Investigation of the Mechanisms of Monoclonal Antibody-Induced Platelet Activation

By Peter Horsewood, Catherine P.M. Hayward, Theodore E. Warkentin, and John G. Kelton

Antiplatelet antibodies can activate platelets causing platelet aggregation and the release reaction. However, the pathway of activation by these antibodies is unknown and several potential mechanisms are possible. In this report, we describe studies investigating potential pathways of platelet activation by IgG antibodies. We tested 16 different IgG monoclonal antibodies (MoAbs) against a variety of platelet surface components and found that six antibodies were capable of causing platelet aggregation and release. These included MoAbs against glycoprotein (GP) IIb/IIIa, CD9, GPIV, and two other not well-characterized platelet components. There was no relationship between the number of platelet binding sites and the ability of an MoAb to activate the platelets. By adding intact and F(ab')2 preparations of the MoAb to control or Fc receptor-blocked platelets, we found that in all instances the MoAbs initiated platelet activation via interacting with the platelet Fc receptors. Clustering of the platelet protein components using a secondary antibody did not cause activation. Studies into the pathway of Fc-dependent activation demonstrated that the MoAbs were capable of activating platelets by occupying Fc receptors on adjacent platelets (interplatelet activation), as well as on the same platelet (intraplatelet activation).

GPLATELETS RESPOND to a variety of agonists by undergoing platelet aggregation and the release reaction. One family of agonists of interest includes IgG antibodies that bind to platelets. IgG can bind to platelet glycoproteins (GPs) or to platelet adsorbed proteins via the Fab portion of the IgG molecule. Antiplatelet autoantibodies, alloantibodies, and monoclonal antibodies (MoAbs) all have been reported to initiate platelet activation as measured by platelet aggregation and the release reaction. IgG can also bind to the platelet Fc receptor (FcR) and this reaction can cause platelet activation and the release reaction.

While very little is known about the possible mechanisms of platelet activation by autoantibodies, several details have emerged recently regarding activation by MoAbs. Thus, anti-CD9 MoAb has been shown to activate platelets through stimulation of a phospholipase C and through the secondary messengers inositol triphosphate and diacyl glycerol. Several earlier studies indicated an Ig Fc component requirement in MoAb-mediated platelet aggregation and release responses and Worthington et al have shown that the activation by anti-CD9 MoAbs is mediated by the FcγRII receptor. However, it is still not understood how the binding of both Fab and Fc portions of antiplatelet antibodies participate in receptor stimulatory events and why only some MoAbs cause activation.

In this report, we used a variety of MoAbs to investigate IgG-mediated platelet activation. We found that the platelet target of the antibody and not the absolute number of MoAbs that bound to the platelet surface determined whether platelet activation and the release reaction occurred. We also found that MoAb activation was mediated via Fc receptor (FcR) activation, occurring by both interplatelet and intraplatelet pathways.

MATERIALS AND METHODS

MoAbs

A variety of MoAbs (Table 1) were tested for their ability to induce platelet activation as measured by both platelet aggregation and the release reaction. MoAbs were selected that could bind to a wide variety of platelet proteins. These MoAbs included three MoAbs with activity against GPIb (6D1, a gift of Dr Barry Coller, Stonybrook, NY, and TW-1 and TW-2 produced in our own laboratory), three MoAbs against GPIIb/IIIa (10E5 and 7E3, gifts from Dr Barry Coller, and an MoAb, Raj-1, produced in our laboratory), two MoAbs against GPIII (Beb-1 and PH-1 produced in our laboratory), an MoAb against GPIV (OM5 from Ortho Diagnostics, Don Mills, Ontario, Canada), three MoAbs against thrombospondin (TSP) (CH-1, CH-2, and CH-3 produced in our laboratory) and two MoAbs against CD9 (AG-1, a gift from Dr Jonathan Miller, Syracuse, NY, and Alb-6, purchased from Bio Can, Mississauga, Ontario, Canada). Other MoAbs tested included one specific for the FcγRII receptor (CDw32) found on platelets (IV.3, a gift from Dr Clark Anderson, Columbus, OH) and two other not well characterized MoAbs, Jun-1 and JS-1, produced in our laboratory.

