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M H Ginsberg, ... , G Marguerie, E F Plow

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**Research Article**

An antiplatelet monoclonal antibody, PMI-1, reacts with glycoproteins (GP) GPIIb, free GPIIb, and the GPIIb-IIIa complex. This antibody binds to 40,900 sites per platelet, with a  $K_d = 0.95$   $\mu\text{M}$ , and its binding is inhibited by the presence of magnesium or calcium in the suspending medium (50% suppression at approximately 0.5 mM divalent cation). Regulation of the PMI-1 epitope is independent of disassembly of the GPIIb-IIIa heterodimer, because it occurred at 22 degrees C and in response to mM magnesium as well as calcium. PMI-1 binding inversely correlated with fibrinogen binding. In addition, we identified a variant of Glanzmann's thrombasthenia with near-normal platelet content of the GPIIb-IIIa heterodimer as judged by crossed immunoelectrophoresis and surface labeling. Binding of PMI-1 to these patients' platelets was not dependent on reduction of the divalent cation concentration. These data suggest that the surface orientation of GPIIb is important in the capacity of platelets to bind fibrinogen.

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# Divalent Cation Regulation of the Surface Orientation of Platelet Membrane Glycoprotein IIb

## Correlation with Fibrinogen Binding Function and Definition of a Novel Variant of Glanzmann's Thrombasthenia

Mark H. Ginsberg, Alton Lightsey,\* Thomas J. Kunicki,‡ Adam Kaufmann,§ Gerard Marguerie,<sup>||</sup> and Edward F. Plow  
Research Institute of Scripps Clinic, La Jolla, California 92037; \*Department of Pediatrics, F. Edward Herbert School of Medicine, Uniformed Services University of the Health Sciences (USUHS), Bethesda, Maryland 20814; ‡Blood Center of Southeastern Wisconsin, Milwaukee, Wisconsin 53233; §University of Cincinnati, Cincinnati, Ohio 45267; and <sup>||</sup>Centre d'Études Nucléaires de Grenoble, 38041 Grenoble, France

### Abstract

An antiplatelet monoclonal antibody, PMI-1, reacts with glycoproteins (GP) GPIIb, free GPIIb, and the GPIIb-IIIa complex. This antibody binds to 40,900 sites per platelet, with a  $K_d = 0.95 \mu\text{M}$ , and its binding is inhibited by the presence of magnesium or calcium in the suspending medium (50% suppression at  $\sim 0.5 \text{ mM}$  divalent cation). Regulation of the PMI-1 epitope is independent of disassembly of the GPIIb-IIIa heterodimer, because it occurred at  $22^\circ\text{C}$  and in response to mM magnesium as well as calcium. PMI-1 binding inversely correlated with fibrinogen binding. In addition, we identified a variant of Glanzmann's thrombasthenia with near-normal platelet content of the GPIIb-IIIa heterodimer as judged by crossed immunoelectrophoresis and surface labeling. Binding of PMI-1 to these patients' platelets was not dependent on reduction of the divalent cation concentration. These data suggest that the surface orientation of GPIIb is important in the capacity of platelets to bind fibrinogen.

### Introduction

Platelet aggregation is essential for normal hemostasis and is dependent upon the presence of fibrinogen (Fg)<sup>1</sup> (1). A molecular explanation for this Fg requirement has been provided by the observation that platelets stimulated with aggregation-promoting agonists specifically and saturably bind Fg (2, 3). Further, the capacity of platelets to bind Fg correlates closely with their aggregation (4). The absence of Fg-binding function in platelets congenitally deficient in membrane glycoproteins (GP) IIb and IIIa (GP IIb-IIIa) in patients with Glanzmann's thrombasthenia implicated these GP in Fg binding (3-5). Additional evidence for their role in this process has been derived from inhibition of Fg binding by certain antibodies against GPIIb-IIIa (6-10), cross-

linking of GPIIb-IIIa to derivatized Fg (11, 12), and the demonstration of association of partially purified GPIIb-IIIa with Fg in vitro (13-15). Thus, GPIIb-IIIa is clearly implicated in the ability of platelets to bind Fg and to aggregate.

GPIIb-IIIa exists as a calcium-dependent heterodimer in detergent solution (16, 17); Fujimura and Phillips (18) found that at  $>10 \mu\text{M}$   $\text{Ca}^{++}$ , the complex was fully associated and that  $\text{Mg}^{++}$  was much less effective than  $\text{Ca}^{++}$  in this regard. The complex exists in isolated platelet membranes as judged by resistance to thrombin hydrolysis (18) and in intact resting platelets as judged by the binding of "complex-specific" monoclonal antibodies (6, 7, 9) where it accounts for most of the specific high-affinity surface  $\text{Ca}^{++}$  binding sites (19). Incubation of intact platelets at  $37^\circ\text{C}$  in the presence of strong  $\text{Ca}^{++}$ -chelating agents ( $[\text{free } \text{Ca}^{++}] < 0.1 \mu\text{M}$ ) results in loss of binding of complex-specific antibodies (6, 7) apparently due to disassembly of the heterodimer (20, 21); loss of aggregability (4, 20, 21) is associated with such treatment. Phillips and Baughan (22) also observed an absolute requirement for  $>1 \mu\text{M}$   $\text{Ca}^{++}$  for Fg binding to isolated platelet membranes, and suggested that  $\text{Ca}^{++}$  at these concentrations supported Fg binding by maintenance of the GPIIb-IIIa heterodimer. These data, taken together, suggest that extracellular  $\text{Ca}^{++}$  is required for maintenance of the GPIIb-IIIa heterodimer, which appears to be a prerequisite for Fg binding and aggregation. The critical  $\text{Ca}^{++}$  concentrations are in the micromolar range; thus, heterodimer formation is unlikely to be regulated physiologically by this mechanism, because the normal extracellular free  $[\text{Ca}^{++}]$  is maintained in the millimolar range.

In addition to the above-noted absolute  $\text{Ca}^{++}$  requirement, several workers have observed that millimolar concentrations of  $\text{Ca}^{++}$  or  $\text{Mg}^{++}$  are required for optimal aggregation and Fg binding to intact cells (3, 23) and membranes (22), and Phillips and Baughan (22) have clearly distinguished this "nonselective" millimolar divalent cation requirement from the "selective" micromolar  $\text{Ca}^{++}$  requirement. It is uncertain whether this second, possibly physiologically relevant, divalent cation requirement is mediated through GPIIb-IIIa, other sites on the platelet, or divalent cation-binding sites in the ligand (24). We now have identified regulation of the surface exposure of a region of GPIIb by  $\text{Ca}^{++}$  or  $\text{Mg}^{++}$  in the millimolar concentration range by use of a monoclonal antibody. This regulation appears to be functionally significant because it correlates with the capacity of platelets to bind Fg in the presence of various concentrations of divalent cations and in a novel variant of Glanzmann's thrombasthenia.

