A Defect of Platelet Aggregation Associated With an Abnormal Distribution of Glycoprotein IIb-IIIa Complexes Within the Platelet: The Cause of a Lifelong Bleeding Disorder

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A young Italian man (A.P.) has a lifelong history of bleeding from gums and mucocutaneous tissue. Electron microscopy showed a wide diversity of platelet size including giant forms. In citrated platelet-rich plasma (PRP), platelet aggregation induced by adenosine diphosphate (ADP) and other agonists was much reduced. Both secretion and clot retraction were normal. The aggregation of washed platelets with ADP was improved but remained subnormal, as was aggregation with collagen and thrombin. Fibrinogen-binding was analyzed by flow cytometry using platelets in whole blood or PRP and was markedly decreased. Crossed immunoelectrophoresis of Triton X-100 extracts of (A.P.) platelets showed that GP IIb-IIIa levels were 40% to 50% of normal. Glycoprotein (GP) IIb and GP IIIa were of usual migration in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, but their labeling was much reduced during lactoperoxidase-catalyzed iodination. Binding to (A.P.) platelets of four different 125I-labeled monoclonal antibodies to GP IIb-IIIa complexes was reduced to 12% to 20% of normal levels. However, when the patient’s platelets were stimulated with α-thrombin, monoclonal antibody binding showed the same increase (approximately 20,000 sites) as normal platelets. Both flow cytometry and immunocytochemical studies showed that the distribution of residual surface GP IIb-IIIa within the total (A.P.) platelet population was heterogeneous and not related to platelet size. Staining of ultrathin sections confirmed the presence of an internal pool of GP IIb-IIIa. Monoclonal antibodies to other membrane glycoproteins bound normally to (A.P.) platelets. The patient has a selective deficiency of the surface pool of GP IIb-IIIa complexes that is manifested clinically by a mild Glanzmann’s thrombasthenia-like syndrome.

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platelets unable to “turn on” GP IIb-IIIa complexes when activated, but where the complexes bind fibrinogen in vitro test systems.3,4,13,14

We now describe a young Italian man with a lifelong history of bleeding from gums and mucocutaneous tissue and who appears to represent a new category of variant. His platelets exhibit a partial deficiency of GP IIb-IIIa complexes, a decrease that particularly affects the surface pool. The patient poses a number of interesting questions about the levels of GP IIb-IIIa required to support platelet aggregation and concerning the definition of the Glanzmann’s thrombasthenia phenotype.

MATERIALS AND METHODS

Subject

The patient (A.P.) is a 21-year-old man who is the elder son of unrelated Italian parents. He is patient 47 in a previous report.4 He has suffered since early childhood from epistaxis, gingival bleeding, and petechiae, and has had occasional hematoma and melena. At the age of 8, he bled for 48 hours after a tooth extraction. His platelet count, measured on many occasions over the last 10 years, has varied between 100 and 160 x 10^9/L. His Ivy bleeding time has been repeatedly greater than 11 minutes (normal range < 8 minutes). Platelet volume was measured using a Coulter counter ZBI (Channelizer C-1000; Coulter, Luton, England) and a 70-μm aperture. A mean platelet volume of 10.3 μm³ was obtained (control range 8.6 ± 1), and both phase contrast and electron microscopy confirmed the presence of anisocytosis (see Results section). Platelet nucleotides were normal, adenosine triphosphate (ATP) 4.92 nmol/10^8 platelets, ADP 3.41 nmol/10^8 platelets, ATP:ADP ratio 1:4. Other normal findings were platelet factor 3 availability, platelet β-thromboglobulin (see Results section) and vWF:Ag contents, thromboxane B2 generation after platelet stimulation with collagen and arachidonic acid, and uptake of 14C-serotonin. Other aspects of platelet function testing, including a description of the platelet aggregation defect, are detailed in the Results section. There was no evidence for the presence of antiplatelet antibody in the patient’s serum, platelet-associated IgG, IgM, and C3 were all within the normal range. Routine tests showed no plasma or coagulation factor disorder. In particular, factor VIIIc, vWF:Ag, and ristocetin cofactor activities were normal. The patient has never received blood transfusions. White blood cell and red blood cell counts were normal. Neither of the patient's two parents, two sisters, or brother have experienced abnormal bleeding. His mother had normal sized platelets, a typical platelet count being 340 x 10^9/L. The only hematologic parameters that were abnormal were a VIIc level of 52% and vWF cofactor activity of 36%. His father showed platelet anisocytosis, a typical platelet count was 230 x 10^9/L. His father’s bleeding time was moderately prolonged, but factor VIIc and vWF cofactor activities were normal. The patient has been studied on several occasions both in London and Paris. Controls were members of our respective hospital staffs.