MoAbs from our laboratory were prepared from fusions of SP2/0 myeloma cells with spleen cells isolated from Balb/c mice. The mice were immunized with three intraperitoneal injections of 5 × 10⁷ washed, human platelets or 25 μg of purified antigen (for glycocalcin and TSP) given 3 weeks apart. Spleen cells were harvested 3 days after the last boost. Fusions were performed by the method of Galfre et al and culture supernatants were screened for reactivity against platelets or immunizing antigen bound to wells of microtitre plates using an enzyme immunoassay (EIA). Positive cultures were selected, cloned twice by limiting dilutions, and hybridomas grown as ascitic fluid in pristane-primed Balb/c mice.

Radioimmunoprecipitation

Platelets were prepared from whole blood collected in acid citrate dextrose (ACD) (6:1, vol:vol) containing 0.3 μmol/L prostaglandin E₁ (PGE₁). The platelets were washed three times in...
Table 1. Platelet Aggregation and Release Responses of MoAbs

<table>
<thead>
<tr>
<th>MoAb</th>
<th>Antibody Specificity</th>
<th>Antigen Sites per Platelet*</th>
<th>Aggregation Response (%)</th>
<th>Release Response (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10E5</td>
<td>GPIIb/IIIa</td>
<td>40,000 (10)</td>
<td>&lt; 2</td>
<td>0</td>
</tr>
<tr>
<td>7E3</td>
<td>GPIIIa</td>
<td>23,400</td>
<td>&lt; 2</td>
<td>4</td>
</tr>
<tr>
<td>Raj-1</td>
<td>GPIIb/IIIa</td>
<td>95</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Beb-1</td>
<td>GPIIX</td>
<td>22,000 (22)</td>
<td>&lt; 2</td>
<td>2</td>
</tr>
<tr>
<td>PH-1</td>
<td>GPIIIa</td>
<td>26,000 (9)</td>
<td>&lt; 2</td>
<td>3</td>
</tr>
<tr>
<td>6D1</td>
<td>GPIib</td>
<td>3,000 (23)</td>
<td>&lt; 2</td>
<td>8</td>
</tr>
<tr>
<td>TW-1</td>
<td>GPIib</td>
<td>2,000 (15)</td>
<td>&lt; 2</td>
<td>8</td>
</tr>
<tr>
<td>TW-2</td>
<td>GPIib</td>
<td>25,000 (24)</td>
<td>85</td>
<td>90</td>
</tr>
<tr>
<td>CH-1</td>
<td>TSP</td>
<td>66,000 (14)</td>
<td>95</td>
<td>99</td>
</tr>
<tr>
<td>CH-2</td>
<td>BSP</td>
<td>800 (13)</td>
<td>90</td>
<td>96</td>
</tr>
<tr>
<td>CH-3</td>
<td>TSP</td>
<td>900</td>
<td>90</td>
<td>91</td>
</tr>
</tbody>
</table>

Aggregation and release responses are representative of results from two or more experiments.

*Number of antigen sites reported per platelet are from MoAb binding curves done in the authors' laboratory (Beb-1, JS-1, and Jun-1) or are representative examples taken from published data (reference in parentheses).

Platelet Aggregation and Release Studies

Platelets from two healthy donors were labeled with 14C serotonin and the release assay performed as described previously. Briefly, labeled platelets (80 μL, 300,000/mL) were added to wells of a 96-well microtiter plate containing buffer or inhibitor (10 μL). MoAb or agonist (10 μL) was added after 10 minutes and the mixture gently stirred (15 rpm) with a siliconized stir bar for 1 hour. Release was terminated by the addition of 10 mmol/L EDTA in PBS (100 μL), the platelets pelleted at 1,600g for 5 minutes, and the supernatant fluid (50 μL) was removed and counted. All tests were performed in triplicate. Percent release was determined relative to Triton X-100 (BioRad, Richmond, CA) lysed platelets and corrected for spontaneous release (buffer control), which was always less than 10%.