### Methods

*Production of PMI-1.* The production of this monoclonal antibody and its characterization have been described in detail (25). Briefly stated, it

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Address reprint requests to Dr. Ginsberg.

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1. Abbreviations used in this paper: Fg, fibrinogen; GP, glycoprotein; PAGE, polyacrylamide gel electrophoresis.

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was produced as a consequence of immunization of Balb C mice with a platelet membrane preparation in Freund's adjuvant. The washed spleen cell suspension from these mice were fused with P3X63-AG8.653 myeloma cells at a 5:1 ratio and the fused cells were grown in hypoxanthine-aminopterin-thymidine medium prior to being distributed in microtiter wells. A single colony of cells secreting an antibody that reacted with platelet membranes and inhibited platelet attachment to collagen (25) was selected and subcloned twice at limiting dilutions of 0.5 cell per well. This cell line, designated PMI-1, was injected into pristine primed mice (ascites fluid developed within 1 wk). Ascites from this subclone exhibited a single band of gamma mobility or cellulose acetate electrophoresis. IgG was prepared from ascitic fluid of by affinity chromatography on protein A-agarose (Pharmacia Fine Chemicals, Piscataway, NJ) in 0.1 ml of sodium phosphate buffer, pH 8, as previously described (25). This antibody was an IgG 1 kappa antibody by Ouchterlony immunodiffusion.

**Binding assays and platelet isolation.** The platelets were isolated from acid citrate dextrose anticoagulated fresh human blood by differential centrifugation and gel filtration as previously described (2). The platelets were ultimately suspended in a Tyrode's buffer containing 0.1% bovine serum albumin, pH 7.4. The ligands employed were <sup>125</sup>I-monoclonal IgG or <sup>125</sup>I-Fg labeled to specific activities of 0.5–3  $\mu$ ci/ $\mu$ g by a chloramine-T procedure as described (2). Detailed descriptions of methodologies employed in binding assays have been published (2). In a typical assay, platelets were diluted in modified Tyrode's buffer to  $8 \times 10^8$  per ml. 50  $\mu$ l of the platelet suspension was added to 110  $\mu$ l of buffer containing the ligand, followed by 40  $\mu$ l of buffer. In some experiments ADP (Sigma Chemical Co., St. Louis, MO), human thrombin (the gift of Dr. John Fenton, N.Y. State Department of Health), or acid-soluble collagen (Millipore Corp., Bedford, MA) was added in place of the buffer. Unless otherwise indicated, incubations were performed at 37°C. At selected time points, triplicate 50- $\mu$ l aliquots were layered onto 300  $\mu$ l of 20% sucrose in modified Tyrode's buffer and centrifuged for 3 min in a Beckman microfuge (Beckman Instruments, Inc., Palo Alto, CA). Tips were amputated and the radioactivity contained was counted in a gamma scintillation spectrometer (Searle, Chicago, IL); the quantity of platelet-bound ligand was calculated from the specific activity utilizing molecular weights of 340,000 for Fg, and 150,000 for IgG. Binding isotherms were analyzed for a one-site model, by use of the nonlinear least squares curve-fitting program of Munson and Robard (Ligand) (26) implemented on the Apple II computer by T. Jackson, Middlesex Hospital, United Kingdom. This program estimates nonsaturable binding as a constant fraction of free ligand.

**Analysis of the surface protein content of thrombasthenic platelets.** Platelets were labeled with <sup>125</sup>I by use of a modified lactoperoxidase technique of Phillips and PohAgin (27) as published (28). The labeled protein content of these surfaced labeled cells was analyzed by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the technique of Laemmli with use of 10% mercaptoethanol to fully reduce surface glycoproteins. In nonreduced gels, 10 mM N-ethylmaleimide was added to prevent disulfide interchange (27). Proteins were visualized by staining with Coomassie blue 250, gels were subsequently dried in vacuum and exposed for 3 h to 5 d at -70°C using Cronex Hiplus enhancing screen (DuPont Co., Wilmington, DE), and film was developed using a Kodak Xomat processor. Standards of reduced fibronectin (225,000 mol wt) and IgG H and L chains (55,000, 25,000 mol wt) were purified in our laboratory. Lactoperoxidase (78,000 mol wt) was provided by Calbiochem-Behring Corp. (La Jolla, CA) and lactic dehydrogenase (140,000 mol wt), phosphorylase B (94,000 mol wt), bovine serum albumin (68,000 mol wt), ovalbumin (43,000 mol wt), and carbonic anhydrase (30,000 mol wt) were purchased from Pharmacia Fine Chemicals. To quantitate intensity of stained or autoradiographic bands, we utilized a soft laser densitometer no. SE504 (Biomed Instruments, Inc., Chicago, IL).

For two-dimensional gel electrophoresis we utilized a variant of the O'Farrell technique as previously published (28). In this system, the proteins are reduced prior to isoelectric focussing. The pH gradients were

determined by slicing the gel into 5 mM segments that were extracted in boiled deionized water; the pH of each fraction was measured on a pH meter. Coomassie blue staining and autoradiography were performed as described for Laemmli gels.

Two-dimensional peptide mapping of labeled peptides were performed exactly as described by Elder et al. (29) with the following modifications. A suspension containing a 1% Triton X-100 platelet lysate ( $10^9$  cells/ml) and 5 mM EDTA 0.01 M Tris 0.15 M NaCl, pH 7.4, was passed through a lentil lectin Sepharose (Pharmacia Fine Chemicals). Bound proteins were eluted in 0.1 M alpha methyl mannoside in the same buffer, and radioiodinated in detergent utilizing chloramine and <sup>125</sup>I (2). The radioiodinated material was then immunoprecipitated by use of a goat polyclonal anti-GPIIb-IIIa and protein A containing staphylococci exactly as described previously (12). The radiolabeled immunoprecipitate was eluted in sample buffer (28) and analyzed by SDS-PAGE under reducing conditions, and the major bands of mol wt 123,000 (GPIIb alpha) and 105,000 (GPIII) were cut out, trypsin was digested, and the resultant peptides mapped by high-voltage electrophoresis followed by thin-layer chromatography exactly as described by Elder et al. (29). Labeled spots were visualized by autoradiography.