Platelet Function Testing

Platelet aggregation was studied in a Payton platelet aggregometer (Payton Scientific Inc, Buffalo, NY) using standard procedures. Initial studies were performed using platelet-rich plasma (PRP) prepared by centrifuging whole blood anticoagulated with 3.8% sodium citrate (1 vol blood:9 vol anticoagulant) at 250g for 10 minutes. On one occasion, platelet aggregation was studied in PRP anticoagulated with 3 U/mL heparin. Washed platelets were prepared from acid-citrate-dextrose (ACD, NIH formula A) anticoagulated blood as described by Legrand et al.15 The platelets were finally resuspended in a modified Tyrode buffer consisting of 137 mmol/L NaCl, 3 mmol/L KCl, 12 mmol/L NaHCO₃, 0.3 mmol/L NaH₂PO₄, 2 mmol/L CaCl₂, 1 mmol/L MgCl₂, 5.5 mmol/L glucose, 5 mmol/L HEPES (pH 7.4) and 0.35% wt/vol bovine serum albumin (BSA, Fraction V; Sigma Chemical Co, St Louis, MO) (HEPES-buffered modified Tyrode, HBMT). Aggregation was studied at 37°C with constant stirring (1,100 rpm). Platelet concentrations were mainly in the range 2 to 3 x 10^10/mL (see Results). The platelet count in the control PRP was adjusted to that of the patient’s with autologous platelet-poor plasma. Stimulation were 1 to 10 μmol/L ADP (disodium salt, Sigma), 1 to 4 μmol/L epinephrine (bitartrate salt, Sigma), 1 mmol/L sodium arachidonate (Sigma), 2.5 μg/mL of the endoperoxide analogue U46619 (kindly provided by Dr J.E. Pike, Upjohn Corp, Kalazamoo, MI), and 1 to 10 μmol/L ionophore A23187 (Sigma). Two preparations of collagen were used: 1 to 4 μg/mL collagen (Hormon-Chemie, Munich, Germany) and 12.5 to 50 μg/mL polymerized type I collagen (provided by Dr Y. Legrand, Hôpital Saint-Louis, Paris, France). With washed platelets, ADP-induced platelet aggregation was performed in the presence of 270 μg/mL dialyzed human fibrinogen (Kabi Diagnostics, Stockholm, Sweden). Aggregation of washed platelets was also studied with 0.05 to 1 U/mL human α-thrombin (3,000 NIH U/mg protein, Sigma). On occasion, the platelets were prelabeled by incubating the PRP with 0.6 μmol/L (14C)-serotonin (40 to 60 mCi/ml, Radiolabeled Chemical Center, Amersham, UK) for 30 minutes at 37°C, and samples were taken 3 minutes after platelet stimulation for (14C)-serotonin release.15 Thrombin-induced clot retraction was assessed using citrated PRP in glass tubes as previously detailed by us.11

Flow Cytometry

These studies were performed in London using a Coulter EPICS Profile flow cytometer.

Fibrinogen. Fibrinogen binding to platelets activated with ADP was studied in citrated whole blood as described by Warkentin et al.16 and in citrated PRP by a modification of the method of Jackson and Jennings,16 using a fluorescein isothiocyanate (FITC)-conjugated polyclonal rabbit antifibrinogen antibody (Dakopatts, Glostrup, Denmark). The platelet population was identified on the basis of its forward and side-scatter profiles (see below).

Monoclonal antibodies (MoAbs). Aliquots of a 1:10 dilution of whole citrated blood in HBMT were incubated at room temperature for 30 minutes in the presence or absence of α-thrombin (final concentrations 0.01 to 0.25 U/mL), together with a saturating concentration of each of the following FITC-conjugated MoAbs, raised in the laboratory of one of us (R.H.): RFGP56 directed against GP IIb-IIIa, and RFGP37, directed against GP Ib. Control experiments were performed in the presence of equivalent amounts of nonimmune mouse IgG. For the experiments on whole blood, the peptide Gly-Pro-Arg-Pro (Sigma) was added to the reaction mixtures at a final concentration of 1 mmol/L, to inhibit fibrin polymerization. At the end of the incubation period, samples were diluted and fixed in 2% (wt/vol) paraformaldehyde and analyzed on the Coulter EPICS Profile flow cytometer. The samples were examined by forward and wide-angled light scatter and the gates set to include the majority of the platelets and to exclude platelet aggregates and other blood cells. Green fluorescence was measured after passage through a 530-nm band pass interference filter and the fluorescence signals expressed using logarithmic amplification. Fluorescence histograms were obtained for 10,000 cells. Positive cells were defined as those that expressed fluorescence levels greater than 98% of platelets exposed to nonimmune mouse or rabbit IgG.
Radiolabeling of Platelet Membrane Glycoproteins