Identification of MoAbs Capable of Causing Platelet Activation and Release

MoAbs in increasing concentrations (0 to 100 μg/mL) were added to the platelets and the platelets monitored for aggregation for 15 minutes. In other experiments, the platelet release reaction was measured as described previously.

The antibodies capable of initiating platelet aggregation and the release reaction were then investigated to determine if the F(ab')2 portion of the MoAb also was capable of causing the aggregation and release reaction. To determine if clustering of the target platelet GP by the antibody was capable of initiating platelet aggregation and release, F(ab')2 preparation of a goat anti-murine antibody was added to the platelets to which the F(ab')2 murine MoAb had already been added.

Study of the Participation of Fc Interactions in Platelet Aggregation and Release

Several MoAbs were shown to initiate platelet aggregation and the release reaction. For those positive MoAbs, the test platelets were preincubated with a saturating concentration of MoAb IV.3, which binds to the platelet Fc receptor. This MoAb can inhibit Fc-mediated platelet activation and the release reaction. 15

Studies Investigating the Contribution of Interplatelet Fc-Dependent Activation

As will be described (Results section), we found that the antibody, Raj-1, specific for GP Ib/IIIa was capable of causing platelet aggregation. Other studies (Results section) also indicated that it was the binding of the MoAbs to the FcR that initiated platelet activation. In the next series of studies, platelets from a patient completely deficient in GP Ib/IIIa (Glanzmann's thrombasthenia) were loaded with 14C-serotonin, and then incubated with control platelets to which antibody IV.3 (anti-FcR) had been added. Hence, any release of the 14C-serotonin would require initial binding of the anti-Iib/IIIa MoAb to the nonradiolabeled control platelets that carried normal amounts of GP Ib/IIa, and that also had their Fc receptors blocked by the monoclonal IV.3. Under these conditions, the MoAb could only cause release if it bridged and bound to the FcR on the Glanzmann's platelets.

Interplatelet Release

PRF was prepared using blood from normal volunteers and from a patient with Glanzmann's thrombasthenia, and platelets labeled with serotonin. Previous studies have shown that this patient bound normal amounts of heat-aggregated IgG and had normal release responses. 1 FcR blocked platelets were prepared as follows: ascites...
fluid, containing MoAb IV.3, was added to labeled and unlabeled normal platelets (1/500 final dilution) and incubated at room temperature for 20 minutes. Unbound antibody was removed by washing in calcium and albumin-free Tyrode's buffer, pH 6.2. The FcR-blocked platelets were resuspended in Tyrode's buffer containing calcium and magnesium, pH 7.4. Buffer or agonist or Triton X-100 (20 μL) was added to platelet populations of Glanzmann’s or FcR-blocked, normal platelets (80 μL). The same reagents (buffer or agonist or Triton X-100) were added also to platelet populations (40 μL + 40 μL) of Glanzmann’s platelets plus FcR-blocked, normal platelets. The Glanzmann’s or the FcR-blocked, normal platelets of the mixed populations were either labeled or unlabeled and used in various combinations (Results section). After 1 hour of incubation at room temperature, the platelet release was measured. Release was measured relative to the Triton X-100 positive control (100%) and corrected for spontaneous release (buffer).

Intraplatelet Release

The mixed population experiments were contrived to restrict MoAb binding to interplatelet interactions. However, the question of whether an intraplatelet interaction could occur remained unanswered. The reason was that in a completely unrestricted system both interplatelet and intraplatelet interactions are possible. The only situation where an intraplatelet mechanism can occur independent of an interplatelet one is with individual platelets in isolation.

