Exposure of Platelet Fibrinogen-Binding Sites by Collagen, Arachidonic Acid, and ADP: Inhibition by a Monoclonal Antibody to the Glycoprotein IIb–IIIa Complex

By Giovanni Di Minno, Perumal Thiagarajan, Bice Perussia, Jose Martinez, Sandor Shapiro, Giorgio Trinchieri, and Scott Murphy

Following stimulation with adenosine diphosphate (ADP), collagen, or arachidonic acid, unstimulated human platelet suspensions bind $^{125}$I-fibrinogen in a reaction that reaches completion within 30 min. Scatchard analysis of these binding data reveals two sets of binding sites with all 3 agents: a high affinity site ($K_d$ 0.029–0.045 μM) binding 1000–1600 fibrinogen molecules per platelet, and a lower affinity site ($K_d$ 1.2–2.0 μM) binding 46,000–76,000 fibrinogen molecules per platelet. At a concentration of apyrase that inhibited ADP-induced fibrinogen binding by $>85\%$, fibrinogen binding induced by collagen and arachidonic acid was only partially affected. This suggests that fibrinogen binding induced by collagen or arachidonic acid does not require released ADP. We isolated a monoclonal antibody, B59.2, which precipitated the glycoprotein IIb–IIIa complex from solubilized platelet membranes. Binding of labeled antibody to platelets before or after exposure to ADP, collagen, or arachidonic acid showed a single class of approximately 22,000 binding sites with $K_d$ 0.019 μM. Binding of B59.2 was complete within 1 min and was not inhibited by EDTA. Preincubation of platelet suspensions with a 2.1 μM concentration of B59.2 caused inhibition of secretion and aggregation, but not of thromboxane-$B_2$ synthesis, in response to 1 μg/ml collagen, 40 μM arachidonic acid, or 4 μM ADP. Concentrations of aggregating agents that produced complete aggregation and secretion in the absence of B59.2. At this concentration of B59.2, fibrinogen binding to stimulated platelets was inhibited by approximately 45–55%. These data demonstrate that collagen and arachidonic acid can expose fibrinogen binding sites independently of released ADP; and that the glycoprotein IIb–IIIa complex is involved in secretion, aggregation, and fibrinogen binding, but not in thromboxane synthesis occurring in response to collagen, arachidonic acid, or ADP.

MATERIALS AND METHODS

Coomassie Brilliant Blue R-250 was from LKB Instruments, Inc, Rockville, Md. Silicon oils (methyl silicon 1.0 DC 200 and Hi phenyl silicon 125 DC 550) were purchased from W.F. Nye Inc., Specialty Lubricants, New Bedford, Mass. Mixtures of DC 200 and DC 550 were prepared as described by Niewiarowski et al.6 Disodium ethylenediaminotetracetate (EDTA) was obtained from Fisher Scientific Company, King of Prussia, Pa. Bovine serum albumin (Pentex, fraction V) was purchased from Miles Laboratories Inc., Elkart, Ind. Pepsin was obtained from Worthington Biochemical, Freehold, N.J. ADP (sodium salt), phenylmethyl-sulphonylfluoride (PMSF), lactoperoxidase (70–100 U/mg protein), and potato apyrase (grade I, partially purified, approximately 60% protein, 1.5–5.0 U/ml protein) were purchased from Sigma Chemical Co., St. Louis, Mo. A quantity of 50 μg/ml of this apyrase preparation completely suppressed aggregation of PRP in response to 4 μM ADP inhibited second-wave aggregation in response to 10 and 20 μM ADP. Equine tendon collagen (1 mg/ml of native collagen fibrils suspended in isotonic glucose solution, pH 2.7) was obtained from Hormon Chemie, Munich, West Germany. AA (>99% pure) was obtained from Nuchek Prep., Elysin, Minn. Solutions of the sodium salt of AA were prepared as described by Silver et al.21 Nonidet P40 was purchased from Particle Data Laboratories, Ltd., Elmhurst, Ill. $^{31}$I (sodium iodide) was from Cambridge Nuclear, Billerica, Mass. Triton X-100 was purchased from Amersham Searle Corp., Arlington Heights, Ill. Sepharose 4B, protein-A-Sepharose, and cyanogen bromide activated Sepharose were obtained from Pharmacia Fine Chemicals, Piscataway, N.J. Sodium dodecylsulfate (SDS) was...
obtained from Pierce Chemical Company, Rockford, Ill. Acrylamide, N,N-methylene-bis-acrylamide, and molecular weight standards were obtained from Bio-Rad Laboratories, Richmond, Calif.

