Quantitation of Membrane Glycoprotein IIIa on Intact Human Platelets Using the Monoclonal Antibody, AP-3


A murine monoclonal antibody specific for glycoprotein (GP)IIla was prepared by immunization with a GPIIb- and GPIIla-enriched Triton X-114 extract of platelet membranes. This antibody, designated AP-3, was shown by indirect immunoprecipitation to react solely with GPIIla derived from either P1A1-positive or -negative individuals. The epitope on GPIIla recognized by AP-3 is expressed on dissociated GPIIla as well as on Ca++-dependent complexes of GPIIb and GPIIla, as shown by crossed immunoelectrophoresis in the presence or absence of EDTA. A previously described monoclonal antibody specific for the GPIIb/IIIa complex (AP-2) inhibited platelet aggregation induced by ADP, thrombin, collagen, or arachidonic acid (Pidard et al, J Biol Chem 258:12582–12586, 1983). In contrast, AP-3 had no effect on aggregation induced by any of these reagents, a finding similar to that previously reported for the GPIIb-specific monoclonal antibody, Tab (McEver et al, J Clin Invest 66:1311–1318, 1980). At saturation, 40,200 AP-3 molecules were bound per platelet, a value similar to that obtained for AP-2 or Tab. Thus, data derived using AP-3 indicate that significant amounts of free GPIIla are not present, thereby supporting the hypothesis that GPIIb and GPIIla exist complexed in a 1:1 stoichiometry in the plasma membrane of intact, nonactivated platelets.

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THE HUMAN platelet membrane glycoprotein IIb/IIIa complex is thought to mediate fibrinogen binding and platelet aggregation induced by adenosine diphosphate, thrombin, arachidonic acid, and a number of other physiologic agents. This complex has been shown by a number of investigators to be dissociable into its glycoprotein components in the presence of divalent cation chelating agents such as EDTA or EGTA. Several investigators have prepared murine monoclonal antibodies that interact specifically with the intact GPIIb/IIIa complex and are able to inhibit both fibrinogen binding and platelet aggregation induced by the above-mentioned agonists. On the other hand, one monoclonal antibody, Tab, has been described that has specificity for only GPIIb. Unlike the GPIIb/IIIa complex-specific antibodies, this antibody does not inhibit either platelet aggregation or fibrinogen binding, suggesting that either GPIIIa alone or the intact IIb/IIIa complex mediates these functions.

Both Tab and several complex-specific antibodies described above have been used to determine the number of GPIIb molecules or GPIIb/IIIa complexes, respectively, on the platelet surface, and have in general found 40,000 to 60,000 antibody binding sites per platelet. Since the number of GPIIb molecules was similar to the number IIb/IIIa complexes, these data provided evidence that GPIIb and IIla exist in a 1:1 stoichiometry on the platelet surface. Due to the lack of a specific probe for GPIIIa, however, the possibility that a greater number of GPIIIa molecules could exist, either free or bound to the complex, has not been investigated.

Using a Triton X-114 (Sigma, St Louis) solubilized extract of platelet membranes highly enriched in GPIIb and GPIIIa as the immunogen, we have prepared a monoclonal antibody specific for GPIIIa.

This report describes the specificity of this antibody, its binding characteristics, and its effect on selected platelet functions.

MATERIALS AND METHODS

Production and Characterization of Monoclonal Antibodies

Human platelet membranes, prepared by methods previously described, were solubilized in Triton X-114, and the integral membrane proteins selectively extracted utilizing the unique temperature-dependent phase-separation properties of the detergent. The resulting detergent phase (D-phase), composed largely of glycoproteins IIb and IIIa (GPIIb and GPIIIa) was made detergent free with BioBeads Sm-2 (Bio-Rad, Richmond, Calif), using procedures suggested by the manufacturer. Ten micrograms of detergent-free D-phase in 100 μL of PBS was mixed with an equal volume of complete Freund’s adjuvant and injected intraperitoneally into BALB/c mice. At weekly intervals, mice were boosted intraperitoneally with 10 μg of detergent-free D-phase in 50% incomplete Freund’s adjuvant for a total of four weeks. Three days after the last immunization, the splenocytes were harvested from the mice and

