Fc-Independent Cross-Linking of a Novel Platelet Membrane Protein by a Monoclonal Antibody Causes Platelet Activation

By Stefan De Reys, Marc F. Hoylaerts, Marc De Ley, Jos Vermylen, and Hans Deckmyn

A monoclonal antiplatelet antibody (MA-13G8E1) is described that dose-dependently induces platelet aggregation and serotonin release in an Fc-independent fashion. Whereas platelets were equally aggregated by Fab' fragments of this monoclonal antibody (MoAb), its Fab fragments, on the other hand, were inactive, indicating that divalent interaction is an essential requirement to induce platelet activation by MA-13G8E1. In addition, we could show that platelet epitope cross-linking by MA-13G8E1 occurred on the same platelet. MA-13G8E1 stimulated platelet phospholipase C (PLC) and induced activation of protein kinase C (PKC), both of which were almost unaffected by aspirin pretreatment. Furthermore, PLC activation appeared to be a direct antibody-mediated effect, since intracellular Ca\(^{2+}\) rises were not inhibited by EGTA, cytochalasin B, or aggregation-blocking MA-16N7C2 (antigliycoprotein [anti-GPIIb/IIIa]). The MA-13G8E1 antigen is constitutively expressed on resting platelets of different species (7,100 ± 800 molecules per human platelet), but not on other cell types tested. Both immunoprecipitation and affinity isolation by MA-13G8E1 showed two low-molecular weight proteins (46 and 36 kD), having slightly acidic isoelectric pH levels (4.5 to 5.5) and forming multimolecular complexes. In conclusion, we found an MoAb that is able to induce platelet activation in an Fc-independent fashion. The mechanism involves cross-linking of a hitherto undescribed platelet membrane protein, leading to PLC and PKC stimulation.

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APART FROM THEIR activation by physiologic agonists, platelets can aggregate and release the contents of their granules following stimulation by monoclonal antiplatelet antibodies. Although many activating monoclonal antibodies (MoAbs) directed against different platelet membrane proteins have been described, aggregation induced by the vast majority is mediated by cross-linking the antigen to the platelet Fc receptor (FcγRII).\(^1\)\(^,\)\(^2\) We previously reported on this type of activation and concluded that this cross-linking occurs on the same platelet and that the activation pathway followed depends on the subtype of the activating MoAb.\(^3\) However, a number of papers show that FcγRII-dependent cross-linking of adjacent platelets by MoAbs also leads to platelet aggregation.\(^4\)\(^,\)\(^5\) The signals generated following FcγRII cross-linking were repeatedly studied and involve phospholipase C (PLC) activation, thromboxane A\(_2\) (TXA\(_2\)) generation,\(^6\) and phosphorylations, i.e., tyrosine phosphorylation of different proteins, including FcγRII itself\(^7\) and phosphorylation by protein kinase C (PKC).\(^5\)

A much smaller group of MoAbs are able to induce platelet aggregation independently of Fc interactions. For example, Fab binding of some anti-GPIIb/IIIa antibodies induce conformational changes in the receptor molecule, allowing fibrinogen to bind. However, these examples only comprise antiglycoprotein [anti-GPIIb/IIIa MoAbs and do not involve general signal transduction mechanisms.\(^8\)\(^,\)\(^9\)

In this report, we describe an MoAb that initiates platelet activation by yet another Fc-independent mechanism, i.e., cross-linking of a hitherto unknown platelet membrane protein. Although this type of activation by MoAbs has rarely been shown for platelet membrane glycoproteins, cross-linking of a substantial number of antigens on other cells (eg, T-cell differentiation antigens,\(^1\) epidermal growth factor [EGF] receptor,\(^1\) insulin receptor,\(^1\) and prolactin receptor\(^1\)) readily induces activation. Interestingly, some studies suggested that cross-linking mimics the physiologic agonist to the receptor.\(^1\)\(^,\)\(^2\) Therefore, it is tempting to believe that this might also occur in our example, implying that the membrane protein which is recognized might well be a potential platelet receptor.

