On the association of the platelet-specific alloantigen, Pena, with glycoprotein IIIa. Evidence for heterogeneity of glycoprotein IIIa.

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J Clin Invest. 1987;80(6):1624-1630. https://doi.org/10.1172/JCI113250.

Research Article

Neonatal alloimmune thrombocytopenic purpura associated with a new platelet-specific alloantigen Pena has been reported. We now provide direct evidence that the Pena determinant is associated with glycoprotein (GP) IIIa, but that it is distinct from epitopes that define the PlA system. By ELISA wherein monoclonal antibodies specific for GPIIb (Tab) and specific for GPIIIa (AP3) were used to capture and hold antigens from a platelet lysate prepared under conditions that generate free GPIIb and GPIIIa, anti-Pena reacted with GPIIIa held by AP3 but not with GPIIb held by Tab. In an alternative ELISA where purified GPIIIa from both PlA1-positive and PlA1-negative platelets were used individually as antigen, anti-Pena reacted with both allelic forms of GPIIIa. By radioimmuno-precipitation, anti-Pena precipitated a single surface-labeled membrane protein with electrophoretic characteristics in sodium dodecyl sulfate-polyacrylamide gels, under nonreduced or reduced conditions, identical to those of GPIIIa. By fluorocytometry, platelets from several donors, regardless of PlA phenotype, bound an amount of anti-Pena roughly equivalent to one-half that amount of anti-PlA1 bound by PlA1 homozygous (A1/A1) platelets and roughly equal to that amount of anti-PlA1 bound by PlA1 heterozygous (A1/A2) platelets. Using platelets from donors typed homozygous for PlA1 and Pena in a quantitative indirect binding assay, 14-24,000 molecules of anti-Pena and 41-51,000 molecules of anti-PlA1 were bound per platelet at saturation. Anti-Pena completely inhibited ADP-induced […]

On the Association of the Platelet-specific Alloantigen, Pen^a, with Glycoprotein IIIa

Evidence for Heterogeneity of Glycoprotein IIIa

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Abstract

Neonatal alloimmune thrombocytopenic purpura associated with a new platelet-specific alloantigen Pen^a has been reported. We now provide direct evidence that the Pen^a determinant is associated with glycoprotein (GP) IIIa, but that it is distinct from epitopes that define the PI^A system. By ELISA wherein monoclonal antibodies specific for GPIIb (Tab) and specific for GPIIIa (AP3) were used to capture and hold antigens from a platelet lysate prepared under conditions that generate free GPIIb and GPIIIa, anti-Pen^a reacted with GPIIIa held by AP3 but not with GPIIb held by Tab. In an alternative ELISA where purified GPIIIa from both PI^{A1}-positive and PI^{A1}-negative platelets were used individually as antigen, anti-Pen^a reacted with both allelic forms of GPIIIa. By radioimmunoprecipitation, anti-Pen^a precipitated a single surface-labeled membrane protein with electrophoretic characteristics in sodium dodecyl sulfate–polyacrylamide gels, under nonreduced or reduced conditions, identical to those of GPIIIa. By fluorocytometry, platelets from several donors, regardless of PI^A phenotype, bound an amount of anti-Pen^a roughly equivalent to one-half that amount of anti-PI^{A1} bound by PI^{A1} homozygous (A1/A1) platelets and roughly equal to that amount of anti-PI^{A1} bound by PI^{A1} heterozygous (A1/A2) platelets. Using platelets from donors typed homozygous for PI^{A1} and Pen^a in a quantitative indirect binding assay, 14–24,000 molecules of anti-Pen^a and 41–51,000 molecules of anti-PI^{A1} were bound per platelet at saturation. Anti-Pen^a completely inhibited ADP-induced aggregation of Pen^a-positive platelets, regardless of PI^A phenotype. These results indicate that the Pen^a determinant is associated with GPIIIa but distinct from PI^A.

Introduction

Friedman and Aster (1) reported a new platelet-specific alloantigen designated Pen^a that has been implicated in at least two cases of neonatal alloimmune thrombocytopenic purpura (NATP).[1] The entire family of the proband is positive for PI^{A1}, PI^{E1}, and Bak^a, and the proband herself is positive for PI^{A1} and PI^{E1}, but negative for Bak^a. Antibody in serum of the proband does not react with any previously recognized platelet-specific alloantigens, including PI^{A1}, PI^{E2}, and Bak^a. The antibody reacted with 300 of 301 normal platelet preparations tested, but not with her own platelets or those from a patient with Glanzmann's thrombasthenia, deficient in platelet membrane glycoproteins (GP) IIb and IIIa. The single normal, unrelated platelet donor (Tru) nonreactive with anti-Pen^a was herself sensitized against Pen^a through blood (platelet) transfusion and thus produced the second antiserum recognized as specific for Pen^a (2).

The GPIIb-IIIa complex is known to mediate platelet cohesion in concert with fibrinogen and calcium (reviewed in reference 3) and to carry target antigens for auto (4–6), allo (7, 8), and isoantibodies (9, 10). Four platelet-specific alloantigen systems have been defined, PI^A, PI^E, Ko, and Bak (Lek) (reviewed in reference 11). Among these alloantigens, PI^{A1} and Bak^a are those most often implicated in the etiology of post-transfusion purpura (PTP) and NATP (12), and have been localized to GPIIIa and GPIIb, respectively (7, 8). We now provide evidence that the alloantigen Pen^a, a causative factor in both NATP and PTP, is also associated with GPIIIa, but is an epitope distinct from PI^A.

