Platelet–Collagen Adhesion: Inhibition by a Monoclonal Antibody That Binds Glycoprotein IIb

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ABSTRACT To identify platelet surface structures involved in adhesion to collagen, the effect of 16 murine antiplatelet membrane hybridoma antibodies were tested in a defined, in vitro assay. Four of these antibodies inhibited platelet–collagen adhesion and reacted with a polypeptide with M_r ~ 125,000, as determined by immunoblots after gel electrophoresis under reducing conditions. Through detailed studies with one of these antibodies, the monoclonal antibody PMI-1, the relevant antigen was identified as platelet glycoprotein IIbα, based upon (*a*) co-migration with this glycoprotein in two-dimensional gel electrophoresis and (*b*) co-purification by immunoaffinity chromatography with a protein with apparent M_r identical to that of glycoprotein III, under conditions in which glycoproteins IIb and III form a complex.

Univalent antibody fragments prepared from monoclonal antibody PMI-1 inhibited >80% of platelet–collagen adhesion, and inhibition was completely blocked by the immunopurified antigen. These results indicate that glycoprotein IIb participates in some aspect of platelet–collagen adhesion. In contrast, the purified antigen only partially neutralized a polyclonal antiserum that blocked platelet–collagen adhesion, to a maximum of ~25%, at saturating antigen concentrations. Thus, by these immunological criteria, glycoprotein IIb is not the only molecule involved in this process.

Antibodies are a major tool for identification of molecules that participate in cell adhesion. Iterative absorption of polyclonal antibodies has permitted identification of several cell adhesion molecules (1). An additional approach has employed monoclonal antibodies, which offer the advantage of exquisite immunologic specificity. These have been used to identify molecules involved in the adhesion of myoblasts (2) and melanoma cells (3) to surfaces in tissue culture, and of *Dictyostelium discoideum* cells to each other (4).

In the preceding article, we described a polyclonal antiserum raised against whole human blood platelets that was employed to identify and partially purify neutralizing antigens involved in platelet–collagen adhesion (5). This work was, however, greatly complicated by the finding that the adhesion that was measured in this assay apparently involved several immunologically distinct molecular entities, each of which only partially neutralized the polyclonal antiserum. Because of the complexities of the system, we turned to monoclonal antibodies to develop monospecific reagents that might react with the relevant entities individually, permitting their clear identification and independent characterization. We describe here a monoclonal antibody that reacts specifically with platelet membrane glycoprotein IIb and blocks platelet–collagen adhesion. The platelet antigen purified by immunoaffinity chromatography, using immobilized monoclonal antibody, only partly neutralizes the polyclonal antiserum described in the preceding article (5), supporting the conclusion that multiple entities are involved in platelet–collagen adhesion.

MATERIALS AND METHODS

Hybridoma Production: Balb C mice were immunized biweekly with 50 μg of platelet membranes (6), emulsified alternately in complete and incomplete Freund's adjuvant. After four immunizations, mice were bled from the retroorbital plexus, and sera were screened for antiplatelet membrane antibodies utilizing a solid-phase radioimmunoassay (see below). The animals showing the highest titers were boosted with 300 μg platelet membranes intravenously 6, 3, and 1 d before sacrifice. Mice were killed, and washed spleen cell suspensions containing 1.5 × 10^8 cells/ml fused with P3X63-Ag8.653

THE JOURNAL OF CELL BIOLOGY · VOLUME 99 DECEMBER 1984 2056–2060
 · 0021-9525/84/11/2056/06 $1.00

myeloma cells at a 5:1 myeloma:spleen cell ratio with 30% (wt/vol) polyethylene glycol-1000 for 7 min as described (7). The fused cells were grown in HAT medium (7) for 3 d before being distributed in 32 microtiter plates, each containing 96 wells.

