POST-TRANSFUSION purpura (PTP) and neonatal isoimmune thrombocytopenic purpura (NITP) are two clinical platelet disorders that arise from an incompatibility between the platelets of a donor and a recipient, or a mother and her fetus, respectively. The incompatibility arises from the presence or absence of a specific antigen, usually PLA1 (also designated Zw4). PLA1 is present on a platelet membrane glycoprotein designated GPlIa, which has an apparent mol wt of 100,000. For purposes of studying the PLA1 antigen on GPlIa, we have produced four monoclonal antibodies (MoAbs) against GPlIa (one against the PLA1 epitope) and have obtained a high titer polyclonal antibody against PLA1 from a patient with PTP.

The purpose of this communication is to report the presence of the platelet PLA1 antigen on a 120,000-mol wt as well as 100,000-mol wt GPlIa band on platelets, a 100,000-mol wt band on endothelial cells, and a 120,000-mol wt and 105,000-mol wt band on megakaryocytes when visualized on immunoblot. The 100,000-mol wt and 120,000-mol wt band on platelets share antigenic determinants with GPlIa as well as PLA1. Enzymatic digestion of both the 120,000- and 100,000-mol wt species produces glycopeptide fragments of apparent mol wt of 55,000 to 65,000 that retain both the PLA1 and GPlIa epitopes.

MATERIALS AND METHODS

MoAb product. MoAbs were raised against GPlIa by modification of the method of Kohler and Milstein. GPlIa was obtained following mechanical elution from a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) slab gel of purified platelet membranes. The antigen (50 μg) was emulsified with complete Freund’s adjuvant (Sigma Chemical Co, St. Louis) and injected intraperitoneally (IP) into Balb/c mice (Taconic, German-town, NY), followed by three weekly injections of 10 μg of antigen in incomplete Freund’s adjuvant (Sigma). Splenocytes were harvested five days after the last injection, minced, and the cells fused with P3U1 mouse myeloma cells (kindly supplied by Dr V. Nussensweig, New York University Medical School). Supernatants were assayed for antibody reactivity against platelets by an enzyme-linked immunosorbent assay (ELISA) procedure (see below).

ELISA assay of hybridoma supernatants on platelets. Human platelets were washed three times in a modified human Ringer’s solution containing 2 mmol/L EDTA and then applied to U-bottom microtiter plates (Dynatech, Chantilly, VA) at a concentration of 107 platelets in 40 μL of Ringer’s solution. The plates were blocked for one hour at 37°C with 1% bovine serum albumin (BSA) in 0.01 mol/L phosphate-buffered saline (PBS) 0.154 mol/L NaCl, pH 7.4. Forty microliters of cell culture supernatant was then placed in each well and assayed by an ELISA employing an antimosive Ig-alkaline phosphatase conjugate (Sigma).

Immunoblot assay. Platelet membranes were prepared as described previously. Whole platelet lysates were prepared by solubilization of washed platelets in 10% SDS in 60 mmol/L Tris buffer, pH 6.8, or in 1% Triton X-100 in 0.15 mol/L NaCl, 0.01 mL/L tris buffer, pH 7.4. PLA1 negative platelets were obtained from females who had given birth to children with neonatal isoimmune thrombocytopenic purpura. The PLA1 negative phenotype was determined by two different laboratories using specific anti-PLA1 antibodies. Platelet membranes or whole platelet lysates were electrophoresed on 10% SDS-PAGE gels and then transferred to nitrocellulose paper. The nitrocellulose paper was blocked with BLOTTO before incubation with either polyclonal anti-PLA1

From the Department of Medicine, New York University Medical School, 550 First Ave, New York.

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Address reprint requests to S. Karpatkin, MD, Department of Medicine, New York University Medical School, 550 First Ave, New York, NY 10016.