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fused to one tenth their number of NS1/Ag4-I14 myeloma cells using standard methodology. Culture wells showing vigorous growth in HAT-medium were screened for the production of anti-GPIIIa/GPIIIb antibodies by an indirect radioimmunoassay employing the immunogen bonded to polyvinyl chloride microtiter plates and using rabbit anti-mouse IgG followed by 125I-protein A as the probe. Positive wells were repeatedly subcloned by limiting dilution to ensure monoclonality of each cell line, and large quantities of one monoclonal antibody, AP-3, were prepared in ascitic fluid by passaging the hybridomas through pristane-primed Balb/c mice. The subclass of AP-3 was determined by double-diffusion of hybridoma culture supernatant against anti-mouse subclass-specific antisera (Miles, Elkhart, Ind), and was found to be an IgG1.

**Purification of Mouse IgG**

For many procedures, IgG was isolated from ascites fluid using caprylic acid by a modification of the technique of Steinbuch and Audran.17 Briefly, ascites fluid containing monoclonal AP-3 was diluted with two volumes of 0.06 μmol/L acetic acid, pH 4.0 (adjusted with NaOH) and 100% caprylic acid (Sigma) was added dropwise to a final concentration of 1.1% (vol/vol). The mixture was stirred at room temperature for 30 minutes, and the precipitate was removed by centrifugation. The supernatant was then dialyzed at 4°C ± 50 mmol/L NaPO4, 150 mmol/L NaCl, pH 7.4 (PBS). Recovery of monoclonal antibody was nearly quantitative, resulting in a preparation that was greater than 80% mouse IgG, as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For quantitative binding assays, AP-3 was further purified by DEAE Affi-gel (BioRad) blue chromatography18 when necessary to yield homogeneous IgG. Protein concentration was measured using 

\[
E_{380} = 14.3 \text{ and was normally 0.5 to 1.0 mg/mL.}
\]

**Radiiodination of Proteins in Solution**

Fifty microliters of D-phase (1 mg/mL) was iodinated with 1 mCi of carrier-free Na125I (New England Nuclear, Boston), using insolubilized lactoperoxidase (Enzymobeads, BioRad Laboratories) according to the manufacturer’s directions. Unbound 125I was removed by gel filtration through Sephadex G-25 equilibrated in PBS containing 0.1% NP-40, pH 7.0. AP-3 and protein A were labeled in a similar fashion, except that NP-40 was left out of the gel filtration buffer. The specific activity of the AP-3 used in binding assays was approximately 0.25 μCi/μg protein. Labeled antibodies were stored in small aliquots at −80°C until use.

**Indirect Immunoprecipitation**

Two to ten microliters containing 500,000 cpm of 125I-labeled D-phase was diluted into 150 μL of immunoprecipitation buffer (IPB) (25 mmol/L NaPO4, 150 mmol/L NaCl, 10 mmol/L EDTA, 0.1% NP-40, 0.1% bovine serum albumin, pH 7.0) was incubated with 50 μL of antibody, and allowed to react for two hours at room temperature. Both rabbit anti-platelet and rabbit preimmune sera were used at dilutions of one to ten. Anti-platelet and control monoclonal antibodies were added as undiluted culture supernatants. In order to immunoprecipitate mouse IgG, monoclonal antibodies, which do not bind well to protein A, 5 μg of rabbit anti-mouse IgG (1 mg/mL) was added and allowed to incubate for an additional 60 minutes. Finally, 50 μL of a 10% suspension of formalin-fixed protein A bearing Staphylococcus aureus cells (Cowan Strain I)19 was added. Following a 30-minute incubation, the cells, containing bound immune complexes, were washed four times in IPB, and the final pellet suspended in 50 mmol/L TRIS, 2% SDS, 5% glycerol, pH 6.8. The mixture was boiled for three minutes, and the cells were removed by centrifugation at 5000 g for ten minutes. The clear supernatants, containing the immunoprecipitated antigens, were applied to polyacrylamide gels for electrophoresis, which was performed as previously described.19 Radiolabeled proteins were visualized by exposing the dried gel to Kodak XRP-1 film at −70°C for 12 to 36 hours in the presence of a DuPont Lightening-Plus Image Intensifying Screen (DuPont, Wilmington, Del).