Growing single colonies of cells were screened in the radioimmunoassay, and 58 culture supernatants, which gave signals greater than twice background, were cloned by limiting dilution at a density of 0.5 cells per well. 21 d after subcloning, position subclones were selected utilizing the radioimmunoassay on culture supernatants. 16 of these were subsequently expanded and frozen in liquid nitrogen with cryoprotection. In addition, positive subclones of well 4D6, which secreted the monoclonal antibody designated PMI-1, were recloned on two separate occasions, then expanded, and 5×10^7 cells/mouse injected intraperitoneally into pristane-primed mice (8). Ascites fluid developed within 1 wk of injection and was harvested and stored at −70°. Ascites from all subclones exhibited a single band in the γ region on cellulose acetate electrophoresis.

Radioimmunoassay: Polyvinyl chloride microtiter plates (#3911, Falcon Plastics, Oxnard, CA) were coated with 5 μg/well platelet membranes in phosphate-buffered saline at 4° for 24–72 h. After washing and blocking with 2% bovine serum albumin, the plates were exposed to culture supernatants, ascites or sera for 3 h at 22°, washed in phosphate-buffered saline containing 0.05% Tween 20 and 0.1% bovine serum albumin, and developed utilizing affinity-purified goat anti-mouse IgG radioiodinated to a specific activity of 2–5 μCi/μg by the chloramine-T technique (8). After washing the wells were cut out with a hot wire and counted in a γ counter. Positive controls contained serum from mice immunized with platelet membranes, and negative controls contained normal mouse serum, culture medium, and medium from the parent myeloma line.

Preparation of Fab from Clone PMI-1: IgG was prepared from the ascites fluid of clone PMI-1 by affinity chromatography on a column of protein A–agarose (Pharmacia Fine Chemicals, Piscataway, NJ) in 0.1 M sodium phosphate buffer, pH 8.0. Fab was prepared by digestion with papain as described previously (5) and Fc fragments were removed by rechromatographing the solution on protein A–agarose. Fabs were tested for purity by PAGE in SDS under nonreducing conditions (9). They were also tested for reactivity with goat anti-mouse Fab (Cappel Laboratories, Philadelphia, PA) by immunodiffusion.

PAGE and Immunoblotting: PAGE in slab gels under standard reducing conditions was performed as described by Laemmli (9) using prestained protein standards (Bethesda Research Laboratories, Rockville, MD) as measures of apparent molecular weights. Proteins were located with a silver stain (10). Immunoblots of proteins electrophoretically transferred to nitrocellulose paper were performed as described previously (4). Antibodies bound to the antigens on the nitrocellulose transfers were visualized by incubation with goat anti-mouse peroxidase conjugated IgG (Cappel Laboratories) followed by development in 0.16 mg/ml 4-*o*-chloronaphthol containing 0.3% H_2O_2, as described previously (4).

Two-dimensional gel electrophoresis under reducing and nonreducing conditions was performed in 7.5% acrylamide gels by the technique of Phillips and Agin (11). In these experiments, the platelet membrane proteins were iodinated by the lactoperoxidase catalyzed iodination technique (11) as described (12).

Immunoaffinity Chromatography: 5 mg of monoclonal antibody PMI-1 was dialyzed against 0.1 M bicarbonate buffer, pH 8.0, and coupled to 2 ml of cyanogen bromide-activated Sepharose (Pharmacia Fine Chemicals) as described by the manufacturer. After coupling, active groups were blocked by incubation in 0.1 M ethanolamine, pH 9.0, and the gel was extensively washed.

Immunoaffinity chromatography was performed essentially as described by McEver et al. (13). Detergent extracts of outdated platelets were prepared using 1% octylglucoside or 1% Triton X-100 as described (5). Samples were dialyzed against Tris saline (15 mN Tris-HCl, pH 7.4, 126 mM NaCl, 5.4 mM KCl, 5×10^{-5} M $CaCl_2$, 1 mM $MgCl_2$, 0.1% glucose) to reduce detergent concentration, applied to the column, and recirculated through the column for 24 h at 4°C to maximize specific binding. Washing of the column was done by successively eluting with: 0.15 M NaCl, 20 mM Tris-HCl, pH 7.4; 1 M NaCl, 20 mM Tris-HCl, pH 7.4; 0.1 M Na borate, pH 8.0. All buffers contained 0.1% octylglucoside. Bound platelet protein was then eluted using 0.2 M diethylamine, pH 11.5. The eluate was immediately neutralized with 1 M Tris-HCl, pH 6.8, and dialyzed against Tris saline.