For crossed immunoelectrophoresis, platelets were suspended to a concentration of  $5 \times 10^9$ /ml in 0.038 M Tris, 0.1 M glycine, pH 8.7. They were solubilized by agitation for 30 min at 4°C in the presence of 1% (vol/vol) Triton X-100, and insoluble material was pelleted by centrifugation at 20,000 g for 1 h at 4°C. These extracts were snap-frozen in liquid nitrogen, and stored at -70°C prior to being transported by air freight, without thawing, to the Blood Center of Southeastern Wisconsin, Milwaukee where analysis was performed. Crossed immunoelectrophoresis was performed as described (16) utilizing an intermediate gel. Radioimmunoassays for platelet factor 4, beta-thromboglobulin, and Fg have all been previously described (30). These analyses were performed on 1% Triton lysates of washed platelets in the presence of 5 mM EDTA.

## Results

**PMI-1 specificity.** As reported previously, this monoclonal antibody blots to a single polypeptide with mobility identical to GPIIb-alpha in electrophoretic transfers of nonreduced-reduced gels of whole platelet proteins (25). To further establish its specificity for this membrane protein, we analyzed the interaction of this antibody with two patients with known hereditary deficiency of GPIIb-IIIa. At the tested concentration, the patient's platelets bound 1 order of magnitude less PMI-1 than control platelets (Table I). Thus, the species immunoblotted by this antibody is authentic GPIIb rather than a coelectrophoresed minor polypeptide. GPIIb exists as a heterodimer complex with GPIIIa in detergent extracts of platelets when the  $[Ca^{++}]$  is greater than micromolar (16, 17). To determine whether PMI-1 recognizes GPIIb in the complex, we performed crossed immunoelectrophoresis in which the radiolabeled antibody was incorporated in the intermediate gel. The antibody labeled band 16, which is

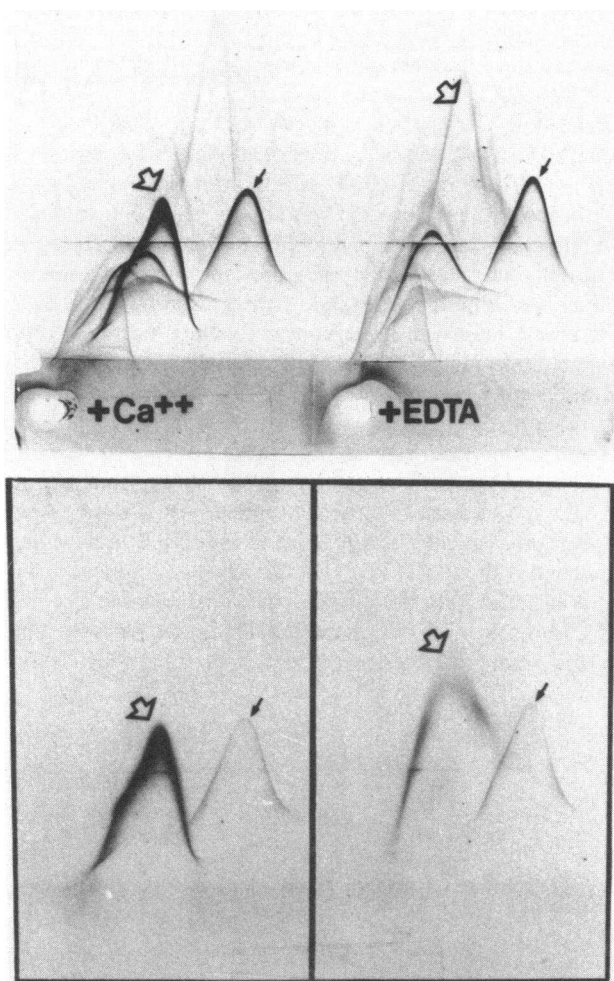
Table I. Binding of PMI-1 to Thrombasthenic Platelets

Subject	PMI-1 bound molecules/platelet
N.L.	4,512±712
M.M.	3,534±41
Control	36,877±16

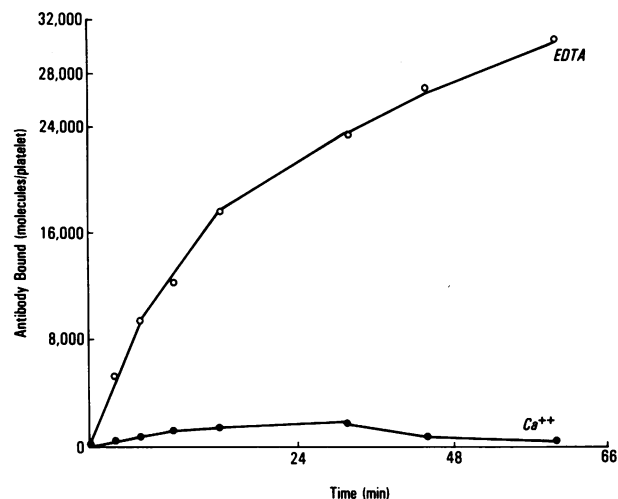
<sup>125</sup>I-PMI-1 ( $8.3 \times 10^{-7}$  M) was incubated at 37°C with the indicated donor's platelets at  $10^8$  cells/ml in the presence of 5 mM EDTA for 30 min, and binding was measured as described in Methods. Results are  $\pm$ SEM of triplicates.

known to contain the GPIIb-IIIa complex (16, 31). When the cells were solubilized in the presence of 5 mM EDTA, band 16 was markedly reduced in height, and the antibody labeled a new more anodal arc which has previously been identified as containing GPIIb (16) (Fig. 1).