MATERIALS AND METHODS

Materials. MoAbs MA-13G8E1 (IgG\(_2\_a\)), MA-16N7C2 (IgG\(_2\_a\), anti-GPIIb/IIIa), and MA-8L4A12 (IgG\(_i\)) were developed in our laboratory; MoAb IV-3 (IgG\(_2\_a\), anti-FcγRII) was purchased from Medarex Inc (Annadale, NJ) and MoAb Raj-1 (Ig\(_i\), anti-GPIIb/IIIa) was a kind gift of Dr Peter Horsewood (McMaster University Medical Center, Hamilton, Canada). Pepsin, thrombin, cytochalasin B, Fura-2/AM, 3-[(1-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) and Agarose (low gelling temperature) were purchased from Sigma Chemical Co (St Louis, MO). Iodo beads (N-chloro-benzensulfonamide)-polystyrene beads were from Pierce Europe BV (Oud Beijerland, The Netherlands), prostacyclin (PGI\(_2\))-sodium salt from Upjohn Diagnostics (Kalamazoo, MI), lysine-acetylsalicyclic acid (ASA) from Synthelabo Beneux (Brussels, Belgium), papain from Fluka Chemie (Buochs, Switzerland), and Triton X-100 from Boehringer Mannheim (Mannheim, Germany). Radiolabeled products came from Amersham (Amersham, UK). Standard chromatography media were supplied by Pharmacia LKB Biotechnology (Uppsala, Sweden). All other materials were of analytical grade.

Platelet aggregations were performed in an Elvi-840 dual-channel aggregometer from Pabisch (Brussels, Belgium). For single-platelet counting techniques, platelets were stirred in a Variamag Telesystem HP60 from HVL (Brussels, Belgium) and counted in a Cell Dyn 610 Counter (Sequioa-Turner Corp, Mountain View, CA).

One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional electrophoresis (isoelectric focusing in the first and SDS-PAGE in the second dimension) were performed using horizontal systems (Phast System and Multiphor II System, respectively) both from Pharmacia LKB Biotechnology, according to the manufacturer’s instructions. Band intensities of proteins separated on SDS-PAGE were measured by gel scanning on an Ultroscan XL laser densitometer from Pharmacia LKB Biotechnology.

Production and purification of MA-13G8E1. Balb/C mice were immunized intraperitoneally with 1 × 10\(^6\) washed human platelets

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(see below), pooled from eight healthy volunteers. The generated hybridoma cells were screened for antiplatelet antibody production in an enzyme-linked immunosorbent assay (ELISA) using microtiter plates coated with human platelets. One of the positive hybridomas, 13G8EI, was injected into pristane-primed Balb/C mice. After 3 weeks, ascitic fluid was collected and the antibody was purified on protein A Sepharose as described previously.

Digestion of MA-13G8EI. For the preparation of F(ab')2 fragments, MA-13G8EI was incubated with pepsin in 0.2 mol/L acetic acid buffer (pH 4.7) (IgG:pepsin:25/1 [wt/wt]) for 24 hours at 37°C. The reaction was stopped by the addition of 1 vol 1 mol/L Tris-HCl buffer (pH 8.3).

Fab fragments were prepared by papain digestion. To this end, papain was mixed with IgG (IgG:papain:25/1 [wt/wt]) in 0.1 mol/L phosphate buffer (pH 7.3) in the presence of 10 mmol/L cysteine and 2 mmol/L ethylenediaminetetraacetic acid (EDTA) for 4 hours at 37°C, after which the reaction was stopped by the addition of ICH2COOH (30 mmol/L).

All fragments were dialyzed against 0.1 mol/L Tris-HCl buffer (pH 8.3) and separated from Fe: fragments and undigested IgG on protein A Sepharose. Their purity was evaluated by SDS-PAGE and the concentration was determined from absorption measurements (A280 (1 cm) (1 mg/mL) = 1.35 mL mg⁻¹ cm⁻¹).