Platelet–Collagen Adhesion Assay: This was performed as described previously (5, 14). Screening the effects of tissue culture supernatants from hybridoma clones was done by preincubating ^{51}Cr-labeled platelets with dilutions of the supernatants that had been heat inactivated at 56°C for 20 min, then measuring platelet–collagen adhesion in the normal way. Measurement of the effects of Fab prepared from monoclonal antibodies on platelet–collagen adhesion, and neutralizing effects of various antigens was done as described previously (5).

RESULTS

In a search for monoclonal antibodies that block specific molecules involved in platelet–collagen adhesion, we screened a series of antiplatelet membrane hybridoma supernatants as inhibitors of our adhesion assay. For these initial experiments, we used intact immunoglobulins in culture medium, to obviate the need to prepare univalent antibody fragments. Many of these antibodies agglutinated the platelets at high concentrations and were, therefore, screened at the highest concentrations that did not cause agglutination. Of 16 hybridoma supernatants screened in this assay, four had significant effects on platelet–collagen adhesion, inhibiting at least 20%, whereas the others had no apparent effect. All four of the active hybridoma supernatants reacted with a protein band of $M_r \sim$ 125,000 in whole-platelet extracts, when examined by the nitrocellulose-transfer technique (Fig. 1, *a–d*). In contrast, a hybridoma supernatant that did not block platelet–collagen adhesion reacted with several other bands (Fig. 1 *e*). Supernatants from the parent cell line used to make the hybridomas, or from a cell line making an unrelated monoclonal antibody did not bind platelet proteins (Fig. 1, *f* and *g*).

We selected one of the adhesion-blocking cell lines, PMI-1 for further experiments. It was recloned by limiting dilution and injected into a mouse peritoneum. The resultant ascites fluid contained an IgG_1k antibody that was homogeneous by cellulose acetate electrophoresis. To obviate effects of the Fc region or bivalency, Fab fragments were prepared from the purified antibody. The Fab fragments inhibited ~50% of platelet–collagen adhesion at 20 μg/ml, and inhibited >80% of adhesion at higher concentrations (Fig. 2). In contrast, Fab fragments prepared from another monoclonal antibody,

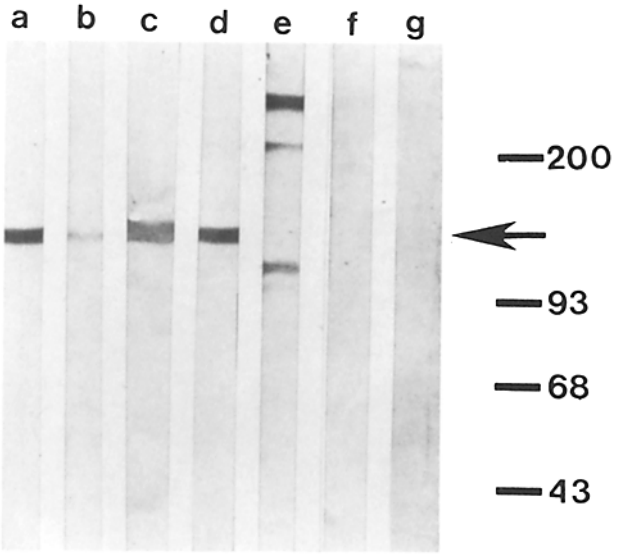

FIGURE 1 Immunoblotting of whole-platelet extract with five hybridoma supernatants that bind platelet surface antigens. Tissue culture supernatants of antiplatelet hybridoma clones were diluted 1:4, incubated with a nitrocellulose blot of a 7.5% SDS polyacrylamide slab gel of whole human platelets, and developed using goat anti-mouse peroxidase conjugate. Supernatants were derived from the following: (lane *a*) clone 23B7; (lane *b*) clone 25B5; (lane *c*) clone PMI-1; (lane *d*) clone 6D2; (lane *e*) clone 23E7; (lane *f*) myeloma MP-8 ascites fluid; and (lane *g*) a clone producing a monoclonal antibody directed against ferritin. Bars represent the positions of prestained molecular weight standards with apparent $M_r \sim$ 200,000, 93,000, 68,000, and 43,000. The arrow indicates the migration of a protein with apparent $M_r \sim$ 125,000.