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antibody at a dilution of 1:5,000 or MoAbs DEK-1, DEK-16, DEK-10, DEK-2c at dilutions of 1:50 to 1:1,000 in BLOTTO for two hours on a shaker at 37°C. The unadsorbed antibody was washed away and the bound antibody detected with an alkaline-phosphatase conjugate of goat-antimouse Ig (1:350, Sigma) or F(ab')2 fragments of goat antihuman IgG (gamma chain specific; 1:1,000, Sigma), which were developed with the enzymatic substrate provided by Bio-Rad Laboratories, Richmond, CA.

Human umbilical endothelial cells were a gift of Dr Richard Levin, New York University Medical Center. They were passed one to two times, grown to near confluence, washed three times with Tyrode's-NaHCO3 buffer, solubilized in 0.5% Triton, and run on immunoblot. The preparation was devoid of platelets.

Human megakaryocytes were a gift of Dr Richard F. Levine, Veterans Administration Hospital, Washington, DC. Megakaryocytes were separated from normal bone marrow aspirates by velocity sedimentation followed by cell elutriation to a purity of approximately 95% megakaryocytes by cell number. 1 x 107 megakaryocytes were solubilized in 10% SDS and run on immunoblot.

Subtyping of mouse MoAbs. This was performed by an ELISA with reagents provided by Boehringer-Mannheim Biochemicals, Indianapolis, according to their instructions.

Silver staining. This was performed with reagents (Gelcode) supplied by Pierce Chemical Co, Rockford, IL, according to their instructions.

MoAb affinity columns. DEK-16 was purified by diethyl aminoethyl (DEAE) chromatography12 and coupled to an Affi-gel-10 column (Bio-Rad) at an antibody concentration of 4 to 5 mg/mL. Coupling was performed as suggested by the manufacturer after washing with isopropanol, ice-cold distilled water, and 0.2 mol/L NaHCO3 buffer, pH 8.0. Antibody, dialyzed against the same buffer, was added to the Affi-gel and incubated overnight at 4°C on a rocker. Remaining active binding sites were inactivated by incubation with 0.1 mol/L Tris for four hours at room temperature. The Affi-gel was then washed once in human Ringer’s 2 mmol/L EDTA solution and 4 to 5 mL. of the platelet lysate (in 0.1 mol/L Tris, 0.5 mol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100) applied to a column or mixed with the Affi-gel, rocking overnight at 4°C. The column or slurry was then washed extensively with 0.01 mol/L Tris, 0.5 mol/L NaCl, 0.2% triton, 5 mmol/L EDTA, followed by 0.01 mol/L Tris, 0.15 mol/L NaCl, 0.1% Triton. The adsorbed platelet membrane antigen(s) was then eluted with 0.2 N acetic acid in 0.15 mol/L NaCl, 0.15% Triton, pH 2.5, and the eluate neutralized with 1.5 mol/L Tris.

Protease digestions. Proteases were incubated with platelet preparations or affinity purified GPIIIa in PBS, pH 7.6, at 37°C for 2 to 24 hours. The enzymes employed were chymotrypsin TLCK (Sigma, type VII), trypsin DPC (Sigma, type XI) at an enzyme platelet protein concentration ratio of 1:10 by weight; Subtilisin Carlsberg (Sigma, type VIII) at a ratio of 1:100; Staphylococcus aureus, strain V8 (Sigma, type XVII) at a ratio of 1:40; and plasmin (Sigma) at a ratio of 1:100. All reactions were stopped with 0.1 mmol/L PMSF, final concentration.

Results

Table 1 demonstrates the subclass and antigen specificity of four mouse MoAbs and one human polyclonal antibody on immunoblot.