Preliminary investigations were performed to determine the optimum conditions that allowed supernatant to be separated from platelets after centrifugation of an agarose gel containing isolated platelets. A low gelling temperature (<3°C), low fragility agarose (type VII; Sigma Chemical Co, St Louis, MO) was chosen and used at a final concentration of 0.15%. The agarose gel (0.6%) was dissolved in boiling water, cooled to 35°C, and diluted with an equal volume of twice concentrated Tyrode’s buffer containing calcium and magnesium, pH 7.4. Equal volumes of the diluted agarose and 14C-serotonin-labeled platelets (500,000/μL) were mixed and added (160 μL) to wells of a 24-well plate (Nunc, GIBCO Canada, Burlington, Ontario, Canada). The plate was placed on a level surface in a refrigerator to allow the rapid gelling of the agarose. After 5 minutes the plate was removed, allowed to equilibrate to room temperature, and agonist, buffer, or Triton X-100 added (40 μL). The plate was covered and the solutions were allowed to diffuse into the gel over 2 hours at room temperature, after which the reaction was terminated. Termination was achieved by the addition of 100 μL of either 10 mmol/L EDTA in PBS or PGE1 (4 μmol/L final) plus IV.3 MoAb (1/1,000 ascites final) followed by centrifugation at 1,600g for 10 minutes. Supernatant fluid (100 μL) was carefully removed and counted. To ensure that the platelets immobile in the agarose gel were indeed isolated, mixing experiments were conducted. Labeled, thrombathenic platelets and FcR-blocked platelets were co-immobilized in agarose and release by MoAbs monitored. Under these conditions, release by Raj-1 would require contact between the two platelet populations.

RESULTS

Characterization of MoAbs

MoAb Raj-1 immunoprecipitated two bands of M, 90,000 and 140,000 from the lysate of surface 125I-labeled platelets as analyzed by SDS-PAGE under reducing conditions. Under nonreducing conditions Raj-1 immunoprecipitated bands of M, 90,000 and 140,000. These data, taken together with identity with the bands immunoprecipitated by an anti-P1 Ali alloantiserum, indicated Raj-1 to be specific for the GPIIb/IIIa surface GP complex. Raj-1 failed to react with SDS-PAGE-separated platelet proteins electrophoretically transferred to nitrocellulose, precluding direct assignment of a specificity to one or the other of the GPIIb/IIIa proteins. Immunoprecipitation of labeled lysate precipitated with EDTA at 37°C and pH 8.0 for 30 minutes showed no precipitable bands indicating specificity for the intact GPIIb/IIIa complex. Under the same conditions, anti-P1 Ali and anti-Bak* alloantisera immunoprecipitated GPIIa and GPIIIb, respectively. These latter conditions are known to dissociate the GPIIb/IIIa heterodimer into its constituent components. Neither Raj-1 nor anti-P1 Ali alloantisera immunoprecipitated proteins from the lysate of radiolabeled platelets from a patient with Glanzmann’s thrombocytopenia.

Immunoprecipitation of labeled platelet lysate using MoAb JS-1 showed a major band at M, 155,000 on SDS-PAGE when electrophoresis was performed under reducing conditions. Immunoprecipitation under nonreducing conditions on a 3% to 6% gradient gel showed several high molecular weight bands. Comparison of these bands with the bands precipitated by all of our other MoAbs or alloantisera failed to show any identity with known platelet GPs. Direct binding studies with radiolabeled F(ab’2) of JS-1 indicated that there are about 600 copies of the JS-1 antigen on resting platelets and this increases to about 4,000 copies after thrombin stimulation. Details of the characterization of JS-1-specific antigen will be reported elsewhere.

Jun-1 MoAb bound to a platelet surface determinant as shown using a whole platelet EIA method. However, the antibody failed to show any bands on autoradiography after SDS-PAGE immunoprecipitation studies with lysate from either 125I or 3H surface-labeled platelets. Immunoblot analysis also showed that Jun-1 was unreactive with SDS-PAGE-separated platelet proteins electrophoretically transferred to nitrocellulose. Direct binding studies with radiolabeled F(ab’2) fragment of Jun-1 indicated that there are about 900 copies of the Jun-1-specific antigen on resting platelets.