22C4, that bound to another platelet membrane molecule, glycoprotein III, had no effect on platelet–collagen adhesion at up to 240 μg/ml (Fig. 2).

In that antibody PMI-1 reacted with a unique platelet antigen, we covalently linked it to a solid support and used it to purify the relevant antigen by immunoaffinity chromatography. With this procedure, we obtained a preparation enriched in a major protein band with M_r ~ 125,000 as well as a fainter band with M_r ~ 100,000 upon PAGE in SDS under reducing conditions (Fig. 3). These bands had mobilities like those of glycoproteins IIb and III. Since the antibody bound only to the band with M_r ~ 125,000, it seemed likely that we had immunopurified the glycoprotein IIb/III complex, as had others (13, 15, 16) with other monoclonal antibodies.

To further analyze antibody specificity, membranes of platelets that had been surface labeled with ^{125}I were separated by two-dimensional electrophoresis and electrophoretically transferred to nitrocellulose paper. The separated proteins were then analyzed by immunoblotting with PMI-1, which was developed with an immunoperoxidase procedure and by autoradiography to determine the location of the platelet surface proteins that had incorporated ^{125}I. PMI-1 bound to a single spot of apparent M_r ~ 133,000 nonreduced, ~123,000 reduced, confirming its monospecificity (Fig. 4). This spot exactly coincided with a spot heavily labeled with ^{125}I that ran beneath the diagonal (Fig. 4). It is identified as the glycoprotein IIbα band, because of its mobility in this gel system and the intensity of labeling.

The antigen, immunopurified on a PMI-1 column, reacted well in immunoblots not only with monoclonal antibody PMI-1 (Fig. 5, lane *g*) but also with a partially purified antiplatelet antibody preparation (Fig. 5, lane *b*) that blocked platelet–collagen adhesion (5). The same protein was apparently present in the "enriched neutralizing antigens" preparation (Fig. 5, lanes *e* and *f*) that partially inhibited the adhesion-blocking activity of the polyclonal antiserum (5). It was also prominent in the detergent phase of a 1% Triton X-114 partition of an extract of whole-platelet membranes (Fig. 5, lane *h*), indicating that it is hydrophobic and appears to be an integral membrane protein (17).

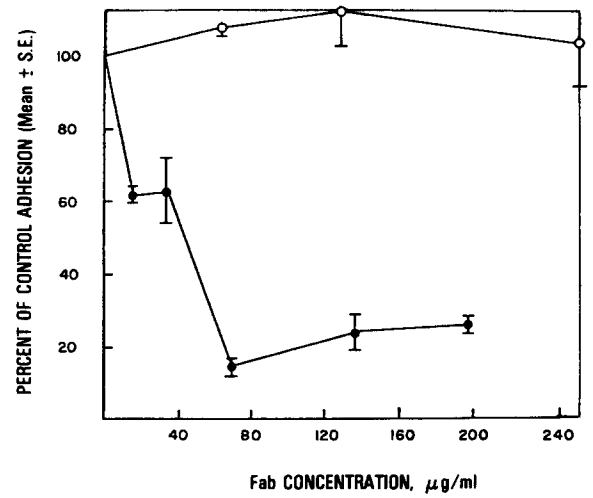

FIGURE 2 Effects on platelet–collagen adhesion of Fabs of monoclonal antibodies PMI-1 (●) and 22C4 (○). The Fabs were prepared from ascites fluid dialyzed into modified Tyrode's buffer, pH 7.4, and tested in the platelet–collagen adhesion assay. Fabs were preincubated with ^{51}Cr-labeled platelets in assay buffer for 10 min at 37°C, and platelet–collagen adhesion was assayed as described in Materials and Methods.

FIGURE 3 PAGE of platelet proteins purified from a crude octylglucoside extract by binding to a column containing immobilized monoclonal antibody PMI-1. Proteins were visualized with a silver stain. The upper arrow indicates the migration of glycoprotein IIb, and the lower migration of a glycoprotein IIIa, electrophoresed in parallel.