Figure 1 demonstrates the specificity of polyclonal BE for PLA1 and stronger reactivity of monoclonal DEK-1 for PLA1. Both antibodies as well as DEK-16 react with a 100,000-mol wt band seen on PLA1 positive platelets (lanes A, C, and E) that has the properties of GPIIa. (Reduction of the 100,000-mol wt band results in an apparent higher mol wt on SDS-PAGE,13 viewed with silver stain.) Note the absence of reactivity of polyclonal BE (lane B) and diminished reactivity of monoclonal DEK-1 (lane F) with the 100,000-mol wt band of PLA1 negative platelets. This diminished reactivity was a consistent finding. Similar relative low reactivity with intact PLA1 negative platelets was noted with an ELISA assay (data not shown). Monoclonal DEK-16 (lanes C and D), however, reacted equally with a 100,000-mol wt band of both PLA1 positive (lane C) and negative (lane D) platelets, indicating this antibody’s specificity for a GPIIia epitope and indicating that equal amounts of solubilized platelet extracts were added to each electrophoresis lane. BE, DEK-16, and DEK-1 also reacted with a 120,000-mol wt band on PLA1-positive platelets (see below). The apparent reactivity of BE with PLA1-negative platelets (lane B) at mol wt greater than 100,000 is an artifact due to reactivity of the second goat antihuman IgG antibody (probably with the platelet Fc receptor). DEK-16, however, did react with the 120,000-mol wt band of PLA1 negative platelets (lane D); DEK-1 did not (lane F).

Figure 2 demonstrates the patterns produced when antibodies were reacted with platelet membrane preparations on immunoblot. Platelet membranes and extracts were prepared from whole blood collected in a modified human Ringer’s solution containing 5 mol/L EDTA, 0.01% soybean trypsin inhibitor, 10 mol/L benzamidine, 0.1 mol/L PMSF, and 0.1% leupeptin. Platelets were prepared and washed with the same protease inhibitors. MoAbs DEK-16 (lane A2), DEK-10 (lane A3), and DEK-10 (lane A4) as well as polyclonal BE (lanes A1 and B1) react with the 100,000-mol wt band that has the properties of GPIIia. All of the above consistently reacted with an additional 120,000-mol wt band on each of the second goat antihuman IgG antibody (probably with the platelet Fc receptor). DEK-16, however, did react with the 120,000-mol wt band of PLA1 negative platelets (lane D); DEK-1 did not (lane F).
Immunoblot of platelets of PLA1 positive and negative subjects employing polyclonal anti-PLA1 (BE), monoclonal anti-GPIIIa (DEK-16), and monoclonal anti-PLA1 (DEK-1) antibodies. Platelets were solubilized in Triton X-100, and equal quantities (determined by platelet number) run on 10% SDS-PAGE lanes, electrophoretically transferred to nitrocellulose paper, and reacted with the various antibodies in BLOTTO at dilutions of 1:5,000, 1:1,000, and 1:100, respectively. Nonspecific binding was washed off the paper and specific binding detected with a goat-antimouse or goat-antihuman lgG antibody coupled to alkaline phosphatase (1:350 and 1:1,000 dilution respectively). Standard mol wt markers were simultaneously run and monitored with Ponceau Red; mol wt in kD is cited next to the appropriate bands. Lanes A, C, and E refer to PLA1-positive platelets reacted with BE (polyclonal anti-PLA1), DEK-16 (monoclonal anti-GPIIIa), and DEK-1 (monoclonal anti-PLA1), respectively. Lanes B, D, and F refer to PLA1-negative platelets run simultaneously with the respective antibodies.

PLA1 positive platelets. The 120,000-mol wt band was shown not to be reduced GPIIb, employing a MoAb 3B2 that is specific for GPIIb and the GPIIb-GPIIIa complex (data not shown). MoAb DEK-2c (lane B2) reacted very weakly or not at all with the 120,000-mol wt band. In addition, some of the antibodies occasionally reacted with a 70,000-mol wt band on the immunoblot. Reactivity of all the antibodies was seen at the top of the gel, which probably reflects the platelet Fc receptor, since these bands were also visible when the alkaline-phosphatase–conjugated second antibody was used alone. Following reduction of the platelet membrane with 5 mmol/L dithiothreitol, none of the five antibodies reacted on immunoblot.

Figure 3 demonstrates that the 120,000-mol wt band copurifies with the 100,000-mol wt GPIIIa band on a monoclonal DEK-16 affinity column. Note that the 120,000-mol wt band was not seen following Coomassie blue staining (lane B), whereas it was seen following silver staining (lane D) of the gel, indicating its relatively low concentration compared to the 100,000-mol wt GPIIIa band.