Preparation of Washed Platelets

Blood was obtained from normal volunteers who had not taken any drug for at least 10 days before venipuncture and were collected into 1/9 volume of citrate-phosphate-dextrose (CPD) anticoagulant, 22 pH 7.4. PRP, platelet-poor plasma (PPP), and platelet concentrate (PC) were prepared as previously described. 23 Freshly prepared PC was cooled in ice for 30 min and 100 mM EDTA (pH 7.4 in distilled water) was added to give final concentration of 8.6 mM. The PC was centrifuged at 2000 g for 6 min at 4°C in a Sorvall centrifuge (Sorvall Superspeed/RC-2, Ivan Sorvall Inc., Norwalk, Conn.). The platelet pellet was resuspended in buffer (0.14 M NaCl, 0.02 M Tris, 0.005 M glucose, 0.01 M EDTA, pH 7.4) equal in volume to the discarded plasma. The platelet suspension was then centrifuged at 900g for 6 min at room temperature and finally resuspended in Tyrode's buffer (0.14 M NaCl, 0.027 M KCl, 0.012 M NaHCO3, 0.0004 M NaH2PO4, pH 7.4) containing 0.005 M EDTA, 0.001 M CaCl2, and 3.5 mg/ml bovine serum albumin. When trace amounts of 125I-fibrinogen were added to the PC, 99% of the fibrinogen originally present in the PC was removed by this washing procedure. Platelets were counted in a model F Coulter Counter (Coulter Electronic Inc., Hialeah, Fla.). Platelets washed and resuspended in this manner showed "swirling" as a gross indication of maintenance of discoid shape. After 2-hr incubation at room temperature, the pH of these platelet suspensions ranged between 7.7 and 7.8. However, after 30-min incubation (which was the time period within which binding of fibrinogen reaches completion), the pH was never higher than 7.4, and it was not changed by the addition of 1 μg/ml collagen, 10 μM ADP, or 40 μM AA. The aggregating response of these platelets, in the presence of fibrinogen, was 80%–90% of that seen in autologous PRP and was unchanged for at least an additional 2 hr. All aggregation studies were conducted within this 2-hr period.

Fibrinogen Purification and Labeling

Fibrinogen was purified from normal donor CPD plasma by the method of Kazal et al., 24 modified by the addition of α-amino caproic acid to a final concentration of 0.1 M in plasma and in buffers. 25 The purified fibrinogen, containing <0.8% immunossayable fibronectin and von Willebrand factor, was dissolved in 0.055 M sodium citrate, pH 7.4, and was quick-frozen in acetone–dry-ice and stored at −80°C. The purified fibrinogen was 95% clottable. Fibrinogen labeled with 125I by the iodine monochloride method of MacFarlane, 26 modified as previously described, 25 had a specific activity of 60 μCi/mg protein. Labeled protein was further purified by gel-filtration on a Sepharose 4B column (100 x 2.5 cm). Unlabeled and labeled fibrinogen showed the characteristic Aα, Bβ, and γ peaks, without any degradation products, when electrophoresed on reduced SDS-polyacrylamide gels (SDS-PAGE). 25 Further details of the properties of this fibrinogen before and after labeling have been previously reported. 25