**Crossed Immunoelectrophoresis**

Crossed immunoelectrophoresis (CIE) was performed according to the method of Kunicki et al. Briefly, 100 μg of Triton X-100 solubilized platelet proteins (platelet lysates) were electrophoresed at 10 V/cm for 75 minutes at 16°C in 1% agarose dissolved in 38 mmol/L TRIS, 100 mmol/L glycine, 0.5% Triton X-100, pH 8.8. Strips of agarose containing the separated proteins were then run at 2 V/cm for 18 hours into a 2-cm intermediate gel containing 1 x 100 cpm of iodinated AP-3 followed by a 5-cm gel containing rabbit anti-whole platelet antibody. Immunoprecipitation arcs were visualized by staining with Coomassie blue R-250. Radiolabeled protein complexes were identified by exposing the dried CIE plates to Kodak X-Omat RP-1 film (Kodak, Rochester, NY) as described above.

**Binding Assays**

Binding assays were performed in triplicate at room temperature using platelet rich plasma (PRP) prepared from whole blood anticoagulated with acid citrate dextrose and used within three hours of venipuncture. Varying concentrations of 125I-AP-3 diluted into platelet poor plasma were added to PRP such that the final platelet concentration was 1 x 106/mL and the final volume 300 μL. After an incubation time of 60 minutes, a 150 μL aliquot was layered onto 500 μL of 20% sucrose dissolved in 20 mmol/L TRIS, 150 mmol/L NaCl pH 7.4 and centrifuged at 12,000 g for two minutes. After removing the supernatant, centrifuge tube tips were amputated and the bound radioactivity determined in a gamma counter. Bound radiolabeled material was pure mouse IgG, as judged by SDS-PAGE and autoradiographic analysis of the pellets. Total radioactivity was determined by counting an identical 100-μL aliquot of each reaction mixture. Nonspecific binding was determined in parallel tubes using a 250:1 ratio of simultaneously added unlabeled-to-labeled AP-3. This value, which was typically less than 5%, was subtracted from the bound cpm to yield total specific bound cpm. In separate experiments, increasing concentrations of platelets were added to a constant amount (200 ng) of 125I-AP-3, thereby increasing the platelet/AP-3 ratio until saturation was reached. Once the maximum percentage of bindable antibody was determined, this value was used to determine the precise amount of unbound (free) active antibody for Scatchard20 and Klotz21 plot analyses.

**Platelet Aggregometry**

Aggregometry was performed in a Biodata PAP-4 aggregometer (Bio/Data Corp, Hatboro, Pa) at 37°C with a stirring rate of 1200 rpm. PRP at 300,000 platelets/μL was prepared from citrated whole blood and used within three hours after venipuncture. Adenosine diphosphate (ADP) was used to induce aggregation at concentrations of 1.3, 2.2, and 4.4 μmol/L. Arachidonic acid- and ristocetin-induced aggregation were performed at concentrations of 1.6 mmol/L and 30 mmol/L, respectively. The effect of monoclonal antibody on platelet aggregation induced by these agents was measured by preincubating the PRP with antibody at concentrations of 10, 20, or 40 μg/μL for five minutes at 37°C with stirring before the induction of aggregation.