Lactoperoxidase-catalyzed $^{125}$I-labeling of the platelet surface glycoproteins was performed as described by Pidard et al.\textsuperscript{17} Washed platelets at $10^9$/mL were incubated with 1 $\mu$Ci/mL sodium $^{125}$I (Amersham-France, Les Ulis, France).

Single- and Two-dimensional Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Washed, unlabeled, or $^{125}$I-labeled platelets were resuspended at $2 \times 10^6$ platelets/mL in 10 mmol/L Tris-HCl, 150 mmol/L NaCl, 3 mmol/L EDTA, pH 7.0. A one-fifth volume of a solution containing 12% (vol/vol) SDS and 30 mmol/L N-ethylymaleimide, pH 7.0, was added and the samples solubilized by heating at 100°C for 5 minutes. When performed, disulfides were reduced by incubating SDS-soluble extracts at 100°C for 5 minutes with 5% (vol/vol) 2-mercaptoethanol. For single-dimension SDS-PAGE, samples were electrophoresed on 7% to 12% gradient polyacrylamide slab gels as detailed previously by us.\textsuperscript{11,17} Two-dimensional nonreduced/reduced SDS-PAGE was also performed according to our previously described procedures.\textsuperscript{11,17} Proteins were located by Coomassie blue R-250 (CB-R) staining, $^{125}$I-labeled proteins on dried gels by autoradiography.\textsuperscript{11}

Crossed Immunoelectrophoresis (CIE)

CIE was performed as described by Hagen et al\textsuperscript{6} and subsequently detailed by us.\textsuperscript{7,11} In brief, washed unlabeled or $^{125}$I-labeled platelets resuspended at $5 \times 10^9$/mL in 38 mmol/L Tris-HCl, 100 mmol/L glycine, pH 8.6 (Tris-glycine) were solubilized in 1% (wt/vol) Triton X-100 (BDH, Poole, UK) for 30 minutes at 4°C. Triton X-100 soluble proteins were clarified by centrifugation at 150,000g for 10 minutes in a Beckmann airfuge (Beckmann Instruments, Gagny, France) and samples (100 $\mu$g protein) subjected to first-dimension agarose gel electrophoresis. Second-dimension electrophoresis was performed in the presence of an intermediate gel and a superior gel containing the isolated IgG of a rabbit antiplatelet antisemum.\textsuperscript{7,11} On occasion, trace amounts of $^{125}$I-labeled MoAbs (detailed below) to platelet membrane glycoproteins were incorporated into the intermediate gel.\textsuperscript{11} Binding of $^{125}$I-fibrinogen to GP IIb-IIIa complexes in CIE was studied as previously described.\textsuperscript{11} Immunoprecipitates were revealed by CB-R staining and those containing antigens reactive with radiolabeled MoAbs or fibrinogen by autoradiography.\textsuperscript{7,11} Peak areas were quantified by planimetry.