Isolation, Purification, and Specific Binding of the Monoclonal Antibody

The procedures used for fusion, screening of hybridomas, cloning, production of ascites, purification, and specificity of antibody B59.2 have been reported in detail elsewhere. 27 Monoclonal antibody B59.2 (IgG2a) was purified from ascites fluid using a protein-A-Sepharose column. The antibody was labeled with 125I by the iodine monochloride method 26 and had a specific activity of 450 μCi/mg protein. The F(ab′)2 fragments were prepared by papain digestion 25 followed by removal of intact immunoglobulin and residual Fc fragments on a protein-A-Sepharose column and characterized as described by Goding. 30 Both B59.2 and its F(ab′)2 fragments showed a single band of 160,000 and 55,000 daltons, respectively, in a 7.5% SDS-polyacrylamide gel. 31 Antibody B33.1 (IgG2a anti-HLA-DR common determinant) did not react with platelets and was used as a negative control throughout. Using 125I-goat anti-mouse IgG, the unlabeled monoclonal mouse antibody was shown to bind to human platelets but not to other human cells. 28 To determine antibody specificity, 4 x 107 washed platelets were radiolabeled with 1 μCi 125I in the presence of 1 mg lactoperoxidase, 29 washed twice with tyrode buffer, and lysed with 0.5% NP40 for 15 min at 4°C. The lysate was centrifuged and aliquots of the supernatant (containing approximately 1.5 x 106 cpm) were incubated with 25 μl of purified B59.2 for 1 hr at 4°C. The complexed antigens were bound to 25 μl protein-A-Sepharose, washed, boiled in 3% SDS, and applied to a 7.5% SDS-polyacrylamide gel prepared according to the Laemmli method. 32 The immunoprecipitates were identified by autoradiography using Kodak X-Omat R film and a Dupont cronex lighting plus intensifying screen. 25 The antibody B59.2 was found to precipitate GpIb–IIIa from normal platelets in the presence or absence of Ca++. However, more than the platelets of three patients with Glanzmann's thrombasthenia. Clinical and laboratory profiles of these patients have been reported by Kornecki et al. 33 These experiments are described in detail in another communication. 25 In another series of experiments, 10 mg of B59.2 were coupled to 4 ml of cyanogen-bromide-activated Sepharose 4B. 34 Washed platelets (5 x 108) were solubilized by suspending in a buffer containing 0.15 M NaCl, 0.02 M Tris, 0.5 mM EDTA, 1 mM PMSF, and 1% Triton, pH 7.5. The solubilized platelet suspensions were centrifuged at 100,000 g at 4°C for 30 min in a Beckman ultracentrifuge (model L3-50 Ultracentrifuge, Beckman Instruments Inc., Palo Alto, Calif.). To remove nonspecifically absorbed proteins, the supernatant solution were pre-cycled through a 1 x 4 cm Sepharose 4B column equilibrated in the same buffer before being applied to the antibody-coupled affinity column. After extensive washing in several volumes of the column buffer, the affinity-bound proteins were eluted with 5 ml of 3% SDS, heated in a boiling water bath for 2 hr, and subjected to SDS-PAGE in 1-mm thick slab gels according to the method of Laemmli. 33 Prolonged boiling in SDS was found necessary to dissociate the GpIb–IIIa complex maximally.

Platelet Aggregation

Platelet aggregation and ATP secretion were measured in a Lumi-aggregometer (Chronolog Corp., Havertown, Pa.) 34 The apparatus was calibrated so that platelet suspensions and Tyrode's solution produced 10 and 100 light transmission units (LTU), respectively. The extent of platelet aggregation was measured 3 min after addition of each aggregating agent, at a time when aggregation and secretion were complete, and expressed in LTU. ATP secretion from platelets was measured by comparing the luminescence produced by platelets 3 min after addition of aggregating agent with that produced by known amounts of ATP added to unstimulated platelet suspensions. The sensitivity was less than 0.1 μM ATP.

Inhibition of Platelet Aggregation and Secretion

Increasing amounts of monoclonal antibody B59.2 (from 1.2 nM to 2.1 μM final concentration) or 4 μM antibody B33.1 were incubated with 0.5 ml platelet suspension (5 x 107/ml), which had been stirring at 37°C for 1 min at 1000 rpm. After a further minute of stirring, 25 μl unlabeled fibrinogen (0.5 μM final concentration) was added, and 1 min later, the aggregating agent was added.
Thromboxane-B₂ Measurement

Thromboxane-B₂ (TXB₂) levels were measured by radioimmunoassay, in the presence or in the absence of the antibody, 3 min after addition of aggregating agents. This radioimmunoassay detects as little as 0.5 pmole TXB₂/ml. Neither the reagents used for the measurement of ATP secretion nor the antibody itself interfered with TXB₂ measurement. Specific antibodies against TXB₂ were kindly provided by Dr. J. B. Smith (Cardeza Foundation, Philadelphia, Pa.).