Methods

Antibodies. The antibodies reactive with the Pen^a alloantigen used in this study were from the proband studied by Friedman and Aster (1) and from a second patient with PTP due to anti-Pen^a (2). Antisera reactive with the Yuk^a and Yuk^b allelic platelet alloantigens (13, 14) were a generous gift from Dr. Y. Shibata (Department of Immunohematology, Toranomon Hospital and Okinaka Memorial Institute for Medical Research, Tokyo, Japan). All antisera and plasmas were heat inactivated (56°C for 30 min) before use. Preliminary studies indicate that Pen and Yuk are identical alloantigen systems, such that Pen^a is equivalent to Yuk^b, whereas Pen^b is identical to Yuk^a (Aster, R. H., and Y. Shibata, unpublished observations). By the antigen-capture ELISA described below, platelets from the proband as well as patient Tru were reactive with anti-Yuk^a (anti-Pen^b) but not with anti-Yuk^b (anti-Pen^a).

The murine monoclonal antibodies, AP1 (specific for GPIbα) (15), AP2 (specific for the GPIIb-GPIIIa complex) (16), AP3 (specific for GPIIIa) (17), Tab (specific for GPIIb) (18), and HB 43 (specific for the Fc portion of human IgG) were isolated from ascites fluid as previously described (15, 16). Ascites fluid containing the monoclonal antibody Tab was a generous gift from Dr. R. McEver (University of Texas Health Science Center, San Antonio, TX). The cell line HB 43 was obtained from the American Type Culture Collection (Rockville, MD).

Using a monoclonal antibody–based direct binding assay (19) on both whole blood and platelet-rich plasma (PRP), platelets from the proband and patient Tru reacted normally with monoclonal antibody AP2 that recognizes the GPIIb–IIIa complex. Also, both patients typed positive for PI^{A1}, using the platelet suspension immunofluorescence test (1). These results indicate that platelets from both individuals contain normal levels of GPIIb-IIIa.

Biotinylation of HB 43. Purified HB 43 IgG (1 mg/ml) was dialyzed against 0.1 M $NaHCO_3$, pH 8.2–8.6, containing no preservatives for

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Received for publication 23 December 1986 and in revised form 24 April 1987.

1. *Abbreviations used in this paper:* GEL, gelatin; IPB, immunoprecipitation buffer; NATP, neonatal alloimmune thrombocytopenic purpura; PBS-TW, 67 mM PBS, pH 7.5, containing 0.15 M NaCl and 0.05% Tween 20; PTP, posttransfusion purpura; RCD, Ringer's citrate–dextrose; TG, 0.038 M Tris and 0.1 M glycine, pH 8.7.

J. Clin. Invest.

0021-9738/87/12/1624/07 $2.00
Volume 80, December 1987, 1624–1630

18 h at 4°C. NHS-LC-Biotin (sulfosuccinimidyl 6-[biotinamido]hexanoate) (Pierce Chemical Co., Rockford, IL) was dissolved in 0.1 M $NaHCO_3$, at a concentration of 1 mg/ml. 120 μl of the biotin solution was added to 1 ml of IgG, and the mixture was incubated at ambient temperature for 4 h. The reaction was terminated by dialysis of the reaction mixture against PBS, pH 7.5, containing 0.05% sodium azide.

Platelet isolation. 6 vol of whole blood were mixed with 1 vol of acid–citrate–dextrose (National Institutes of Health formula A) and centrifuged at 250 *g* for 12 min at ambient temperature to obtain PRP. PGE_1 (Sigma Chemical Co., St. Louis, MO) was added to the PRP to a final concentration of 20 ng/ml. After a 15-min incubation, the mixture was centrifuged at 750 *g* for 12 min to sediment the platelets. The platelet pellet was resuspended in Ringer's citrate–dextrose (RCD) buffer, pH 6.5, (20) containing 20 ng/ml PGE_1 and washed three times in the same buffer. The final platelet concentration was determined electronically using a counter (Coulter Electronics, Inc., Hialeah, FL).

Solubilization of platelets in Triton X-100. Washed platelets were resuspended in nine parts of 0.038 M Tris and 0.1 M glycine, pH 8.7 (TG), mixed with one part of 10% (vol/vol) Triton X-100 in TG in the presence of 2.5 mM EDTA (to affect dissociation of GPIIb from GPIIIa), and agitated for 30 min at 4°C. Final platelet concentration was adjusted to 5×10^9/ml. The lysates were then centrifuged for 30 min (4°C) at 80,000 *g* to pellet the insoluble material. The protein concentration of the supernatant was determined by the method of Markwell (21) and ranged from 8 to 10 mg/ml. The lysate was diluted 1:10 in TG containing 2.5 mM EDTA, and precleared of residual GPIIb–IIIa complexes by incubation with the complex-specific monoclonal antibody AP2 at ambient temperature overnight and by subsequent incubation with Protein A–Sepharose CL-4B at ambient temperature for 30 min. Preclearing in this fashion was performed to remove GPIIb-IIIa complexes from the lysate so that the only GPIIIa present would be the dissociated form. After centrifugation, the supernatant was further diluted 1:10 in TG containing 2.5 mM EDTA and then used as the source of soluble platelet antigens. By crossed immunoelectrophoresis, no precipitin arc characteristic of that given by the GPIIb-IIIa complex was detected in these precleared lysates (data not shown).