Direct Binding of $^{125}$I-Labeled MoAbs to Platelets

A number of different antibodies were used in these experiments which were performed in Paris. AP-2 is directed against the GP IIb-IIIa complex\textsuperscript{18} and was kindly made available by Dr T. Kunicki (Blood Center of Southeastern Wisconsin, Milwaukee, WI), Tab is directed against GP Ib\textsuperscript{19} and was from Dr R. McEver (University of Oklahoma Health Sciences Center, Oklahoma City, OK), SZ-21 is directed against GP IIa\textsuperscript{20} and was from Dr C. Ruan (Suzhou Medical College, Suzhou, China), WM-23 is directed against GP Ib\textsuperscript{21} and was from Dr M. Berndt (Westmead Medical Centre, Sydney, Australia), FA6-152 is directed against GP IV\textsuperscript{22} and was from Dr L. Edelman (Institut Pasteur, Paris, France), and 5G11, which is specific for thrombospodin,\textsuperscript{23} was a gift of Immunotech Inc (Luminy, France). The Fab fragments of a polyclonal rabbit antibody to fibrinogen have been previously characterized by us.\textsuperscript{11} Samples of the isolated IgG of each MoAb, or the Fab fragments of the rabbit antifibrinogen IgG, were radiolabeled with $^{125}$I using the chloramine T procedure according to described procedures.\textsuperscript{13,18} Free $^{125}$I was separated from the radiolabeled IgG or Fab by gel filtration through a Sephadex G25 column (Pharmacia France SA, Saint Quentin en Yvelines, France). Specific activities of the radiolabeled IgG were of the order of 100 to 700 cpm/ng protein. Binding of the labeled antibodies to platelets was measured using an adaptation of the procedure of Pidard et al.\textsuperscript{18} Unless otherwise stated, studies were performed using washed platelets resuspended in HBMT at $1.5 \times 10^9$/mL. Aliquots (0.3 mL) of platelets were incubated at room temperature for 30 minutes with $^{125}$I-antibody (0.5 to 10 $\mu$g/mL) added in a total volume of 0.025 mL. Duplicate volumes (0.16 mL) of each reaction mixture were then layered over 0.5 mL of a 1:25:1 (vol/vol) mixture (density [d] = 1.014 at 20°C) of dibutyrylphosphate and dioctylphthalate oils (Aldrich-Chemie, Strasbourg, France). The platelets were separated by centrifugation at 12,000g for 4 minutes. The radioactivities in both pellets and supernatants were measured in a gamma counter (Beckman Instruments). Nonspecific binding was assessed in parallel tubes using equivalent amounts of $^{125}$I-labeled nonimmune mouse IgG and was typically on the order of 500 molecules per platelet. Binding isotherms were constructed as previously described.\textsuperscript{18} Studies were also performed using platelets stimulated for 6 minutes at 25°C with 0.1 U/mL human a-thrombin. Here, thrombin activity was stopped with a tenfold excess of hirudin (Sigma) before the addition of the radiolabeled antibody. Binding of selected MoAbs to platelets in citrated PRP was also performed. In this case, the platelet count was adjusted to $1.5 \times 10^9$/mL with autologous plasma and the platelets were separated from the incubation mixture by centrifugation across 0.5 mL of 20% (wt/vol) sucrose dissolved in 20 mmol/L Tris-HCl and 0.15 mol/L NaCl, pH 7.2.

Western Blot Analysis

Single-dimension SDS-PAGE of nonreduced samples (50 $\mu$g protein), electrophoretic transfer of the separated proteins to nitrocellulose membrane, incubation of the membranes with a mixture of rabbit antibodies to GP Ib and GP IIIa (a gift of Dr M. McEver), and detection of bound IgG using $^{125}$I-protein A (Amersham) were all performed according to procedures previously detailed by us.\textsuperscript{24} Bound $^{125}$I was detected by autoradiography (see above).

Electron Microscopy and Immunocytochemical Studies

Immunogold staining before embedding. Immunogold staining with the MoAb AP-2 was performed on glutaraldehyde-fixed washed platelets according to the procedures of Hourdill et al.\textsuperscript{25} Controls were performed by omitting AP-2 or by using an equivalent concentration of nonimmune mouse IgG. Bound murine MoAb was located using affinity-purified goat anti-mouse IgG adsorbed onto 5-nm gold particles (Janssen Pharmaceutica, Beerse, Belgium). Incubation was for 4 hours at room temperature followed by overnight at 4°C. The samples, postfixed in osmium tetroxide, were dehydrated by treatment with graded alcohols and propylene oxide before being embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and examined with a Phillips EM 201 electron microscope (Eindhoven, Holland) at 80 kV.

