Inhibition of Platelet Functions by a Monoclonal Antibody (LYP20) Directed Against a Granule Membrane Glycoprotein (GMP-140/PADGEM)

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Granule membrane protein (GMP-140), also known as platelet activation-dependent granule-external membrane (PADGEM), is an integral membrane glycoprotein that is expressed on the platelet surface following degranulation. GMP-140, also expressed by endothelial cells, is a part of a new family of cell adhesion molecules (selectins) related to the endothelial leukocyte adhesion molecule (ELAM-1) and to the lymphocyte homing receptors in humans (Leu-8/TQ1) and in mouse (gp90Mel-14). The role of GMP-140 in platelet functions remains to be elucidated. In this study, a monoclonal antibody, LYP20, was raised against GMP-140. LYP20, directed against a disulphide bridge-dependent epitope, significantly binds to thrombin-stimulated platelets (12,200 ± 1,184 bound molecules/platelet, kd = 5.0 ± 0.61 nmol/L) compared with controls (2,400 ± 266 molecules/platelet, kd = 2.3 ± 0.54 nmol/L) and inhibits collagen or thrombin-induced aggregation of washed platelets or platelets in platelet-rich plasma. In addition, LYP20 inhibits rosetting of thrombin-activated platelets to U937 cells. These results strongly suggest that GMP-140 plays an important role in platelet aggregation and platelet interaction with other blood cells. © 1991 by The American Society of Hematology.

MATERIALS AND METHODS

Isolation and labeling of platelets. Blood from normal donors was anticoagulated in acid/citrate/dextrose (ACD). Platelets, used in binding, aggregation, and rosetting studies, were isolated and washed according to the technique of Mustard et al with slight modifications: 20 ng/mL protaglandin E1, (PGE1), and 10−5 M D-phenylalanine/L-aromatic chloromethyl ketone (PPACK, Behring Diagnostics, La Jolla, CA) were added in the first wash. Platelet-rich plasma (PRP) was obtained from heparinized (5 U/mL) blood. Surface proteins were iodinated as described by Phillips and Poh Agim, using the lactoperoxidase method.

Production of MoAbs. LYP20 was obtained by immunizing mice with chymotrypsin-treated platelets. Briefly, washed platelets were resuspended at 107/mL and treated with 0.2 mg/mL of a-chymotrypsin (Pierce, Rockford, IL) at 37°C for 30 minutes. Platelets were washed twice in phosphate-buffered saline (PBS) before injection (107 platelets/mouse) into BALB/c mice (6-week-old females, provided by IFFA CREDO, L’Arbresle, France) in the presence of Complete Freund’s Adjuvant (GIBCO Laboratories, Grand Island, NY). The immunization procedure was repeated twice before production of hybridomas. Hybridomas were obtained by the fusion of spleen cells of immunized mice with sp20-Ag14 as previously described. Secreting hybridomas were selected on resting or activated formaldehyde-fixed platelets or against platelet lysates using an enzyme-linked immunsorbent assay (ELISA) system. Further characterization of LYP20 was performed by the use of Western blots, immunoprecipitation as described by McEver and Martin and sequential immunoprecipitation techniques. Secreting hybridomas were cloned and subcloned by limiting dilution. LYP20 was found to be IgG1 as determined by the Amersham isotyping kit (Amersham International, UK). MoAbs were purified on a Protein A-Sepharose column (Pharmacia, Uppsala, Sweden). F(ab) fragments of MoAb LYP20 were prepared according to the technique described by Lamoyi and Nisonoff.

Binding studies. The number of 125I-LYP20 molecules bound to unstimulated and stimulated (adenosine diphosphate [ADP] or a-thrombin, a generous gift from Dr Aronson, Bureau of Biologics, US Drug Administration, Bethesda, MD) platelets was assessed as previously described. The number of binding sites and the dissociation constant kd were calculated by Scatchard analysis. The binding of 125I-fibrinogen to platelets stimulated with ADP or thrombin was measured in the presence or absence of LYP20, as previously described.

Aggregation and secretion. Aggregation studies were performed with washed platelets loaded with 5C-Serotonin as previously described. Platelets, adjusted to 2 × 107 platelets/mL, were aggregated with 2.5 μmol/L ADP, 1.5 μg/mL collagen, or 0.016 U/mL of thrombin in the presence or absence of LYP20 (whole
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Fig 1. Binding of "25I-LYP20 to resting or stimulated platelets. Various amounts of "25I-LYP20 (0 to 4 µg/mL) were added to resting or thrombin-stimulated platelets (1 U/mL). The radioactivity associated to the platelet pellet was expressed in number of molecules of "25I-LYP20 bound per platelet.