Purification of GPIIIa. AP3 (3.5 mg) and nonimmune mouse IgG (5 mg) were coupled to 25 ml of Affi-Gel 10 (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions. Washed, pooled platelets (5×10^{11}) were solubilized in 50 mM Tris–HCl, pH 7.5, containing 0.2 M NaCl, 0.4 mM phenylmethylsulphonyl fluoride, and 1% Triton X-100 and 10 mM EDTA at 4°C. After centrifugation at 100,000 *g* for 30 min, the lysate was precleared by incubation with nonimmune mouse IgG coupled to Affi-Gel 10 at ambient temperature for 60 min. The slurry was centrifuged at 1,100 rpm for 10 min, and the supernatant was aspirated and then incubated with AP3 coupled to Affi-Gel 10 at 4°C overnight. The AP3–Affi-Gel 10 beads were then washed with Tris–HCl, pH 7.5, containing 0.5% Triton X-100 and 10 mM EDTA, and GPIIIa was eluted by perfusion of the beads with 0.05 M diethanolamine buffer, pH 11.2, containing 0.02% SDS. The eluate was immediately neutralized with 0.5 M Tris–HCl, pH 6.8.

ELISA. 50 μl of Tab IgG or AP3 IgG in carbonate buffer, pH 9.6 at a concentration of 5 μg/ml, were added to each well of a 96-well microtiter plate (Immulon I; Dynatech Laboratories, Inc., Alexandria, VA) and incubated at 4°C overnight. The wells were then filled with 67 mM PBS, pH 7.5, containing 0.15 M NaCl, 0.05% Tween 20, and 1% gelatin (PBS-TW–1% GEL), and incubated at ambient temperature for 30 min.

The microtiter plates were then washed twice with PBS-TW (same buffer minus GEL) using a Nunc Immunowash 12 apparatus (Vangard International Inc., Neptune, NJ) and incubated with the platelet lysate precleared of residual GPIIb-IIIa complex, as described above, containing 2.5 mM EDTA (50 μl per well) at ambient temperature for 60 min. After three subsequent washes, 50 μl of anti-Pen^a, anti-Pl^{A1}, anti-Bak^a, or control plasma, serially diluted in PBS-TW–0.1% GEL, were added to duplicate or triplicate wells. The wells were washed three additional times, and biotinylated HB 43 diluted 1:500 in PBS-TW–0.1% GEL was added. The microtiter plates were then incubated at ambient temperature for 60 min, each well was washed an additional three times and incubated with avidin–biotin–alkaline phosphatase complex (Vectastain ABC-AP kit; Vector Laboratories, Inc., Burlingame, CA) diluted 1:250 in PBS-TW–0.1% GEL for 15 min. After an additional four washes, substrate (*p*-nitrophenylphosphate) in 0.9 M diethanolamine buffer, pH 9.8, containing 0.5 mM $MgCl_2$, was added, and the plates were incubated at 37°C for 30 min. The absorbance at 405 nm was recorded using a microplate reader (MR 600; Dynatech Laboratories, Inc.). In the AP2-based antigen-capture ELISA, platelet lysates prepared in the absence of EDTA represented the source of GPIIb–GPIIIa complexes.

In selected experiments, purified GPIIIa from either homozygous Pl^{A1}-positive (A1/A1) or homozygous Pl^{A1}-negative (A2/A2) platelets was directly conjugated to microtiter wells. Pl^A phenotype of donor platelets was determined independently by reactivity with anti-Pl^{A1} vs. anti-Pl^{A2} using both ^{51}Cr release and the AP2-based antigen-capture ELISA. 50 μl of the respective, purified GPIIIa, diluted in carbonate buffer, pH 9.6, to a concentration of 2 μg/ml, was added to each well of the microtiter plates and incubated at 4°C overnight. Antigen-coated plates were then blocked and washed in preparation for ELISA as described above.

Radioimmunoprecipitation. Washed platelets were radiolabeled with ^{125}I by the lactoperoxidase-catalyzed method of Phillips and Poh Agin (22) and solubilized in 50 mM Tris–HCl, pH 7.5, containing 0.15 M NaCl, 10 mM 3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate, and 5 mM EDTA. After centrifugation at 10,000 *g* for 15 min, the lysate was diluted 1:10 in 50 mM Tris–HCl, pH 7.5, containing 0.15 M NaCl, 2.5 mM 3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate, 5 mM EDTA, and 0.5% gelatin (immunoprecipitation buffer [IPB]). 200 μl of ^{125}I-labeled platelet extracts were mixed with 25 μl of normal human plasma. After a 1-h incubation at ambient temperature, 25 μl of a 1:1 slurry of Protein A–Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ) in IPB was added, and the mixture was incubated for 30 min. After centrifugation at 750 *g* for 3 min, the supernatant was obtained and used as the source of antigens. 25 μl of plasma containing anti-platelet alloantibody was added to 200 μl of the antigen source (10^6 cpm), and the mixture was incubated at ambient temperature for 2 h with constant agitation. 25 μl of Protein A–Sepharose CL-4B were then added, and the mixture was incubated an additional 30 min at ambient temperature. The Protein A beads were pelleted by centrifugation, washed three times with IPB, and then once more with IPB containing 0.2% SDS. Bound ^{125}I-labeled, platelet proteins were eluted by resuspending the final pellet in 50 μl of SDS-PAGE sample buffer (2% SDS with or without 5% 2-mercaptoethanol) and incubating the mixture at 95–100°C for 5 min. The mixture was then centrifuged at 750 *g* for 5 min, and radiolabeled platelet proteins in the supernatant were analyzed by SDS-PAGE using a 7–15% exponential-gradient acrylamide gel. Gels were stained, destained, dried, and then exposed to Kodak XAR-5 film at −70°C for 5–7 d in the presence of image-intensifying screen (Cronex Hi-Plus; DuPont Co., Wilmington, DE).