Immunogold staining performed postembedding. Procedures previously used by us\textsuperscript{26} were modified as described by Berryman and Rodewald.\textsuperscript{27} Washed platelets were fixed for 2 hours at room temperature in 0.1 mol/L phosphate buffer containing 4% (wt/vol) paraformaldehyde, 1% (vol/vol) glutaraldehyde, 0.2% (wt/vol) picric acid, 0.5 mmol/L CaCl$_2$, pH 7.4. Fixed platelets were washed three times in 0.1 mol/L phosphate buffer containing 3.5% (wt/vol) sucrose, 0.5 mmol/L CaCl$_2$, pH 7.4 (sucrose-phosphate buffer). Free aldehydes were quenched with 50 mmol/L glycine in sucrose-phosphate buffer, pH 7.4, for 1 hour at room temperature. Platelets were then washed four times in cold 0.1 mmol/L maleate buffer. From www.bloodjournal.org by guest on April 1, 2017. For personal use only.
buffer containing 3.5% (wt/vol) sucrose, pH 6.5 (sucrose-maleate buffer). They were postfixed for 2 hours in ice with 2% (wt/vol) uranyl acetate in sucrose-maleate buffer, pH 6.5. Platelets were then pre-embedded in 1% (wt/vol) low-temperature gelling agarose in 50 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.4, dehydrated through a series of graded acetone solutions at −20°C before being embedded in Lowicryl K4M (Agar Scientific Ltd, Stansted, England) at this temperature. The resin was photopolymerized at 4°C for 48 hours. Ultrathin sections were mounted on collodium-coated nickel grids as previously described. Rabbit antibodies to GP IIb and GP IIIa were prepared in the laboratory of one of us (D.P.) and used at a 1/300 dilution as previously described. Sections were then transferred to a solution containing 1/70 dilution of goat antirabbit IgG conjugated to 5 or 10 nm gold particles. After 2 hours at room temperature, the grids were rinsed and stained for 15 minutes in 2% (wt/vol) osmium tetroxide. After further rinsing and counterstaining with lead citrate, the sections were examined at 80 kV in the electron microscope.

RESULTS

Platelet Function Testing

Citrated PRP from the patient was studied on six occasions over a 10-year period. A slow and much reduced platelet aggregation response, without subsequent disaggregation, was consistently observed. This is illustrated for ADP and epinephrine in Fig 1A. Similar results were observed for two preparations of collagen, arachidonic acid, the endoperoxide analogue U46619, and ionophore A23187 at a range of concentrations (see Materials and Methods). A combination of ADP plus epinephrine gave only a marginally improved response. Aggregation remained defective when ADP was added to PRP anticoagulated with heparin, and when measured in citrated whole blood by impedance aggregometry in response to ADP, collagen, and arachidonic acid. When ADP was added to washed platelets together with fibrinogen, the aggregation response was somewhat improved (Fig 1B). An improved aggregation response was also seen when washed platelets were incubated with collagen (not shown). However, with α-thrombin, a slow and irreversible aggregation was again observed. It took several minutes for the characteristic oscillations given by large platelet aggregates to be observed on the aggregometer recorder, and with all agonists the size of the aggregates appeared smaller than usual. In contrast, the kinetics of secretion of [14C]-serotonin was normal with collagen or thrombin. As with the control, ADP induced little or no [14C]-serotonin release from washed (A.P.) platelets. The total β-thromboglobulin content of (A.P.) platelets was 7.2 μg/10^8 platelets, as compared with a control platelet range of 7.6 ± 1.6 μg/10^8 platelets (n = 8). Thrombin-induced release of β-thromboglobulin from the patient’s platelets was maximal at 84% and showed no differences from control platelets. Thrombin-induced clot retraction, performed using citrated PRP in glass tubes, began within 10 minutes and was completed after 1 hour when it was indistinguishable from the control.

In view of the low content of GP IIb-IIIa complexes in (A.P.) platelets (see below), we considered the possibility that the different response to ADP between PRP and washed platelets might have been a function of the fibrinogen concentration in the medium. It was hypothesized that the high fibrinogen content in plasma (3 mg/mL) might have resulted in inhibition of platelet aggregation through monovalent occupancy of the reduced number of receptors. Therefore, washed platelets were challenged with 10 μmol/L ADP in the presence of varying amounts of fibrinogen (0.02 to 0.5 mg/mL). In fact, washed (A.P.) platelets aggregated at about half the normal rate whatever the fibrinogen concentration (not illustrated). ADP-induced aggregation of control platelets in the presence of patient’s plasma was normal.

Radiolabeling of Platelet Surface Glycoproteins

The analysis of 125I-labeled platelets was first performed by single-dimension SDS-PAGE both in the absence of and after reduction of disulfides with 2-mercaptoethanol. The results showed that the major membrane glycoproteins were all present in (A.P.) platelets but that GP IIb and GP
IIIa were of reduced concentration. The decreased radiolabeling of GP IIb and GP IIIa was most clearly shown following two-dimensional nonreduced/reduced SDS-PAGE (Fig 2). Here, a limited mapping of the platelet surface components was achieved by first migrating nonreduced samples of SDS-soluble protein, reducing disulfides in the first dimension gel, then following this by a second dimension separation of the now reduced proteins on a gradient slab gel. The spots given by GP IIbα, GP IIbβ and GP IIIa are clearly diminished on the patient’s profile in comparison with the other membrane glycoproteins. CB-R staining detected no abnormalities in the migration of the major platelet polypeptides, but confirmed that GP IIb (both the α- and β-subunits) and GP IIIa were reduced in amount (not illustrated). Western blot analysis following single-dimension nonreduced SDS-PAGE of unlabeled platelet proteins performed using a mixture of polyclonal antibodies to GP IIb and GP IIIa showed no abnormalities in the migration of either protein, while additional low mol weight bands indicative of proteolytic degradation were not seen (results not shown).

