Monoclonal Antibodies against Human Platelet Membrane Glycoprotein IIIa and Collagen-Induced Platelet Activation

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Two monoclonal antibodies against human platelet membrane glycoprotein IIIa (GPIIIa) were obtained. One monoclonal antibody, designated as 1B1, was found to inhibit both collagen-induced platelet aggregation and release reactions. This antibody also inhibited the binding of ^{125}I-labeled collagen to human platelets. On the other hand, the other antibody, designated as B10, had no effect on platelet activation induced by a number of physiological stimulants including collagen. Direct binding studies involving ^{125}I-labeled 1B1 or B10 demonstrated that the binding sites for these antibodies on unstimulated platelets have dissociation constants of 4.2 and 14.0 nM, respectively. The binding of ^{125}I-labeled 1B1 or B10 to platelets was not inhibited by the other antibody. Purified 1B1 and B10 were covalently coupled to Affi-Gel and then proteolytic fragments of GPIIIa were applied to the Affi-Gel immunoadsorbent columns. Of the several proteolytic fragments, the 56 kilodaltons (kDa) fragment obtained on digestion with V8 protease bound to both of the columns. The 69 and 55 kDa fragments obtained with BrCN bound to only the 1B1 Affi-Gel column, while the 63 kDa fragment obtained with chymotrypsin only bound to the B10-Affi-Gel column. Based on the partial amino acid sequences of these fragments and the amino acid sequence of GPIIIa (C. A. Fitzgerald, B. Steiner, S. C. Rall, Jr., S. Lo and D. R. Phillips, *J. Biol. Chem.*, 262, 3936 (1987)), the epitopes for 1B1 and B10 were concluded to be located at amino acids 335 to 582 and 206 to 335, respectively. These results indicate that a certain epitope of GPIIIa or the GPIIb/IIIa complex on the platelet cell surface constitutes a receptor for collagen, and that the epitope for 1B1 is located near or in the collagen-binding site of GPIIIa. Thus, the collagen binding site is located near the transmembrane portion of GPIIIa, namely, amino acid residues 335 to 582. Furthermore, the 1B1 antibody was found to induce the aggregation of washed platelets, suggesting that GPIIIa is directly involved in the activation of platelets.

Keywords monoclonal antibody; human platelet; membrane glycoprotein; collagen; platelet activation

Platelets play important roles in hemostasis and thrombosis. After vascular injury, circulating platelets adhere to the exposed subendothelial connective tissue, where they aggregate with the concomitant release of several biologically active substances, the aggregation eventually becoming irreversible. Fibrillar collagen in the underlying connective tissue is thought to be the primary component that interacts with platelets in this process. However, the chemical nature of the receptors on human platelets for collagen has not been fully elucidated. Monoclonal antibodies that bind to platelet membrane components, primarily glycoproteins, have been used to investigate the molecules involved in platelet activation. This immunological technique allows the identification of receptors on the platelet surface for several biologically active substances, such as von Willebrand factor and fibrinogen.[1-5] Recently, Shadle *et al.*[6] obtained a monoclonal antibody that reacts specifically with human platelet membrane glycoprotein IIb (GPIIb) and which blocks platelet-collagen adhesion, suggesting that GPIIb participates in a certain phase of platelet-collagen adhesion. We previously examined the interactions of collagen and plant lectins with platelet membrane glycoproteins and found that lentil lectin and collagen share a common receptor on platelet membranes.[7] Since GPIIb comprises the primary binding site on platelet membranes for lentil lectin, we assumed that GPIIb is most likely the receptor for collagen on the platelet cell surface. GPIIb is known to form a complex with another major human platelet membrane glycoprotein, glycoprotein IIIa (GPIIIa), and this complex has been proposed to serve as a receptor for fibrinogen,[8,12] von Willebrand factor,[13] fibronectin[14] and adenosine diphosphate (ADP). This complex is a Ca^{2+}-dependent heterodimer[3,9,15,16] that still exhibits the receptor function when reconstituted into phospholipid vesicles.[17] Recently, membrane glycoprotein complexes, which are immunologically and biochemically similar to the membrane glycoprotein complex, GPIIb/IIIa, of platelets, were reported in endothelial cells[18-21] and other cell lines.[22,23] The α-subunits of the LFA-1 and Mac-1 antigens on leucocytes, which are involved in leucocyte adhesion, were found to contain an amino acid sequence similar to that of GPIIb.[22,24] The amino acid sequences of GPIIb[25] and GPIIIa[26] deduced from complementary deoxyribonucleic acid (cDNAs) showed homology to those of the fibronectin and vitronectin receptors,[27] indicating that GPIIb/IIIa is a member of a family of cell-surface adhesion receptors called "integrins".[28] Fibrinogen, von Willebrand factor and fibronectin binding to GPIIb/IIIa is inhibited by any one of the others and the binding of these adhesion molecules to GPIIb/IIIa is mediated by the Arg–Gly–Asp sequence, which is common in these extracellular ligands.[29] Collagen contains the Arg–Gly–Asp sequence,[30] but it is not known whether the GPIIb/IIIa complex binds to this matrix protein or not.

We report here the possibility of GPIIIa or the GPIIb/IIIa complex being a receptor for collagen. Furthermore, the direct involvement of GPIIIa or the GPIIb/IIIa complex in platelet activation was also demonstrated.