Fluorocytometry

Platelets were obtained from EDTA-anticoagulated whole blood (0.3 ml of 5% EDTA per 10 ml blood) by differential centrifugation, washed three times in RCD, pH 6.5, containing 50 ng/ml PGE_1 and 0.01% sodium azide, and resuspended in the same buffer to a final concentration of 1×10^8 per ml. In each well of a flat-bottom, 96-well plastic microtiter tray (Costar Data Packaging Corp., Cambridge, MA), 50 μl of the platelet suspension was incubated with 50 μl of plasma containing test antibody for 2 h at ambient temperature. Platelets were then washed twice with RCD and incubated for 20 min at ambient temperature with fluorescein-conjugated goat anti-human IgG plus IgM (Meloy Laboratories, Inc., Springfield, VA) (200 μl of a 1:40 dilution per well). Platelets were then washed once in RCD; the pellet in each well was resuspended in 200 μl of RCD, made to 500 μl total

volume with RCD, and analyzed in a flow cytometer (FACS II; Becton Dickinson & Co., Mountain View, CA).

Quantitative indirect binding assay

Quantification of platelet-bound alloantibodies was performed according to a modification of the method of LoBuglio et al. (23). Briefly, 0.1 ml of PRP (2.5×10^8 platelets/ml) obtained from normal donors whose PI^A and Bak phenotype is known was incubated with 0.1 ml of plasma containing alloantibody and 10 μl (2 μg) of ^{131}I-labeled AP3 for 30 min at ambient temperature. The platelets were washed three times in 10 mM PBS, pH 6.5, containing 10 mM EDTA plus 50 ng/ml of PGE_1 and resuspended in 0.2 ml of the same buffer containing 1% BSA (PBS-BSA). 2 μg of purified, ^{125}I-labeled, murine monoclonal antibody specific for the Fc portion of human IgG (HB 43) in a volume of 0.05 ml was then added, and the mixture was incubated for an additional 30 min at ambient temperature.

PBS-BSA (0.25 ml) was then added, and 0.1 ml aliquots of this mixture were layered onto a 0.2-ml cushion of 20% Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) in 0.4 ml soft polypropylene centrifuge tubes (Sarstedt, Inc., St. Louis, MO). The tubes were centrifuged at 10,000 *g* for 4 min in a centrifuge (Microfuge II; Beckman Instruments, Inc., Fullerton, CA). The tubes were then clamped with a hemostat at a point just above the platelet button, the tube tip was removed with a scalpel and placed in a glass tube, and the amount of radioactivity incorporated into the pellet was counted in a gamma scintillation counter. The number of molecules of radiolabeled probe bound to each target platelet was determined from the number of counts bound per platelet and the specific radioactivity (counts per minute/microgram protein) of the probe as described by LoBuglio et al. (23). Platelet yield in the pellet was calculated from the recovery of ^{131}I-labeled AP3. Bound alloantibody per platelet was then recalculated based upon the determination of platelet recovery.

Inhibition of ADP-induced platelet aggregation

PRP was obtained from freshly drawn whole blood anticoagulated with sodium citrate (1 ml of 3.8% sodium citrate per 9 ml whole blood). Various donors were selected who were known to be homozygous PI^{A1}-positive (A1/A1), or homozygous PI^{A1}-negative (A2/A2), or heterozygous for PI^{A1} (A1/A2), but all positive for the Pen^a allele. The platelet count was adjusted to 3×10^8/ml with autologous platelet-poor plasma. 300 μl of PRP was mixed with 100 μl of normal, anti-Pen^a, or anti-PI^{A1} plasma that was serially diluted in normal plasma, and the mixture was incubated at 37°C for 30 min. After addition of ADP (5 μM), the change in light transmission was recorded in a Lumi-aggregometer (Chrono-Log Corp., Havertown, PA) at 37°C with continuous stirring.

Results

ELISA. The monoclonal antibody-based, antigen-capture ELISA represents a straightforward way to determine the specificity of alloantibodies reactive with purified glycoprotein antigens. As shown in Fig. 1 *A*, anti-Pen^a and anti-PI^{A1} IgG bound specifically to GPIIIa held by AP3, whereas anti-Bak^a and control IgG did not. Conversely (Fig. 1 *B*), anti-Bak^a IgG bound specifically to GPIIb held by Tab, whereas anti-Pen^a, anti-PI^{A1}, and control IgG did not. The data presented in Fig. 1 is representative of four independent experiments.

Using this assay, lysates from all of 96 normal donors screened to date have been positive for anti-Pen^a but negative for anti-Yuk^a (Pen^b). Therefore, all of the platelet preparations used in the studies described below are considered to be homozygous Pen^a/Pen^a. In subsequent studies, GPIIIa purified from either PI^{A1}-positive (A1/A1) or PI^{A1}-negative (A2/A2) platelets by AP3 immunoaffinity chromatography was directly conjugated to microtiter wells as antigen. As shown in Fig. 2 *A* and *B* anti-PI^{A1} IgG bound specifically to PI^{A1}-positive GPIIIa but not to PI^{A1}-negative GPIIIa. On the other hand, anti-Pen^a reacted equally well with both PI^{A1}-positive and PI^{A1}-negative GPIIIa.