**Binding of PMI-1 to platelets.** We next evaluated the interaction of radiolabeled PMI-1 with intact platelets. Surprisingly, very little antibody bound saturably to resting platelets in the presence of physiologic  $Mg^{++}$  and  $Ca^{++}$  concentrations (Fig. 2). Stimulation in suspension with up to 100  $\mu M$  ADP or epinephrine, 1 U/ml thrombin, or 50  $\mu g/ml$  calf skin collagen did not increase binding (not shown). In contrast, addition of 5 mM EDTA to the platelet suspension resulted in time-dependent



**Figure 1.** Crossed immunoelectrophoretic analysis of antigenic targets of the PMI-1 antibody. Platelet extracts in the presence of 5 mM  $CaCl_2$  (+ $Ca^{++}$ ) or 5 mM EDTA (+EDTA) were electrophoresed left to right, and then from bottom to top. The top gel contained a high-titer polyclonal rabbit anti-human whole platelet antibody; the intermediate gel contained  $^{125}I$ -labeled PMI-1. (Upper panels) Coomassie blue staining. (Lower panels) Autoradiograms of the same gel. Note the densely staining arc (open arrow) which reacts with the PMI-1 antibody (lower panel) in the presence of calcium. In the presence of EDTA, that arc disappears, and a new arc, indicated by an open arrow in the +EDTA figure, appears. This arc, corresponding to GP IIb, is labeled with PMI-1. Note that the small black arrow indicates platelet albumin which is artifactually labeled.



**Figure 2.** Kinetics of binding of PMI-1 antibody to platelets. PMI-1 (1  $\mu M$ ) was incubated with  $2 \times 10^8$  platelets at 37°C for the indicated time, in the presence of 5 mM calcium ( $\bullet$ ) or 5 mM EDTA ( $\circ$ ); bound was separated from free as described in Methods.

binding (Fig. 2), which approached steady state by 30 min incubation. To further characterize the interaction of this antibody with platelets, we measured binding across a broad dose range. Binding was saturable (Fig. 3) and analysis by a nonlinear least squares curve-fitting program (26) was consistent with binding to a single class of sites ( $K_d = 0.95 \mu M$ , 40,900 sites per cell, nonsaturable binding = 0.001, mean square error = 9.1). The number of sites estimated is in good agreement with measurements of the binding of GPIIb-specific antibodies to platelets (32), suggesting the antibody does not recognize a subspecies of GPIIb.

Detailed analyses of divalent cation requirements for suppression of the PMI-1 epitope have been performed. When platelets were prepared in medium containing  $<0.1 \mu M$   $Ca^{++}$  (23), PMI-1 binding was comparable to that noted in the presence of 1 mM EDTA (Fig. 4). Addition of either  $Mg^{++}$  or  $Ca^{++}$  as the chloride salt suppressed PMI-1 binding; binding was 50% suppressed at  $\sim 400 \mu M$  of either divalent cation and fully suppressed near physiologic concentrations (Fig. 4). Thus, variations in local concentrations of  $Ca^{++}$  and  $Mg^{++}$  near the physiologic range may regulate expression of the PMI-1 epitope.

To determine whether PMI-1 epitope exposure is reversible, we bound the antibody to platelets in the presence of EGTA for 30 min and then recalcified the platelet suspension. Addition of calcium eluted most ( $>90\%$ ) of platelet bound PMI-1 by 60 min (Table II). Thus, availability of the PMI-1 epitope on the platelet surface is reduced by  $Ca^{++}$  even in the continuous presence of the antibody.

**Relationship of PMI-1 binding to binding of Fg and a complex-specific anti-GPIIb-IIIa.** Fg binding to platelets is divalent ion dependent, and maintenance of the GPIIb-IIIa complex is calcium dependent. To explore potential relationships among these events, we compared the regulation of the binding of the complex-specific anti-GPIIb-IIIa, AP-2 (9), with PMI-1 and Fg. At room temperature, the AP-2-binding site was maintained even at extracellular  $Ca^{++}$  concentrations below  $0.1 \mu M$ , consistent with the (20, 21) behavior of the GPIIb-IIIa complex-specific antibody binding to intact platelets. Under these con-

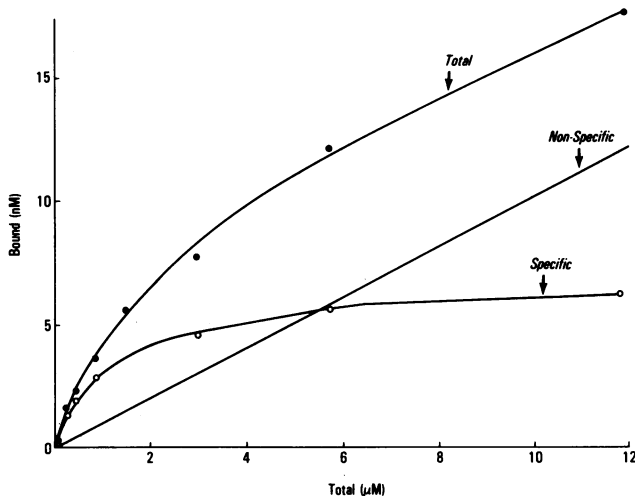


Figure 3. Relationship of PMI-1 bound to input antibody concentration. Platelets ( $10^8/\text{ml}$ ) were incubated for 30 min, at  $37^\circ\text{C}$  in the presence of 5 mM EDTA and the indicated input concentration of PMI-1 antibody. Bound antibody was measured as described in Methods. Total bound PMI-1 is indicated by the upper data points ( $\bullet$ ); the curve represents the best fit of the data to a single class of binding sites ( $K_d = 0.95 \mu\text{M}$ , 40,600 sites per cell, and nonsaturable binding = 0.001) utilizing the LIGAND computer program. The line with no symbols represents the computer estimated nonsaturable binding. This was validated in 11 experiments by addition of a 100-fold molar excess cold ligand at 0.1  $\mu\text{M}$  input PMI-1, in this case nonsaturable binding was estimated at  $0.001 \pm 0.00015$  ( $n = 11$ ). Saturable binding, calculated as the difference between total and nonsaturable binding, appears on the lower curve ( $\circ$ ). 1 nM bound PMI-1 =  $6.02 \times 10^3$  molecules per platelet.

ditions, the PMI-1 epitope was fully expressed and Fg binding fully inhibited (Fig. 5). Addition of  $\text{Mg}^{++}$  restored Fg binding and suppressed the PMI-1 epitope in concert (Fig. 5). This result confirms a divalent cation requirement for Fg binding above that required for maintenance of the GPIIb-IIIa complex and

indicates that capacity to bind Fg closely correlates with suppression of the PMI-1 epitope.

