 wk before fusion, when it was thawed and maintained in the culture medium routinely used (RPMI 1640 supplemented with 10% fetal calf serum and 1,000 U of penicillin and 100 μg of streptomycin/ml). Fusion was carried out according to a modification of the method of Levy et al. (33). Briefly, $\sim 2.7 \times 10^8$ spleen cells and 7×10^7 myeloma cells were pelleted together, the pellet was gently suspended in 2 ml of 35% polyethylene glycol in RPMI 1640 medium and the cells immediately centrifuged at 500 *g* at 22°C for 6 min. The solution was then diluted with RPMI 1640 to 9% polyethylene glycol, the cells resuspended and immediately centrifuged at 230 *g* for 6 min at 22°C. The supernatant fluid was then aspirated and the fused cells suspended in RPMI 1640 medium supplemented with 20% fetal calf serum and 10% 109 medium (National Collection of Type Cultures). The cells were placed in a flask and incubated overnight at 37°C in a 5% CO_2, 95% air atmosphere. The following day, the medium was made selective for successfully hybridized cells by adding hypoxanthine (10^{-4} M), aminopterin (4×10^{-7} M), and thymidine (1.6×10^{-5} M), after which the cells were aliquoted into 960 microtiter wells (Costar, Data Packaging, Cambridge, MA). 2 wk later, 574 wells showed growth and the supernatant fluids from 59 wells were positive in a screening assay for antifibrinogen receptor activity (see below). After an additional 2 wk in culture, the positive clones were transferred to 24-well microtiter dishes (Costar) and fed with the same medium as above, but without the aminopterin. The clones were expanded and the cells that continued to produce antifibrinogen receptor antibody were suspended in 90% fetal calf serum-10% DMSO and frozen in liquid nitrogen. The three clones having the most potent antibodies were also continued in culture and one of them (10E5) was subcloned twice by the limiting dilution technique (34), resulting in a statistical probability of monoclonality of >99%. The original clone has remained stable in culture for more than 12 mo. All studies were performed with antibody derived from either the stable clone or one of its subclones. Ascitic fluid rich in 10E5 antibody was prepared by intraperitoneal injection of Pristane-pretreated BALB/c mice with 5×10^6 hybrid cells that had been washed twice in 0.15 M NaCl, 10 mM sodium phosphate, pH 7.4 (PBS).

Screening assay. 35 μl of PRP (adjusted to 3×10^{11} platelets/liter) and 35 μl of the supernatant culture medium (or ascitic fluid) to be assayed were incubated together for 2 min in a well of a round-bottomed microtiter plate (Linbro Chemical Co., Hamden, CT). 5 μl of the fibrinogen-coated bead suspension was then added and the plate was mixed on a rotator (Tekator V, American Scientific Products, Edison, NJ) for 5 min at ~280 rpm. The wells were observed from the bottom with the aid of a magnifying mirror apparatus (Cooke Microtiter System, Dynatech Laboratories, Inc., Alexandria, VA). Wells containing culture medium that had not been used for growing cells showed marked agglutination of the beads (rated as 4+), whereas the supernatant culture media from positive clones (or ascitic fluid) inhibited the agglutination, resulting in lower readings (0–3+).

Antibody purification. Culture supernatants were precipitated at 4°C with 50% saturated ammonium sulfate and resuspended to between $^1/_{20}$ and $^1/_{10}$ of their original volume in 0.1 M sodium phosphate buffer, pH 8.0. After dialysis against the same buffer, the sample was applied to a 0.8 × 15.9 cm column of protein A Sepharose CL-4B that had been equilibrated with the phosphate buffer (after having been washed with both the phosphate buffer and a 0.1 M citrate buffer, pH 3.0). The column was eluted with the phosphate buffer until the optical density of the eluate returned to base line, after which stepwise elution was accomplished with 0.1 M citrate buffers of pH 6.0, 4.5, 3.5, and 3.0, as described by Ey et al. (35). Immunoglublins from control culture medium were eluted at pH 6.0, whereas 10E5 antibody did not elute until the pH was decreased to 4.5. Protein elution was monitored by optical density at 280 nm and appropriate fractions were pooled and dialyzed against T-S containing 0.05% sodium azide. Antibody concentration was estimated by absorption of 280 nm, assuming $A^{1\%}_{280} = 15$.

F(ab')$_2$ preparation. Purified antibody was dialyzed overnight at 4°C against 0.2 M NaCl, 0.2 M acetate, pH 4.0, after which freshly prepared pepsin (1 mg/ml) was added in an amount equal to 2% of the antibody's weight. The solution was incubated at 37°C for 18 h. Digestion was stopped by dialyzing the solution against PBS, pH 7.4. Analysis by polyacrylamide gel electrophoresis indicated that the digestion was essentially complete. In some experiments, the F(ab')$_2$ were further purified by chromatography on protein A Sepharose CL-4B to be certain that any remaining traces of whole IgG were removed; this additional step did not reduce the potency of the F(ab')$_2$ preparation in the screening assay.

Antibody iodination. ^{125}I was covalently bound to 0.52 mg of whole antibody, using immobilized lactoperoxidase and glucose oxidase according to the manufacturer's recommendations (Protein Iodination Kit, New England Nuclear, Boston, MA), except that all of the reagents were doubled, 2 mCi of ^{125}I were used, the reaction proceeded for 80 min, the sodium metabisulfite step was omitted, and the reaction was terminated (and the protein separated from free ^{125}I) by chromatography on a column of Sephadex G-25; the latter had been prerun with 50 ml of T-S, 0.05% sodium azide containing 0.2% bovine serum albumin, and then equilibrated and eluted with T-S buffer containing 0.05% sodium azide but without albumin. The specific activity of the radiolabeled antibody was 144 cpm/ng. Greater than 89% of the radioactivity in the fractions used for binding studies was precipitable by 10% trichloroacetic acid and polyacrylamide gel electrophoresis analysis demonstrated that >90% of the radioactivity migrated with the intact antibody.

Binding of ^{125}I-antibody to human platelets. Binding was assessed at 22°C, using PRP ($\sim 3 \times 10^{11}$ platelets/liter) prepared from whole blood collected into 3.2% sodium citrate. Increasing

amounts of a mixture of labeled and unlabeled antibody ranging in specific activity from 21 to 144 cpm/ng were added to 0.2 ml PRP. To establish equilibrium binding conditions, duplicate 0.1-ml aliquots were layered onto silicone oil (Contour Chemical Co., Inc., North Reading, MA; specific gravity 1.040) after 0.5, 5, 10, and 20 min. They were then centrifuged at 22°C for 2 min at 12,000 *g* in a microcentrifuge (Beckman Instruments, Inc., Irvine, CA) to separate platelets with bound antibody from unbound antibody. The tubes were inverted, and the tips containing the platelet pellets sliced off with a dog nail cutter and counted. Total radioactivity of the samples was determined by counting duplicate 10-μl aliquots of the remaining platelet suspensions. Binding was measured after 5 min as it did not increase thereafter.