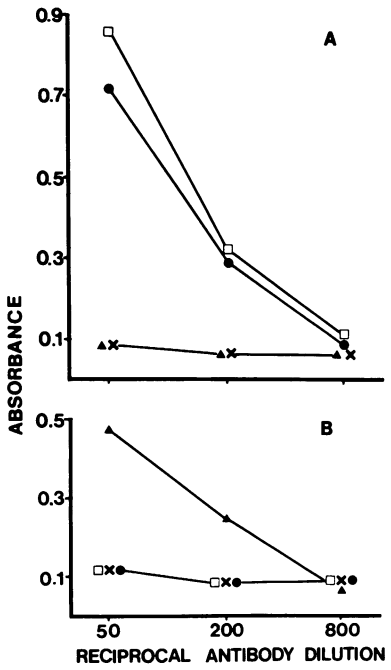

Figure 1. ELISA using (*A*) solid phase AP3 to adsorb GPIIIa and (*B*) solid phase Tab to adsorb GPIIb. A lysate containing only dissociated GPIIb and GPIIIa was used as source of antigens and was obtained by detergent lysis in the presence of EDTA and by preclearing with AP2-Sepharose to remove GPIIb-IIIa complexes. Anti-PI^{A1} (●) reacted with GPIIIa held by AP3 but not with GPIIb held by Tab, whereas anti-Bak^a (▲) reacted solely with GPIIb. Anti-Pen^a (□) reacted solely with GPIIIa. Control IgG (×) reacted with neither glycoprotein.

Radioimmunoprecipitation. Using the lactoperoxidase-catalyzed procedure, several platelet membrane proteins are radiolabeled with ^{125}I, but GPIIb and GPIIIa incorporate a particularly high amount of the radioisotope (Fig. 3, lanes *1* and *5*). Both glycoproteins are readily detectable after SDS-PAGE and subsequent autoradiography. Using lysates derived from such radioiodinated platelets, anti-PI^{A1} antibody precipitated a surface protein with electrophoretic characteristics, under re-

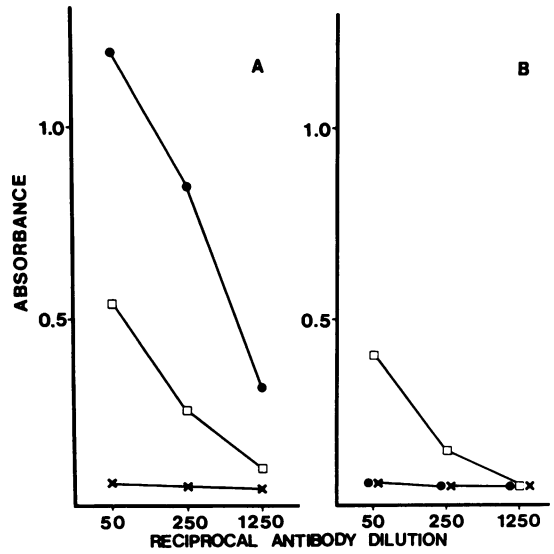

Figure 2. ELISA wherein purified GPIIIa from either (*A*) homozygous PI^{A1}-positive (A1/A1) or (*B*) PI^{A1}-negative (A2/A2) platelets was directly conjugated to microtiter plates. Anti-PI^{A1} (●) reacted only with PI^{A1}-positive GPIIIa, whereas anti-Pen^a (□) reacted with either phenotype of GPIIIa. Control IgG (×) reacted with neither phenotype of GPIIIa.

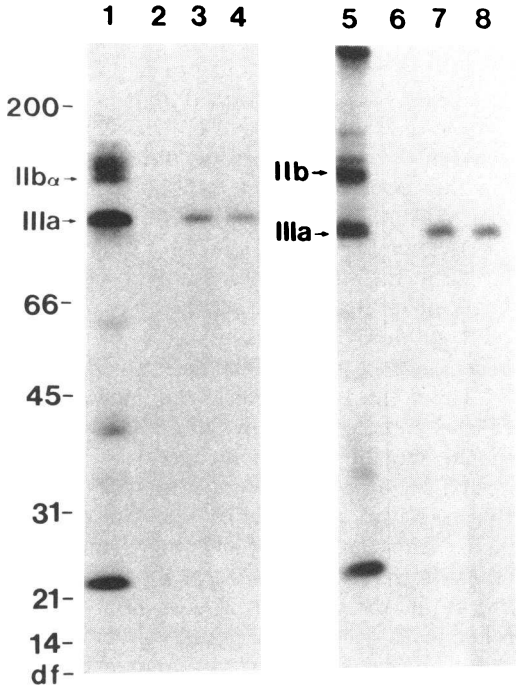

Figure 3. Autoradiograph of ^{125}I-labeled solubilized platelet membrane proteins immunoprecipitated by alloantibodies and analyzed by SDS-PAGE under reduced (lanes *1–4*) and nonreduced (lanes *5–8*) conditions. Several major glycoproteins are surface-labeled with ^{125}I, and GPIIb and GPIIIa are labeled strongly as shown in the total protein lysate (lanes *1* and *5*). Anti-Pl^{A1} antibody (lanes *3* and *7*) and anti-Pen^a antibody (lanes *4* and *8*) both precipitated a protein with an apparent M_r of ~ 120,000 under reduced conditions and M_r of 100,000 under nonreduced conditions, corresponding to that of GPIIIa. Nonimmune IgG did not precipitate any surface-labeled protein (lanes *2* and *6*). Molecular weights are shown as $M_r \times 10^{-3}$.