*Identification of a variant of Glanzmann's thrombasthenia with abnormal regulation of the PMI-1 epitope.* The experiments described above indicate that divalent cation suppression of PMI-1 epitope expression correlates with the ability of platelets to bind Fg. Because Fg possesses  $\text{Ca}^{++}$ -binding sites (24), it is possible that the divalent cation effects are mediated through Fg, and that PMI-1 epitope suppression is an epiphenomenon. To address this issue, we have identified a novel variant of Glanzmann's thrombasthenia in which the GPIIb-IIIa complex is present, but Fg binding is negligible and the PMI-1 epitope is not suppressed at physiologic concentrations of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ . Affected individuals are two male and one female siblings in a Guamanian family in which one male and one female sibling and both parents are clinically unaffected. The affected individuals have suffered with epistaxis, mucous membrane bleeding, gastrointestinal hemorrhage, and soft-tissue bleeding since neonatal life. Bleeding episodes have only been controllable by platelet transfusion. Laboratory investigation of affected individuals revealed a complete absence of platelet aggregation in response to ADP or thrombin, whereas the initial phase of ristocetin-induced agglutination was normal (Fig. 6). In addition, clot retraction was absent and bleeding times were greater than 20 min in each patient on several occasions. Plasma Fg concentrations, prothrombin times, and partial thromboplastin times have always been within normal limits. Platelet content of Fg was  $1 \pm 0.2 \mu\text{g}/10^9$  cells ( $n = 3$ ) whereas three simultaneously assayed normal controls had  $31.3 \pm 7.7 \mu\text{g}/10^9$  cells. In contrast, content of platelet factor 4 in patient cells was  $14.8 \mu\text{g}/10^9$  cells whereas the three normal cell preparations contained  $15.9 \pm 1.4 \mu\text{g}/10^9$  cells. Platelet size and morphology were normal in both the light and electron microscope (not shown). On more than 10 occasions, patient platelets failed to bind Fg saturably when stimulated with ADP (Fig. 7). Binding of Fg, fibronectin, and von Willebrand factor to thrombin-activated cells was also negligible (not shown). Thus, these individuals have a congenital, probably autosomal recessive platelet abnormality which is clin-

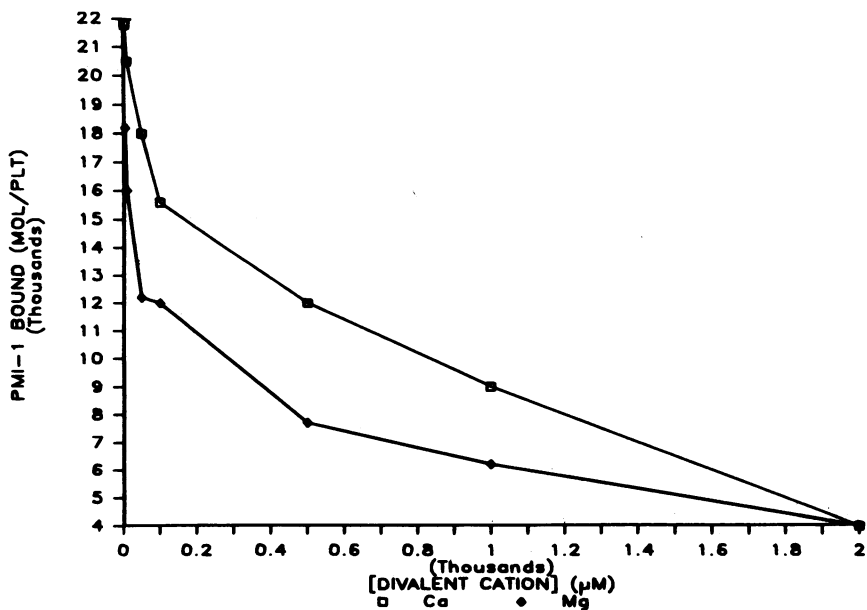


Figure 4. Influence of divalent cations on PMI-1 binding. Platelets ( $10^8/\text{ml}$ ) were suspended in divalent cation-free Tyrode's solution. The indicated concentrations of calcium ( $\square$ ) or magnesium ( $\diamond$ ) were added, and the cells were incubated at  $37^\circ\text{C}$  for 30 min with 1  $\mu\text{M}$  PMI-1 antibody. Bound ligand was measured (mol/plt, molecules per platelet) as described in Methods.

Table II. Dissociation of Platelet Bound PMI-1 by Ca<sup>++</sup>

Divalent cations	PMI-1 bound	
	35 min	60 min
	<i>molecules/platelet</i>	<i>molecules/platelet</i>
5 mM EGTA	18,300±600	20,900±650
25 mM Ca <sup>++</sup>	670±40	850±10
5 mM EGTA addition of 25 mM Ca <sup>++</sup> at 30 min	10,800±610	3,000±120

A suspension of washed platelets ( $5 \times 10^8$ /ml) was incubated at 22°C in the presence of 0.5 μM PMI-1 in the indicated divalent cation conditions. Samples were removed for measurement of PMI-1 binding at 35 and 60 min of incubation.

ically and functionally typical of severe Glanzmann's thrombasthenia.

To further characterize the abnormality in this kindred, their platelet membrane proteins were analyzed by lactoperoxidase-catalyzed iodination followed by SDS-PAGE and autoradiography. No difference was apparent in the surface-labeling patterns (Fig. 8), and densitometric quantitation of the GPIIIa/GPIIb ratios revealed a value of  $8.8 \pm 3.3$  in normal ( $n = 5$ ) and  $7.3 \pm 1.7$  ( $n = 4$ ) in an affected member of this family. In contrast, similar analysis of four previously reported (33) thrombasthenia patients gave a mean ratio of  $0.16 \pm 0.11$ . We considered the possibility that the patients lacked GPIIb-IIIa, but possessed another surface-labelable species of similar size present in increased quantities or labeled with a higher specific activity as a consequence of their abnormality. To exclude this, their surface-labeled platelets were analyzed by O'Farrell two-dimensional gel electrophoresis. The major spots at apparent pI = 4.5 and  $M_r = 123,000$  and 105,000 were not appreciably reduced relative to the normal (Fig. 9). These spots have previously been identified as GPIIb alpha and GPIIIa in part by their deficiency in typical patients with thrombasthenia (28). To establish definitively the identity

of these species as GPIIb and IIIa, two-dimensional tryptic peptide maps of the patients' proteins were compared with those of normal controls. Substantial differences between the maps of GPIIb and IIIa were apparent (Fig. 10) confirming previous work (34, 35). Nevertheless, there was no consistent difference between the normals and the patients with respect to the maps for either protein. To determine whether there were any other abnormality in platelet protein content, the patients platelets were analyzed by crossed immunoelectrophoresis. In this analysis the absence of platelet Fg was apparent (Fig. 11). Band 16 was present, but appeared of reduced intensity. In addition, there was a suggestion of subtle anodic splitting of this arc (Fig. 11). Free GPIIb and GPIIIa were not readily identified.