Trapped fluid was determined using ^{14}C-sorbitol (Amersham Corp., Arlington Heights, IL) in separate samples as previously described (15). Nonspecific binding was assessed by incubating PRP with labeled antibody in the presence of a 1,000-fold excess of unlabeled antibody in ascites fluid. Greater than 97% of the binding of labeled antibody was inhibited by the presence of ascites fluid, indicating that, with the exception of radioactivity trapped in the platelet pellets, virtually all of the binding observed represented specific interactions between platelets and the antibody. To establish that the Fc portion of the antibody was not contributing to the binding, the binding of radiolabeled antibody was measured in the presence of increasing amounts of either cold whole antibody or cold $F(ab')_2$ fragments of antibody. As both of the cold preparations produced the same inhibition of binding of the radiolabeled antibody, it was concluded that the whole antibody and $F(ab')_2$ fragments have identical binding characteristics.

Affinity chromatography. Approximately 5 mg of purified 10E5 antibody and 3 mg of control monoclonal antibody were each coupled to ~3 ml of Affigel 10 according to the manufacturer's recommendations using a 0.1 M Hepes buffer, pH 7.5. The columns were washed with the same series of buffers used by McEver et al. (36) (except that 1.5 M NaCl was substituted for 1 M NaCl) and were kept at 4°C in 0.15 M NaCl, 0.02 M Tris/Cl, 1% Triton X-100, 0.05% sodium azide, pH 7.4.

Immunoprecipitation of platelet membrane proteins (37). A freshly drawn unit of platelet concentrate was radiolabeled with ^{3}H by washing twice in TS-E, resuspending in 5 ml PBS at 0°C (~5×10^9 platelets/ml), adding 50 μl of freshly prepared sodium metaperiodate (200 mM; final concentration 2 mM), incubating for 10 min at 0°C in the dark, adding 1 ml 0.2 M glycerol, washing twice in TS-E, resuspending in ~5 ml PBS, adding 25 mCi of NaB_3H_4 in 0.01 M NaOH, incubating for 15 min at 22°C, washing four times in TS-E, solubilizing the pellet in 2.5 ml 1% Triton X-100, 1 mM DFP, 1 mM leupeptin for 15 min at 4°C with sonication (thrice for 15–20 s, power reading 30–40, model 9100 with probe model MT-1, Lab-Line Instruments, Inc., Melrose Park, IL), centrifuging at 4°C for 15 min at 5,000 *g* and recentrifuging the supernatant at 4°C for 1 h at 40,000 *g*. A solid phase immunoprecipitin was produced by washing 2 ml of a 10% suspension of protein A-containing staphylococci thrice in PBS containing 1% Triton X-100, 0.02% sodium azide, 0.1% sodium dodecyl sulfate (SDS), 0.1% bovine serum albumin, pH 8.6; adding 2 ml of affinity-purified goat anti-mouse IgG plus IgM; incubating for 30 min at 22°C; washing eight times in the above buffer; dividing the suspension into seven aliquots; centrifuging the suspension at 12,000 *g* for 2 min at 22°C; adding 600 μl of 10E5 or control culture supernatant to the pellets; incubating for 40 min at 22°C and 10 min at 37°C; washing four times in the above buffer and resuspending in 0.3 ml of buffer. The solubilized platelet proteins (0.3 ml) and the antibody-coated staphylococci (0.3 ml) were then incubated for 64 h at 4°C and centrifuged at 12,000 *g* for 2 min. The pellet was washed seven times in the above buffer and then air dried. To release the bound antibody and antigen from the staphylococci, 0.1 ml of a solution consisting of 1 part 3.3% SDS, 6 mM *N*-ethylmaleimide and 1 part 1% SDS, 12.5 mM Tris/Cl, 20% glycerol, 0.025% Bromophenol blue, pH 6.8, was added to the pellet and heated to 100°C for 3 min. After centrifuging the sample at 12,000 *g* for 2 min, the supernatant was incubated with 5% (vol/vol) 2-mercaptoethanol for 3 min at 100°C and electrophoresed on a 5–15% exponential gradient polyacrylamide gel according to the method of Laemmli (38). Gels were fixed, stained for protein with Coomassie Blue, and prepared for fluorography either by the method of Bonner and Laskey (39) or by soaking for 1 h in 50 ml of a fluorography-enhancement solution (EN^{3}HANCE, New England Nuclear) and 1 h in water before drying and placing in a cassette with X-ray film (XAR-5, Eastman Kodak) at −70°C. Another immunoprecipitation experiment was performed on washed platelets that had been radiolabeled with ^{125}I by reaction with lactoperoxidase as described by Phillips et al. (17). The platelets were solubilized as above, reacted first with control or 10E5 antibody for 18 h at 4°C and then with a 10% suspension of protein A-containing staphylococci for 6 h at 4°C. The pellet was washed extensively and the bound protein released by heating to 100°C as indicated above. The released proteins were electrophoresed in gels either with or without 2-mercaptoethanol reduction as above and the radioactive bands were identified by autoradiography after 7 d of development at 22°C.

Solubilized platelet proteins for affinity chromatography. A platelet concentrate was washed five times in TS-E and once in T-S. The pellet was then solubilized in T-S containing 1% Triton X-100 and 1 mM leupeptin overnight at 4°C. The suspension was sonicated as above at 0°C, centrifuged at 2,000 *g* for 20 min, and the supernatant then centrifuged at 40,000 *g* for 1 h at 4°C. Electrophoresis of the supernatant in 7.5% polyacrylamide stick gels and staining with the periodic acid-Schiff reagent as described previously (40) revealed the three major platelet glycoprotein bands.

RESULTS

Physicochemical characterization of the antibody. The antibody that was most potent in inhibiting the agglutination of fibrinogen-coated beads by PRP (clone 10E5) eluted from protein A-Sepharose at pH 4.5, suggesting that it was of the IgG_{2a} subclass (35). Confirmation of this subclass identification was obtained when Ouchterlony immunodiffusion analysis against anti-IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgM, and IgA sera indicated the exclusive presence of IgG_{2a}. Immunoelectrophoresis of ascites fluid and antibody purified from the supernatant culture medium of 10E5 and its subclones all revealed the identical, markedly restricted electrophoretic mobility, with the antibody migrating to the anodal side of the application well. Thin-layer electrofocusing of intact 10E5 antibody also revealed marked restriction, but with some microheterogeneity, as judged by the presence of several closely spaced bands focusing at ~pH 5.5.