Platelet aggregations and platelet counting techniques. Platelet-rich plasma (PRP) and gel-filtered platelets (GFP) were prepared as described previously. Aggregation of platelet suspensions (300,000 platelets/μL; 250 μL) was performed in plastic microcuvettes under stirring conditions (1000 rpm) in the presence of protease inhibitors (2 pg/mL aprotinin, 2 pg/mL leupeptin, and 100 μg/mL phenylmethylsulfonyl fluoride). The platelets were lysed with 0.5% Triton X-100. One volume of protein A Sepharose (4B) was bound, washed and treated with 0.1% vol/vol phosphate-buffered saline (PBS) and coated on 96-well polystyrene plates at 4°C for 16 hours. The plates were further treated according to standard procedures using MA-13G8EI coupled to horseradish peroxidase, prepared according to Nakane and Kawaoi. Gel filtration of the MA-13G8EI antigen. An aliquot of the antigen was purified by affinity chromatography on MA-13G8EI–Sepharose 4B, prepared from activated CNBr-Sepharose according to the manufacturer’s instructions. Washed platelets (5 × 10⁹ platelets/mL) in modified Ernes buffer supplemented with 10 mmol/L EDTA, 3 mmol/L MgCl₂, and protease inhibitors (see above) (MEPAL buffer) were lysed with 0.1% vol/vol Triton X-100 and eluted with 2.5 mol/L potassium thiocyanate added to the buffer. The mixture was incubated with 0.5 μL Triton X-100 for 30 minutes and incubated overnight at 4°C. After centrifugation (1 hour, 50,000g) at 4°C, the supernatant was removed and the pellet was washed extensively with 0.1% vol/vol glycine buffer (pH 2.8). After centrifugation (10 minutes, 10,000g), the immunoprecipitated proteins were separated by SDS-PAGE (10% gel) and visualized by autoradiography.
Aggregation of platelets by MA-13G8E1 and its F(ab')2 fragments. The murine antiplatelet MoAb, MA-13G8E1, aggregated human platelets and stimulated the release of serotonin both in plasma and buffer. The observed responses were completely inhibited by prostacyclin (50 nmoL/L) or an MoAb against GPIIb/IIIa (MA-16N7C2, 50 μg/mL), indicating that MA-13G8E1 induces aggregation rather than agglutination of platelets (Fig 1A). This platelet aggregation was dose-dependent, aggregation rate and lag time being increased and decreased, respectively, upon raising the MoAb concentration. However, maximal aggregation remained constant. F(ab')2 fragments of MA-13G8E1 also elicited strong aggregation responses, comparable to the ones obtained with equivalent amounts of intact antibody (inset of Fig 1A) and likewise inhibited by prostacyclin or MA-16N7C2 (data not shown). Additionally, preincubation of platelets (15 minutes) with the Fc receptor blocking MoAb IV.3 (10 μg/mL) did not have any effect on MA-13G8E1-induced aggregations (Fig 1A).

Finally, rabbit platelets, lacking functional Fc receptors,27 aggregated upon addition of MA-13G8E1 or its F(ab')2 fragments. On the other hand, MA-7R4E7, which aggregates human platelets through cross-linking GPIIb/IIIa with the Fc receptor,2 bound rabbit platelets, but failed to aggregate them (Fig 1B). Altogether, these experiments provide strong evidence that MA-13G8E1-induced aggregations are not dependent on Fc receptor binding.