The data presented above indicate that these individuals possess GPIIb and IIIa, which exist, at least in part, in a complex, yet have severe Glanzmann's phenotype. To determine whether they manifested abnormal regulation of the PMI-1 epitope, we studied the interaction of this antibody with their cells. In two of the affected patients, studied on four occasions, the binding of PMI-1 to platelets was not influenced by the presence of divalent cations (Fig. 12). Moreover, the affected individuals specifically bound >20,000 molecules per platelet confirming that they had at least 50% the normal content of GPIIb.

## Discussion

The data reported here have defined a divalent cation dependent regulation of the surface exposure of the heavy chain of platelet membrane GPIIb. This regulation occurs in physiologic concentrations of extracellular Ca<sup>++</sup> and Mg<sup>++</sup>, and closely correlates with the capacity of platelets to bind Fg. It is presently not clear whether the divalent cations interact with the GP to render the PMI-1 epitope cryptic or whether the epitope is modulated through indirect effects of divalent cations at other sites on the platelet membrane. Nevertheless, abnormal regulation of the PMI-1 epitope appears to be a distinguishing feature of a novel variant of Glanzmann's thrombasthenia described herein. In addition, because the PMI-1 antibody inhibits platelet-collagen

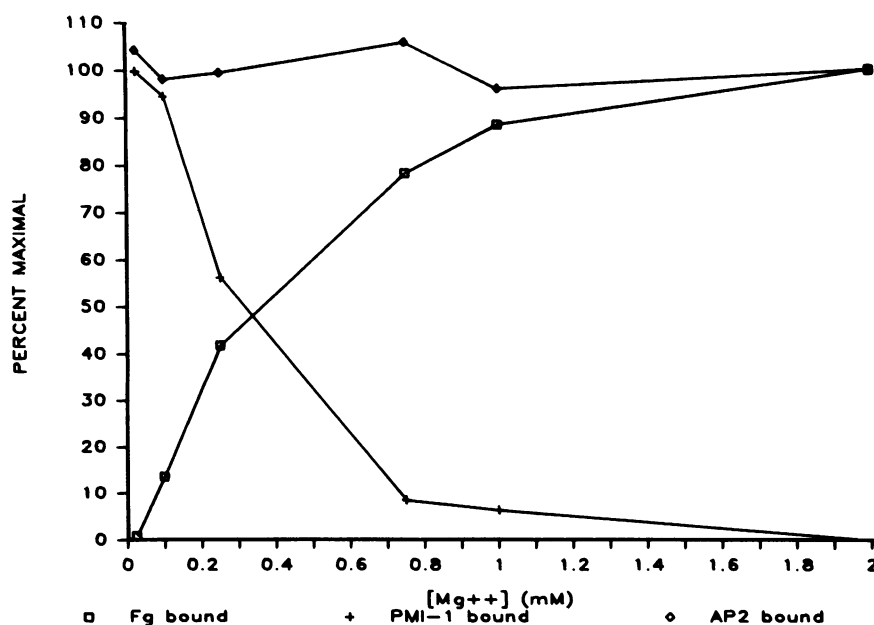
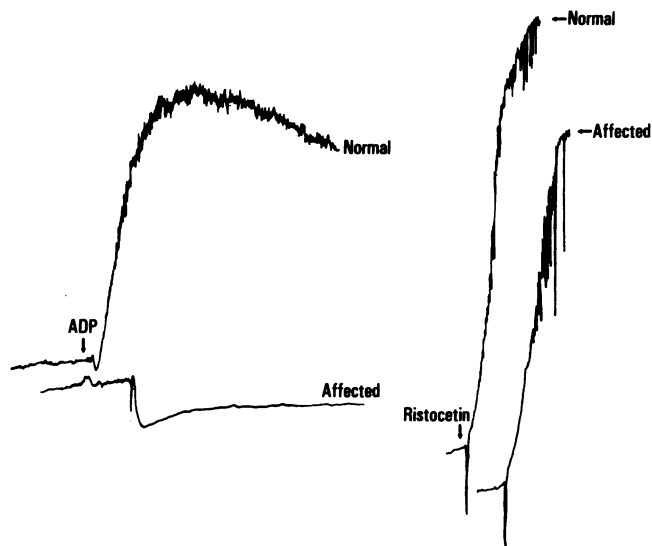


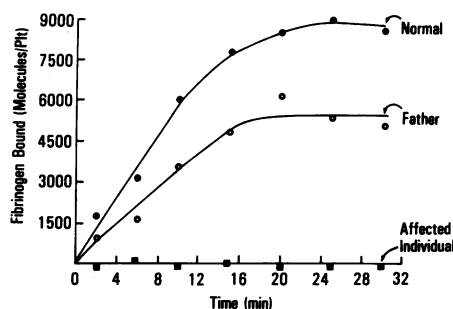
Figure 5. Correlation between PMI-1 binding, AP2 binding, and Fg binding.  $2 \times 10^8$  platelets per ml were suspended in divalent cation-free Tyrode's solution containing the indicated concentration of added MgCl<sub>2</sub>. In three parallel assays, the binding of 1 μM PMI-1 (x), 1 nM AP2 (o), and 0.3 μM Fg (□) in the presence of 10 μM ADP was measured after incubation at 22°C for 30 min. Maximal binding (100%) was taken as that value observed in the absence of added divalent cations for PMI-1, and in the presence of 2 mM magnesium for AP2 and Fg. Approximately 20,000 molecules per platelet of each ligand were maximally bound.



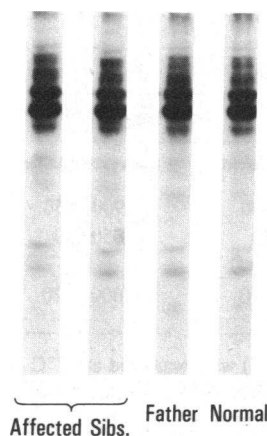
**Figure 6.** Abnormal platelet aggregation in variant thrombasthenia. Platelet aggregation assays were in 0.38% citrated platelet-rich plasma containing  $2 \times 10^8$  platelets/ml, at  $37^\circ\text{C}$ , and with stirring at 1,100 rpm in a Sienco (Morrison, CO) dual-channel aggregometer. The left panel shows the response to  $10 \mu\text{M}$  ADP, in which the normal platelet showed typical shape change and aggregation whereas the thrombasthenic (affected) show only shape change. The right panel shows similar aggregation responses to  $100 \mu\text{g/ml}$  ristocetin.

adhesion in a  $\text{Mg}^{++}$ -dependent system, it may represent a prototype for a novel class of thrombus-directed antibodies.