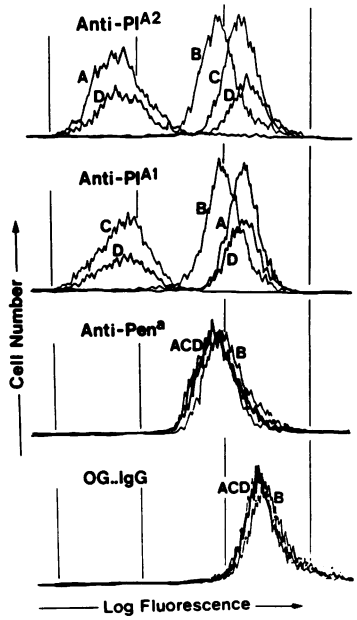

Figure 4. Fluorocytometry. Washed platelets from donors that had been typed with respect to Pl^{A1} and Pl^{A2} in independent assays were incubated with plasma containing (*top* to *bottom*) anti-Pl^{A2}, anti-Pl^{A1}, anti-Pen^a, or OG IgG (a GPIIIa-specific isoantibody), washed, and then incubated with fluorescein-conjugated, goat anti-human IgG plus IgM. The amount of platelet-bound fluorescence was then determined by fluorocytometry. The ordinate indicates the number of platelets analyzed, and the abscissa shows the log of platelet-bound fluorescence. The following samples are depicted: (*A*) A1/A1 platelets alone; (*B*) A1/A2 platelets alone; (*C*) A2/A2 platelets alone; (*D*) a 50:50 mixture of A1/A1 and A2/A2 platelets (*A* plus *C*). In each assay, 10,000 platelets were analyzed.

duced (lane *3*) or nonreduced (lane *7*) conditions, identical to those of GPIIIa. Anti-Pen^a antibody also precipitated one major surface-labeled protein (lanes *4* and *8*) that exhibited the same mobility as that of GPIIIa. Nonimmune IgG did not precipitate any proteins (lanes *2* and *6*). When radioiodinated Pl^{A1}-negative platelets were used as the source of antigen, anti-Pen^a still precipitated GPIIIa but anti-Pl^{A1} did not (data not shown).

Fluorocytometry. An estimate of the relative amount of human GPIIb and/or GPIIIa-specific antibodies bound per platelet at saturation was obtained by fluorocytometry. Using this method, we compared the binding of anti-Pl^{A1}, anti-Pl^{A2}, anti-Pen^a, and a GPIIIa-specific isoantibody produced by a transfused patient with Glanzmann's thrombasthenia (OG IgG) (24). OG IgG binds to GPIIIa but not to GPIIb in immunoblot assays using nonreduced protein (data not shown). In Fig. 4, the binding of these antibodies to platelets of the following known phenotypes are compared: (*A*) A1/A1; (*B*) A1/A2; (*C*) A2/A2; and (*D*) a 50:50 mixture of *A* plus *C*. As expected, a reciprocal binding of anti-Pl^{A1} and anti-Pl^{A2} to these platelets was observed. The reactivity of either antibody to the mixture of homozygous Pl^{A1}-positive and Pl^{A1}-negative platelets confirmed the supposition, that in Pl^{A1}-heterozygous individuals, each platelet expresses both A1 and A2 alleles.

All of the platelet samples tested bound an amount of anti-Pen^a roughly equivalent to that amount of anti-Pl^{A1} or anti-Pl^{A2} that is associated with A1/A2 platelets at saturation. On the other hand, all platelet samples bound an amount of the isoantibody, OG IgG, roughly equivalent to that amount of anti-Pl^{A1} bound to A1/A1 platelets at saturation. These results indicate that there is a twofold difference in the quantitative expression of Pl^A and Pen on human platelets, even though both alloantigens are associated with the same glycoprotein, GPIIIa.

Quantitative indirect binding assay

The results of our quantitative binding assay confirmed the observation made in the fluorocytometry assay, namely, that there are roughly twice as many binding sites per platelet for anti-Pl^{A1} when compared with anti-Pen^a.

As shown in Table I, at saturation, platelets from individuals homozygous for A1 bind an average of 46,500 molecules of antibody per platelet, whereas platelets from individuals heterozygous for A1 bind roughly one-half that amount (21,200 molecules per platelet). These values agree well with previously reported results using a similar assay (25). Unex-

Table 1. Quantitation of Alloantibodies Bound by Platelets

Antibody tested	Platelet type	Molecules of antibody bound per platelet*			
		N‡	Mean	SD	Range
Anti-Pl^{A1}	A1/A1	3	46,553	5,520	40,596–51,495
	A1/A2	5	21,151	5,476	14,627–28,501
	A2/A2	3	1,508	441	1,234–2,016
Anti-Pen^a	a/a§	15	17,302	3,141	13,872–24,238

* According to the method of Lobuglio et al. (22).
‡ Number of donors.
§ Genotype based upon absence of reactivity with anti-Yuk^a (Pen^b) in the antigen-capture ELISA described in this report.

pectedly, when platelets typed homozygous for Pen^a were studied, the maximum amount of anti-Pen^a bound per platelet (17,300) was essentially one-half the maximum amount of platelet-bindable anti-PI^{A1}.

Inhibition of ADP-induced platelet aggregation. All of the platelet preparations tested, including PI^{A1}-homozygous (A1/A1), PI^{A1}-heterozygous (A1/A2), and PI^{A1}-negative (A2/A2) platelets, were positive for Pen^a, and each aggregated to an equivalent extent in response to the addition of 5 μM ADP (Fig. 5 *A*). Prior incubation with anti-PI^{A1} plasma at a 1:4 dilution completely inhibited aggregation of A1/A1 platelets, partially inhibited aggregation of A1/A2 platelets, but did not inhibit aggregation of A2/A2 platelets (Fig. 5 *B*). The same findings were obtained with this source of anti-PI^{A1} at a dilution of 1:8. Each of numerous anti-PI^{A1} plasmas tested inhibited aggregation at a final dilution characteristic of the amount of anti-PI^{A1} activity detected in that plasma. However, regardless of the strength of the anti-PI^{A1} plasma used, we only observed complete inhibition of platelet aggregation when platelets homozygous for PI^{A1} (A1/A1) were used. Anti-PI^{A1} could only partially inhibit aggregation of PI^{A1}-heterozygous platelets, and in this case, the degree of residual aggregation was related more to the platelet source than the antibody source. On the other hand, prior incubation with anti-Pen^a plasma at 1:4 dilution consistently inhibited platelet aggregation regardless of the PI^A-phenotype of the platelets used (Fig. 5 *C*). Identical inhibition was observed with a 1:8 dilution of this anti-Pen^a plasma.