Materials and Methods

Hybridomas Eight-week-old BALB/c female mice were immunized i.p. with 200 μg of human platelet membranes, emulsified in complete Freund's adjuvant (Nakarai Chemicals, Ltd., Kyoto, Japan). Two weeks later, a second immunization of 10^8 washed platelets without any adjuvant was given, and then repeated injections of washed platelets were given three or four times weekly. Spleen cells harvested 3 d after the last injection were mixed with SP-2 or NS-1 murine myeloma cells in the ratio of 7:1

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(myeloma cells: spleen cells) and then treated with 45% (w/v) polyethylene glycol 4000 for 7 min. After the polyethylene glycol treatment, the cells were cultured in hypoxanthine (1×10^{-4} M)–aminopterine (4×10^{-7} M)–thymidine (1.6×10^{-5} M) (HAT) medium in 24-well plates at a density of 1.5×10^{-6} spleen cells per well. After 7—10 d, colonies were observed in almost all the wells and the culture medium was subjected to radioimmunoassay. The hybridoma cells that produced antibodies against platelets were cloned by limiting dilution at a density of 0.5 cells per well in 96-well plates in the presence of thymocytes, as the feeder layer. Usually, limiting dilution was performed repeatedly, two or three times. The hybridoma clones thus obtained were injected i.p. into pristane-primed mice. Ascites fluid that developed within 2 weeks after the injection was harvested.

Purification of the Antibodies Ascites fluid containing a monoclonal antibody was diluted 1 : 1 with 10 mM sodium phosphate buffer, pH 7.3, containing 0.85% NaCl (PBS), followed by centrifugation at $1300 \times g$ for 10 min at room temperature. To the supernatant was added an equal volume of saturated $(NH_4)_2SO_4$ and then the mixture was incubated at 4°C for 18 h. The precipitate formed was collected by centrifugation at $40000 \times g$ for 30 min and then suspended in a minimum volume of PBS. The ammonium sulfate precipitation was repeated twice. The crude antibody solution thus obtained was dialyzed at 4°C for 18 h against 0.1 M Tris-phosphate, pH 8.5, and applied to a column of diethyl aminoethyl (DEAE)-cellulose which had been equilibrated with the same buffer. The flow-through fraction was collected and concentrated. The purity of immunoglobulin G (IgG) in the resultant preparation was found to be more than 95% by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

Radioimmunoassay Aliquots (50 μl) of a washed platelet suspension containing 5×10^7 cells were distributed into 96-well polyvinyl chloride microtiter plates that had been soaked for 12 h in 3% bovine serum albumin (BSA) before use. Next, 50 μl aliquots of culture supernatant, ascites fluid or serum were added to the wells, and the plates were incubated for 1 h at room temperature. After washing with PBS, ^{125}I-labeled goat anti-mouse IgG (350 Ci/ml, 1×10^5 cpm/well; Amersham International Ltd., U.K) was added to each well and then incubation was continued for a further 30 min at room temperature. After washing out of the unbound materials, the radioactivity in each well was counted with a gamma counter. The background level of radioactivity in each well was estimated by the use of non-immune normal mouse serum or the conditioned medium of the parent myeloma cell line.

Preparation of Platelets Blood was collected from healthy volunteers with 0.1 volume of 8.3% sodium citrate as the anticoagulant. Platelet-rich plasma was obtained by removing erythrocytes and leucocytes by slow centrifugation ($200 \times g$, 10 min). Platelet-poor plasma was prepared by further centrifugation at $1300 \times g$ for 10 min. The platelet-rich plasma and platelet-poor plasma thus obtained were used for the aggregation and release assays, respectively. The platelets were then pelleted by centrifugation of platelet-rich plasma after the addition of 1/100 volume of 1 M citric acid. In some experiments, platelets were isolated from platelet concentrates obtained from the Blood Transfusion Service, University Hospital, University of Tokyo, as described above. Platelet membranes were prepared by the glycerol lysis technique described by Barber and Jamieson[31] in the presence of phenylmethylsulfonyl fluoride (PMSF) at a final concentration of 0.5 mM. Washed platelets were obtained from platelet-rich plasma by the method of Mustard *et al.*[32] The twice-washed platelets were finally resuspended in Ca^{2+}-free-Tyrode's solution, pH 7.4, containing 0.35% BSA (type V, Sigma) and 0.1% glucose to 2×10^8 platelets/ml.

Platelet Aggregation and Release Assays Platelet aggregation was measured with a dual-channel aggregometer (Niko Bioscience Inc., Tokyo). Usually, 10—20 μl of a stimulant was added to 200 μl of platelet-rich plasma (2.5×10^8/ml). For examination of the effect of a monoclonal antibody on platelet aggregation, platelet-rich plasma was incubated with the monoclonal antibody (0—50 μg/ml) for 4 min and then a stimulant was added. The release of serotonin was measured with platelets preloaded with ^{14}C-serotonin (60 mCi/mmol; Amersham). Platelet-rich plasma was incubated with ^{14}C-serotonin (0.4 μCi/ml) for 30 min at 22°C and then the platelets were pelleted by centrifugation after adding 1/100 volume of 1 M citric acid. The platelets loaded with ^{14}C-serotonin were resuspended in platelet-poor plasma and then collagen (Collagen reagent Horm; Hormon-Chemie, Munich, F.R.G.), ADP (Sigma), ristocetin (Boehringer Mannheim) or von Willebrand factor (Sigma) was added. First, aggregation of the platelets was measured in an aggregometer. After 5 min, 20 μl of 1.5 M formaldehyde was added to the reaction mixture to stop the release reaction, and then the mixture was cooled on ice and centrifuged at $10500 \times g$ for 2 min in an Eppendorf centrifuge. The amount of radioactivity in the supernatant was determined and expressed as a percentage of the total counts in control platelets.

Collagen Binding Studies Collagen binding studies were performed as described previously.[7] Collagen from calf skin (type III, Sigma) or Collagen reagent Horm (Hormon-Chemie) was used for these studies. Radioiodination of collagen was carried out by the chloramine T method.[33] The specific radioactivity of the collagen was about 2.5×10^5 cpm/μg in each case. Na^{125}I (350 mCi/ml) was purchased from New England Nuclear (Boston, Mass., U.S.A.).