The PMI-1 antibody unequivocally reacts with GPIIb alpha based upon Western blotting of two dimensional gels (25), crossed immunoelectrophoresis, and reduced reactivity with platelets deficient in GPIIb-IIIa. Moreover, McEver et al. (7, 32) have reported that Tab, an antibody directed against GPIIb, binds to 40,000 sites per platelet as did PMI-1; it therefore seems likely that the antibody does not recognize a subpopulation of the GP. Unlike Tab (7), PMI-1 recognizes a divalent cation-regulated epitope. Several laboratories have recently reported (6, 7) that  $\text{Ca}^{++}$  may regulate the binding of complex-specific antibodies to intact platelets. When this was examined in detail (20), reduction of antibody binding required lowering of the



**Figure 7.** Reduced Fg binding to variant thrombasthenic platelets. Platelets from the affected individual ( $\blacksquare$ ), the patient's father ( $\circ$ ), and a normal individual ( $\bullet$ ) were incubated at  $2 \times 10^8$  cells/ml in modified Tyrode's solution at  $22^\circ\text{C}$  in the presence of  $0.3 \mu\text{M}$  Fg and  $10 \mu\text{M}$  ADP for the indicated time. Bound ligand was measured as indicated in Methods. Note the absence of detectable Fg binding in the case of the affected individual.

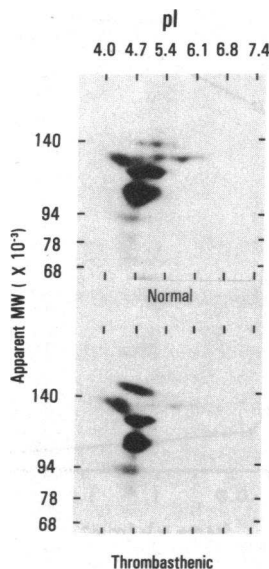


**Figure 8.** SDS-PAGE analysis of the surface membrane proteins of individuals with variant thrombasthenia. Lactoperoxidase catalyzed iodinated platelets of two affected individuals, their father, and a simultaneously prepared normal individual electrophoresed under reducing conditions in 7.5% polyacrylamide gels. Autoradiograms of these gels are shown. Note the two most heavily labeled bands which contain GP IIb-IIIa.

extracellular  $\text{Ca}^{++}$  to  $<1 \mu\text{M}$ , did not proceed rapidly at  $22^\circ\text{C}$ , and could not be abrogated by  $\text{Mg}^{++}$ . In contrast, PMI-1 is regulated at  $22^\circ\text{C}$  as well as  $37^\circ\text{C}$  and by  $\text{Mg}^{++}$  as well as by  $\text{Ca}^{++}$  in the millimolar range. Moreover, PMI-1 bound to the complex as well as the free GPIIb (Fig. 1), and it could be regulated by  $\text{Mg}^{++}$  under conditions in which the binding of a "complex-specific" antibody did not vary.

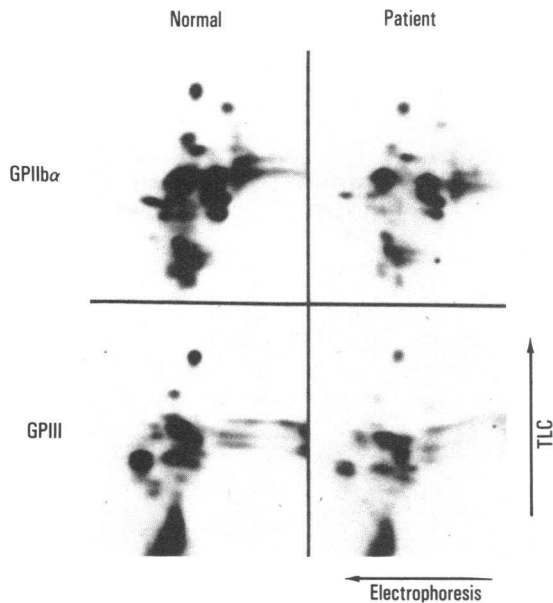
One explanation for PMI-1 regulation is that the antibody recognizes a feature of the conformation of GPIIb. Brass and Shattil (19) have reported marked deficiency of high-affinity ( $K_d = 9 \text{ nM}$  and  $400 \text{ nM}$ ) selective  $\text{Ca}^{++}$  binding sites in Glanzmann's thrombasthenia, suggesting these sites reside on the GPIIb-IIIa complex. In view of the different cation selectivity and concentration requirements, these high-affinity sites are unlikely to be those involved in PMI-1 regulation. Nevertheless, the conformation of GPIIb could be regulated by lower-affinity divalent cation-binding sites on GPIIb, GPIIIa, or indirectly via some other site on the platelet membrane. Alternatively, this antibody may recognize a site that is uncovered by reorganization of the platelet surface which may occur as a consequence of a reduction of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  concentrations or of platelet interaction with certain surfaces.

Numerous investigators have identified a divalent cation requirement for platelet aggregation and stimulus induced binding



**Figure 9.** O'Farrell gel electrophoresis of surface-labeled proteins from variant thrombasthenia. Cells were labeled as described in Fig. 8, and analyzed by O'Farrell electrophoresis with isoelectric focusing in the horizontal direction and SDS-PAGE in the vertical direction. The samples were reduced in both directions. Illustrated are autoradiograms. The major spots of  $\text{pI} = 4.5$  ( $M_r = 123,000$  (GP IIb alpha) and  $\text{pI} = 4.5$  ( $M_r = 105,000$  (GP IIIa) were not appreciably reduced relative to normal. Minor differences observed between the normal and patients, were not reproducible from gel to gel.