Fibrinogen Binding to Platelets

Binding of ¹²⁵I-fibrinogen to platelet suspensions was performed as reported by Niewiarowski et al., with some modifications. Aliquots of 0.5 ml of unstimred platelet suspensions (5 x 10⁸/ml) were incubated at room temperature with 4 or 10 µM ADP, 1 µg/ml collagen, or 40 µM AA. After 3-min incubation, 25 µl (0.5 µl of 0.5 ml of unstirred platelet suspensions (5 x 10⁸/ml) were added together at the initiation of the binding reaction. At 5 and 15 min after stimulation, addition of a 100-fold excess of unlabeled fibrinogen displaced 96% and 86% of the labeled protein, respectively. However, by 30 min, only 34% of the label could be displaced, indicating late irreversible binding of fibrinogen to platelets. EDTA (10 mM) inhibited 92%-94% of the fibrinogen binding to ADP, collagen-, and AA-stimulated platelets. Binding of fibrinogen to stimulated platelets was studied quantitatively by incubating platelet suspensions with increasing concentrations (3–1200 nM) of fibrinogen for 30 min (Fig. 1). The binding of fibrinogen to platelets stimulated with all 3 agents was similar and reached saturation at 300 nM.

Antibody Binding to Platelets

Binding of the labeled B59.2 to platelets was studied in a manner similar to that described for fibrinogen. Platelet suspensions were incubated in Tyrode’s buffer (pH 7.4) containing 2 mM Ca++ or 10 mM EDTA, in the presence or absence of platelet-aggregating agents. Specific binding was calculated by subtracting from the total amount of fibrinogen bound in the presence of 10 mM EDTA, as described by Peerschke et al. For fibrinogen binding, the data were analyzed after 20-min and 30-min incubation, where binding was 90% and 100% complete, respectively.

Inhibition of ¹²⁵I-Fibrinogen Binding to Platelets

Concentrations of B59.2, ranging from 1.2 nM to 2.1 µM, were incubated with platelet suspension in the absence of stirring at room temperature. After 1 min, ¹²⁵I-fibrinogen was added (0.5 µM final concentration) and binding measured as previously described. Similar experiments were performed using EDTA (10 mM) or apyrase (50 µg/ml) or 4 µM of monoclonal antibody B33.1.

Protein Concentration

Protein concentration was calculated from absorbance at 280 nm using an extinction coefficient of 15.1 for fibrinogen and 14.3 for IgG.

Lactate Dehydrogenase (LDH)

Lactate dehydrogenase (LDH) loss from platelet suspensions was measured at the end of incubation procedures according to the method of Doery et al.

Statistical Analysis

Statistical analysis was done using Student’s t test for paired comparisons.

RESULTS

Fibrinogen Binding to Platelet Suspensions

After exposure to ADP, collagen, or AA, an immediate time-dependent binding of fibrinogen occurred in a reaction that was complete within 30 min. Labeled and unlabeled fibrinogen behaved identically when added together at the initiation of the binding reaction. At 5 and 15 min after stimulation, addition of a 100-fold excess of unlabeled fibrinogen displaced 96% and 86% of the labeled protein, respectively. However, by 30 min, only 34% of the label could be displaced, indicating late irreversible binding of fibrinogen to platelets. EDTA (10 mM) inhibited 92%-94% of the fibrinogen binding to ADP, collagen-, and AA-stimulated platelets. Binding of fibrinogen to stimulated platelets was studied quantitatively by incubating platelet suspensions with increasing concentrations (3–1200 nM) of fibrinogen for 30 min (Fig. 1). The binding of fibrinogen to platelets stimulated with all 3 agents was similar and reached saturation at 300 nM. Scatchard analysis of the fibrinogen binding data is shown in Fig. 2. We interpreted these data to indicate the presence of 1100–1600 high-affinity fibrinogen-binding sites (Kd 0.029–0.045 µM) and 46,000–76,000 low-affinity fibrinogen-binding sites (Kd 1.2–2.0 µM) per platelet. In the absence of stimulation, fibrinogen binding to platelets was less than 0.1% of that seen after stimulation.