MA-13G8E1-induced aggregations are triggered by cross-linking receptors on the same platelet. In contrast to the activating ability of divalent IgG and F(ab')2, monovalent Fab fragments were devoid of any platelet aggregating effect even at high concentrations (1 μmol/L; see inset of Fig 1A). This cannot be explained by a marked decrease in affinity for the corresponding platelet epitope, because Fab fragments strongly inhibited platelet aggregation (inhibiton constant [Ki], 11.6 ± 1.4 nmoL/L) induced by the native IgG (effective dose to yield 50% of maximal response [ED50], 9.3 ± 2.7 nmoL/L; n = 5). Thus, it seems that cross-linking of two equivalent platelet epitopes by divalent MA-13G8E1 is an absolute requirement to trigger activation. This is further substantiated by the observation that addition of high amounts of MA-13G8E1 results in decreased aggregation or
Platelet activation via cross-linking of GPIIb/IIIa on one dependency of these phenomena. Horsewood et al² (see Materials and Methods). Two control experiments were performed. First, MA-13G8E1 was tested in an intraplatelet activation assay, as originally described by Horsewood et al² (see Materials and Methods). Two control inducers were used: thrombin (1 IU/mL), which activates single platelets, and Raj-1, an MoAb reported to induce platelet activation via cross-linking of GPIIb/IIIa on one platelet and the FcγRII on an adjacent platelet. Because Raj-1 can also induce activation by cross-linking both receptors on the same platelet surface, an additional experimental set-up was required. Two platelet populations were combined: Fc receptor–blocked normal platelets and [³⁵S]serotonin-loaded Glanzmann’s platelets. When these two platelet populations are mixed together, complete activation appears to be the direct consequence of F(ab')₂-platelet interactions, rather than the result of an aggregation process. PGI₂ completely inhibited aggregation, but not release. Thus, the release reaction proceeds through the activation pathways responsible for the observed Ca²⁺ elevations. One of the major pathways in platelets involves the activation of PLC,²⁹,³⁰ resulting in IP₃ formation. Figure 3A indeed shows that MA-13G8E1-F(ab')₂ induced a significant dose-dependent rise in IP₃, which was paralleled by a rise in Ca²⁺ (Fig 3B). In addition, the possibility that intracellular Ca²⁺ increased solely due to Ca²⁺ influxes was eliminated by experiments in the presence of 5 mmol/L EGTA. Addition of this Ca²⁺-chelator resulted in a Ca²⁺

### Table 1. Effect of High MA-13G8E1 Concentrations on Aggregation and Intracellular Calcium Release

<table>
<thead>
<tr>
<th>Parameter Studied</th>
<th>IV.3 Absent</th>
<th>IV.3 Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Ca²⁺] (nmol/L)*</td>
<td>448 ± 34</td>
<td>312 ± 22†</td>
</tr>
<tr>
<td>Slope of aggregation (%/min)</td>
<td>73.6 ± 1.2</td>
<td>51.4 ± 6.7†</td>
</tr>
</tbody>
</table>

Values represent the mean ± SE for three experiments using 150,000 platelets/μL from different donors.

* Intracellular Ca²⁺-concentrations were determined as the peak value above basal Ca²⁺ levels.

† Statistical differences (P < .05) relative to 44 nmol/L MA-13G8E1 (maximal aggregation rate in the inset of Fig. 1).

### Table 2. Serotonin Release From Platelets Immobile in Agarose and in Suspension

<table>
<thead>
<tr>
<th>Inducer</th>
<th>% Serotonin Release</th>
<th>Platelets in Agarose</th>
<th>Platelets in buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin (1 IU/mL)</td>
<td>80 ± 5</td>
<td>77 ± 2</td>
<td></td>
</tr>
<tr>
<td>MA-13G8E1 (10 µg/mL)</td>
<td>52 ± 9</td>
<td>60 ± 11</td>
<td></td>
</tr>
<tr>
<td>Raj-1 (10 µg/mL)</td>
<td>11 ± 2</td>
<td>49 ± 4</td>
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</table>

* Percentage release was calculated as described.¹

† Platelet population obtained by mixing equal volumes of [³⁵S]serotonin-labeled Glanzmann’s platelets (250,000 platelets/μL) and unlabeled IV.3-blocked normal platelets (250,000 platelets/μL).
F(ab')2-INDUCED PLATELET ACTIVATION

**Figure 2.** Platelet cross-linking experiments as measured by single-platelet counting. (A) Residual platelet numbers determined at set time intervals after addition of different inducers in the presence of GP Ib/IIIa-blocking MA-16N7C2. Values represent the mean ± SE of five experiments using platelets of different donors. (B) Platelet volume distribution at the end of each experiment displayed in A. Tracings are representative of five experiments and are determined by the Cell Dyn 810 Coulter counter, which normalizes the relative number of cells and plots it on the vertical y-axis.