Effects of 10E5 antibody on screening assay, platelet aggregation, and platelet secretion. Supernatant culture medium from the 10E5 clone completely inhibited the agglutination of the fibrinogen-coated beads by PRP in the screening assay at dilutions ranging between 1/4 and 1/16. Ascites fluid obtained from

a mouse injected with cells from 10E5 was much more potent, remaining positive at dilutions of 1/5,120. The effects of 10E5 antibody on platelet aggregation was assessed in 26 experiments conducted on the platelets of 10 different donors. When added to the sample for at least 30 s before stimulation, the purified antibody inhibited ADP-induced platelet aggregation in a dose-dependent fashion, with complete inhibition of the initial slope achieved at ~6 μg/ml. In one experiment in which the effect was titred, there was 39% inhibition of the initial slope at 2.1 μg/ml, 64% inhibition at 3.1 μg/ml, and 100% inhibition at 5.2 μg/ml. Despite the total lack of aggregation, the platelets still underwent their characteristic shape change, as evidenced by the transient increase in optical density after ADP was added. To avoid any potentially confounding effects of the antibody's Fc fragment in inhibiting aggregation, the studies were repeated with $F(ab')_2$ fragments from both the 10E5 antibody and a control murine monoclonal antibody (Fig. 1). The results were essentially identical to those obtained with whole antibody; complete inhibition occurred at ~7.8 μg/ml. The 10E5 antibody (and its $F[ab']_2$ fragments) also completely inhibited aggregation induced by epinephrine (2.5 μM) and thrombin (both human and bovine, 0.2 U/ml) (Fig. 1). As with ADP, shape change produced by thrombin was not inhibited. No shape change was seen when epinephrine was added to PRP containing 10E5 antibody, consistent with previous studies indicating that this agonist produces aggregation without causing shape-change (41). The effect of 10E5 antibody on collagen-induced platelet aggregation varied with the preparation and dose. At limiting concentrations of particulate collagen, 10E5 antibody was able to prevent any decrease in optical density in the aggregometer tracing, although microscopic examination of the cuvette contents revealed the presence of occasional small aggregates. As with other reagents, the antibody did not inhibit shape change. At higher doses of the particulate collagen suspension, the 10E5 antibody did not prevent a decrease in optical density, but the response was always markedly inhibited and the aggregates remained very small when compared with the control, as judged by both visual inspection and the amplitude of oscillations of the aggregometer tracings. Even with limiting doses of the soluble collagen, the 10E5 antibody could not completely prevent a decrease in optical density, but there was a prolongation of the lag time and marked decreases in both the slope of aggregation and the size of the aggregates that formed (Fig. 1). When the release of ATP was measured simultaneously with aggregation, it was found that the 10E5 antibody completely inhibited release induced by ADP and epinephrine, but only partially inhibited release induced by thrombin and collagen (Fig. 1).

The partial inhibition of collagen and thrombin-induced release of ATP from platelets by 10E5 antibody was investigated in more detail (Table I). It was found that the inhibition of release depended upon the dose of the agonist used, with marked inhibition at low concentrations and little or no inhibition when high doses were used. This was interpreted as indicating that the binding of fibrinogen and/or the resultant platelet aggregation may act to enhance release at low doses of these agonists, whereas at high doses release is independent of both fibrinogen binding and aggregation. To test this hypothesis further, the effects of 5 mM EDTA was also assessed, since this agent prevents fibrinogen binding to platelets by chelating calcium. As shown in Table I, the results with EDTA were virtually identical to those obtained with 10E5 antibody, with inhibition of release induced by thrombin and collagen evident only at low agonist concentrations. The effect of 10E5 antibody on release induced by ionophore A23187 also demonstrated a marked dose dependence, an observation consistent with the notion that agents known to be able to induce release by one or more mechanisms that are independent of aggregation at high doses may show enhanced release at low concentrations if fibrinogen binding and aggregation occur (42, 43).

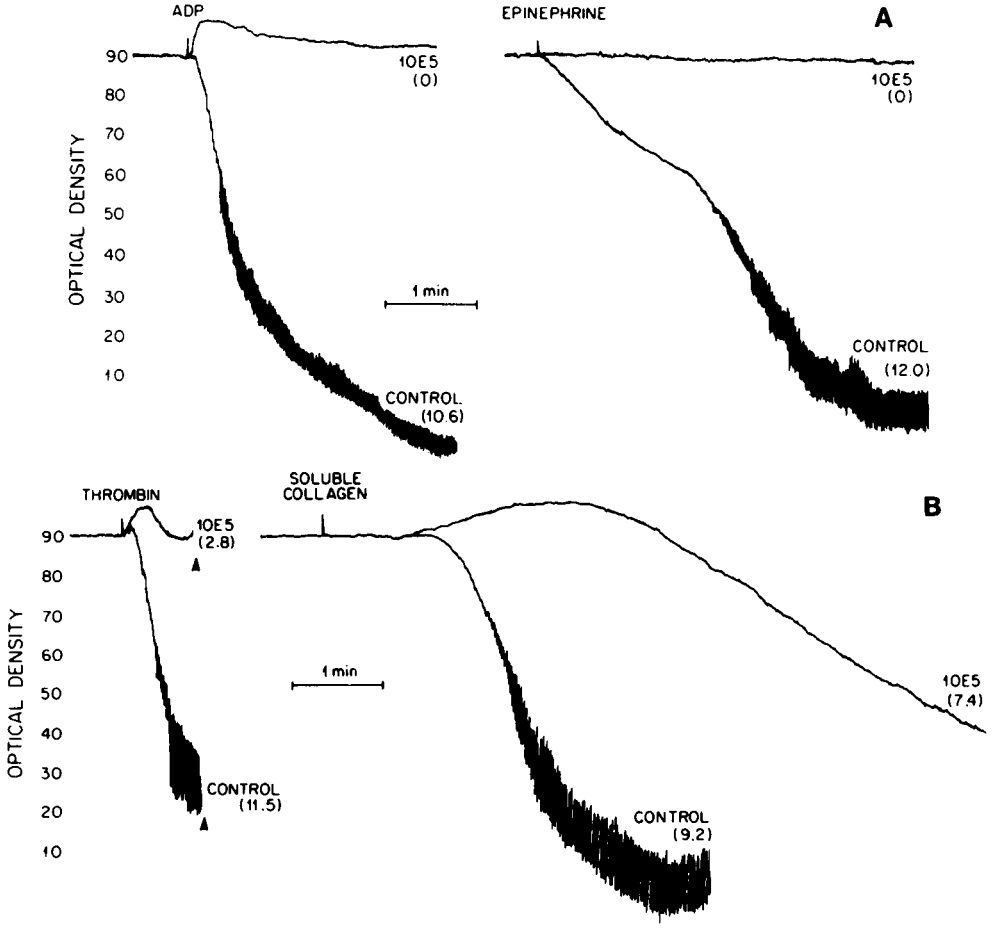
In contrast to its effects on aggregation induced by agonists that require fibrinogen as a cofactor, 10E5 antibody did not inhibit ristocetin-induced agglutination of formaldehyde-fixed platelets. When assessed in PRP, however, concentrations of 10E5 antibody that completely inhibited ADP-, epinephrine-, and thrombin-induced aggregation did produce some decrease in the initial slope of ristocetin-induced platelet aggregation (24±7%, mean±SEM, $n = 7$). Moreover, when the dose of ristocetin chosen produced two relatively distinct waves of aggregation, the second wave was completely inhibited by the 10E5 antibody. Similar results were obtained with bovine von Willebrand factor. With only a single exception, the 10E5 antibody alone did not produce direct aggregation of platelets, even when tested at concentrations severalfold higher than that required to completely block aggregation. The exception was the donor showing the briskest aggregation and release responses whose platelets underwent shape change, slight aggregation (4% change in optical density), and release (11% of maximum) when stimulated with 12 μg/ml of 10E5 antibody.