Immunoblot Procedure Human platelet membranes isolated by the glycerol-lysis technique[32] were electrophoresed on acrylamide gels in the presence of SDS according to the method of Laemmli[34] and then electroblotted onto nitrocellulose membrane strips for 20—40 h at 6 V/cm. The blotted membrane strips were incubated in PBS containing 3% BSA for 1 h at room temperature, followed by exposure to an ^{125}I-labeled monoclonal antibody (1×10^6 cpm/strip) for 2 h at room temperature. The unbound antibody was removed by washing with PBS containing 0.05% Tween 20 several times. Autoradiography was conducted with Kodak X-Omat S film at −80°C and the film was developed after 1—10 d.

Effect of Direct Binding of Monoclonal Antibodies to Platelets 1B1 and B10 were radiolabeled with Na^{125}I by the iodogen method[35] and then separated from free Na^{125}I by gel filtration on a Sephadex G-25 column. All the steps in the binding assay were performed at room temperature. Five hundred microliters of platelet-rich plasma, which had been pretreated with 10 μl/ml prostaglandin E_1 (PGE_1) and 4 μl of PMSF, was transferred to a tube containing 50 μl of an ^{125}I-labeled monoclonal antibody in the presence or absence of the non-labeled monoclonal antibody. After incubation of the platelets with the antibody for 40 min, the platelets were separated from the free antibody by centrifugation and then the radioactivity was counted with a gamma counter.

Purification of GPIIIa Purification of GPIIIa was performed as described previously.[36] Human platelet membranes were dissolved in 50 mM Tris–HCl, pH 7.4, containing 1% Tween 20 and then applied to a concanavalin A (Con A)-Sepharose column equilibrated with PBS, pH 7.4, containing 0.1% Tween 20. The column was washed with the same buffer until the absorbance at 280 nm of the effluent had returned to the baseline. The bound glycoprotein material was then eluted with the buffer containing 0.2 M α-methyl-D-mannoside and 0.1% Tween 20. The Con A-bound fraction was further purified by SDS-polyacrylamide gel electrophoresis according to the method of Laemmli.[34] GPIIIa (molecular weight: 110 kilodaltons (kDa)) was electrophoretically eluted from polyacrylamide gel slices according to the method of Stralfors and Belfrage.[37] The eluate was dialyzed against PBS containing 0.05% Tween 20.

Cleavage of GPIIIa with Lysylendopeptidase, Arginylendopeptidase, V8 Protease, Chymotrypsin, Trypsin or BrCN The purified GPIIIa was radioiodinated with Na^{125}I by the iodogen method.[35] The radiolabeled protein was separated from free Na^{125} I by gel filtration on a Bio-Rad P-6DG column with PBS containing 0.05% Tween 20 as the elution buffer. The final concentration of GPIIIa was adjusted to 2.5×10^8 cpm/mg. Fifty microliters of ^{125}I-GPIIIa (2.5×10^5 cpm, 1 μg) was added to 20 μl of lysylendopeptidase (3 units/ml; Boehringer Mannheim), arginylendopeptidase (200 units/ml; Boehringer Mannheim) or V8 protease (80 units/ml; Boehringer Mannheim). Immobilized chymotrypsin from bovine pancreas (20 units/mg gel; Boehringer Mannheim) or immobilized trypsin from bovine pancreas (30 units/mg gel; Boehringer Mannheim) was added in the ratio of 1 mg of enzyme-gel to 50 μl (2.5×10^5, 1 μg) of GPIIIa. These mixtures were incubated at 37°C for appropriate times and then the reaction was stopped by adding a protease inhibitor or by centrifugation in the case of enzyme-gels.

Affinity Chromatography on B10- and 1B1-Affi-Gel Each of the two monoclonal antibodies, B10 and 1B1, was purified from mouse ascites tumor fluid by successive ammonium sulfate precipitation and DEAE-Sephadex A-50 ion-exchange chromatography according to Onoue *et al.*[38] The purified B10 and 1B1 were covalently coupled with the affinity-gel (Affi-Gel 10; Bio-Rad Laboratories, Richmond, Calif., U.S.A.). Monoclonal antibodies (3 mg/ml) were mixed with Affi-Gel 10 (10 ml) with gentle agitation, in 50 ml of 0.1 M ethanolamine–HCl. One hundred microliters of the enzymically digested GPIIIa was applied to a column (0.8×2 cm) of B10- or 1B1-Affi-Gel equilibrated with PBS containing 0.1% Tween 20. After washing of the column with 5 column volumes of the same buffer, the bound materials were eluted with 50 mM diethylamine, pH 11.0, containing 0.1% Tween 20. The eluate was neutralized by adding

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1 M Tris–HCl, pH 7.4. The bound and unbound fractions were separately collected and concentrated with a Centricon-10 (Amicon Corp., Danvers, Mass., U.S.A.), and then subjected to SDS-polyacrylamide gel electrophoresis on 10% slab gels.

Autoradiography After the electrophoresis, the slab gels were fixed and then stained with 0.05% Coomassie brilliant blue. The gels loaded with ^{125}I-labeled materials were dried under vacuum and then left in contact with Kodak X-Omat AR film (Eastman Kodak Co., Rochester, N. Y., U.S.A.) at −80°C in a cassette with a DuPont Cronex Lighting Plus intensifying screen.

Determination of the Partial Amino acid Sequences of Several Proteolytic Fragments The purified GPIIIa (0.1 mg) in PBS containing 0.1% Tween 20 was digested with *Staphylococcus aureus* protease V8 at the concentrations given above. The reaction mixture was subjected to SDS-polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue. The GPIIIa fragments were recovered from gel slices electrophoretically according to the method of Stralfors and Belfrage.[37] To remove Tris and glycine, each sample was applied to a Superose 12 (Pharmacia, Uppsala, Sweden) gel filtration column equilibrated with 50 mM ammonium acetate containing 0.1% SDS. Chromatography was performed at a flow rate of 0.5 ml/min and the protein was monitored by measuring the absorbance at 280 nm, with checking by polyacrylamide-gel electrophoresis. Ammonium acetate was removed by repeated evaporation. The amino acid sequence was determined with a protein sequencer (model 477A; Applied Biosystems, Foster, Calif., U.S.A.). A BrCN solution (10 mg/ml in 70% formic acid) was added to 500 μl of the purified GPIIIa solution and then the mixture was agitated in the dark at room temperature. After 20 h, the reaction mixture was lyophilized and subjected to amino acid sequence analysis in the same manner.