There is a transient increase, in contrast to the sustained Ca²⁺ elevations in the absence of EGTA. This suggests that Ca²⁺ influxes were indeed necessary to obtain a full response, but are not involved in the initial phase of platelet activation.

Neither aspirin (Fig 3C) nor inhibition of fibrinogen binding by MA-16N7C2 had major effects on the rise of intracellular Ca²⁺ induced by MA-13G8E1. These results suggest that the activation of PLC by MA-13G8E1 precedes formation of TXA₂ and fibrinogen binding to activated GP Ib/IIIa, respectively. Furthermore, the Ca²⁺ rise induced by MA-13G8E1-F(ab')₂ took place in the absence of actin polymerization, since cytochalasin B had only minor inhibitory effects, whereas it completely inhibited collagen-induced Ca²⁺ rises in similar circumstances.

Increased levels of intracellular Ca²⁺ activate several kinases, such as PKC, leading to the phosphorylation of intraplatelet proteins. Figure 4 shows a significant phosphorylation of the 47-kD substrate of PKC and the myosin light chain (20-kD band). These experiments were performed under nonstirring conditions, further confirming that MA-13G8E1 directly induced protein phosphorylations without the involvement of platelet aggregation. In the presence of aspirin, an apparent drop in the mean intensity of the phosphorylated bands was noticed, which was not significant (P > .05), indicating that TXA₂ is not or only mildly involved in phosphorylation of P47 and P20.

**Characterization of the MA-13G8E1 epitope.** MA-13G8E1 aggregates rabbit, pig, dog, and baboon platelets, as well as human platelets. No binding to endothelial cells, granulocytes, and smooth muscle cells or to activated or resting peripheral blood mononuclear cells could be detected (data not shown). The binding of iodine-labeled MA-13G8E1 to human platelets yielded a saturable dose-response curve. Scatchard analysis (Fig 5) resulted in a linear plot and showed 3,700 ± 600 binding sites per platelet to which MA-13G8E1 bound with a moderately high affinity (kd = 1.6 ± 0.9 nM/L). Labeled monovalent Fab fragments bound in a similar fashion to platelets. However, they recognized twice as many binding sites [Bₘₐₓ(Fab)/Bₘₐₓ(lgG) = 1.98 ± 0.13; n = 3]. Consequently, these data further support the concept that MA-13G8E1 binds to platelets in a divalent manner. Binding studies with thrombin-activated platelets showed a comparable number of binding sites for MA-13G8E1, suggesting that the epitope is already fully exposed on resting platelets.

In a first attempt to characterize the platelet antigen recognized by MA-13G8E1, we performed inhibition experiments with MA-13G8E1-Fab on aggregations induced by thrombin, collagen, adenosine diphosphate (ADP), U46619, platelet-activating factor (PAF), arachidonic acid, adrenaline or ristocetin. However, no inhibitory effects were observed, not even after addition of high Fab concentrations up to 1 mg/mL.

Immunoprecipitation of ¹²⁵I-labeled platelet surface proteins from one healthy donor, separated by SDS-PAGE, yielded two bands of 45 and 36 kD, both under reducing and nonreducing conditions. The same bands were obtained when platelet lysates were made and applied onto an MA-13G8E1 Sepharose 4B column (Fig 6). Two-dimensional gel electrophoresis revealed the acidic nature of the two protein bands (isoelectric pH 4.5 to 5.5). Moreover, the diffuseness of the two bands, especially in the first dimension, could indicate a certain degree of heteroglycosylation (data not shown). MA-13G8E1 failed to detect the purified antigen after Western blotting, indicating that either the epitope is conformationally determined or the antibody recognizes an

| Table 3. Influence of Platelet Function Inhibitors on Platelet Aggregation and Serotonin Release Induced by MA-13G8E1-F(ab')₂ (10 µg/mL) |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | PG1 (10 nM/L)  | ASA (200 µM/L) | MA-16N7C2 (50 µg/mL) | Cytochalasin B (20 µM/L) |
| Effect           | % Inhibition by * |
| Slope of aggregation | 130 ± 0         | 35 ± 11         | 96 ± 4          | 13 ± 4          |
| Serotonin release | 99 ± 1          | 23 ± 4          | 4 ± 2           | 12 ± 4          |