Inhibition of platelet retention and adhesion to glass. The addition of 10E5 $F(ab')_2$ fragments at a concentration of 6 μg/ml reduced the platelet retention of normal blood from 96 to 20%. The control value is based on the identical retention of two samples containing either 6 μg/ml of control $F(ab')_2$ fragments or buffer that were tested before and after the sample containing the 10E5 $F(ab')_2$ fragments, so as to exclude

an effect of temporal drift. When PRP was allowed to incubate for 30 min on a glass slide that had been precoated with platelet-poor plasma for 5 s, the platelets formed a dense lawn on the slide (Fig. 2). Inspection of individual platelets at high magnification revealed that many had undergone extensive spreading on the surface. The addition of 10 μg/ml of control antibody to the PRP before incubation had no effect on the adhesion or spreading. In contrast, the addition of 10 μg/ml of 10E5 antibody to the PRP resulted in a dramatic and, in fact, nearly total inhibition of the adhesion of platelets to the glass surface. Detailed observation of the rare platelets that did adhere failed to reveal significant spreading. A control slide in which the 10E5 antibody was added to the platelet-poor plasma used for the precoating, but not to the subsequent PRP, had as dense a lawn of platelets as when untreated platelet-poor plasma was used, indicating that the antibody was affecting the platelets and not the deposition of plasma proteins.

Inhibition of clot retraction. The addition of 10 μg/ml of 10E5 antibody significantly decreased the rate of clot retraction of a diluted suspension of PRP that had been clotted with thrombin (Fig. 3). The clot in the control antibody tube underwent significantly more retraction in the first 15 min than was evident in the 10E5 antibody sample at 1 h. Clot retraction in the control sample was essentially complete in <1 h, but the sample containing 10E5 antibody did not achieve complete retraction even after overnight incubation.

Immunofluorescent studies. Normal platelets gave bright immunofluorescence when reacted first with 10E5 antibody and then with a FITC-labeled goat anti-mouse IgG antibody; none of the other cells in the washed PRP preparation (primarily lymphocytes) stained positively. The platelets of two patients with thrombasthenia gave much less or no fluorescent staining in three separate experiments, whereas the platelets from two patients with Bernard-Soulier syndrome were as intensely fluorescent as the control platelets. Staining of bone marrow smears with 10E5 antibody revealed staining of large cells with nuclear and cytoplasmic characteristics of megakaryocytes, but no



staining of cells that could be identified as being in the myeloid or erythroid cells lines. A rare small cell was also labeled, but as these cells could not be identified as belonging to a specific cell lineage, they were suspected of being megakaryocyte precursors (44).

Binding of ^{125}I-antibody to platelets. The radiolabeled 10E5 antibody bound to gel-filtered platelets and platelets in PRP, with equilibrium established by 5 min. Binding studies employing mixtures of the radiolabeled antibody and either unlabeled antibody or $F(ab')_2$ fragments of antibody demonstrated that the labeled material bound exactly as the unlabeled material. Fig. 4 shows the relationship between the amount of 10E5 added and the amount bound to gel-filtered platelets. The curve reveals a pattern approaching saturation at ~6 μg/ml of 10E5 antibody. It should be noted that no correction of the raw data for nonspecific binding was required, since the addition of excess unlabeled 10E5 antibody reduced the amount of labeled antibody bound to the platelets to just above background levels. Analysis of the data derived from the binding of 10E5 antibody to platelets in PRP or gel-filtered platelets by the method recently recommended by Klotz (45) indicated that a reliable estimation of the total number of sites could be made because the higher concentrations yielded data points well above the inflection point (Fig. 5). This method was used to estimate the maximum number of sites on the platelets of 10 separate individuals. In 7 of the 10, the mean value was 40,000 with a standard deviation of 8,000 sites; the other three values (12,000, 75,000, and 83,000) were >3 SD from the mean. An estimation of the K_d of 10E5 antibody for platelets was obtained from the graphs by determining the amount of antibody added at the inflection point of the binding curve; in 10 separate experiments, the K_d was 16.2±6 nM (mean±SD). The platelets of one of the patients with thrombasthenia (N.L.) were also tested; at an antibody concentration approaching saturation (10 μg/ml), her platelets bound only 1,960 molecules of 10E5 antibody, or 5.3% of the mean number of molecules bound by normal platelets tested at the same time. This same patient's platelets were tested for their ability to bind ^{125}I-fibrinogen (see below); her platelets bound 2,383 molecules of fibrinogen per platelet, or 3.2% of that bound by a sample of control platelets tested at the same time.

Inhibition of ^{125}I-fibrinogen binding to platelets by 10E5 antibody; correlation with inhibition of ADP-induced aggregation and binding of antibody to platelets. The addition of increasing concentrations of unlabeled 10E5 antibody to 1 ml of gel-filtered platelets (~3×10^{11}/liter) produced a dose-dependent inhibition of ADP-induced specific binding of ^{125}I-fibrinogen (Fig. 4). Inhibition was essentially complete at concentrations between 6 and 12 μg/ml (n = 4). Samples of the same gel-filtered platelet preparation were tested for binding of 10E5 antibody (see above) and platelet aggregation induced by ADP (10 μM) after addition of fibrinogen (0.1 mg/ml final concentration). Platelet aggregation was shown to be inhibited in parallel with both the inhibition of fibrinogen binding and the increased binding of 10E5 antibody to the platelets (Fig. 4).