In order to determine the effect of secreted ADP on collagen and AA-induced fibrinogen binding, platelet suspensions were incubated with apyrase (50 µg/ml) for 1 min before or for 1 min after addition of ADP, collagen, or AA. After 3 min, 0.5 µM (final concentration) ¹²⁵I-fibrinogen was added, and 30 min later, fibrinogen binding was measured as described. Results were independent of the order of addition of apyrase, and are shown in Table 1. At concentrations of apyrase that suppressed ADP-induced fibrinogen binding by more than 85%, binding induced by AA (40 µM) was decreased only 45%. Binding induced by 1 µg/ml collagen was only slightly, if at all, reduced, although it was reduced by 50% when 0.25 µg/ml collagen was used.

The role of prostaglandins and thromboxanes in collagen-, AA-, and ADP-induced fibrinogen binding

Statistical analysis was done using Student’s t test for paired comparisons.

Analysis of Binding Data

The binding of fibrinogen and B59.2 to platelets was plotted according to Scatchard and analyzed by the method of Feldman. For fibrinogen binding, the data were analyzed after 20-min and 30-min incubation, where binding was 90% and 100% complete, respectively.

Inhibition of ¹²⁵I-Fibrinogen Binding to Platelets

Concentrations of B59.2, ranging from 1.2 nM to 2.1 µM, were incubated with platelet suspension in the absence of stirring at room temperature. After 1 min, ¹²⁵I-fibrinogen was added (0.5 µM final concentration) and binding measured as previously described. Similar experiments were performed using EDTA (10 mM) or apyrase (50 µg/ml) or 4 µM of monoclonal antibody B33.1.

Protein Concentration

Protein concentration was calculated from absorbance at 280 nm using an extinction coefficient of 15.1 for fibrinogen and 14.3 for IgG.
FIBRINOGEN BINDING TO PLATELETS

30
60
90
200
250
300
600
900
200

AA
400M

COL 1 mg/ml

ADP 10 μM

EDTA 10 mM

FIBRINOGEN MOLECULES BOUND/PLATELET

FIBRINOGEN ADDED (FINAL CONCENTRATION nM)

Fig. 1. Dose-response of 125I-fibrinogen binding to platelets stimulated with ADP, collagen, or AA. The data are the mean of 7 different determinations.

was determined in platelet suspensions from 3 subjects who had ingested 1 g aspirin (Bayer, West Germany) 4 hr before donating blood. It appeared, that while binding in response to 40 μM AA was completely abolished, binding occurring in response to 10 μM ADP or 1 μg/ml collagen was inhibited by 50%–63% (data not shown). Identical results were obtained when binding was determined in suspensions of platelets incubated in vitro with 0.5 mM aspirin for 60 min (data not shown).

Specificity of the B59.2 Monoclonal Antibody

Triton-solubilized platelet membranes were applied to a B59.2 affinity column, an the column eluted with SDS. Electrophoresis of the eluate gave the pattern shown in Fig. 3. Two peptides of apparent molecular weight 130,000 and 95,000 were present, as well as a 45,000-dalton band comigrating with actin. Some material was variably present at the origin, its extent depending on the length of time the sample was boiled in SDS.

Binding of 125I-Labeled B59.2 to Platelets

Binding of B59.2 to both unstimulated and stimulated washed platelet suspensions reached equilibrium in 1 min. Up to 120-min incubation, >98% of the bound radioactivity could be displaced within 3 min by a 100-fold excess of unlabeled antibody. The extent of binding was the same for unstimulated platelets as for platelets stimulated by ADP, collagen, or arachidonic acid. There was no difference in binding in the presence of 2 mM Ca2+ or 10 mM EDTA. The binding isotherm for unstimulated platelets, together with the Scatchard analysis of these data, are shown in Fig. 4. One class of 21,380 binding sites (1 SEM = 3732) was found, with an apparent Ka of 0.019 μM.