Platelet Activation by MoAbs

In Table 1 is shown the various MoAbs tested and the presence or absence of the aggregation and release reaction. This table illustrates that none of the MoAbs against the Ib and IX GP complex (a total of five different MoAbs) caused platelet aggregation or release. Only one of the three MoAbs against GPIIb/IIIa caused platelet aggregation and release, as did the MoAb against GPIV. The MoAb against the p155 GP, JS-1, caused aggregation and release, and also the MoAb, Jun-1, against the uncharacterized platelet membrane component. The two different MoAbs against the CD9 antigen both caused aggregation and release. Also shown in Table 1 are the approximate number of copies of the various GPs per platelet, as
determined either by ourselves or taken from the literature. There was no relationship between the number of copies of the GP on the platelet surface and the ability of the MoAb to induce platelet aggregation and the release reaction.

The MoAbs Raj-1, Jun-1, and JS-1 were all of IgG, \( \kappa \) type and the FcRII has been shown to specifically bind this subclass in responder individuals.\(^\text{23}\) All of the other MoAbs tested were of the IgG, isotype except antibody 10E5, which is of the IgG\(_3\) isotype.

**Aggregation Studies**

Addition of the MoAbs Raj-1, Jun-1, or JS-1 to PRP induced strong aggregation (Fig 1). A dose-response effect was seen for all three antibodies. Increasing amounts of antibody caused increasing aggregation and a decreasing lag time. The FcR requirement of the aggregation reaction was shown when addition of F(ab’\(_2\)) fragments of the MoAbs failed to cause aggregation of PRP. The F(ab’\(_2\)) fragments not only did not cause aggregation, but they effectively blocked aggregation of their corresponding intact antibodies (Table 2). The addition of 20 \( \mu \)g/mL of F(ab’\(_2\)) fragment of Raj-1 caused complete inhibition of aggregation initiated by intact Raj-1. Addition of 0.25 \( \mu \)g/mL F(ab’\(_2\)) fragment caused only 10% inhibition. The specificity of the inhibition was substantiated when prior incubation with PRP were dibutyl cyclic AMP, EDTA, and thrombin and ADP.\(^\text{19-21}\) 10E5 consistently failed to inhibit aggregation caused by the GPIIb/IIIa-specific MoAb Raj-1. It is possible that binding of Raj-1 competes with 10E5, or causes a conformational change in GPIIIa, such that 10E5 can no longer bind but fibrinogen is still able to mediate in aggregation.

**Platelet Release Studies**

Serotonin was released from washed platelets when incubated with any of the MoAbs Raj-1, Jun-1, and JS-1. The release was shown to be dose-dependent (Fig 2) and could be inhibited by PGE\(_2\), F(ab’\(_2\)) fragments of the antibodies did not cause release of serotonin, indicating that the FcR portion of the IgG molecule was required. Incubation with MoAb IV.3 caused complete inhibition of the release induced by the antibodies (Fig 2). Antibody IV.3 was shown also to inhibit release caused by MoAbs OKM5, AG-1, and Alb-6 (data not shown). OKM5 is specific for GPIV and has been reported previously to cause platelet activation.\(^\text{26}\) AG-1 and Alb-6 are both specific for the CD9 antigen and previously have been shown to cause release of platelet granule contents.\(^\text{27}\)

**Pathways of MoAb-Induced Platelet Aggregation and Release**

Investigation of whether receptor clustering was capable of causing platelet aggregation and release. Previous studies described by Rosenfeld et al\(^\text{27}\) have shown that cross-linking of mouse antibody against the FcR (IV.3) by the F(ab’\(_2\)) fragment of goat antime Ig can induce platelet aggregation. We confirmed this observation and showed that platelet release occurred with either whole or Fab fragment of anti-FcR antibody (IV.3) when cross-linked with F(ab’\(_2\)) fragments of goat antime IgG. Cross-linking of bound anti-GPIIb/IIIa MoAbs 10E5 and 7E3, which by themselves did not cause release, with F(ab’\(_2\)) fragments of goat antime IgG did not cause platelet release. Additionally,
none of the MoAbs, Raj-1, Jun-1, or JS-1, that by themselves caused platelet aggregation and release, were capable of causing release when their bound F(ab')2 fragments were cross-linked by the goat antimouse Ig (Table 3). These data support a mechanism of platelet activation through FcR clustering but not MoAb-induced platelet GP clustering.