Displacement of ^{125}I-fibrinogen from ADP-stimulated platelets. The addition of 10E5 antibody (10

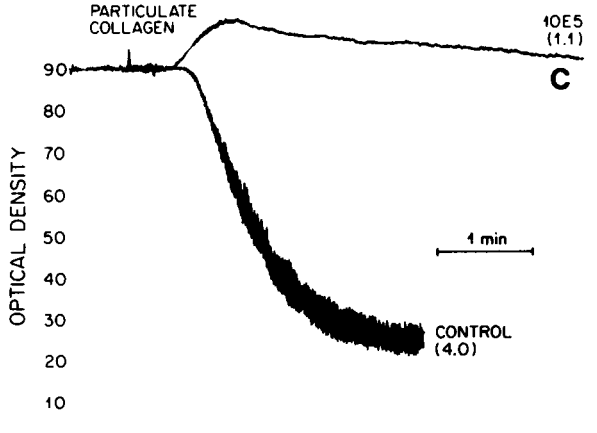


FIGURE 1 Inhibition of platelet aggregation and release of ATP by 10E5 antibody. For all but the particulate collagen study, citrated PRP and the ATP luminescence reagent were incubated with 10E5 $F(ab')_2$ fragments (7.8 μg/ml final concentration) or control antibody $F(ab')_2$ fragments (8.6 μg/ml final concentration) for 1 min at 37°C. At the indicated points, ADP (2.5 μM final concentration), epinephrine (2.5 μM final concentration), bovine thrombin (0.2 U/ml final concentration), and soluble collagen (135 μg/ml final concentration) were added and both aggregation and ATP release were monitored. The numbers in parentheses indicate the peak release of ATP in nanomoles per 10^9 platelets. The results from the donor showing the briskest aggregation and release reactions were chosen to emphasize the inhibition produced by the 10E5 $F(ab')_2$ fragments; most donors released much less ATP with ADP and epinephrine stimulation than with thrombin. The particulate collagen experiment was performed as above on the citrated PRP from another donor, except that intact 10E5 (5.7 μg/ml) and control antibody (6.3 μg/ml) were used; aggregation was initiated with 10 μl of the collagen suspension. Note the dramatic inhibition of aggregation induced by all of the reagents, but the lack of inhibition of shape change (manifested as a rapid increase in optical density) induced by ADP and thrombin. The decrease in optical density seen with the soluble collagen is probably more a reflection of platelet adhesion than aggregation (see Discussion). Note that the inhibition of release induced by ADP and epinephrine was complete whereas the inhibition of release induced by collagen and thrombin was only partial.

TABLE I

Effect of 10E5 Antibody and EDTA on Release of ATP from Platelets Induced by Collagen, Thrombin, and Ionophore

Antibody	EDTA	Agonist	Peak release
			nmol ATP/10⁸ platelets
Control	−	Collagen (160 μg/ml)	7.6
10E5	−	Collagen (160 μg/ml)	2.4
Control	+	Collagen (160 μg/ml)	2.7
10E5	+	Collagen (160 μg/ml)	2.4
Control	−	Collagen (680 μg/ml)	11.6
10E5	−	Collagen (680 μg/ml)	10.2
Control	+	Collagen (680 μg/ml)	10
10E5	+	Collagen (680 μg/ml)	9.8
Control	−	Thrombin (0.2 U/ml)	11.6
10E5	−	Thrombin (0.2 U/ml)	2.0
Control	+	Thrombin (0.2 U/ml)	2.3
10E5	+	Thrombin (0.2 U/ml)	2.7
Control	−	Thrombin (1 U/ml)	12.5
10E5	−	Thrombin (1 U/ml)	11.6
Control	+	Thrombin (1 U/ml)	14.7
10E5	+	Thrombin (1 U/ml)	11.8
Control	−	Ionophore A23187 (5 μM)	9.9
10E5	−	Ionophore A23187 (5 μM)	0.2
Control	−	Ionophore A23187 (25 μM)	9.5
10E5	−	Ionophore A23187 (25 μM)	9.6

PRP (0.45 ml) was incubated with 0.05 ml of the luciferin-luciferase reagent and, in some cases, with Na_2EDTA (5 mM final concentration). After it was certain that the pH was 7.70±0.05, the cuvette was placed in a Lumi-aggregometer and stirred until a base line was established. Either purified control or 10E5 antibody (37 μl of a 140-μg/ml solution) was then added, and after 2 min so was the indicated agonist. The stock solutions were 6.7 mg/ml soluble collagen, 74 U/ml thrombin, and 5 mM ionophore A23187 (in ethanol). After maximal release occurred, 1 μl of a 1 mM, freshly prepared solution of ATP was added as an internal standard.

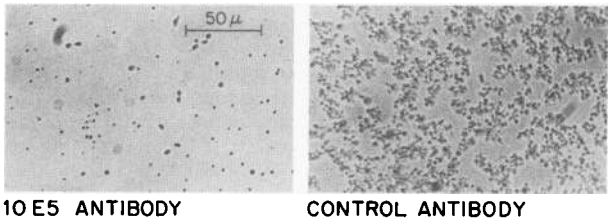


FIGURE 2 Inhibition of platelet adhesion to glass by 10E5 antibody. Glass microscope slides were precoated with platelet-poor plasma for 5 s, washed with T-S, and air dried. Citrated PRP containing 10 μg/ml of control or 10E5 antibody was then added and incubated for 30 min at 22°C. The slides were then washed with T-S, fixed in methanol, and observed by phase-contrast microscopy. Note the dense lawn for adherent platelets in the presence of the control antibody, but the virtual absence of adherent platelets in the presence of 10E5 antibody.

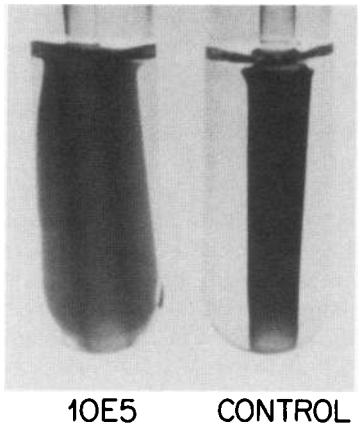


FIGURE 3 Inhibition of clot retraction by 10E5 antibody. Citrated PRP was diluted with phosphate buffer containing control antibody or 10E5 antibody (final concentration of 10 μg/ml for both). After 10 min at 37°C, 5 μl of erythrocytes and 0.1 ml of bovine thrombin were added and the tubes incubated at 37°C for 45 min. Note that 10E5 antibody dramatically inhibited the retraction of the clot.