Figure 4 demonstrates the effect of chymotrypsin digestion of a platelet membrane preparation (panel A) as well as an affinity purified GPIIIa/120,000-mol wt band preparation (panel B) following digestion for one-half hour to 24 hours at 37°C. Note the disappearance of the 120,000-mol wt band (lanes A1 and B1), partial to complete disappear-
as the chymotrypsin digest fragments (lane C). Note the decrease in mol wt of the 100,000 species (lane B), as well as the 58,000- to 71,000-mol wt digest fragments (lane D).

All the lanes were developed with polyclonal anti-PLA1 antibody BE. Thus carbohydrate is retained on the chymotrypsin digest fragments containing the PLA1 epitope (lane C), and that which is removed is not required for reactivity of the epitope with anti-PLA1 antibody (lane D).

Figure 6 demonstrates the presence of PLA1 antigenic determinants on platelets (lane A2), megakaryocytes (lane A1), and endothelial cells (panel B). On platelets the PLA1 antigen is usually seen on immunoblot as a 100,000-mol wt and 120,000-mol wt band (the 120,000-mol wt band is not well visualized due to a transfer artifact). On megakaryocytes it is seen as a 105,000-mol wt and 120,000-mol wt band as the chymotrypsin digest fragments (lane C). Note the decrease in mol wt of the 100,000 species (lane B), as well as the 58,000- to 71,000-mol wt digest fragments (lane D). All the lanes were developed with polyclonal anti-PLA1 antibody BE. Thus carbohydrate is retained on the chymotrypsin digest fragments containing the PLA1 epitope (lane C), and that which is removed is not required for reactivity of the epitope with anti-PLA1 antibody (lane D).

Figure 5 demonstrates the effect of endoglycosidase H digestion (lane B) of affinity purified GPIIIa (lane A) as well as the chymotrypsin digest fragments (lane C). Note the decrease in mol wt of the 100,000 species (lane B), as well as the 58,000- to 71,000-mol wt digest fragments (lane D). All the lanes were developed with polyclonal anti-PLA1 antibody BE. Thus carbohydrate is retained on the chymotrypsin digest fragments containing the PLA1 epitope (lane C), and that which is removed is not required for reactivity of the epitope with anti-PLA1 antibody (lane D).

Figure 6 demonstrates the presence of PLA1 antigenic determinants on platelets (lane A2), megakaryocytes (lane A1), and endothelial cells (panel B). On platelets the PLA1 antigen is usually seen on immunoblot as a 100,000-mol wt and 120,000-mol wt band (the 120,000-mol wt band is not well visualized due to a transfer artifact). On megakaryocytes it is seen as a 105,000-mol wt and 120,000-mol wt band.
compared to PLA1 and sent Fc receptors. On endothelial cells it is seen as a possible higher mol wt bands, which may represent GPIIIa. It is also of interest that Stricker et al have recently reported the presence of an autoantibody against a 120,000-mol wt band in a patient with post-transfusion purpura. The 120,000-mol wt antigen was present on PLA1 positive as well as negative platelets. The properties of their 120,000-mol wt antigen are both similar and dissimilar to the one that we described. It is similar with respect to its presence on Glanzmann’s thrombasthenic platelets and its apparent presence on PLA1 negative platelets. However, it is dissimilar with respect to its reactivity with polyclonal anti-PLA1 antibody: we cannot detect the antigen in the reduced state, whereas they can; they cannot demonstrate the sharing of epitopes for the 120,000- and 100,000-mol wt GPIIIa glycoproteins, whereas we can. Nevertheless, it is still possible that we are both observing the same 120,000-mol wt glycoprotein, since the polyclonal antibody that they have employed is different from ours.

A MoAb reagent capable of differentiating PLA1 positive from negative platelets would be clinically valuable in predicting the risk of PLA1 isoimmune neonatal alloimmunity. A recent abstract has similarly reported PLA1 on endothelial cells.