Studies investigating whether the Fc-dependent platelet aggregation and release was interplatelet or intraplatelet. The addition of intact MoAbs (Raj-1, Jun-1, JS-1) to 14C-serotonin-labeled platelets that previously had been incubated with anti-FcR antibodies (IV.3) prevented these platelets from undergoing aggregation and the release reaction (Figs 1 and 2).

MoAbs Jun-1 and JS-1 both caused release from the labeled thrombasthenic platelets, but no release occurred with the anti-GPIIb/IIIa-specific MoAb Raj-1. Similarly, Raj-1 did not cause release with the FcR-blocked normal platelets (Fig 3).

Mixtures of serotonin-labeled, FcR-blocked platelets and labeled Glanzmann’s platelets gave release with all three antibodies. The positive result with the Raj-1 antibody can only be interpreted as binding of the antibody to GPIIb/IIIa antigen binding sites on the IV.3-blocked platelets and simultaneously on the FcR on the Glanzmann’s platelets. This is an interplatelet MoAb bridging. That the 

\[ \text{Table 3. Release by Antibody Cross-Linking} \]

<table>
<thead>
<tr>
<th>MoAb</th>
<th>F(ab')2, Goat Antimouse IgG</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>7E3</td>
<td></td>
<td>(1)</td>
<td>(1)</td>
</tr>
<tr>
<td>10E5</td>
<td></td>
<td>(0)</td>
<td>(1)</td>
</tr>
<tr>
<td>Raj-1 F(ab')2</td>
<td></td>
<td>(2)</td>
<td>(0)</td>
</tr>
<tr>
<td>Jun-1 F(ab')2</td>
<td></td>
<td>(0)</td>
<td>(0)</td>
</tr>
<tr>
<td>JS-1 F(ab')2</td>
<td></td>
<td>(0)</td>
<td>(0)</td>
</tr>
<tr>
<td>IV.3</td>
<td></td>
<td>(92)</td>
<td>(0)</td>
</tr>
<tr>
<td>IV.3 Fab</td>
<td></td>
<td>(100)</td>
<td>(0)</td>
</tr>
</tbody>
</table>

Platelet serotonin release by MoAbs or their fragments was measured with or without added F(ab')2 fragments of a goat antimouse IgG secondary antibody.

and not the antigen-bearing platelets was shown when Raj-1 was added to a mixture of unlabeled Glanzmann’s platelets with labeled FcR-blocked platelets. Under these circumstances, no release occurred (Fig 3). Conversely, release did occur when Raj-1 was incubated with a mixture of labeled Glanzmann’s platelets and unlabeled FcR-blocked platelets. This indicated that clustering of FcR mediated release.

To examine antibody interactions with isolated platelets, the platelets were immobilized in a suspension of agarose. The labeled platelets were shown by size analysis to be free of aggregates and calculations showed that the platelets represented only 0.2% of the gel volume. MoAbs Raj-1, Jun-1, and JS-1 all released 14C-serotonin when allowed to diffuse into a gel containing immobilized, labeled platelets. Heat-aggregated human IgG and thrombin also caused release (Fig 4). No release occurred with either buffer or with an MoAb, Beb-1, specific for platelet GPIX (Fig 4).
These experiments show that release can occur through an intraplatelet mechanism.

To ensure that release did not occur via interplatelet contact of antibody-sensitized platelets during the centrifugation step, we terminated the reaction by adding a mixture of PGE, and IV.3 MoAb. These experiments gave identical results to the experiments terminated with EDTA. From previous release experiments (Fig 2), IV.3 antibody was shown to be able to inhibit platelet release that could occur on centrifugation of sensitized platelets.