μg/ml) to gel-filtered platelets that had previously been stimulated with 10 μM ADP to bind ^{125}I-fibrinogen resulted in rapid displacement of the bound fibrinogen. Table II shows the results of four experiments in which platelets were incubated with 0.4–0.5

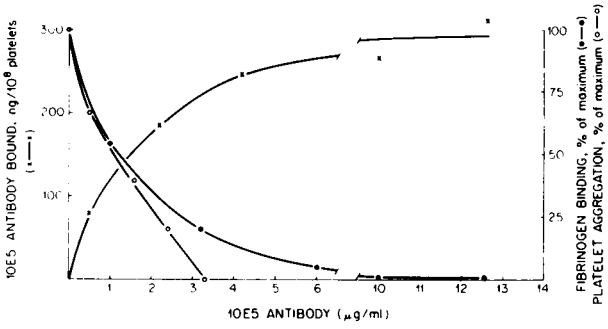


FIGURE 4 Correlation of 10E5 antibody binding to platelets with inhibition of fibrinogen binding and platelet aggregation. A single preparation of gel-filtered platelets (~3×10^{11} platelets/liter) was pretreated with increasing concentrations of labeled or unlabeled 10E5 antibody for 5 min and then assessed in three different systems: (*a*) binding of 10E5 antibody to platelets, (*b*) inhibition of ADP-induced binding of ^{125}I-fibrinogen to platelets, and (*c*) inhibition of ADP-induced platelet aggregation in the presence of 0.1 mg/ml fibrinogen; the initial slopes of platelet aggregation were measured and the results are expressed as percentages of the maximal initial slope of aggregation. Note that increasing concentrations of 10E5 antibody resulted in a decrease in both the amount of fibrinogen bound to platelets and the aggregation induced by ADP. There was virtually no detectable aggregation when fibrinogen binding was reduced to <20% of control and virtually no fibrinogen bound when the 10E5 concentration was ≥10 μg/ml.

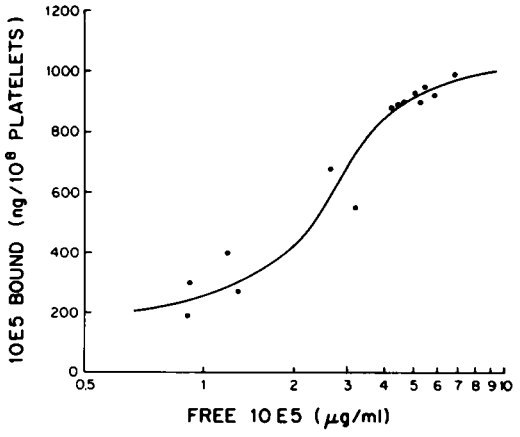


FIGURE 5 Binding of ^{125}I-10E5 antibody to platelets. ^{125}I-10E5 antibody was incubated at increasing concentrations with platelets in platelet-rich plasma for 5 min at 22°C, after which bound antibody was separated from free antibody. Data are plotted as recommended by Klotz (45) and show that at high concentration of antibody the data points are well above the inflection point.

mg/ml fibrinogen. Similar results were obtained with 0.8 mg/ml fibrinogen and a trace concentration of fibrinogen. Control samples incubated for the same period of time without antibody did not show any dissociation of bound fibrinogen.

Immunoprecipitation and affinity chromatography. The immunoprecipitate formed by the reaction of 10E5 antibody with periodate/NaB_3H_4-labeled platelets was subjected to electrophoresis in a polyacrylamide gel after solubilization in SDS and reduction with 5% 2-mercaptoethanol. When analyzed by fluorography, it was found to contain two major radioactive bands of M_r ~125,000 and ~110,000 and two minor bands of M_r ~225,000 and ~23,000 (Fig. 6 *A*). The two major radioactive bands corresponded to Coomassie Blue-positive bands on the gel. Preliminary analysis based on published values for the molecular weights and staining characteristics of platelet glycoproteins (46) suggested that the two major bands were GPIIb α and GPIIIa, respectively, and the M_r-23,000 band was GPIIb β. The M_r-225,000 band could not be identified, but, since it was similar in molecular weight to the sum of GPIIb and GPIIIa, it is possible that it represents some residual complex of these proteins that resisted dissociation by the SDS. In contrast to the results with 10E5 antibody, fluorograms of precipitates of solubilized platelets incubated with the control monoclonal antibody failed to reveal any radioactive bands entering the gel, although a very weak band was present at the top of the gel; a similarly weak

TABLE II
Dissociation of 10E5 Antibody of ^{125}I-fibrinogen Bound to ADP-stimulated Platelets

Time	Bound fibrinogen	% Dissociated
min	*molecules/platelet*	
0	12,586±3,773*	—
5	3,957±4,779	69
20	3,457±4,034	73
60	1,513±3,027	88

Gel-filtered platelets were incubated with 0.4–0.5 mg/ml ^{125}I-fibrinogen and then stimulated with 10 μM ADP. A sample was then removed (0 time) to assess maximal binding and 10E5 antibody (10 μg/ml) was added. Samples were removed at the indicated times and the remaining bound fibrinogen determined. Control samples incubated for the same period of time without antibody showed no dissociation of fibrinogen.

* Mean±SD, n = 4.

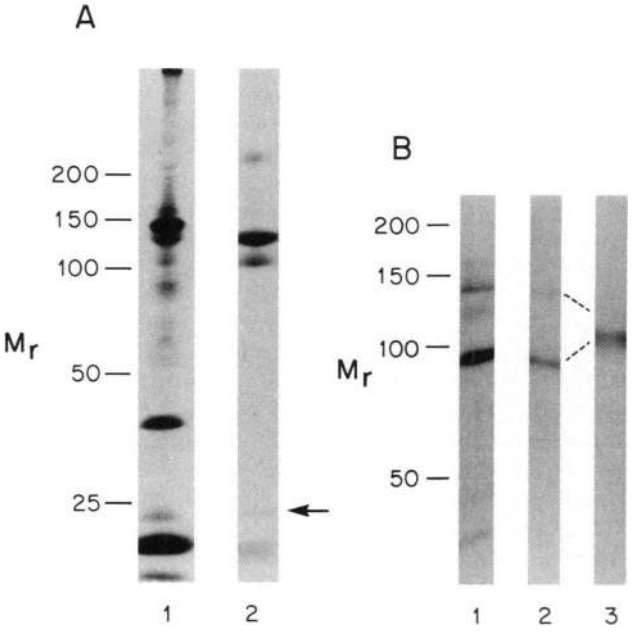


FIGURE 6 Analysis of platelet membrane glycoproteins that bind to 10E5 antibody. (*A*) Platelet membrane glycoproteins were labeled with sodium metaperiodate/NaB_3H_4, solubilized, and immunoprecipitated as described in Methods. The immunoprecipitate was solubilized in SDS, reduced with 2-mercaptoethanol and electrophoresed in a 5–15% polyacrylamide gel. After suitable preparation, the gel was dried and exposed to X-ray film for 10 d at −70°C. Lane *1* contains the whole platelet preparation and lane *2* contains the immunoprecipitate showing the two major bands of M_r 125,000 and 110,000 and the two minor bands of M_r 225,000 and 23,000 (arrow). The diffuse patch of radioactivity below the band indicated by the arrow was felt to be carryover radioactivity from a heavily staining band in an adjacent lane. (*B*) Similar to *A*, except platelets were labeled with ^{125}I by reaction with lactoperoxidase. Lane *1* contains the whole platelet preparation without reduction of disulfide bonds. Lane *2* contains the 10E5 precipitate without reduction. Lane *3* contains the 10E5 precipitate in the presence of 10% 2-mercaptoethanol.