Discussion

The Pen alloantigen system was first described by Friedman and Aster as the causative factor in a case of NATP (1). Subsequently, one case of PTP associated with an antibody specific for the same antigen, Pen^a, was reported by Simon et al. (2). Also, a recently reported platelet alloantigen system, Yuk, which was the causative factor of several cases of NATP in Japan (13, 14) has turned out to be identical to the Pen system (Aster, R. H., and Y. Shibata, unpublished observations). The Pen^a allele is identical to the Yuk^b allele, whereas Pen^b is equivalent to Yuk^a.

Both Shibata et al. (13, 14) and we (1) have previously shown that anti-Pen^a (Yuk^b) antibodies fail to react with platelets of patients with Glanzmann's thrombasthenia, suggesting that this alloantigen is associated with either or both GPIIb and GPIIIa. In this report, we show that the Pen^a determinant is associated with GPIIIa and that it is distinct from the alleles of the PI^A system. This conclusion is based on the following lines of evidence. First, by ELISA wherein AP3 and Tab are used to hold corresponding antigens derived from a platelet lysate obtained in the presence of EDTA, anti-Pen^a specifically bound to GPIIIa held by AP3 but not to GPIIb held by Tab. These results suggested that GPIIb and GPIIIa were completely dissociated in the antigen source used in this ELISA and that Pen^a is thus associated with GPIIIa but not GPIIb. Second, by an ELISA wherein purified GPIIIa from either PI^{A1}-positive (A1/A1) or PI^{A1}-negative (A2/A2) subjects was directly conjugated to microtiter plates, anti-Pen^a bound to both allelic forms of GPIIIa. In the same assay, anti-PI^{A1} bound only to PI^{A1}-positive GPIIIa.

This evidence indicated that the Pen^a determinant is distinct from PI^{A1}. Third, anti-Pen^a specifically immunoprecipitated a surface-labeled protein that, by SDS-PAGE, has the same electrophoretic mobility under nonreduced and reduced conditions as that ascribed to GPIIIa. Anti-Pen^a immunoprecipitated GPIIIa from lysates of either PI^{A1}-positive or PI^{A1}-negative platelets. Neither anti-Pen^a nor anti-PI^{A1} immunoprecipitated GPIIb from lysates prepared in the presence of EDTA. Fourth, by fluorocytometry, platelets from several donors, regardless of PI^A phenotype, bound an amount of anti-Pen^a roughly equivalent to one-half that amount of anti-PI^{A1} that binds to PI^A homozygous (A1/A1) platelets and roughly equal to that amount of anti-PI^{A1} that binds to PI^A heterozygous (A1/A2) platelets. This result was supported by the data obtained by a quantitative indirect binding assay. Using plaelets from donors typed homozygous for PI^{A1} and Pen^a in this assay, 14–24,000 molecules of anti-Pen^a, and 41–51,000 molecules of anti-PI^{A1} were bound per platelet at saturation. Lastly, anti-Pen^a completely inhibits the aggregation of platelets induced by ADP regardless of PI^{A1} phenotype under conditions where phenotype-restricted inhibition by anti-PI^{A1} is observed. GPIIIa, the protein expressing Pen and PI^A epitopes, is one component of the heterodimer complex of human platelets that binds to fibrinogen and thereby mediates platelet aggregation.

It is intriguing that, at saturation, the maximum number of bindable anti-PI^{A1} molecules is essentially twice the number of bindable anti-Pen^a molecules. The numbers of GPIIb, GPIIIa, and GPIIb-IIIa complex molecules per platelet have been estimated to range from 40,000 to 60,000 by direct binding of murine monoclonal antibodies specific for GPIIb, such as Tab (18), or specific for GPIIIa, such as AP3 (17), or specific for the GPIIb-IIIa complex, such as AP2 (16). Several studies of the expression of complex-dependent epitopes on intact platelets would indicate that GPIIb and GPIIIa always exist as a complex in the intact platelet membrane (reviewed in reference

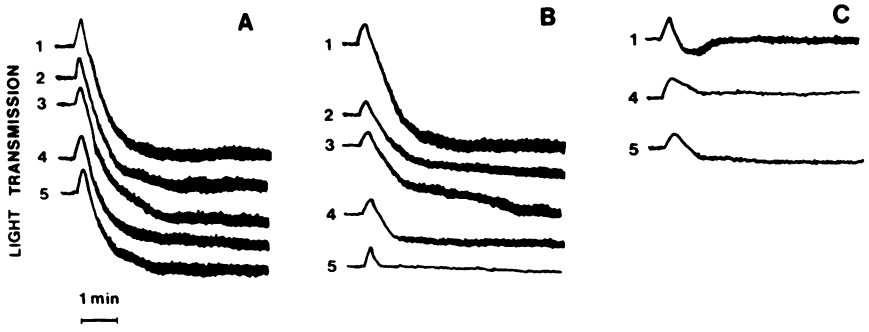

Figure 5. Inhibition of ADP-induced platelet aggregation by anti-platelet alloantibodies. All platelet preparations tested, including PI^{A1}-homozygous (A1/A1), PI^{A1}-heterozygous (A1/A2), and PI^{A1}-negative (A2/A2) platelets, were homozygous for Pen^a and aggregated to the same extent in response to addition of 5 μM ADP (*A*). The inhibitory effect of the binding of anti-PI^{A1} (*B*) or anti-Pen^a (*C*) on ADP-induced platelet aggregation was recorded using a Lumi-aggregometer. The following platelet donors and their PI^A phenotype are indicated: (*1*) A2/A2; (*2–4*) A1/A2; and (*5*) A1/A1.