The 120,000-mol wt band seen on immunoblot is a consistent finding that does not appear to be related to proteolysis or methods of platelet preparation. The findings were reproducible following meticulous collection of blood and separation of platelets by gel filtration in the presence of an array of protease inhibitors. The 120,000-mol wt band was seen with three of four MoAbs raised against the 100,000-mol wt GPIIIa as well as a polyclonal anti-PLA1 antibody. It is unlikely that it is due to partially reduced 100,000-mol wt GPIIIa (which is known to raise its apparent position on SDS-PAGE due to intramolecular disulfide bonds) because none of our anti-PLA1 antibodies react with the epitope in the fully reduced state. However, we cannot rule out the possibility that the apparent 120,000-mol wt band may be a GPIIIa precursor that does not have its full complement of intradisulfide bonds and is therefore partially reduced. This could, in turn, be responsible for its aberrant mobility on SDS-PAGE. An alternative possibility is that the 120,000-mol wt band represents a different glycoprotein adhesive protein receptor with homology for GPIIIa. It is not due to dimerization of a 55,000-mol wt chymotrypsin digest product because the preparation is prepared in the presence of protease inhibitors and the 110,000-mol wt dimer reported by Kornecki et al is different from the 120,000-mol wt band that we are describing (data not shown).

The physiologic significance of this 120,000-mol wt band seen on immunoblot of PLA1-positive platelets and that shares three or more epitopes with GPIIIa as well as an epitope with PLA1 remains to be determined. However, it is of interest that Coller et al have subsequently also noted this band on immunoblot in a study of control platelets employing our antibodies BE and DEK-16, as well as platelets from Glanzmann’s thrombasthenic in Iraqi Jews employing their polyclonal rabbit anti-GPIIIa antibody. One of the patients studied had an absent GPIIIa band in the presence of a higher mol wt band that was 20,000 dalton greater than their GPIIIa band. These data are consistent with the postulate that the 120,000-mol wt glycoprotein may be a precursor of GPIIIa. However, it is still possible that we are both observing the same 120,000-mol wt glycoprotein, since the polyclonal antibody that they have employed is different from ours.

A MoAb reagent capable of differentiating PLA1 positive from negative platelets would be clinically valuable in predicting the risk of PLA1 isoimmune neonatal alloimmunity. A recent abstract has similarly reported PLA1 on endothelial cells. The absence of the 120,000-mol wt band on three different endothelial cell preparations is consistent with recent data suggesting that the endothelial GPIIIa gene may be different from the platelet GPIIIa gene. For example, Giltay et al have noted normal synthesis and expression of GPIIIa on endothelial cells of Glanzmann’s thrombasthenic platelets. Thus the 120,000-mol wt band appears to be tissue specific.

The PLA1-reactive chymotrypsin, subtilisin, or trypsin digest products of 55,000 to 65,000 mol wt were noted following one hour of digestion and were resistant to further digestion by these enzymes for an additional 23 hours. Protease V8 digestion produced a fragment of 82,000-mol wt at one-half hour that remained resistant to digestion for an additional 11 hours. Reduction of the 55,000- to 65,000-mol wt fragments resulted in loss of PLA1 reactivity. These data are compatible with the presence of a PLA1 epitope on GPIIIa that is resistant to digestion and is determined by a conformation that involves intramolecular sulfhydryl groups.

Endoglycosidase H had no effect on PLA1 antigenicity, confirming the observation of Newman et al, who also noted no effect following treatment with endoglycosidases O and D. Our data extend these observations by noting a loss of mol wt of 2,000 to 6,000 daltons following endoglycosidase-H digestion. However, we are unable to confirm the presence of a 17,000-mol wt PLA1 epitope reported by Newman et al following trypsin digestion. In our hands trypsin had no further effect on the 55,000- to 65,000-mol wt PLA1 epitope after one to 24 hours of digestion at room temperature. The reason for this discrepancy is unclear. However, one proce-
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GPIIIa-related PLA1 antigens with different molecular weights: studies in platelets, endothelial cells, and megakaryocytes

A Dancis, C Ehmann, R Ferziger, K Grima and S Karpatkin