IgG or F(ab')2). After each aggregation, the suspension was microfuged, and 100 µL of the supernatant was counted in a β-counter, to measure the level of secretion.

Platelet-monocyte binding assay. U937 cells (a generous gift from Dr T. Jungi, Bern, Switzerland) in exponential growth were washed by the method of Jungi et al12 as modified by Silverstein et al12 and kept on ice at a concentration of 3 x 10⁶/mL. Washed platelets (10⁸/mL) were activated with (0.3 U/mL) thrombin; after 10 minutes, 10⁻⁵ mol/L PPACK was added; and after a further 10 minutes, platelets were incubated either with LYP20 or with a nonimmune IgG, for 5 minutes at room temperature. Fifty microliters of the platelet suspension, incubated with an antibody, was added to 100 µL of resuspended U937 in Minisorb tubes (Nunc, Denmark), gently mixed, and left on ice for 30 minutes. An aliquot of the platelet-U937 suspension was counted in a Neubauer

Fig 2. Western blot of platelet proteins with LYP20. Platelet lysates, electrophoresed under nonreducing (NR) or reducing (R) conditions on a 7.5% gradient SDS-PAGE, were transferred onto an Immobilon (PVDF) membrane that was incubated with LYP20 (lane P20) or with a nonimmune IgG (lane C). Western blots were developed with a goat antimouse antibody conjugated to horseradish peroxidase.

Fig 3. Immunoprecipitation of platelet GPs with LYP20. Lysates from "25I-labeled platelets were incubated with nonimmune IgG of the same isotype as LYP20 (lane C), with an anti-GPllb-IIIa MoAb (LYP18) (lane P18), with LYP20 (lane P20), and with an anti-GMP-140 MoAb (S12) (lane S12). Whole labeled platelet lysate were electrophoresed under nonreducing conditions (lane A). All other samples were electrophoresed under nonreducing (NR) or reducing (R) conditions, run on a 5% to 15% gradient SDS-polyacrylamide gel and autoradiographed.
counting chamber (Schreck, Hafheim, Germany). U937 bearing more than two platelets were considered as rosetting cells. ^12\textsuperscript{h}

RESULTS

Characterization of MoAb LYP20. In binding studies, performed on four donors, LYP20 bound significantly more to thrombin-stimulated (1 U/mL) washed platelets (12,200 ± 1,184 molecules/platelet, kd = 5 ± 0.61 nmol/L) compared with resting platelets (2,400 ± 266 molecules/platelet, kd = 2.3 ± 0.54 nmol/L) (Fig 1). These results showed that LYP20 recognized an activation-dependent GP.

To identify which GP was recognized by LYP20, platelet extracts were run on a 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Immobilon sheets (Millipore, Bedford, MA) to perform a Western blot. LYP20 recognized an antigen only under nonreducing conditions, migrating at 128 Kd (Fig 2). ^12\textsuperscript{h}-labeled platelet lysates immunoprecipitated by LYP20 were compared, in SDS-PAGE, with those immunoprecipitated by an MoAb (S12) specific for GMP-140 (MoAb S12 is a generous gift from Dr R.P. McEver). Autoradiography of SDS-PAGE (Fig 3) showed that the antigen immunoprecipitated by LYP20 comigrated with the antigen (GMP-140) immunoprecipitated by S12, under nonreducing (128 Kd) or reducing (132 Kd) conditions. When GMP-140 was removed from labeled platelet lysates by two successive immunoprecipitations in the presence of S12, LYP20 did not bring down the 128 Kd protein, as shown by autoradiography (Fig 4), whereas LYP22, an anti-GP\textsubscript{IIa} MoAb, immunoprecipitated its antigens. ^28 Moreover, when platelet lysates were immunoprecipitated with an excess of LYP20, S12 did not immunoprecipitate any GMP-140. These results indicated that LYP20 recognized platelet GMP-140.

In addition, a 100-fold excess of LYP20, as well as LYP2 (an anti-GP\textsubscript{IIb-IIIa} MoAb)\textsuperscript{28} and LYPlO (an anti-thrombospondin MoAb),\textsuperscript{26} did not block the binding of labeled S12 to thrombin-stimulated platelets, and vice versa.