26). Since a Pl^{A1} homozygous platelet binds an amount of anti-Pl^{A1} equivalent to that amount of bound AP3, it is logical to conclude that each GPIIIa molecule should express one Pl^{A1} epitope. By fluorocytometry, the entire platelet population of each individual analyzed was shown to bind the same amount of anti-Pen^a antibody, therefore the Pen^a epitope is uniformly expressed on the entire platelet population at a level equivalent to one-half of what one would presume to be the level of GPIIIa molecules. One explanation for these findings is that on a given platelet there exist two populations of GPIIb-IIIa both expressing the Pl^A antigens, but only one expressing the Pen antigens. Alternatively, the binding of anti-Pen^a may be inherently bivalent thereby restricting the number of antibody molecules bound at saturation. One would expect that this effect could be overcome by increasing the ratio of antibody-to-antigen. However, no increase in molecules of anti-Pen^a bound per platelet is obtained using fivefold higher levels of anti-Pen^a (incubation in undiluted plasma). Lastly, it is possible that actually there are only about 20,000 Pen^a-positive or Pl^{A1}-positive GPIIIa molecules per platelet, and that the discrepancy in binding at saturation lies in the fact that there are two Pl^{A1} epitopes for every one Pen^a epitope on each molecule of GPIIIa. In view of the quantitative binding data obtained with monoclonal antibodies cited above (16–18), this last possibility is the least likely.

In the presence of calcium ions, GPIIIa is complexed with GPIIb constituting a receptor site not only for fibrinogen (27) but also for fibronectin (28) and von Willebrand factor (29) on activated platelets. van Leeuwen et al. (30) reported that anti-Pl^{A1} antibody induces a thrombasthenia-like platelet dysfunction of normal Pl^{A1}-positive platelets, regardless of zygosity (A1/A1 or A1/A2), by inhibiting the binding of fibrinogen to platelets. In our hands, anti-Pl^{A1} completely inhibited ADP-induced aggregation of Pl^{A1}-homozygous (A1/A1) platelets, but only prolonged the lag phase of or partially inhibited the aggregation of Pl^{A1}-heterozygous (A1/A2) platelets and did not inhibit at all the aggregation of Pl^{A1}-negative (A2/A2) platelets. Anti-Pen^a also inhibited ADP-induced aggregation of Pen^a-positive platelets, regardless of Pl^A or Bak phenotype, in a dose-dependent manner. These data suggest that anti-Pl^{A1} antibodies must occupy all possible Pl^{A1} epitopes on GPIIIa ($\sim$ 40,000 molecules per platelet) to completely inhibit ADP-induced platelet aggregation. On the other hand, $\sim$ 20,000 molecules per platelet of anti-Pen^a can completely inhibit platelet aggregation under identical conditions.

GPIIIa has been implicated in several aspects of platelet immunology. The Pl^{A1} alloantigen, which is that most frequently implicated to the etiology of PTP and NATP (11, 12), has been localized to GPIIIa by Kunicki and Aster (7), and further localized to a 17,000-D fragment of GPIIIa by Newman et al. (31). Beardsley et al. (5) have identified autoantibodies in sera from several patients with chronic ITP that react specifically with GPIIIa, and Woods et al. (4) and Niessner et al. (6) also reported autoantibodies against the GPIIb-IIIa complex in patients with the same disorder. Also, isoantibodies directed against components of the GPIIb-IIIa complex have been described in plasma from polytransfused patients with Glanzmann's thrombasthenia (9, 10, and this study). Pen (Yuk) represents the second platelet-specific alloantigen system localized to GPIIIa. In view of the important role of GPIIIa in both platelet physiology and platelet immunology, anti-Pen^a represents yet another valuable reagent for the further characterization of the structure, function, and antigenic makeup of this glycoprotein. In particular, if a previously unrecognized heterogeneity of the GPIIIa molecule exists that may be relevant to its function as a component of the physiologic receptor for platelet cohesion, anti-Pen antibodies can be used to establish and characterize this heterogeneity.

Acknowledgments

We thank Dr. Toby Simon (University of New Mexico and United Blood Services, Albuquerque, NM) for providing antiserum specific for Pen^a, Dr. Shibata for his gift of antisera reactive with Yuk^a (Pen^b) and Yuk^b (Pen^a), Dr. Sherill Slichter (Puget Sound Blood Center and University of Washington School of Medicine, Seattle, WA) for her gift of antiserum specific for Pl^{A2}, Dr. Brian Bull (Loma Linda University Medical Center, Loma Linda, CA) for providing plasma from thrombasthenic patient OG, Dr. McEver for his gift of the monoclonal antibody Tab, Ms. Susan Kristopeit for her excellent technical assistance, and Ms. Anna Kuster for the preparation of this manuscript.

This work was supported by National Heart, Lung, and Blood Institute (NHLBI) grants HL-32279 and HL-37471. Dr. Furihata is a Bradley Scholar in Biomedicine, funded by the Lynde and Harry Bradley Foundation, Milwaukee, WI. Dr. Kunicki is an Established Investigator (83-186) of the American Heart Association, and Dr. Nugent is the recipient of NHLBI Clinical Investigator Award HL-01649.

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