**Crossed Immunoelectrophoresis (CIE)**

A typical CIE analysis of (A.P.) platelets followed by CB-R staining is shown in Fig 3. The GP IIb-IIIa precipitate was estimated by planimetry to be ≈45% of that given by control platelets. Other precipitates were normally present. These include that given by platelet fibrinogen (highlighted on the figure). The use of 125I-labeled MoAbs in the intermediate gel followed by autoradiography confirmed the identity of the GP IIb-IIIa precipitate (AP-2) and showed that additional precipitates corresponding to free GP IIIa (SZ-21) or GP IIb (Tab) were not present (not illustrated). Therefore, it appeared that the residual GP IIb and GP IIIa of (A.P.) platelets were present as GP IIb-IIIa complexes. 125I-Fibrinogen was incorporated into the intermediate gel as previously described by Nurden et al. Autoradiography confirmed that the residual complexes retained the ability to bind fibrinogen in vitro (not illustrated).

**Binding of Radiolabeled Antibodies to Platelets**

We then studied the ability of (A.P.) platelets to bind a series of 125I-labeled MoAbs to platelet glycoproteins. Results of a typical experiment are presented in Fig 4 for washed control and patient’s platelets before and after their stimulation with α-thrombin. The control represents the platelets of a normal donor processed in parallel. A severe decrease was seen in the number of molecules of AP-2 (anti-GP IIb-IIIa), Tab (anti-GP IIb), or SZ-21 (anti-GP IIIa) that bound to unstimulated (A.P.) platelets. For AP-2, the value for the patient was 7,730 molecules/platelet. Previous studies on the binding of AP-2 to platelets of 27 control donors have shown a range of 22,000 to 58,000 binding sites (mean 37,100 ± 8,600) (Table 1 and reference 28). The control donor used in Fig 4 possessed platelets that bound 40,000 molecules of AP-2, close to the mean value referred to above. Tab bound to 7,940 sites on (A.P.) platelets, a number similar to AP-2. In contrast, SZ 21 bound to 3,780 sites: about half the number of sites recognized by Tab and AP-2, a finding that was repeated for the control donor (Fig 4). As expected, thrombin stimulation of control platelets was accompanied by an increase on the order of 50% in the number of molecules of AP-2 bound. This is thought to be caused by the surface exposure of intracellular pools of GP IIb-IIIa complexes (see Discus-
ABNORMAL DISTRIBUTION OF PLATELET GP IIb-IIIa

Fig 3. Analysis of the proteins of (A.P.) platelets by CIE. Washed platelets from (A.P.) and a control donor were solubilized with Triton X-100 and the soluble proteins (70 μg) separated by first-dimension electrophoresis in agarose. Second-dimension electrophoresis was performed in which the superior gel contained 750 μg/cm² of the immunoglobulin fraction of a rabbit antihuman platelet serum. The intermediate gel was without antibody in this experiment. Immunoprecipitates were located by CB-R staining. Note the decreased size of the GP IIb-IIIa precipitate on the patient’s profile.

sion). After thrombin stimulation, the number of AP-2 binding sites on (A.P.) platelets rose to 26,400, an increase on the order of 240%. Results with Tab were again virtually identical to those obtained with AP-2 and the same pattern was also seen with SZ-21. Thus, despite the decreased surface pool of GP IIb-IIIa complexes, thrombin-stimulated (A.P.) platelets showed the same increase in the number of binding sites for each of these monoclonals as did the control platelets. This suggests that the internal pool of GP IIb-IIIa complexes was normally present. In contrast to the above results, binding of WM-23 (anti-GP Ib) and FA-6 (anti-GP IV) to unstimulated and stimulated (A.P.) platelets was similar to, if not slightly increased, compared with their binding to control platelets. Overall, the MoAb binding experiments highlight a selective surface deficiency of GP IIb-IIIa complexes on (A.P.) platelets.