Effect of MoAbs LYP20 and S12 on platelet functions. LYP20 or its F(ab)' fragments inhibited ADP-induced (by 60%) (results not shown), collagen-induced (by 63%), and thrombin-induced (by 50%) aggregation of washed platelets, as compared with controls performed with a nonimmune IgG or with S12 (Fig 5). However, in PRP, LYP20 inhibited platelet aggregation induced by collagen but not by ADP (Fig 6). LYP20, while inhibiting platelet aggregation, had no effect on ^12\textsuperscript{h}-fibrinogen binding to thrombin-stimulated platelets. In three studies, the binding of ^12\textsuperscript{h}-fibrinogen (240 μg/mL added) to thrombin-stimulated
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Effect of LYP20 on platelet-U937 rosetting. LYP20 inhibited (by 40%) interaction of thrombin-activated (0.3 U/mL) platelets with U937 cells (Fig 7). Control experiments were performed with an anti–tissue-type plasminogen activator (tPA) MoAb, an anti–GPIIIb-IIIa MoAb (LYP2)9 and an anti-CD9 MoAb (ALB6),30 all having the same isotype as LYP20. None of these MoAbs had any effect on platelet adhesion to U937 cells.

DISCUSSION

In this study, we have produced an MoAb, LYP20, directed against a protein expressed on thrombin-activated platelets. LYP20 was observed to inhibit platelet functions. Several lines of evidence indicated that MoAb LYP20 was directed against platelet GMP-140 (also known as PAD-GEM): (1) LYP20 immunoprecipitated a protein having the same apparent molecular weight, under nonreducing or reducing conditions, as that precipitated by an anti-GMP-140 MoAb (S12); (2) the protein recognized by LYP20 on a Western blot had the same molecular weight as GMP-140; (3) LYP20 failed to bind this protein when GMP-140 was sequentially immunoprecipitated by S12 from labeled platelet lysates, and vice versa; (4) the number of binding sites of LYP20 on thrombin-activated platelets was similar to that observed for S12 and KC4.12 Having shown that LYP20 was directed against GMP-140, we then observed that these two MoAbs were not directed against the same epitope. Moreover, the conformation of the LYP20 epitope appears to be

platelets was measured in the presence (4 μg/mL) or absence of LYP20. Thrombin-stimulated platelets bound 42,550 ± 7,600 molecules of [125I]-fibrinogen in the absence of LYP20, and 40,200 ± 6,300 molecules in the presence of LYP20. Platelet secretion induced by collagen, but not by thrombin, was significantly inhibited by LYP20 (Fig 5).

Fig 5. Effect of LYP20 on aggregation of washed platelets. Typical aggregation curves are shown with washed platelets, loaded with 14C-Serotonin, aggregated with 1.5 μg/mL collagen (A) or 0.03 U/mL thrombin (B) in the presence (40 μg/mL) or absence of LYP20 F(ab)2 fragments. Controls were performed either in the presence of a nonimmune IgG (control) or S12. Release of 14C-Serotonin was measured on washed platelets prepared from each of five donors. Statistical analysis was performed by paired t-test.

Fig 6. Effect of LYP20 on aggregation in PRP. Typical aggregation curve is shown with platelets in heparinized plasma. Platelets were aggregated with 2 μg/mL collagen, in the presence (50 μg/mL) or absence of LYP20 (control).

Fig 7. Inhibition of platelet rosetting to U937 cells by LYP20. Activated platelets (10⁶/mL) were preincubated with LYP20 (●) (60 μg/mL), or with control IgG (○) (anti-tPA, anti–GPIIIb-IIIa [LYP2], or anti-CD9 [ALB6], 60 μg/mL) for 5 minutes at room temperature before incubation with an equal volume of U937 (3 × 10⁵/mL) on ice for 30 minutes. By using a Neubauer chamber, the percentage of rosetting was estimated by dividing the number of U937 bearing more than two platelets by the total number of U937 in the sample. The experiment was repeated four times. Statistical analysis was performed by paired t-test. P = .001.
LY20, or F(ab)\textsuperscript{2} fragments, inhibited thrombin-, collagen-, or ADP-induced aggregation of washed platelets. The inhibitory effect of LYP20 on platelet aggregation was also observed with collagen in heparinized PRP but not with ADP. Expression of GMP-140 on activated platelets allowed the binding of LYP20 to the platelet surface, which might prevent platelets from linking together without affecting fibrinogen binding. Similar inhibition of platelet aggregation has been previously reported for anti-thrombospondin MoAbs\textsuperscript{31} and polyclonal antibodies.\textsuperscript{32} In addition, S12, when tested under identical conditions as LYP20, did not inhibit platelet aggregation, thus supporting the specific effect of LYP20. It is conceivable that GMP-140 plays a substantial role in events leading to irreversible aggregation of platelets. Such a hypothesis is based on the work of W.M. Isenberg, R.P. McEver, Y.V. Jacques, and D.F. Bainton (unpublished observations), who showed that, 15 minutes after thrombin stimulation, GMP-140 was the only GP found in areas of contact between platelets that had irreversibly aggregated. Results obtained with LYP20 would strongly confirm the role of GMP-140 in platelet-platelet interaction.