The protein with M_r ~ 125,000 is apparently highly antigenic. This is suggested not only by the ease of detection of multiple monoclonal antibodies that apparently react with it (Fig. 1) but also by the abundance of antibodies that react with it in a polyclonal antiserum. This is indicated by the strong reaction of a partially purified polyclonal antiserum (5) with a band with M_r ~ 125,000 in a crude platelet extract (Fig. 5, lane *a*). Furthermore, it is the only prominent protein band in enriched neutralizing antigens (5) that binds this partially purified antiserum (Fig. 5, lane *c*).

Because the proteins purified on the monoclonal antibody column reacted with the adsorbed polyclonal antibody on immunoblots, we sought to determine whether they neutralized the rabbit antibody in adhesion assays. The protein preparation that was purified on the immobilized monoclonal antibody column neutralized up to ~20% of the platelet–collagen adhesion-blocking activity of the polyclonal Fab (Fig. 6), under standard assay conditions in which the polyclonal Fab inhibited 50% of platelet–collagen adhesion. This result was consistently observed in a number of experiments, and in no case was inhibition greater than 25%. In contrast, the enriched neutralizing antigens prepared in the previous report neutralized the polyclonal Fab by a maximum of ~60% (Fig. 6). These results indicate that antibodies against a mixture of glycoproteins IIb and III account for only a part of the adhesion-blocking activity of the polyclonal antibody. In contrast, the immunopurified glycoproteins IIb and III completely neutralized the inhibitory activity of Fab prepared from PMI-1 (Fig. 7).

DISCUSSION

These experiments underscore the power of using monoclonal antibodies to screen for cell adhesion molecules and support

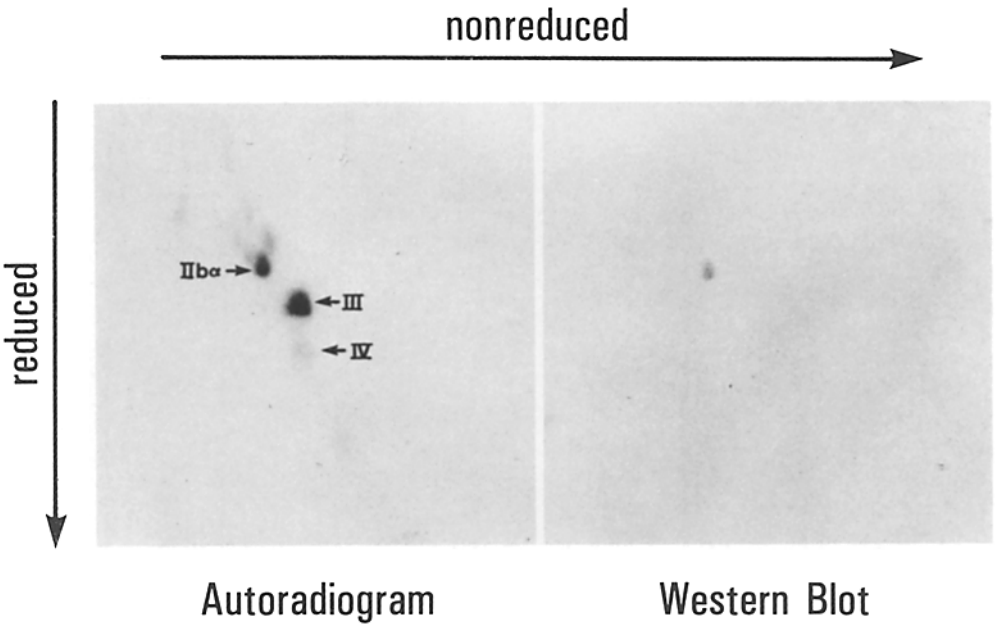

FIGURE 4 Co-migration of the antigen that reacts with monoclonal antibody PMI-1 and the α-subunit of glycoprotein IIb, on two-dimensional PAGE. Electrophoresis was done under nonreducing conditions in one dimension and under reducing conditions in the other. The α-subunit of glycoprotein IIb is indicated by the arrows on the autoradiogram of platelet membrane proteins that had been radioiodinated by surface labeling. The antigen that binds monoclonal antibody PMI-1 is visualized on a nitrocellulose transfer by an immunoperoxidase procedure, designated Western blot.