band was present at the top of the 10E5 gel. Another immunoprecipitation study was performed with platelets labeled with ^{125}I by reaction with lactoperoxidase. When electrophoresed without reducing agent, two major bands of M_r 145,000 and 82,000 were identified in the 10E5 precipitate, whereas there were no detectable radioactive bands in the gel of the control antibody precipitate (Fig. 6 *B*). As in the other immunoprecipitation study, a very light band of M_r ~230,000 was also seen in the gel of the 10E5 precipitate after prolonged exposure. When the 10E5 precipitate was reduced with 10% 2-mercaptoethanol, the M_r-145,000 band changed position to M_r ~122,000 and the M_r-82,000 band changed position to M_r ~115,000. Both the radiolabeling patterns and the changes in M_r with reduction indicate that the two proteins are GPIIb and IIIa (46).

To confirm the identity of the glycoproteins that were immunoprecipitated, affinity columns of control and 10E5 antibody were prepared. Platelets solubilized in Triton X-100 were first passed over the control antibody column and then the 10E5 clumn. The columns were washed extensively and then eluted with a series of buffers (36). After the initial flow-through peak, no other proteins eluted from the control column, indicating the lack of any specific binding. In contrast, the 1.5 M NaCl, 1% Triton X-100 buffer eluted two proteins from the 10E5 column. When electrophoresed unreduced, the proteins were well separated and of M_r 145,000 and 88,000, respectively. However, when the disulfide bonds were reduced with increasing concentrations of 2-mercaptoethanol (0.1, 1, and 10%) the two proteins became much closer in molecular weight; the M_r-145,000 band became 128,000, 125,000, and 126,000 at 0.1, 1, and 10% 2-mercaptoethanol, respectively, whereas the M_r-88,000 band became 110,000, 112,000, and 118,000 at 0.1, 1, and 10% 2-mercaptoethanol, respectively. Both proteins were stained with either Coomassie Blue or periodic acid-Schiff reagent. As indicated above, the observed shifts in M_r upon reduction are characteristic of GPIIb (which loses GPIIb β and so has a lower M_r) and GPIIIa (which is thought to have intramolecular disulfide bonds that, when broken, result in slower migration through the gel; 46).

DISCUSSION

There have been several previous studies of antibodies that either blocked fibrinogen binding to platelets and/or bound to GPIIb and GPIIIa. A patient with thrombasthenia who had received multiple platelet transfusions developed one or more IgG antibodies that inhibited (*a*) the binding of fibrinogen to platelets; (*b*) platelet aggregation induced by ADP, epinephrine, collagen, and thrombin, but not by ristocetin or bovine von Willebrand factor; and (*c*) clot retraction (47–49). An early study indicated that the antibody precipitated only a single protein of M_r 120,000, as judged by polyacrylamide gel electrophoresis after reduction (47), whereas a subsequent study employing crossed immunoelectrophoresis showed that the antibody precipitated a complex composed of GPIIb and GPIIIa (50). Kaplan and Nachman (51) prepared a heterologous antibody to a lectin-purified platelet glycoprotein; the antibody did not inhibit either ADP or collagen-induced aggregation. Jenkins et al. (52) prepared a heterologous antibody against a mixture of GPIIb and GPIIIa that inhibited collagen-induced aggregation, but only the second wave of ADP-induced aggregation. Leung et al. (53) prepared heterologous antisera to GPIIb and IIIa after they had been separated from each other by preparative polyacrylamide gel electrophoresis. Neither antiserum alone nor mixtures of the two antisera inhibited either platelet aggregation or the binding of fibrinogen to platelets. McEver et al. (36) produced a murine monoclonal antibody that precipitated both GPIIb and GPIIIa. The antibody bound to 39,000±4,600 (mean±SD) sites on normal platelets, but to <2,000 sites on the platelets of patients with thrombasthenia; obligate heterozygotes for the thrombasthenic gene tended to have intermediate values. The antibody apparently inhibited neither platelet aggregation nor fibrinogen binding to platelets.

Heterologous antisera produced against whole platelets by several groups have been shown to precipitate GPIIb and IIIa as a single peak in crossed immunoelectrophoresis, indicating that they exist as a complex when platelets are dissoved in nonionic detergents (50, 52, 54–56). Recent evidence indicates that calcium is required for the maintenance of the complex (54–56).

The hybridoma technique permits the development of large amounts of monoclonal antibody directed against a single epitope (21). By immunizing with whole platelets and then employing a functional screening assay based on the inhibition of platelet-induced agglutination of fibrinogen-coated beads, it was possible to select a clone producing an antibody that blocks the binding of fibrinogen to its receptor on the platelet; it is likely, but not certain, that the antibody binds to a site on the receptor protein(s) itself. Analysis of the effects of this antibody on platelet function and biochemical identification of the glycoproteins to which the antibody binds provide insights into normal platelet physiology.

The 10E5 antibody completely blocked the binding of fibrinogen to platelets in two separate systems: agglutination of fibrinogen-coated beads by platelets in PRP (14) and binding of ^{125}I-fibrinogen to gel-filtered platelets after stimulation with ADP (15). In addition, the antibody produced rapid dissociation of fibrinogn from platelets that had been pretreated with ADP. The

fibrinogen-coated bead assay can be performed with native PRP and thus avoids the potential artifacts introduced when platelets are separated from plasma. Moreover, since agonist activation does not appear to be required for the agglutination (14), this assay is presumably measuring fibrinogen receptors directly; thus, it should not be affected by defects in the transduction mechanism that links agonist binding with fibrinogen receptor exposure. It was previously shown that the assay is sensitive to the defect in thrombasthenic platelets (14), and the current study offers further validation, since antibodies selected on the basis of their ability to inhibit this assay also inhibited fibrinogen-dependent platelet aggregation and the binding of ^{125}I-fibrinogen to ADP-stimulated platelets. Thus, even though the fibrinogen is immobilized on the beads, its interaction with platelets mimics that of fluid-phase fibrinogen during ADP-induced aggregation.