Fig 4. Binding of radio-labeled MoAbs, or Fab fragments of a polyclonal antibody, to unstimulated and thrombin-stimulated control and (A.P.) platelets. Washed control or patient’s platelets were incubated at 2.5 x 10⁶/mL in HBMT for 6 minutes at 25°C in the presence or absence of 0.1 U/mL of human α-thrombin. After the addition of a 10-fold excess of hirudin, the platelets were diluted to 1.5 x 10⁶/mL with HBMT and aliquots (0.3 mL) incubated with ¹²⁵I-labeled MoAb (4.5 to 6.0 μg/mL) or Fab polyclonal antibody (0.4 μmol/L) for 30 minutes at room temperature. Duplicate volumes (0.16 mL) of each reaction mixture were then layered over 0.5 mL of an oil mixture and the platelets separated by centrifugation at 12,000 rpm for 4 minutes. The radioactivities in the pellets were measured in a gamma counter. Results are expressed as number of antibody molecules/platelet (x 10⁶). [ ], Control; [ ], control + Th; [ ], A.P.; [ ], A.P. + Th.
Table 1. MoAb Binding to Platelets of the (P.) Family

<table>
<thead>
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<th>Donor</th>
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<th>Tab</th>
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<td>12,000</td>
</tr>
<tr>
<td>Mother</td>
<td>21,500</td>
<td>19,000</td>
<td>—</td>
<td>15,000</td>
</tr>
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Studies performed using 125I-labeled MoAbs as described in Materials and Methods. Results are expressed as number of molecules of antibody bound/platelet.

*Estimates from two different bleedings.

In view of the differences obtained between the aggregation responses to ADP in citrated PRP or with washed platelets, the binding of AP-2 and Tab to (A.P.) platelets was also studied in citrated PRP. Slightly greater values were obtained for AP-2 (9,500) and Tab (11,700) using PRP than for washed platelets. These results show that GP IIb-IIIa complexes were not being translocated onto the surface during the platelet washing procedures.

Other experiments were designed to estimate the ability of the patient's platelets to bind or express secreted α-granule proteins. As with the controls, washed unstimulated (A.P.) platelets failed to bind antibodies to thrombospondin (5G11) or fibrinogen (Fig 4). After platelet stimulation with thrombin, (A.P.) platelets readily bound antibody to thrombospondin. Although the amount of fibrinogen was lower than that present on control platelets, a sufficient amount was detected to imply that residual GP IIb-IIIa complexes on (A.P.) platelets were functionally active. It should be emphasized that 125I-labeled Fab fragments of a rabbit antibody were used here, and that the results are not a direct measure of the number of molecules of fibrinogen present. The number of Fab fragments that bound to each molecule of bound fibrinogen is not known precisely, but was previously estimated by us to be on the order of 20 following the precipitation of 125I-Fab-fibrinogen complexes in solution with polyethylene glycol.13

Flow Cytometry

Experiments were next performed to examine the distribution of surface glycoproteins within the total (A.P.) platelet population in whole blood. As shown in Fig 5, the mean fluorescence representing the binding of RFGP41 (anti-GP IIb-IIIa) to the patient's platelets was markedly decreased as compared with the control. However, there was considerable heterogeneity in the residual GP IIb-IIIa content of individual platelets. A similar result was obtained with PRP and washed platelets. When the number of GP IIb-IIIa complexes, represented by fluorescence, was plotted against platelet size, represented by forward light scatter, a clear correlation between these two parameters was seen for the control platelets, but no such relationship was seen with (A.P.) platelets (a finding illustrated in the electron microscopy studies). Binding of RFGP37 (anti-GP Ib) to (A.P.) platelets was somewhat greater than to the control, and showed a normal distribution and size relation-ship (not illustrated) confirming the results with 125I-labeled antibody (see above). Thrombin-stimulation was performed in whole blood in the presence of the peptide Gly-Pro-Arg-Pro to inhibit fibrin polymerization. Figure 5 shows that (A.P.) platelets responded to the thrombin and that the large increase in GP IIb-IIIa expression (quantitated in Fig 4) extended to the whole platelet population.