MoAb LYP20 F(ab)\textsuperscript{2} fragments significantly inhibited platelet secretion induced by collagen but not by thrombin. Collagen and thrombin stimulation of platelets are mediated by different mechanisms.\textsuperscript{33} It is possible that LYP20, by binding to surface expressed GMP-140, affects the collagen pathway, but not thrombin, to interfere with secretion. Alternatively, LYP20, by inhibiting platelet aggregation, may somehow disrupt the feedback mechanism in controlling collagen-induced secretion.\textsuperscript{34}

LY20 inhibited aggregation induced by ADP of washed platelets but not platelets in heparinized PRP. Differences in these results could be explained by the fact that washed platelets, unlike platelets in heparinized PRP, were slightly activated during the washing procedure and expressed sufficient amounts of GMP-140 to allow the binding of LYP20, whereas platelets in PRP did not express any GMP-140 when stimulated by ADP. Inhibition by LYP20 of ADP-induced aggregation of washed platelets, resuspended in the presence of physiologic levels of Ca\textsuperscript{2+}, appears to be an artifact related to the presence of GMP-140.

Results obtained with LYP20 in inhibiting platelet aggregation are the first to show a functional role of GMP-140 in platelet aggregation. Two anti-GMP-140 MoAbs (KC4 and S12) were observed to have no effect on platelet aggregation.\textsuperscript{1,2} Lack of effect of KC4 and S12 on platelet aggregation might be due to the position of their epitope on GMP-140. Previous work on GPIIb-IIIa showed that the epitope position of anti-IIb-IIIa MoAbs was vital in determining their effect on platelet functions.\textsuperscript{30}

LY20 is the first MoAb to inhibit the rosetting of thrombin-activated platelets to U937 cells. The effect of LYP20 on platelet-U937 rosetting was weaker, as compared with that obtained by Larsen et al,\textsuperscript{11} who used a polyclonal anti-GMP-140 antibody. In contrast, an anti-C9,\textsuperscript{16} an anti-GPIIb-IIIa,\textsuperscript{20} or an anti-tPA MoAb did not inhibit the rosetting. The adhesion of thrombin-activated platelets to U937 cells was also inhibited by a polyclonal anti-GPIIb (CD36) antibody.\textsuperscript{17} Such inhibition was previously reported by Silverstein et al\textsuperscript{32} using anti-IIb MoAbs (OKM5, or 1BlG7). It would appear that platelet-U937 or platelet-monocyte interaction is mediated by more than one receptor, probably involving GPIIb and GMP-140.

LY20 is directed against an epitope bearing disulfide bridges that seems to be involved in platelet-platelet and in platelet-U937 interactions. The occupancy of platelet GMP-140 by LYP20 may prevent the binding of a ligand specific for GMP-140, most probably a membrane GMP-140 or another membrane protein, expressed by platelets and U937. Further work is currently being performed to elucidate the mechanism of action of GMP-140 in mediating platelet-platelet and platelet-U937 interactions.

ACKNOWLEDGMENT

We thank Patrick Vuillot for excellent technical assistance.

REFERENCES

10. Bevilacqua MP, Stengelin S, Gimbrone MA, Seed B: Endothelial leukocyte adhesion molecule 1: An inducible receptor for
neutrophils related to complement regulatory proteins and lectins. Science 243:1160, 1989


23. Pischel KD, Bluestein HG, Woods VL Jr: Platelet glycoproteins Ia, Ic and IIa are physiologically indistinguishable from the very late activation antigens adhesion-related proteins of lymphocytes and other cell types. J Clin Invest 81:505, 1988


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