Others Reagents ADP and von Willebrand factor were products of Sigma. Ristocetin was purchased from Boehringer Mannheim. Protein A was obtained from Nakarai Chemicals, Ltd., Kyoto. Protein A-Sepharose was prepared by coupling Protein A to cyanogen bromide-activated Sepharose 4B. The binding capacity for IgG was estimated to be 10 mg/ml gel. Other reagents were of analytical grade.

Results

Preparation of Monoclonal Anti-GPIIIa Antibodies We have assayed more than twenty kinds of hybridoma cells for the production of IgG antibodies specific for platelets, to obtain monoclonal antibodies that inhibit collagen-induced aggregation of platelets. As shown in Fig. 1, 1B1, at a concentration of 10 μg/μl, totally inhibited collagen (2 μg/μl)-induced platelet aggregation. The binding specificity of 1B1 was analyzed by immunoblotting with the ^{125}I-labeled 1B1 antibody. The results of immunoblotting showed that 1B1 specifically bound to GPIIIa (Fig. 2). GPIIIa is known to be characteristically slow-moving on SDS-polyacrylamide gel electrophoresis under reducing conditions (Fig. 2). We next carried out direct binding studies with the ^{125}I-1B1 antibody to determine the dissociation constant and the number of 1B1 binding sites on platelets.

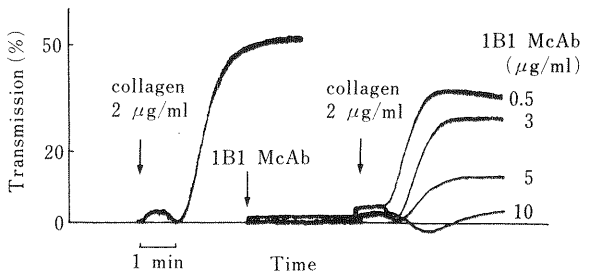

Fig. 1. Inhibitory Effect of Monoclonal Antibody (Mc Ab) 1B1 on Collagen-Induced Platelet Aggregation

Various amounts of monoclonal antibody 1B1 were added to platelet-rich plasma (2.5×10^8 platelets/ml) at zero time and then aggregation was followed. After 4 min, collagen (final concentration, 2 μg/ml) was added.

From the results of Scatchard plot analysis shown in Fig. 3, ^{125}I-labeled 1B1 was found to bind to 4.0×10^4 sites/platelet, with a dissociation constant of 4.2 nM.

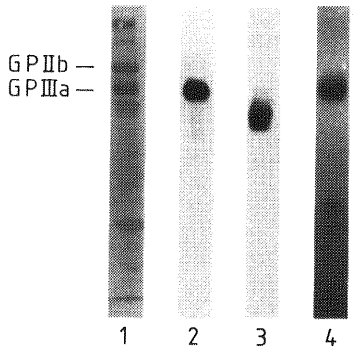

Fig. 2. Immunoblotting of Platelet Proteins with Monoclonal Antibody 1B1 or B10

A radioiodinated monoclonal antibody was incubated with a nitrocellulose blot of a 8% SDS-polyacrylamide slab gel on which whole human platelet membrane proteins had been electrophoresed, followed by autoradiography. Lanes 2 and 3, ^{125}I-1B1; lane 4, ^{125}I-B10. Lane 1, the strip was stained with Amide Black. Lanes 1, 2 and 4, reduced (5% dithiothreitol); lane 3, nonreduced.

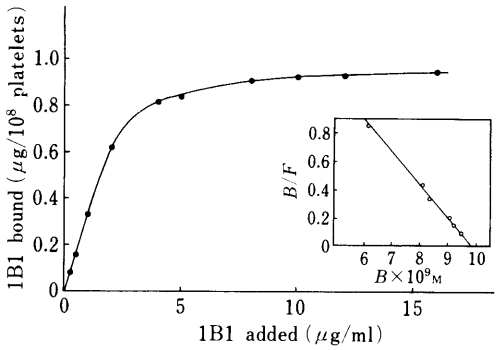

Fig. 3. Steady-State Binding of ^{125}I-1B1 to Human Platelets

The reaction mixtures comprised 100 μl of ^{125}I-1B1, at various concentrations, in 10 mM Tris–HCl, pH 7.4, containing 0.15 M NaCl, 0.38% sodium citrate, 1 mM PMSF, 5 μg/ml PGE_1, 2 mM EDTA and 0.38% BSA. After a 30-min incubation at room temperature, to separate the platelets from the suspension, the incubation mixture was layered onto 500 μl of a 20% sucrose solution, followed by centrifugation at $10500 \times g$ for 2 min in an Eppendorf centrifuge. The supernatant containing unbound ^{125}I-1B1 was removed and then the ^{125}I-1B1 bound to the platelets was measured by counting the radioactivity at the tip of the centrifuge tube. Nonspecific binding of the 1B1 antibody was determined in an identical mixture which contained 100-fold unlabeled monoclonal antibody 1B1 over the amount of the labeled monoclonal antibody 1B1. The inset shows a Scatchard plot[46] of the binding data.