**RESULTS**

**Production of a Monoclonal Antibody to GPIIIa**

Balb/c mice were immunized with a Triton X-114 solubilized platelet membrane protein fraction (D-
Phase) highly enriched in GPIIb and GPIIIa. Following hybridoma production and cell cloning, cell culture supernatants found to contain anti-D-phase antibodies by radioimmunoassay were further characterized by indirect immunoprecipitation of $^{125}$I-labeled D-phase proteins in the presence of 10-mm EDTA. Following immunoprecipitation, solubilized immune complexes were applied to a 5% to 15% SDS polyacrylamide gel and analyzed by autoradiography. As shown in Fig 1, a polyclonal rabbit antiplatelet antiserum immunoprecipitated a number of $^{125}$I-labeled platelet proteins, including GPIIb and GPIIIa, (Fig 1B), while one monoclonal antibody, designated AP-3, specifically immunoprecipitated only GPIIIa (Fig 1D).

The human platelet alloantigen, PI, has previously been shown to be located on GPIIIa. AP-3 was able to bind to GPIIIa derived from either PI positive or PI negative platelets (not shown), indicating that the epitope recognized by this monoclonal antibody is distinct from the PI alloantigenic determinant. This was further supported by the inability of AP-3 to react with a 15-kilodalton tryptic polypeptide fragment of GPIIIa that contains PI (P.J. Newman, unpublished experiments).

In order to further characterize the epitope recognized by AP-3, Triton X-100 platelet lysates prepared in the presence or absence of 5 mmol/L EDTA were analyzed by CIE employing radiolabeled AP-3 in the intermediate gel. In the presence of Ca$^{2+}$, GPIIb and GPIIIa form a single, calcium-dependent, immunoprecipitin arc (Fig 2A). In the presence of either EDTA or EGTA, additional precipitin arcs containing free GPIIb or free GPIIIa are detected (Fig 2B). As shown in the corresponding autoradiograms, AP-3 bound to GPIIIa whether it was complexed to GPIIb (Fig 2C) or separated from it (Fig 2D); however, AP-3 did not bind to the precipitin arc containing free GPIIb.

**Binding at AP-3 to Whole Platelets**

$^{125}$I-AP-3, at final concentrations ranging from 0.1 to 10 $\mu$g/mL, was added to freshly prepared PRP (adjusted to 1 x $10^8$ platelets/mL). Equilibrium was reached in 30 minutes at room temperature and remained constant for at least two hours. Binding data (Fig 3A) analyzed by the methods of Scatchard$^{24}$ (Fig 3B) and Klotz$^{25}$ (not shown) showed that 40,200 molecules of AP-3 were bound per platelet at saturation, with an average dissociation constant ($K_d$) of 0.72 nmol/L.

**Effect of AP-3 on Platelet Aggregation**

In vitro platelet aggregation induced by ADP, arachidonic acid, thrombin, or ristocetin was unaffected by prior addition of AP-3, even at concentrations as high as 40 $\mu$g of monoclonal antibody/mL of PRP. In contrast, a monoclonal antibody specific for GPIb (AP-1) completely abolished thrombin- or ristocetin-induced aggregation, and a GPIIb/IIIa complex...
specific monoclonal antibody (AP-2) inhibited aggregation induced by ADP, thrombin, and arachidonic acid. AP-3 itself did not induce platelet agglutination or aggregation.

**DISCUSSION**

We have prepared a unique murine monoclonal antibody with demonstrated specificity for human platelet membrane glycoprotein IIIa. That GPIIIa contained the epitope recognized by AP-3 was determined by indirect immunoprecipitation of a radiolabeled platelet membrane preparation enriched in GPIIb and GPIIIa. Since antibodies directed against even one component of the GPIIb/IIIa complex can coprecipitate both glycoproteins, certain technical precautions were taken to ensure accurate determination of the specificity of AP-3. First, indirect immunoprecipitation was performed under conditions known to result in maximum complex dissociation (10 mmol/L EDTA for 60 minutes at 25°C). The degree of complex dissociation in such preparations was confirmed by CIE. Second, since GPIIb and GPIIIa have similar mobilities on SDS-PAGE, especially under reducing conditions, immunoprecipitates were analyzed on 5% to 15% linear gradient polyacrylamide gels in order to maximize the resolution of the two glycoproteins. Finally, the technique of crossed immunoelectrophoresis with 125I-AP3 incorporated into the intermediate gel was employed, since this method allows simultaneous analysis of ligand binding to glycoproteins IIb and IIIa in both their free and complexed forms. As we have shown (Fig 2), AP-3 bound to the precipitin arc containing free GPIIIa, but not to the arc containing free GPIIb.