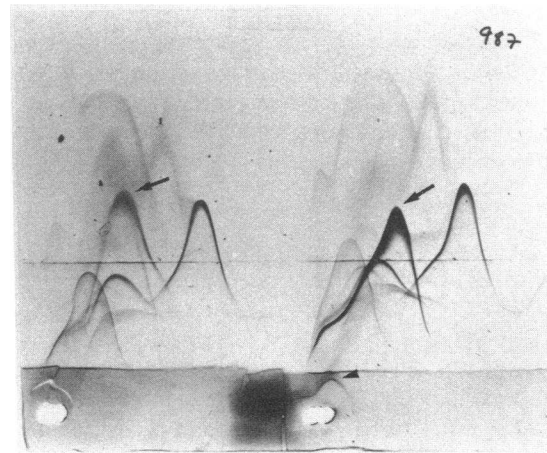




**Figure 10.** Two-dimensional tryptic peptide mapping of patient versus normal GP IIb alpha and GP IIIa. Partially purified GP IIb-IIIa was radiolabeled, and the subunits isolated as described in Methods. Tryptic digests were analyzed by high-voltage electrophoresis followed by thin layer chromatography (TLC). Autoradiograms of these plates are illustrated. Note the near complete concordance of peptides visualized for normal and variant thrombasthenic patient GP IIb alpha and GP IIIa.

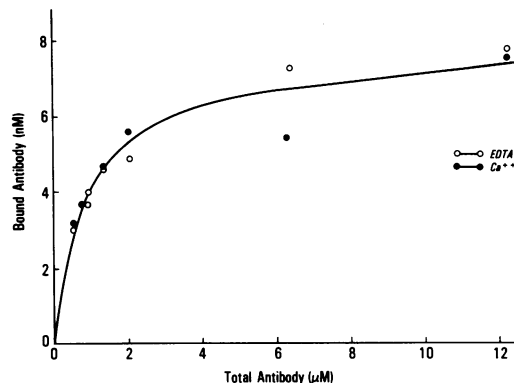
of Fg to platelets (3, 23). As noted in the introduction, the requirement for  $>10 \mu\text{M}$   $\text{Ca}^{++}$  for maintenance of the GPIIb-IIIa complex (18, 20, 21) affords one potential explanation for divalent cation effects on Fg binding, but as also noted there is clear (3, 22, 23) evidence for regulation of Fg binding by millimolar concentrations of  $\text{Mg}^{++}$  or  $\text{Ca}^{++}$  independent of the state of the GPIIb-IIIa heterodimer. Because GPIIb-IIIa play a critical role in Fg receptor function, it seems likely that the changes in surface exposure of GPIIb alpha is involved in this "nonselective" divalent cation regulation. Strong support for this concept comes from the striking correlation between regulation of PMI-1 epitope expression of Fg receptor function by divalent cations. In addition, divalent cations appear to regulate the epitope on the platelet rather than the antigen recognition function of the antibody, inasmuch as the antibody binds to the platelets of the variant of Glanzmann's thrombasthenia in the presence of  $\text{Mg}^{++}$  and  $\text{Ca}^{++}$ . This Glanzmann's variant provides further compelling support for the physiologic significance of the PMI-1 epitope, in that these platelets fail to express binding sites for three adhesive glycoproteins.

The most severely affected patients with Glanzmann's thrombasthenia are nearly completely deficient in GPIIb-IIIa, clot retraction, and Fg binding function and may have reduced intraplatelet Fg (36). The present family has a hereditary, congenital bleeding disorder with absent clot retraction, and  $>95\%$  deficits in platelet Fg content and binding function despite having at least 50% the normal content of GPIIb-IIIa, most of which is in the complex on crossed immunoelectrophoresis. The profound functional abnormalities in these patients clearly distinguish them from heterozygotes or "type II" (36, 37) Glanzmann's patients, who may manifest the presence of reduced levels of GPIIb-IIIa. It is possible that the abnormality of platelet function in



**Figure 11.** Crossed immunoelectrophoresis of variant thrombasthenic platelets versus normal platelets, the anode is to the right. The extracts from normal and variant thrombasthenic platelets were analyzed by crossed immunoelectrophoresis as described in Fig. 1. Coomassie blue-stained plates are illustrated. In the right panel (normal control) note the prominent immunoprecipitin arc adjacent to the sample well which contains platelet Fg (arrowhead), and the major arc at the midpoint of the electrophoretogram (arrow) identified previously as band 16 which contains GP II/IIIa. In the left panel (patient) the former arc is absent. The band 16 arc (arrow) although of similar height, is of reduced staining intensity relative to the normal, and also shows subtle anodic splitting.

the present family is due to a qualitative abnormality in their GPIIb-IIIa. This seems particularly likely because they also manifest abnormal surface orientation of at least a portion of the GPIIb molecule. The lack of difference in tryptic peptide maps does not exclude qualitative difference in the GP in that only one protease was employed, only radiolabeled peptides were visualized, and minor sequence changes may not be visualized by this technique. Case reports have appeared of other families in which the degree of reduction of GPIIb-IIIa did not correlate with functional impairment (38-40), but their relationship to the present abnormality is uncertain. In any event the PMI-1



**Figure 12.** Binding of PMI-1 antibody to variant thrombasthenic platelets in the presence and absence of calcium.  $2 \times 10^8$  variant thrombasthenic platelets per ml were incubated in the presence of 5 mM EDTA (○) or 5 mM calcium (●) at the indicated input concentration of PMI-1 antibody at 22°C for 30 min. Bound ligand was measured as described in Methods. 1 nM bound PMI-1 =  $3.01 \times 10^3$  molecules per platelet.



antibody appears to type for this variant, and in view of its other unusual features, this variant appears to warrant designation as a unique entity. In accord with the wishes of the family, we designate it the Cam variant of Glanzmann's thrombasthenia.

Evidence from diverse sources including lectin inhibition (41), immunoinhibition (30), and cross-linking studies (42) has implicated GPIIb in platelet adhesion to collagen. The work of Shadle and Barondes (43) suggests that GPIIb is one of a multiplicity of platelet proteins that neutralize the ability of anti-platelet antibodies to inhibit Mg-dependent platelet adhesion to trimeric chick skin type I collagen. In addition, PMI-1, an anti-GPIIb, blocked adhesion (25). Clearly, further study will be required to determine whether this antibody also blocks adhesion to other substrata. In addition, the capacity to block adhesion to collagen implies that this antibody bound to platelets under the conditions of the assay (2 mM Mg<sup>++</sup>). If this epitope is exposed during membrane reorganization triggered by adhesion to a collagenous substrate, then this antibody may represent a prototype for novel thrombus-directed diagnostic and therapeutic reagents.

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