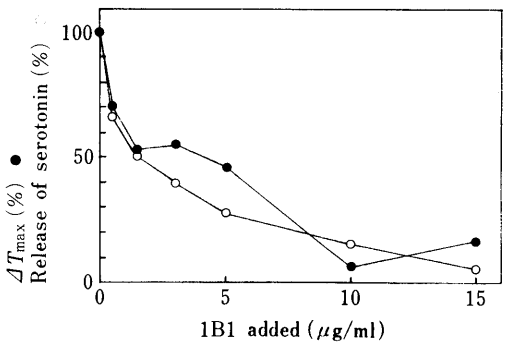

Fig. 4. Effect of Monoclonal Antibody 1B1 on Collagen-Induced Platelet Aggregation and the Release Reaction

^{14}C-Serotonin-loaded platelets (2.5×10^8/ml platelet poor plasma) were preincubated with monoclonal antibody 1B1 for 4 min and then collagen (final concentration, 2 μg/ml) was added. Aggregation was followed for 5 min with an aggregometer and then the platelets were pelleted by centrifugation. Then the amount of radioactivity in the supernatant was determined.

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TABLE I. Characteristics of the 1B1 and B10 Antibodies

	Subclass	Specificity	Dissociation constant (nM)	Number of binding sites (sites/platelet)
1B1	IgG2b	GPIIIa	4.2	4.0×10^4
B10	IgG2a	GPIIIa	14.0	3.7×10^4

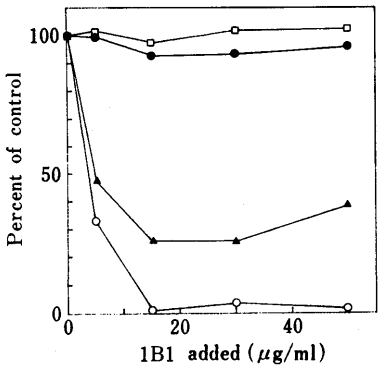

Fig. 5. Effect of Monoclonal Antibody 1B1 on Platelet Aggregation Induced by Various Stimulants

Platelet aggregation was measured as described under Materials and Methods. □—□ A23187, 50 μg/ml; ●—● ristocetin, 1.5 mg/ml; ▲—▲ ADP, 10 μM; ○—○ collagen, 2 μg/ml.

Furthermore, we obtained another monoclonal antibody, designated as B10, which was also found to recognize GPIIIa (Fig. 2, lane 4), but it had no effect on platelet aggregation induced by various physiological stimulants including collagen (data not shown). The results of binding studies with ^{125}I-labeled B10 indicated that this monoclonal antibody bound to 3.7×10^4 sites/platelet, with a dissociation constant of 14.0 nM. The characteristics of these monoclonal antibodies, 1B1 and B10, are summarized in Table I. Newman *et al.* estimated the number of copies of GPIIIa on platelet membranes to be 40200, with the use of a monoclonal antibody, AP-3, which specifically binds to GPIIIa.[39] The number of binding sites for the 1B1 or B10 antibody was in good agreement with the value obtained in the case of AP-3.

Effect of Monoclonal Antibodies on the Platelet Function We examined the effect of monoclonal antibody 1B1 on platelet aggregation induced by collagen. Monoclonal antibody 1B1 inhibited collagen-induced platelet aggregation in a concentration-dependent manner (Fig. 1). Next, we examined the effect of monoclonal antibody 1B1 on the collagen-induced release reaction using platelets preloaded with ^{14}C-serotonin. When platelets were preincubated with 1B1 for 4 min, the collagen-induced release of ^{14}C-serotonin from the platelets was found to be suppressed in parallel with the inhibition of aggregation (Fig. 4). On the other hand, various concentrations of the 1B1 antibody were found to have almost no effect on platelet aggregation induced by ristocetin or A23187, but had a significant suppressive effect on ADP-induced platelet aggregation (Fig. 5). These results suggest that the inhibition of aggregation and the release reaction by 1B1 is due to blocking of collagen receptors on platelet membranes by 1B1.

To verify this assumption, we studied the effect of 1B1 on the binding of ^{125}I-labeled collagen to platelets. Figure 6

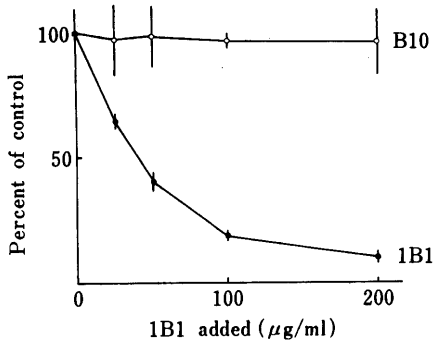

Fig. 6. Effect of Monoclonal Antibodies on the Binding of ^{125}I-Collagen to platelets

Fresh, washed platelets (2.5×10^8/ml) were preincubated with different amounts of the monoclonal antibodies in 10 mM Tris–HCl pH 7.4, containing 0.15 M NaCl, 0.38% sodium citrate, 2 mM PMSF, 5 μg/ml PGE_1, 2 mM EDTA and 0.35% BSA, for 15 min at 22 °C. Then the platelets were incubated with ^{125}I-collagen (4 μg/ml) for 30 min at 22 °C and the amount of collagen bound to the platelets was determined after centrifugation. The experimental details are given under Materials and Methods. Each value represents the mean for three experiments.