* Percentage inhibition was given by (1 − R/R₀) × 100, with R₀ and R being the response in the presence and absence of inhibitor, respectively.
epitope, only present when both proteins are associated, as has been found with a series of antibodies against GPIIb/IIIa. To check the latter possibility, a gel-filtration experiment was performed. The bulk of the purified antigenic material eluted at a position corresponding to 440 kD (Fig 7). Increasing the CHAPS concentration from 0.1% to 0.5% did not alter the elution pattern. Interestingly, additional but smaller immunoreactive peaks were observed at positions corresponding to 150 and 70 kD. This suggests that the two low-molecular weight proteins exist as a complex, which can associate to form multimeric entities. Electrophoretic analysis of the individual fractions and evaluation of the separated proteins by densitometry clearly confirmed the presence of both 45-kD and 36-kD proteins (inset of Fig 7), the levels of which correlated well ($P < .001$) with the observed immunoreactivity in each fraction. These data again confirm that the MA-13G8E1 antigen is to be found within this doublet.
DISCUSSION

Although most activating MoAbs directed against platelet membrane glycoproteins aggregate platelets through Fc receptor cross-linking,\(^1\)\(^-\)\(^5\) Fc-independent pathways of platelet aggregation by monoclonal antibodies also exist. Whereas one pathway involves the direct interaction between the monovalent Fab fragment and its antigen, the other requires cross-linking by divalent F(ab')\(_2\) fragments. The former is exemplified by the ability of some Fab fragments directed against GPIIb/IIIa to induce conformational changes in the glycoprotein, allowing fibrinogen to bind and aggregation to take place.\(^6\)\(^-\)\(^10\)

Evidence for the latter mechanism is exceptional. Although Fc-independent intraplatelet cross-linking has been postulated previously, evidence for the Fc independency\(^12\)

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**Fig 6.** Characterization of MA-13G8E1 antigen by SDS-PAGE (10% to 15% gradient gels). (A) Autoradiography of immunoprecipitated proteins from \(^125\)iodine-labeled platelets by MA-13G8E1 under nonreducing (lane 1) and reducing (lane 2) conditions. (B) Coomassie staining of proteins, purified from Triton X-100 platelet lysates on MA-13G8E1-Sepharose 4B under nonreducing (lanes 1) and reducing (3) conditions. A protein standard run under similar conditions is shown in lane 2. All tracings are representatives of three separate isolations from different donors.

**Fig 7.** Gel filtration of affinity-purified material on Sephacryl S-200 HR in the presence of 0.1% CHAPS. Curves represent binding of MA-13G8E1 to eluted material measured in an ELISA system. The eluted material was analyzed by SDS-PAGE, silver-stained, and the gel scanned by laser densitometry at 633 nm. Bars represent the integrated area of the 45-kD (■) and 36-kD (■) bands. The area was determined as the mean of duplicate integrations over two different sections of each lane, and is expressed in arbitrary units. The inset displays SDS-PAGE analysis of fractions 34 to 49. Protein bands were visualized by silver staining.
or intraplatelet interaction was not clearly established. This study reveals, for the first time, compelling evidence that this mechanism indeed can occur for platelets. From a panel of 93 antiplatelet MoAbs, only one, designated MA-13G8E1, was found capable of inducing this type of cross-linking, indicating the unique character of this MoAb and, presumably, also of its antigen.

Three different observations show the Fc independency of the MA-13G8E1-induced activation. First, MA-13G8E1 could still induce platelet aggregations in the presence of an FcγRII-blocking MoAb, IV.3. Second, F(ab')2 fragments of MA-13G8E1 could mimic all aggregation responses induced by the native IgG. Third, rabbit platelets devoid of a functional Fc receptor also were activated.