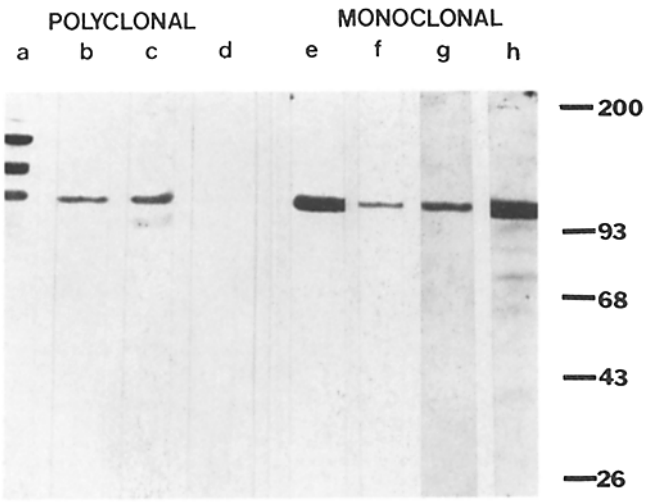

FIGURE 5 Immunoblots with polyclonal immunoglobulins (lanes *a–d*) and monoclonal antibody PMI-1 (lanes *e–h*). Whole platelets and various purified preparations were electrophoresed and transferred to nitrocellulose sheets as described in Materials and Methods. Lanes *a–c* were reacted with a partially purified polyclonal antiplatelet immunoglobulin preparation, which had been adsorbed with the components of an octylglucoside extract of platelets that did not bind to immobilized wheat germ agglutinin (5). Lane *d* was reacted with preimmune rabbit antiserum. Lanes *e–h* were reacted with monoclonal antibody PMI-1 purified from ascites fluid by affinity chromatography on protein A–Sepharose. The antigen preparations used were the following: (lanes *a*, *d*, and *e*) whole platelets; (lanes *b* and *g*) protein purified by immunoadsorption on a column of monoclonal antibody PMI-1; (lanes *c* and *f*) enriched neutralizing antigens (5); and (lane *h*) detergent phase of 1% Triton X-114 partition of an extract of whole platelet membranes. Bound antibodies were visualized by the peroxidase procedure, as described in Materials and Methods.

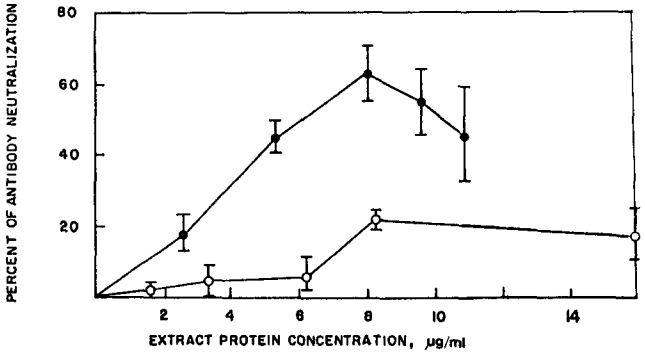

FIGURE 6 Comparative neutralization of antiplatelet Fab by antigens purified on a PMI-1 column, and enriched neutralizing antigens (5). The antiplatelet Fab was added at a concentration that inhibited 50% of platelet–collagen adhesion. Neutralizing activity of enriched neutralizing antigens (5) (●), and antigen eluted from a column of monoclonal antibody PMI-1 (○) were tested at the concentrations indicated. Results are mean ±SE of triplicate determinations.