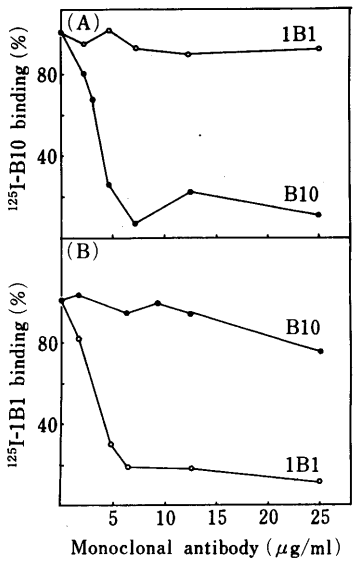

Fig. 7. Competition between B10 and 1B1 for Binding Sites on Platelets

A. Effect of 1B1 on ^{125}I-B10 binding. ^{125}I-Labeled B10 (0.75 μl) was incubated with washed platelets for 30 min and then the binding of the ^{125}I-B10 to the platelets was measured as described under Materials and Methods. Non-specific binding was measured in the presence of 100-fold cold B10 antibody. Open circles denote platelets incubated with various amounts of 1B1 and closed circles, platelets incubated with B10. B. Effect of B10 on ^{125}I-1B1 binding. The same experiments were performed under the same conditions as above. Open circles denote platelets incubated with 1B1 and closed circles, platelets incubated with B10.

shows that preincubation of platelets with 40 μg/ml of 1B1 resulted in about 50% inhibition of the binding of collagen to platelets, while B10 did not affect the binding of collagen to platelets at all. These results suggest that collagen and 1B1 share a common receptor on platelet membranes and that they bind to the same epitope of GPIIIa, but 1B1 and B10 bind to different epitopes of GPIIIa. This assumption was supported by the results of cross-inhibition experiments. The binding of the ^{125}I-labeled 1B1 antibody to platelets was not affected by pretreatment of the platelets with the unlabeled B10 antibody, and *vice versa* (Fig. 7).

Affinity Chromatography on B10- and 1B1-Affi-Gel Then, the distribution of the epitopes for 1B1 and B10 on several enzymatic fragments of GPIIIa was studied by

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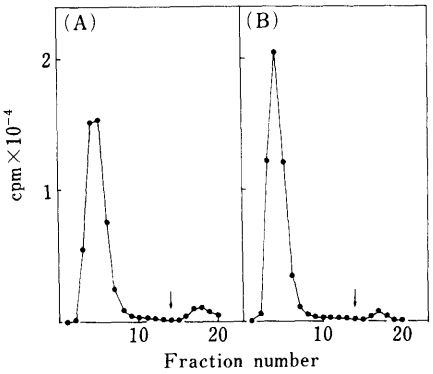

Fig. 8. Affinity Chromatography of Lysylendopeptidase Fragments of GPIIIa on B10- and 1B1-Affi-Gel Columns

Elution was performed with PBS containing 0.1% Tween 20, followed by 50 mM diethylamine, pH 11.0, containing 0.1% Tween 20. The arrows indicate when the eluent was changed. Fractions of 0.6 ml were collected. A) Lysylendopeptidase fragment of GPIIIa on a B10-Affi-Gel column. B) Lysylendopeptidase fragment of GPIIIa on a 1B1-Affi-Gel column.

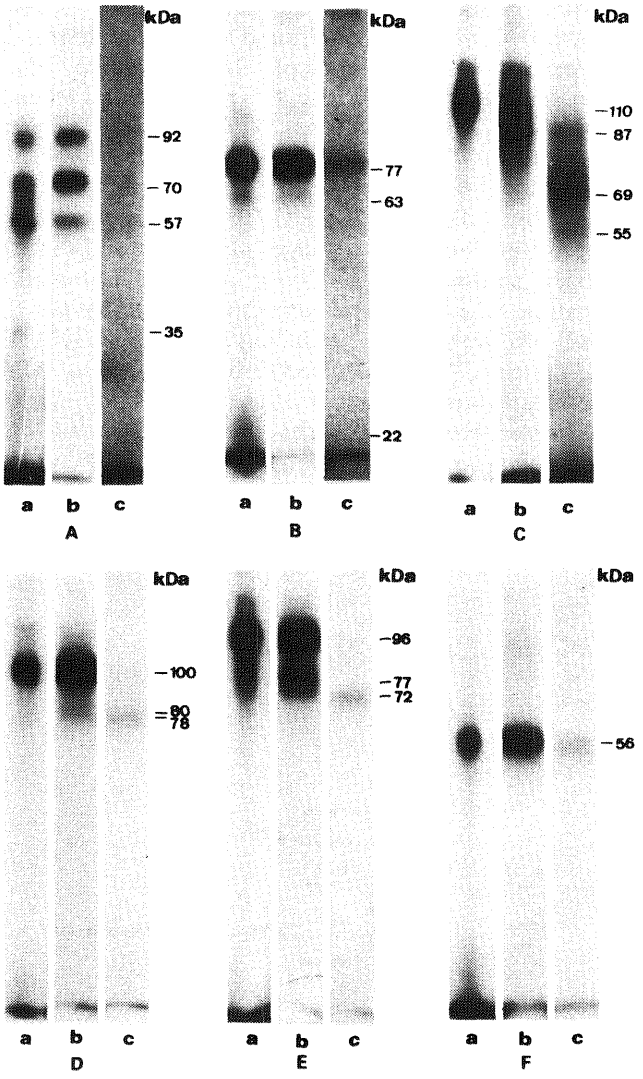

Fig. 9. SDS-Polyacrylamide Gel Electrophoresis of the Affinity-Purified Lysylendopeptidase Fragments of GPIIIa

^{125}I-Labeled GPIIIa was incubated with A) lysylendopeptidase, B) chymotrypsin, C) BrCN, D) trypsin, E) arginylendopeptidase or F) V8 protease for appropriate times. The reaction mixtures were passed through the B10 and 1B1 affinity columns. Lanes: a) the proteolytic fragments before application to the column; b) the fragments bound to B10; c) the fragments bound to 1B1.