Interaction of the antigen-binding domains with one epitope alone did not seem to fulfill all requirements for activation because monovalent Fab fragments were inactive. Thus, cross-linking of two identical platelet epitopes seems to be required. This is further supported by the fact that at high F(ab')2 or IgG concentrations, a decrease in divalency and subsequent decrease in activation rates was observed, suggesting competition between divalent and monovalent binding. These latter effects, resulting in a bell-shaped dose-response aggregation curve, are indicative for antigen cross-linking because monovalent F(ab')2 fragments were inactive. Thus, the MoAb binds in a bivalent manner to platelets.

Experiments in which single platelets were immobilized in an agarose gel strongly suggested that the epitope cross-linking occurs on the same platelet surface. No changes in platelet count or platelet size distribution induced by MA-13G8E1 could be detected in the presence of fibrinogen receptor blockade, further confirming that platelet activation by this MoAb occurs in an intraplatelet mode.

MA-13G8E1 generates a whole cascade of biochemical events resulting in platelet activation and release of serotonin. Among these, we could clearly demonstrate the activation of phospholipase C as measured by intraplatelet elevations of IP3, paralleled with rises in intracellular Ca2++. The latter even persisted in the presence of EGTA, aspirin, MA-167N7C2, or cytochalasin B, strongly suggesting that PLC was activated primarily as the result of MA-13G8E1 binding without major involvement of Ca2+ influxes, TxA2 formation, aggregation, or cytoskeleton reorganization.

Both liberated Ca2+ and diacylglycerol (DAG) can activate different kinases, ie, PKC. We found that MA-13G8E1 induced a fourfold to fivefold increase in phosphorylation of the 47-kD substrate of PKC and a twofold enhancement of phosphorylation of the myosin light chain (20 kD).

The Fc-dependent mechanism of antibody-mediated platelet activation on the one hand, and the mechanism induced by MA-13G8E1 on the other hand, both involve cross-linking. However, the signal transduction pathways generated in both cases differ in their TxA2 involvement and cytoskeleton rearrangements, since aspirin and cytochalasin B markedly inhibited platelet activation after Fc receptor cross-linking.6,9 The existence of these differences suggests that intraplatelet cross-linking per se does not determine the signal transduction pathways to be followed. This implies that the membrane protein that is cross-linked, ie, the MA-13G8E1 antigen, is a signal-transducing platelet receptor.

The antigen appeared to be abundant (7,100 ± 800 molecules/platelet) and platelet-specific. MA-13G8E1 immunoprecipitated two membrane proteins with molecular weights of 45 kD and 36 kD, which also could be isolated on MA-13G8E1-Sepharose 4B. We suggest that the two proteins are present as a complex because, during gel filtration, the majority of immunoreactivity always eluted at a position corresponding to 440 kD despite high detergent concentrations, with additional peaks at 150 kD and 70 kD. In addition, SDS-PAGE and silver staining of the eluted material showed that the immunoreactivity highly correlated with the intensity of the two protein bands.

At this point we cannot formally exclude the idea that the antigen is associated with yet another molecule present in the preparations. However, at present, we do not favor this possibility because the two proteins, which eluted at 440 kD, appeared to be in excess compared with other high-molecular weight proteins (inset of Fig 7), making it more likely that the former are capable of self-association.

The identity of the 45-kD and/or the 36-kD membrane proteins remains to be elucidated. It is highly unlikely that one of the two bands might be the platelet Fc receptor (Mr = 40 kD) because we detected an average of 7,100 molecules/platelet, which is much higher than the highest FcγRII number on platelets ever reported.6 Second, MoAb IV.3 did not react with the purified material in an ELISA system. Recently, Hildreth et al37 reported a newly identified membrane protein with properties (electrophoretic mobility, isoelectric point) similar to our MA-13G8E1 antigen. However, identity between our antigen and theirs, which was later identified as CD63 and as melanoma-associated antigen ME491,39 seems unlikely because CD63 is an activation-dependent molecule, not present on resting platelets.

In conclusion, we have demonstrated the existence of an Fc-independent mechanism of platelet activation by a murine MoAb. Further analysis identified a hitherto unknown signal-transducing receptor, the activation of which depends on cross-linking by the antibody. So far, no physiologic agonist for this receptor has been identified. Further work is currently ongoing to better characterize this receptor and its physiologic relevance.

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