Effect of B59.2 on Platelet Aggregation, Secretion, and TXB2 Synthesis

In the absence of added fibrinogen, washed platelet suspensions did not aggregate or secrete in response to 100 μM ADP. However, these platelets showed partial aggregation (range, 28.1–40.9 LTU) and secretion (range, 1.1–2.0 μM) of ATP in response to 1 μg/ml collagen or 40 μM AA. When fibrinogen was present, aggregation and secretion were complete (i.e., >60%

Table 1. Effect of Apyrase on 125I-Fibrinogen Binding to Platelets

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Specific Fibrinogen Binding (Molecules/Platelet)</th>
<th>Apyrase</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP (4 μM)</td>
<td>2,712 ± 298</td>
<td>23,718 ± 4,016*</td>
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<tr>
<td>ADP (10 μM)</td>
<td>6,140 ± 1,805</td>
<td>41,417 ± 8,428*</td>
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<tr>
<td>Collagen (0.25 μg/ml)</td>
<td>8,921 ± 4,628</td>
<td>18,698 ± 5,212*</td>
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<tr>
<td>Collagen (1 μg/ml)</td>
<td>44,006 ± 6,020</td>
<td>53,216 ± 9,030</td>
<td></td>
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<tr>
<td>AA (40 μM)</td>
<td>38,166 ± 9,030</td>
<td>69,049 ± 11,438*</td>
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</table>

*p < 0.01 for the difference between apyrase and buffer. The values reported are the means ± SEM of 4 determinations.
LTU and >2.5 μM ATP released) in response to 4 μM ADP, 0.25 μg/ml collagen, or 40 μM AA. 121-fibrinogen and unlabeled fibrinogen behaved identically in this system. Pretreatment of platelet suspensions with 10 mM EDTA resulted in complete inhibition of platelet aggregation in response to all the stimuli used, whether or not fibrinogen was present (data not shown). Both in the presence or in the absence of fibrinogen, B59.2 did not cause platelet aggregation at concentrations as high as 8.4 μM. As shown in panels A and B of Fig. 5, when 2.1 μM B59.2 was present, platelet aggregation and secretion in response to 40 μM AA or 4 μM ADP were inhibited by more than 90%. Similar results were obtained with 1 μg/ml collagen (data not shown). When higher concentrations of ADP (10 μM) were used, 2.1 μM B59.2 completely inhibited secretion but only partially affected aggregation (Fig. 5C).

Preincubation of platelet suspensions with B59.2, fibrinogen, or EDTA did not significantly affect TXB2 synthesis in response to 40 μM or to 1 μg/ml collagen (Table 2).

Effects of B59.2 on Fibrinogen Binding to Platelets

Fibrinogen binding to stimulated platelets was inhibited in a dose-dependent fashion by increasing concentrations (from 1.2 nM to 2.1 μM) of B59.2 (Fig. 5). A 2.1-μM concentration of antibody inhibited fibrinogen binding approximately 80% during the first 5 min (the time period within which aggregation occurs) and 45%–55% after 15–30 minutes (Fig. 6). The antibody decreased fibrinogen binding to stimulated platelets at all fibrinogen concentrations tested. Analysis of the data (Table 3) implies that only the low affinity sites were affected. Similar results were obtained when platelet suspensions were incubated with the F(ab')2 fragment of B59.2. In contrast, a concentration of antibody B33.1 as high as 4 μM did not affect platelet aggregation and secretion or fibrinogen binding (data not shown).

LDH Loss From Platelets

No LDH loss occurred when washed platelets were incubated with the antibody alone or in combination with ADP, collagen, or AA, in the presence or absence of fibrinogen or of PPP. Likewise, no LDH was lost from platelet suspensions treated with apyrase alone or in combination with the aggregating agents (data not shown).