immunoaffinity chromatography. ^{125}I-Labeled GPIIIa was partially digested with lysylendopeptidase at 37°C. Four distinct fragments were obserbed on SDS-polyacrylamide gel electrophoresis (Fig. 9-A, lane a). The molecular weights of these major fragments are 92, 70, 57 and 35 kDa. A mixture of these fragments was applied to an affinity column prepared by coupling 1B1 or B10 to Affi-Gel 10, and the bound fragments were eluted with 50 mM diethylamine, pH 11.0, containing 0.1% Tween 20 (Fig. 8). As shown in Fig. 9-A, the lysylendopeptidase fragments with molecular weights of 92, 70 and 57 kDa bound to both (1B1- and B10-Affi-Gel) columns, while the 35 kDa fragment failed to bind to either column. Similar experiments were performed on fragments prepared by digestion of GPIIIa with chymotrypsin, trypsin, arginylendopeptidase, V8 protease or BrCN. The results shown in Fig. 9 are summarized in Table II. The partial amino acid sequences of the BrCN fragments with molecular weights of 55 and 87 kDa were then analyzed with a gas-phase protein sequencer. The sequences of the N-terminal regions of the 55 and 87 kDa fragments were found to be Asp–Ser–Ser–Asn and Gly–(Cys)–Asn–Ile, respectively. On the basis of these sequences, the specificity of the BrCN degradation, and the complete amino acid sequence of GPIIIa deduced from cDNA,[26] the 55 kDa BrCN fragment was assumed to originate from

Table II. Characteristics of the Fragments Obtained on Protease Digestion of GPIIIa

Enzyme	Molecular weight of fragment	Binding to immunoaffinity columns prepared by coupling monoclonal antibody	
		B10	1B1
Lysylendopeptidase	92	(+)[a]	(+)
Lysylendopeptidase	70	(+)	(+)
Lysylendopeptidase	57	(+)	(+)
Lysylendopeptidase	35	(−)	(−)
Chymotrypsin	77	(+)	(+)
Chymotrypsin	63	(+)	(−)
Trypsin	80 (78)	(+)	(+)
Arginylendopeptidase	77 (72)	(+)	(+)
V8 protease	56	(+)	(+)
BrCN	87	(+)	(+)
BrCN	69	(−)	(+)
BrCN	55	(−)	(+)

a) Elution was carried out with 50 mM diethylamine, pH 11.0, containing 0.1% Tween 20. ^{125}I-Labeled proteolytic fragments not bound to the immunoaffinity column are indicated as (−); those bound to the column are indicated as (+).

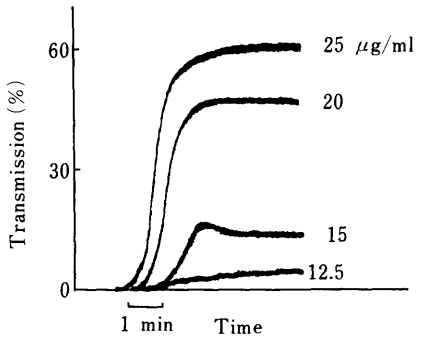

Fig. 10. Platelet Aggregation Induced by Monoclonal Antibody 1B1

A washed platelet suspension was preincubated at 37°C for 3 min and then the reaction was initiated by the addition of monoclonal antibody 1B1.

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amino acid positions 335 to 701 and the 87 kDa fragment was assigned to the N-terminal region of GPIIIa (amino acids 1 to 626). Similarly, the N-terminal sequence of the 56 kDa fragment obtained on treatment of GPIIIa with V8 protease was found to be Val–Lys–Lys–Gln. Thus, this 56 kDa fragment was assumed to originate from amino acid positions 206 to 582 of GPIIIa. From these results and the time course analyses of proteolytic digestions (data not shown), we propose that the epitope for 1B1 is located in the inner fragment (positions 335 to 582), which includes a cysteine-rich region, whereas the epitope for B10 is located in the fragment ranging from amino acid positions 206 to 335 of GPIIIa.

Platelet Activation Induced by Monoclonal Antibody 1B1 with Washed Platelets In contrast with the inhibitory effect of monoclonal antibody 1B1 on platelet aggregation and the release reaction when platelet-rich plasma was used, this antibody was found to induce the aggregation of washed platelets by itself (Fig. 10). In parallel with the aggregation, the release of preloaded ^{14}C-serotonin was also found to occur, depending upon the concentration of the added monoclonal antibody 1B1 (Fig. 11). It is of interest that this monoclonal antibody has apparently opposite effects, depending on the state of the platelets. We then added various amounts of plasma to a suspension of washed platelets and examined the effect of the plasma on the 1B1-induced platelet aggregation and release. As shown in Fig. 12, the addition of plasma was found to suppress both the aggregation and release induced by 1B1 in a dose-dependent manner. These results suggest the existence of certain inhibitory factors in plasma that prevent 1B1-induced platelet activation.

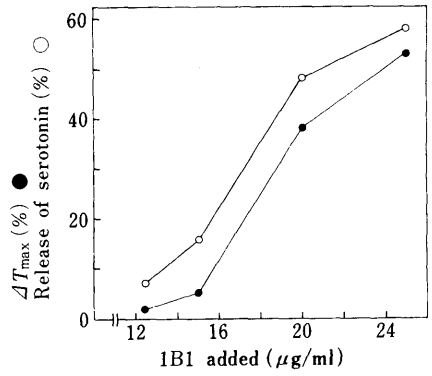

Fig. 11. Platelet Aggregation and the Release Reaction Induced by Monoclonal Antibody 1B1

Aggregation and the release reaction were measured as described under Materials and Methods.

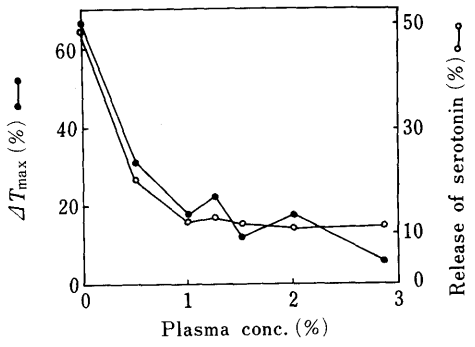

Fig. 12. Inhibitory Effect of Plasma on the Monoclonal Antibody 1B1-Induced Aggregation and Release Reaction

A washed platelet suspension was preincubated with an appropriate concentration of plasma for 3 min at 37 °C and then the reaction was initiated by the addition of monoclonal antibody 1B1. Aggregation and the release reaction were measured as described under Materials and Methods.