In experiments with mixed platelet populations, release with antibody Raj-1 (anti-GPIIb/IIIa) was similar to the buffer background while antibody Jun-1 initiated strong release (Table 4). This experiment shows that there was very little or no platelet-platelet contact in the agar.

### Table 4. Antibody-Mediated Platelet Release of $^{14}$C-Serotonin Using Platelets Suspended in Agarose Gel

<table>
<thead>
<tr>
<th>MoAb</th>
<th>Radiolabeled Platelets</th>
<th>% Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raj-1</td>
<td>Glanzmann's</td>
<td>19</td>
</tr>
<tr>
<td>Jun-1</td>
<td>Glanzmann's</td>
<td>88</td>
</tr>
<tr>
<td>Buffer</td>
<td>Glanzmann's</td>
<td>10</td>
</tr>
<tr>
<td>Raj-1</td>
<td>Glanzmann's + FcR blocked</td>
<td>22</td>
</tr>
<tr>
<td>Jun-1</td>
<td>Glanzmann's + FcR blocked</td>
<td>57</td>
</tr>
<tr>
<td>Buffer</td>
<td>Glanzmann's + FcR blocked</td>
<td>7</td>
</tr>
</tbody>
</table>

The $^{14}$C-serotonin-labeled platelets were from a patient with Glanzmann's thrombasthenia and were tested alone or mixed 1:1 with $^{14}$C-serotonin-labeled, FcR-blocked, normal platelets. The percent release is relative to Triton X-100 lysis (100%) and is the average of triplicates. This study demonstrates that agarose gel prevents platelet-platelet contact, as shown by the inability of the MoAb against GPIIb/IIIa (Raj-1) to activate by binding to the FcR-blocked platelets and then binding to FcR on the Glanzmann's platelets.

### DISCUSSION

A large number of agonists can bind to platelets initiating platelet aggregation and the release reaction. For most, the mechanism is a ligand-receptor coupling with secondary signal transduction. Antiplatelet iso,$^{21}$ auto,$^{29}$ and xeno$^{30}$ antibodies have all been shown to be capable of causing platelet aggregation and release, but the pathway of platelet activation has not been well studied. At least three different potential mechanisms of antibody-mediated platelet activation can be postulated. These are illustrated schematically in Fig 5. In type I, platelet activation occurs via binding of the antibody through its Fab region to a platelet surface membrane component (usually a GP). This represents a classical receptor stimulation which, under normal conditions, occurs via ligand occupancy. A second potential type of antibody-mediated platelet activation (arbitrarily designated as type II) could occur through an antibody-mediated local clustering of platelet surface receptors. The clustering or aggregation of these receptors would be responsible for initiating platelet activation. Aggregation induced by cross-linking may be a normal requirement for signal generation for many receptors and clustering of epidermal growth factor receptors by MoAb has been shown to result in cellular activation.$^{31}$ A third theoretical mechanism of platelet activation is via the binding of the antibody Fab portion of IgG to a platelet surface constituent with activation occurring via occupancy of the platelet FcR by the Fc portion of the IgG. This type of interaction can be further subdivided into the IgG binding to FcR on the same platelet or on adjacent platelets. A survey of the studies describing MoAb-mediated platelet activation does not clarify whether all of these pathways of platelet activation are possible. Some investigators have described results that would be consistent with the activation following the binding of antibody to a platelet protein occurring either by direct stimulation (type I activation) or by antigen clustering (type II).$^{32}$ Others have suggested that it occurs via Fc interaction (type III).$^{44}$

Uncertainty also exists about the general characteristics of a platelet surface protein that would make it more or less likely to initiate platelet activation. The previous reported cases of platelet activation caused by MoAbs have, with one exception, been caused by antibodies against high frequency surface antigens. Thus, MoAbs against CD9 and GPIIb/IIIa can cause platelet activation. Both CD9 and GPIIb/IIIa are the most abundant surface proteins, having approximately 40,000 to 60,000 copies per platelets.