DISCUSSION

It has previously been reported that stimulation with ADP, epinephrine, thrombin, or prostaglandin-H2 exposes fibrinogen binding on the platelet surface.1,9,17,18 In addition to these aggregating agents,
Fig. 5. Dose-dependent inhibition by antibody B59.2 of platelet aggregation, secretion, and fibrinogen binding in platelet suspensions treated with 40 μM AA (A), 4 μM ADP (B), and 10 μM ADP (C). Increasing concentrations of B59.2 were incubated with 0.5 ml platelet suspension (5 x 10^8/ml) that had been stirred at 37°C for 1 min at 1000 rpm. After 1 min, 25 μl unlabeled fibrinogen (0.5 μM final concentration) was added, and 1 min later, 50 μl of a mixture of firefly luciferase and luciferin and 1–5 μl of an aggregating agent was added. In parallel, aliquots of 0.5 ml of unstirred platelet suspension (5 x 10^8/ml) were incubated at room temperature with identical concentrations of aggregating agents. After 3 min, B59.2 (from 1.2 nM to 2.1 μM final concentration) was added, and 1 min later, 125I-fibrinogen (0.5 μM final concentration) was added. After 15 min, free and platelet-bound fibrinogen were separated in silicon oil and counted. The data reported are the mean of 4 determinations. It should be emphasized that the inhibition of fibrinogen binding is underestimated, because at 15 min, 14% of the bound labeled fibrinogen could not be displaced by a 100-fold excess of the cold protein.

treatment of platelets with chymotrypsin or pronase also exposes fibrinogen-binding sites. We have extended these observations to demonstrate similar properties for collagen and AA. We have found 2 classes of fibrinogen-binding sites after treatment with ADP, collagen, and AA, as did Peerschke et al. and Kornecki et al. with ADP, and Niewiarowski et al. and Kornecki et al. with chymotrypsin treatment. Others, however, have described only a single class of fibrinogen-binding sites induced by ADP, epi-nephrine, thrombin, and prostaglandin-H2. The difference in these results may be related to the method of platelet preparation, since 2 classes of binding sites are always seen with washed platelets (the present study). However, both one class and 2 classes of binding sites have been reported with gel-filtered platelets. In this paper we have included both raw fibrinogen data and Scatchard analyses for the sake of comparison with other studies. Scatchard analyses of our data have to be interpreted with
caution, since in order to reach equilibrium, fibrinogen and platelets must be incubated for 30 min. At this time, fibrinogen binding to platelets is 70% irreversible, violating another requirement for Scatchard analysis. However, when we analyzed our data after 15 min, when binding was 90% complete and only 14% irreversible, our results were essentially unchanged. We found 2 classes of binding sites at both times, whether or not irreversible binding is subtracted.

The nature of the fibrinogen-binding sites on the platelet surface is not precisely known, although most evidence suggests that they are, or are closely associated with GpIIb-IIIa.\(^1,3,17-20\) Platelets from patients with Glanzmann’s thrombasthenia are deficient in GpIIb-IIIa, and these platelets have decreased or absent binding of fibrinogen after stimulation with ADP.\(^1,3,17,18\) Using a monoclonal antibody against GpIIb-IIIa, which did not inhibit platelet aggregation, McEver et al.\(^4\) found the number of GpIIb-IIIa per platelet to be similar to the number of fibrinogen molecules bound at saturation. Our experiments using B59.2 detected approximately 22,000 GpIIb-IIIa complexes per platelet. For calculations, we assumed the binding of one molecule of antibody to each GpIIb-IIIa complex. It is possible that divalent antibody could have bound to different, but adjacent, GpIIb-IIIa complexes, causing us to underestimate the number of these complexes. On the other hand, as previously suggested,\(^6\) each GpIIb-IIIa complex might have several similar antigenic sites, resulting in the binding of several antibody molecules and leading to an overestimate of the number of complexes. Further evidence for the role of the GpIIb-IIIa complex in fibrinogen binding was shown using an antibody arising in a thrombasthenic patient that was specific against GpIIb-IIIa. This antibody inhibited fibrinogen binding to ADP-stimulated platelets.\(^17\)