Incubation of normal platelets with nanomolar concentrations of 10E5 antibody produced a thrombasthenic-like state as judged by the major criteria of (*a*) absent primary wave aggregation by ADP, epinephrine, and thrombin (9, 10, 30, 57–59); (*b*) markedly inhibited clot retraction (9, 10, 30, 57, 58); (*c*) absent fibrinogen binding (11, 12, 14, 15); and (*d*) decreased or absent platelet retention and adhesion to glass surfaces (7, 9, 10, 30). In addition, 10E5 antibody-treated platelets retained functions known to be either unaffected or less disturbed in thrombasthenic platelets, including shape change (9, 60) and release induced by high concentrations of thrombin, collagen, and ionophore (9, 10, 30, 57, 61–63). The complete inhibition of release induced by ADP and epinephrine is also consistent with the results found in thrombasthenia (9, 59) and presumably reflects the aggregation-dependence of release induced by these agents (42, 43). The 10E5 antibody dramatically inhibited, but did not completely abolish aggregation induced by collagen when assessed by microscopic observation. Although some reports indicated that thrombasthenic platelets do not aggregate at all in response to collagen (9, 30), studies by several investigators showed that small aggregates are, in fact, produced, even in patients who have no response to ADP or epinephrine (57–59, 63). It is unclear whether these small aggregates are the result of platelets truly attaching to each other or rather result from the adhesion of several platelets to an underlying collagen fibril, which then simulates the appearance of an aggregate. The 10E5 antibody did not inhibit ristocetin-induced agglutination of fixed platelets and this is in accord with the findings using fixed thrombasthenic platelets (58). When ristocetin was added to PRP, 10E5 antibody-treated platelets did not aggregate to the same final extent as normal platelets, presumably reflecting the contribution of fibrinogen binding to platelets after the release reaction was initiated; thrombasthenic platelets are known to behave in a similar way, but the complex oscillatory pattern observed with thrombasthenic platelets stimulated with ristocetin was not found with 10E5-antibody-treated platelets (58). The variable and modest decrease in initial slope of ristocetin- and bovine von Willebrand factor-induced platelet aggregation is in accord with the majority of reports on thrombasthenic platelets (58, 64–66). These studies thus suggest that the abnormality in fibrinogen binding in thrombasthenia is adequate to explain all of the observed abnormalities in platelet function.

The immunofluorescence studies confirmed the specificity of the antibody for an antigen that is uniquely expressed on platelets and megakaryocytes; detectable antigen was decreased or absent from platelets of patients with thrombasthenia, but not from platelets of patients with Bernard-Soulier syndrome. Since immunofluorescent studies are only semiquantitative, the 10E5 antibody was radiolabeled and its ability to bind to platelets measured. Analysis of binding by plotting bound antibody vs. log of free ligand revealed that normal platelets have an average of ~40,000 binding sites/platelet, assuming that the antibody binds to only a single site. This corresponds well with the 39,000 sites/platelet reported by McEver et al. (36) with their monoclonal antibody. In contrast, the platelets from a patient with thrombasthenia had fewer than 2,000 binding sites/platelet and this finding is also in accord with those of McEver et al. (36).

Since monoclonal antibodies are usually directed at a single epitope, it is paradoxical that two glycoproteins (GPIIb and GPIIIa) were precipitated by, and bound to, affinity columns of both 10E5 antibody and the monoclonal antibody prepared by McEver et al. (36). However, since GPIIb and GPIIIa are known to form a complex with each other (18, 50, 52, 54–56), an antibody directed at either of the glycoproteins separately or at an epitope formed when the glycoproteins combine could account for the observed results. Studies on purified HLA-A antigens, which are also composed of a complex of two noncovalently associated proteins, suggest that a complex-dependent epitope may still be localized to one or the other protein (in contrast to an epitope to which both proteins contribute), if one of the proteins undergoes a conformational change when in the complex (67). Completeness requires consideration of the possibilities that either the same epitope is present on each glycoprotein or that the antibody is directed at an epitope on one glycoprotein that, by chance, is cross-reactive with another epitope on the other glycoprotein. The former possibility seems unlikely, given that the tryptic digests of the two glycoproteins are so different (53).

Studies on the platelet fibrinogen receptor and its

role in the pathophysiology of thrombasthenia remain controversial (11–13, 15, 20, 49, 68–71). Some of the unresolved issues are (*a*) whether there is only a single class of receptors or a combination of high and low affinity receptors, (*b*) whether thrombasthenic platelets lack the receptors or have receptors that remain cryptic upon exposure to normal agonists, and (*c*) whether an M_r-70,000 protein involved in fibrinogen receptor function is in addition to, or instead of, GPIIb and GPIIIa. The monoclonal antibody in the present study completely blocked the binding of fibrinogen to platelets, a result that is most compatible with there being a single anatomic site that is crucial to the binding of all fibrinogen molecules. It does not, however, rigorously exclude the possibility of there being multiple classes of receptors since complex mechanism may be operating, as for example, if the binding of the initial fibrinogen molecules produces a conformational change that exposes additional, separate receptor classes. It is clear that our monoclonal antibody recognizes a site on GPIIb and/or GPIIIa. The simplest interpretation of these data is that these glycoproteins are, in fact, the fibrinogen receptor and that thrombasthenic platelets cannot bind fibrinogen because they lack these glycoproteins. The evidence is not conclusive, however, since it is possible that the platelet membrane may be organized such that attachment of the antibody to GPIIb and/or GPIIIa blocks access of fibrinogen to another site that is the actual receptor. It is also possible that the antibody is affecting a molecule involved in fibrinogen receptor exposure, but this seems considerably less likely because (*a*) the antibody does not affect shape change induced by ADP or thrombin, and thus it does not affect the binding of these agonists or the steps involved in changing the platelet's shape, steps that may be common to fibrinogen receptor exposure; (*b*) prostaglandin E_1 at concentrations that prevent ADP from exposing platelet fibrinogen receptors (presumably by affecting the expression mechanism), does not prevent the interaction of platelets with fibrinogen-coated beads while the antibody abolishes this interaction; and (*c*) the antibody was able to dissociate fibrinogen from platelets that had been previously stimulated with ADP. Data derived from additional studies with 10E5 antibody and other monoclonal antibodies that partially or completely block fibrinogen binding to platelets, such as those reported in preliminary form after the current studies were begun (72–77), should help to resolve these issues.

Note added in proof. Since this manuscript was submitted for publication, three full reports of monoclonal antibodies that block fibrinogen binding to platelets have appeared. 10E5 antibody differs from the other antibodies in that it produces 100% inhibition of ADP-induced fibrinogen binding at ~10 μg/ml, whereas the antibody reported by DiMinno G., P. Thiagarajan, B. Perussia, J. Martinez, S. Shapiro, G. Trinchieri, and S. Murphy (1983, *Blood.* 61:140–148) produced a maximum of ~50% inhibition at ~150 μg/ml; the antibody reported by McEver, R. P., E. M. Bennett, and M. N. Martin (1983, *J. Biol. Chem.* 258:5269–5275) produced a maximum of 65% inhibition at 25 μg/ml; and the antibody reported by Bennett, J. S., J. A. Hoxie, S. F. Leitman, G. Vilaire, and D. B. Cines (1983, *Proc. Natl. Acad. Sci. USA.* 80:2417–2421) produced a maximum of 87.5% inhibition at 50 μg/ml.

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