In this report, we attempted to address these issues by...
using a large and representative sample of MoAbs against platelet surface proteins (Table 1). The first observation we made was that there is no apparent relationship between the number of MoAbs bound and the likelihood of activating platelets. An activating MoAb against a surface protein having about 1,000 copies per platelet also has been described.19

The membrane mobility of a platelet protein may also be important in determining whether an MoAb can activate platelets. MoAbs against the GPIIb/IIIa complex and against CD9 caused platelet activation. These surface antigens (IIb/IIIa and CD9) are readily mobile. GPIIb/IIIa complex can patch and cap with either monoclonal or with allo- and autoantibodies.20 In contrast, GPIb and adjacent GPIX are not surface mobile.21 Neither our own, nor any reported MoAbs against this complex activate platelets. At the same time, there are other factors influencing MoAb-initiated platelet activation, and mobility alone is insufficient. For example, only one of the three MoAbs that we tested against GPIIb/IIIa complex were capable of causing platelet activation. Presumably, local topographical factors also contribute to whether an MoAb can cause platelet activation.

The platelet-activating MoAbs directed against GPIIb/IIIa, GPIV, CD9, and the two uncharacterized platelet membrane components (JS-1 and Jun-1) were used to study if there were different pathways of IgG-mediated platelet activation. The platelet-activating MoAbs had a number of different types of specificity that ranged from the abundant GPIIb/IIIa and CD9 to uncommon and not well characterized platelet surface components p155 and the target of MoAb Jun-1. We systematically investigated the three potential pathways of antibody-mediated platelet activation shown in Fig 5. When F(ab')2 fragments were used, none of our MoAbs had a direct activating effect. Invariably, aggregating and release reactivity was lost, excluding a type 1 mechanism. Similarly, when an anti-IgG reagent was used to cross-link the initial antibody, only an MoAb against the FcR (IV.3) could activate platelets following clustering (type 2). This latter result confirms previous observations of Rosenfeld et al that one pathway of platelet activation is via FcR clustering.16

It has long been known that the binding of IgG immune complexes to human platelets can cause platelet activation and the release reaction by binding to platelet FcR. Recent investigators have shown that this also can occur for MoAbs directed against CD9 on platelets.5 The studies described in this report indicate that platelet-activating MoAbs activated platelets via the platelet FcR. This was demonstrated by showing that preincubation of test platelets with IV.3 (anti-FcR) prevented any platelet activation by the MoAbs. Similarly, the use of F(ab')2 alone or in a preincubation stage before adding the intact MoAb resulted in a loss of the platelet activation by their respective MoAbs.

We next determined whether Fc activation occurred across adjacent platelets (interplatelet). The results of these studies were uniformly consistent and indicated that platelet activation occurred when the MoAb bound to the platelet GP on one platelet and initiated the release reaction by occupying the FcR on adjacent platelets (type III, interplatelet).

Although we had demonstrated that MoAbs could activate platelets by an interplatelet FcR-mediated mechanism, it remained uncertain whether the MoAb was capable of causing platelet activation by binding to FcR on the same platelet. To test this possibility, we suspended the platelets in a loose agar that was selected so that the agonist could diffuse into the agar and activate individual platelets, but the platelets could not physically interact with each other. These studies demonstrated that MoAbs can bind via their Fab region to a platelet surface protein and then cause platelet activation by binding to the platelet FcR on the same platelet (the "scorpion" model of platelet activation).

Some MoAbs against GPIIb/IIIa cause platelet activation and some do not. The reasons for the differences is at present unknown but may involve epitope specificity. Similarly, it is likely that some antibodies cause platelet activation by either, or both, interplatelet or intraplatelet mechanisms. The preference for one mechanism over another may depend on the target antigen and/or the epitope specificity.

Our studies using MoAbs indicate that a number of factors determine whether a particular MoAb can cause platelet activation. An important determinant is whether the target protein of the MoAb is mobile and can cluster on the platelet surface. In our hands, every MoAb that caused platelet activation did so via interacting with the platelet FcR and this interaction can occur between platelets or on the same platelet.

REFERENCES

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Investigation of the mechanisms of monoclonal antibody-induced platelet activation

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