Discussion

In this study, we assayed monoclonal antibodies for inhibitory activity toward platelet activation induced by collagen. A monoclonal antibody, 1B1, completely inhibited the collagen-induced platelet aggregation and release (Figs. 5 and 6), but did not affect the platelet aggregation induced by other stimulants including ristocetin and A23187, except for ADP. The results of binding experiments involving ^{125}I-labeled collagen showed that the inhibition of collagen-induced platelet activation by monoclonal antibody 1B1 was apparently caused by blocking of the binding of collagen to its receptor sites on platelet membranes. Immunoblotting analyses showed that 1B1 specifically bound to GPIIIa. On the other hand, the other antibody, B10, which was also found to bind to GPIIIa, did not have a suppressive effect on platelet activation by any of the stimulants mentioned above. It is probable that 1B1 and B10 recognize distinct epitopes of GPIIIa and that the binding site for 1B1 on GPIIIa is located near or in the collagen-binding site.

It has been indicated that the GPIIb/IIIa complex acts as a receptor for fibrinogen on platelets[2-4,8-12] and that the fibrinogen binding is required for ADP-induced platelet aggregation. A preliminary experiment showed that the 1B1 antibody also suppressed the binding of fibrinogen to ADP-treated platelets. This might lead to the inhibition of ADP-induced platelet aggregation by the 1B1 antibody. Melero and Gonzalez-Rodriguez[40] also prepared a monoclonal antibody against GPIIIa, which inhibits ADP-induced platelet aggregation, but they did not examine the effect of the antibody on platelet aggregation induced by other factors.

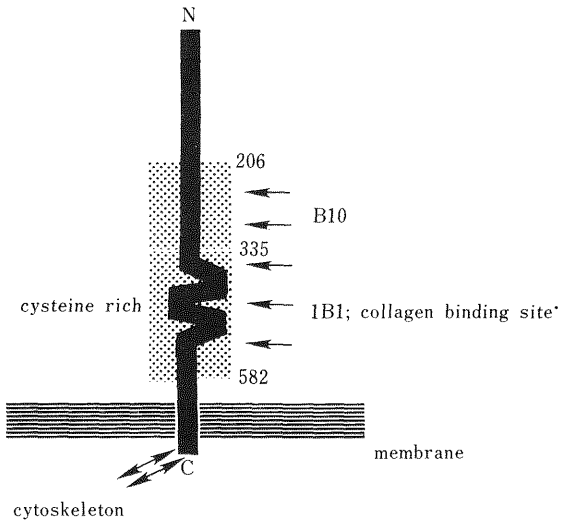

Fig. 13. B10- and 1B1-Binding Sites on GPIIIa, and the Postulated Collagen-Binding Site

The dotted area from amino acid 206 to 335 represents the B10-binding site, and that from 335 to 582 represents the 1B1-binding site.

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In the present study, we tried to localize the 1B1 antibody epitope of GPIIIa and to define the functionally significant sites in GPIIIa. The epitope of GPIIIa for 1B1 is located within a 55 kDa fragment, especially in the region comprising amino acids 335 to 582, and this region is located near a cell surface cysteine-rich region (Fig. 13). Binding of 1B1 to this epitope inhibited collagen-binding to platelets, indicating that the collagen-binding site is located in this region. Shadle *et al.* reported that monoclonal antibody PMI-1 inhibits platelet adhesion to collagen[6]; the epitope for PMI-1 was found to be localized near the carboxyl terminus of the heavy chain of GPIIb and a 17-residue peptide proximal to the carboxyl terminus of the GPIIb heavy chain inhibited PMI-1-binding to GPIIb/IIIa.[41] The PMI-1 epitope is also near the cell surface, suggesting that GPIIIa or the GPIIb/GPIIIa complex forms a collagen receptor site, which is near the cytoplasmic face of platelet membranes. Recently, Calvete *et al.* reported that a 23 kDa N-terminal trypsin fragment of GPIIIa carries an antigenic determinant for monoclonal antibody P37, which inhibits platelet aggregation induced by ADP.[42] The role of GPIIIa or the GPIIb/IIIa complex in ADP-induced platelet aggregation is thought to be as a receptor for fibrinogen,[8-12] and possibly for fibronectin[14] and von Willebrand factor.[13] Therefore, the fibrinogen receptor site is located near the N-terminal region of GPIIIa and is thus quite distinct from the collagen-binding site on GPIIIa.

The results presented here indicate that collagen and 1B1 share a common binding site on GPIIIa or the GPIIb/IIIa complex and that GPIIIa or the GPIIb/IIIa complex most likely serves a receptor site for collagen for platelet activation. The participation of GPIIb and/or GPIIIa in the interaction of collagen with platelets has been suggested, based on the use of monoclonal antibodies, lectins and cross-linking reagents.[6,7,43] However, Saito *et al.* reported that platelet factor XIII located on the surface of platelets may serve as a collagen receptor in collagen-induced platelet aggregation.[44] Further studies are, therefore, necessary to clarify the nature of the collagen receptors on platelets.

It seems of interest that the 1B1 antibody, which inhibits collagen-induced platelet aggregation in platelet-rich plasma, induced the aggregation of washed platelets by itself. This aggregation was found to be accompanied with the release of serotonin (Fig. 11) and was inhibited by the addition of ethylenediaminetetraacetic acid (EDTA) (2 mM) or PGE_1 (10 μg/ml), indicating that the aggregation was induced *via* platelet activation. A monoclonal antibody against GPIIb with similar characteristics to those of the 1B1 antibody has already been reported.[45] Although the mechanism underlying platelet activation induced by the monoclonal antibodies remains unknown, the present observations strongly suggest that the GPIIb/IIIa complex plays an important role in platelet activation.

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