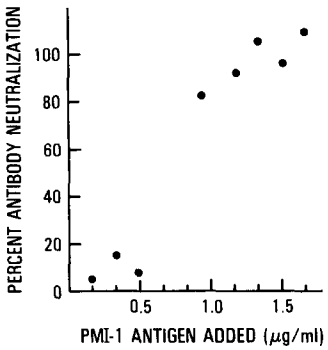

FIGURE 7 Neutralization of monoclonal Fab PMI-1 by antigen affinity-purified on immobilized PMI-1. Fab concentration was adjusted to yield ~50% inhibition of platelet–collagen adhesion, and neutralization by the indicated concentrations of antigen was determined.

previous findings in other systems that such monospecific agents can completely block adhesion (2–4). They also indicate that glycoprotein IIb participates directly or indirectly in some aspect of the platelet–collagen adhesion process.

The best evidence that the target for monoclonal antibody PMI-1 is glycoprotein IIb comes from studies of its migration in two-dimensional PAGE under reducing and nonreducing conditions. After resolving platelet constituents in this way, only one spot reacts with the monoclonal antibody and it migrates exactly with a radioiodinated spot with the mobility of glycoprotein IIbα. Further support for this identification comes from the finding that, upon affinity chromatography on the column of immobilized monoclonal antibody PMI-1, the antigen that binds this antibody co-purifies with another platelet protein that migrates like glycoprotein III on reduced

gels. Others have found such co-purification using monoclonal antibodies directed against glycoprotein IIb (13), presumably because these two glycoproteins associate with each other in detergent extracts of the platelet plasma membrane (13, 15).

In most previous work, glycoprotein IIb has been implicated in platelet aggregation and fibrinogen binding (18–20), but not in platelet adhesion (21). Because our assay measures adhesion rather than aggregation, we conclude that glycoprotein IIb or a sterically related structure is also involved in this platelet function. The finding that glycoprotein IIb and III may become physically associated with the cytoskeleton (22) and might thereby mediate the spreading and shape change of platelet could explain its participation in both adhesion and aggregation. Based on binding studies with *Lens culinaris* agglutinin and collagen, Tsunehisa et al. have also concluded that glycoprotein IIb participates in platelet–collagen adhesion (23).

The present studies provide strong support for the inference, based on polyclonal antibody studies, that platelet–collagen adhesion involves more than one immunologically distinct entity. Using the monoclonal antibody PMI-1, we have purified one of these entities and found that it can neutralize no more than 25% of the platelet–collagen adhesion-blocking activity of a polyclonal antiplatelet antiserum. Based on reasoning like that in the previous paper (5), this suggests that ~25% of the active antibody in that polyclonal preparation is directed against glycoproteins IIb and III but that additional, distinct components of the antibody preparation bind to other relevant platelet membrane substances that are also involved in platelet–collagen adhesion. For the reasons given previously (5), these entities behave as though they are interdependent.

It is notable that, had we begun our work with monoclonal antibody PMI-1, which can block platelet–collagen adhesion completely and be totally neutralized by glycoprotein IIb, we might have erroneously concluded that this glycoprotein is the only "platelet–collagen adhesion molecule." After all, the monoclonal antibody can inhibit platelet adhesion completely, and the neutralizing antigen can completely neutralize it. Yet, the finding that glycoprotein IIb only partially neutralizes the polyclonal antiserum, and that other distinct neutralizing fractions have been identified, invalidates what would have been a reasonable inference. Indeed, there is evidence (24) that another platelet molecule, with M_r ~ 65,000, may participate in platelet–collagen adhesion by binding directly to the α chain of the collagen molecule.

Note Added in Proof: While this paper was in press, Kotite (Kotite, N. J., J. V. Stavos, and L. W. Cunningham) reported (Biochemistry, 1984, 23:3099–3104) that cross-linking glycoprotein IIb/IIIa with a photoaffinity agent specifically blocked collagen-induced platelet aggregation. This verifies our findings by an independent, nonimmunological method and further implicates glycoprotein IIb/IIIa in platelet-collagen interactions.

This research was supported by grants from the U. S. Public Health Service (HD13542, AM27214, and HL16411) and the McKnight Foundation, and by the Veterans Administration Medical Center. Paula Shadle was a graduate student in the Department of Biology and was supported by a predoctoral fellowship from the National Science Foundation, and a predoctoral training grant from the National Institutes of Health.

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