In addition to its reactivity for dissociated GPIIIa, AP-3 was able to bind to calcium-dependent complexes of GPIIb and GPIIIa during CIE, indicating that the region of GPIIIa that is involved in binding to GPIIb during complex formation is not likely to be close to the AP-3 binding site. Since these two molecules can readily be purified to homogeneity,25 AP-3 can be used to immobilize GPIIIa in order to study the mechanism of its interaction with GPIIb.

In the presence of AP-3, platelets were able to aggregate fully in response to ADP, arachidonic acid, thrombin, or ristocetin. Since ADP-induced aggregation was normal, one can assume that AP-3 does not inhibit the binding of fibrinogen to platelets. The monoclonal antibody Tab, which is specific for only GPIIb, also had little effect on platelet aggregation.26 Similarly, monospecific polyclonal rabbit anti-GPIIb and anti-GPIIIa, either singly or in combination, did not inhibit the platelet aggregation response to physiologic stimuli.26 In contrast, all GPIIb/GPIIIa complex-specific monoclonal antibodies reported to date have either partially or completely blocked platelet aggregation.27,28 Similarly, monospecific polyclonal rabbit anti-GPIIb and anti-GPIIIa, either singly or in combination, did not inhibit the platelet aggregation response to physiologic stimuli.26 In contrast, all GPIIb/GPIIIa complex-specific monoclonal antibodies reported to date have either partially or completely blocked platelet aggregation.27,28 Similarly, monospecific polyclonal rabbit anti-GPIIb and anti-GPIIIa, either singly or in combination, did not inhibit the platelet aggregation response to physiologic stimuli.26 In contrast, all GPIIb/GPIIIa 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pleiotropy has not yet been ruled out, since the characterization of C15 and C17 employed methods that are incapable of distinguishing between complex-specific antibodies and antibodies specific for the free glycoproteins. It is also plausible that C15 and C17 may be binding to different GPIIIa sites that are responsible for ADP-induced aggregation. In either event, the conclusions drawn concerning their effect on platelet function await further definition of the specificities of those antibodies.

Since AP-3 binds to both known allelic forms of GPIIIa, PI\(^{A1}\) and PI\(^{A2}\) (\(-\) PI\(^{A1}\) negative), and since GPIIIa is recognized by AP-3 whether it is free of or complexed to GPIIb, we were able to reliably quantitate the total number of GPIIIa molecules present on the platelet surface. As we have shown, AP-3 bound to approximately 40,000 sites per platelet at PRP at a platelet concentration of 1 \(\times\) 10\(^8\)/mL. This value is in close agreement with that observed using monoclonal antibodies directed against both the intact GPIIb/IIIa complex\(^{10-12}\) and GPIIb alone.\(^{10,13}\) Moreover, complex-specific antibodies have been shown to bind the same number of sites on both resting and activated platelets,\(^{10,12}\) suggesting that GPIIb and GPIIIa exist in a preformed complex in unstimulated platelets. Taken together, these data are consistent with the hypothesis that GPIIb and GPIIIa exist in the platelet plasma membrane in a 1:1 stoichiometry and that significant amounts of free GPIIb and GPIIIa do not exist on the platelet surface. The combined use of monoclonal antibodies directed against different sites on GPIIb and GPIIIa should give further insight into the orientation of these two membrane glycoproteins and their specific roles in platelet function.

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