Monoclonal antibody B59.2 inhibited platelet aggregation as well as binding of fibrinogen in response to collagen, AA, and ADP. Assuming that the platelet GpIIb-IIIa complex is at least one of the platelet binding sites for fibrinogen, some differences are apparent between the binding of antibody and of fibrinogen to this complex. While fibrinogen binding requires stimulated platelets and is calcium-dependent,\(^1,9,17,18,20\) the antibody binds equally well to stimulated and unstimulated platelets in a reaction that does not require calcium. It is possible that changes in the disposition of the GpIIb-IIIa complex, following platelet stimulation, are required for fibrinogen binding. Recently, Polley et al.\(^2\) have shown that GpIIb-IIIa may not be the actual fibrinogen-binding site, but may play a more indirect role in exposing fibrinogen-binding sites following platelet stimulation. This would be consistent with the findings of Kornecki et al., that high-affinity fibrinogen-binding sites on thrombasthenic platelets can be exposed by chymotrypsin treatment\(^18\) and that fibrinogen binding may be associated with a 70,000-dalton platelet membrane protein present in thrombasthenic platelets and exposed after

<table>
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<tr>
<th>Stimulus</th>
<th>Without Fibrinogen</th>
<th>With Fibrinogen (0.5 μM)</th>
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<tr>
<td></td>
<td>Buffer</td>
<td>B59.2 (2.1 μM)</td>
</tr>
<tr>
<td>Collagen (1 μg/ml)</td>
<td>1.230 ± 270</td>
<td>1.084 ± 302</td>
</tr>
<tr>
<td>AA (40 μM)</td>
<td>1.768 ± 290</td>
<td>1.710 ± 301</td>
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Mean ± SEM of 7 determinations.

![Fig. 6. Time course of \(^{11}H\)-fibrinogen binding to platelets and effect of B59.2. The data reported are the mean of 7 determinations.](https://example.com/fib.png)
chymotrypsin treatment. In this case, B59.2 may inhibit fibrinogen binding by interfering with GpIIb-IIIa function, in some manner preventing exposure of this site.

Results with antibody B59.2 suggest a complex relationship between fibrinogen binding and platelet aggregation and secretion. The pattern and the extent of this relationship seems dependent on the aggregating agent and on the concentration of the agent employed. When 40 μM AA or 1 μg/ml collagen was used, there was a parallel inhibition of aggregation and secretion. In contrast, when ADP was employed, secretion appeared to be more easily inhibited than aggregation. For example, a 10-nM concentration of B59.2 inhibited almost 40% of the secretion of ATP, but only 14% of the aggregation, occurring in response to 4 or 10 μM ADP. A 2.1-μM concentration of B59.2 totally inhibited secretion and aggregation in response to 4 μM ADP. At this concentration of antibody, platelet secretion in response to 10 μM ADP was totally inhibited, whereas aggregation was only decreased by 30%-35%. Even when aggregation in response to AA and collagen was completely inhibited, fibrinogen binding was only inhibited by 50%. We have no explanation for this finding, but it is interesting that Peerschke and Zucker observed fibrinogen binding in normal and thrombasthenic platelets on exposure to ADP or epinephrine. J Clin Invest 64:1393, 1979

Our results employing apyrase or aspirin suggest that the pattern of biochemical events inducing fibrinogen binding varies with the type and concentration of aggregating agent used. EDTA (10 mM) failed to inhibit TXB2 formation induced by 40 μM AA or 1 μg/ml collagen. This interesting new finding is under intensive investigation in our laboratory. Like EDTA, B59.2 had no effect on TXB2 formation, whether or not fibrinogen was present in the system. It is well known that in Glanzmann’s thrombasthenia, aggregation is absent, whereas platelet TXB2 formation is little affected. However, B59.2 cannot be said to induce a thrombasthenic-like state in normal platelets, since in Glanzmann’s thrombasthenia, platelet secretion is normal in response to AA. Jenkins et al., using a rabbit polyclonal antibody against human GpIIb–IIIa, also reached similar conclusions.

In conclusion, our data demonstrate that collagen and arachidonic acid can expose fibrinogen binding sites on platelets independently of ADP, and that the GpIIb–IIIa complex is involved in both secretion and aggregation in response to these agents. In contrast, thromboxane synthesis appears to be unrelated to GpIIb–IIIa-dependent fibrinogen binding.

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Exposure of platelet fibrinogen-binding sites by collagen, arachidonic acid, and ADP: inhibition by a monoclonal antibody to the glycoprotein IIb-IIIa complex

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