Fibrinogen binding to platelets stimulated with ADP in citrated whole blood was studied. Figure 6A shows that there was an approximate fourfold reduction in the binding of fibrinogen to (A.P.) platelets relative to the control and the expression of the fibrinogen within the total platelet population was heterogeneous. There was a marked similarity between the histograms showing the reduced surface GP IIb-IIIa expression on circulating (A.P.) platelets (Fig 5) and their residual ADP-induced fibrinogen binding capacity (Fig 6A). When log-dose response curves were obtained for the ADP-induced binding of fibrinogen to (A.P.) and

![Fig 5. Platelet glycoprotein analysis by flow cytometry. Whole blood was incubated with FITC-conjugated RFGP41 (anti-GP IIb-IIIa) and the peptide Gly-Pro-Arg-Pro in the absence (O) or presence (0.25) of α-thrombin, 0.25 U/mL, fixed and analyzed on a Coulter EPICS Profile flow cytometer as described in Materials and Methods. Binding of the antibody to (A.P.) platelets is greatly reduced relative to the control, but increases markedly throughout the platelet population after stimulation with thrombin.](image-url)
control platelets (ADP, 0.1 to 10 µmol/L), a maximum of approximately 40% of the platelets expressed levels of fibrinogen greater than that of 98% of unstimulated platelets (Fig 6B). This compared with a maximum value of greater than 80% positive platelets for the control. Other studies showed that, whereas maximal fibrinogen binding to control platelets in response to 10 µmol/L ADP was achieved within 5 minutes, the residual binding to (A.P.) platelets followed a much slower kinetic (data not shown). Very similar results to those presented above were obtained when ADP-induced fibrinogen binding was studied in citrated-PRP.

Platelet Morphology and Immunocytochemical Studies

Electron microscopy confirmed the abnormal morphology of (A.P.) platelets. As shown in Fig 7a, some platelets were of normal size and discoid whereas others were enlarged and spheroid. Although not quantitated, the α-granule content of the platelets appeared quite variable. Some platelets had an abundance of granules, although in others only two or three were visible on the section. The density of residual surface GP IIb-IIIa complexes was first studied by transmission electron microscopy and immunogold staining with the monoclonal antibody AP-2. As shown in Fig 7a, staining density was low and differed considerably from platelet to platelet. The results confirmed the flow cytometry studies. There did not appear to be a direct correlation between the extent of the decreased staining and platelet size. Although many of the enlarged spherical platelets bound little AP-2, this was not an absolute rule; and some normal sized platelets also showed little surface labeling. As illustrated for a single platelet in Fig 7b, thrombin stimulation was followed by a distinct increase in the surface density of AP-2 binding. The dark central mass in this platelet confirms that activation has been followed by secretion. Finally, unstimulated platelets were embedded in the resin Lowicryl K4M and immunogold staining was performed on thin sections using a polyclonal rabbit antibody to GP IIIa. Here, larger (10 nm) gold particles were used to facilitate their detection within the cell. Figure 7c shows that, despite a low surface staining, there was clear labeling of the internal pool of GP IIb-IIIa complexes. This included membranes of both the surface-connected canalicular system and α-granules. This illustration is typical of that seen for (A.P.) platelets.

Family Studies

MoAb binding studies were performed on platelets isolated from both parents (Table 1). Values for the patient (A.P.) in this table were those obtained for two separate bleedings before that which gave rise to the data in Fig 4. These results, which were obtained over a period of 2 years, confirm the consistency of the abnormality. His mother, who possessed normal-sized platelets, bound MoAbs to GP IIb-IIIa complexes at levels that would be expected for an obligate heterozygote for Glanzmann's thrombasthenia. His father, with somewhat large platelets, expressed levels of GP IIb-IIIa complexes at the low end of the normal range. Both parents possessed normal levels of GP Ib. CIE confirmed the intermediate GP IIb-IIIa content of the mother’s platelets and the fact that the GP IIb-IIIa content of his father’s cells was at the low end of the normal range (data not shown). As the father’s platelets included a high percentage of giant forms (see case history), the possibility that he may also be heterozygous for Glanzmann’s thrombasthenia will require further study.

DISCUSSION

Patient (A.P.) has had a lifelong bleeding disorder manifested by occasional bleeding episodes mainly from...
Fig 7. Platelet morphology and immunogold staining of GP IIb-IIIa complexes as shown by electron microscopy. (a) Unstimulated, washed platelets from (A.P.) were fixed with glutaraldehyde before incubation with the MoAb AP-2 (anti-GP IIb-IIIa complex). Bound IgG was detected on ultrathin sections using a commercial preparation of goat antimouse IgG coupled to 5 nm gold particles. Note both the size heterogeneity of (A.P.) platelets and the low but variable density of GP IIb-IIIa complexes. (b) The patient's platelets in HBM were stimulated with thrombin (0.5 U/mL) for 10 minutes at 37°C before fixation. A typical “activated” platelet, showing an increased surface staining with AP-2, is shown. (c) Immunogold staining was performed on ultrathin sections of unstimulated, fixed, (A.P.) platelets pre-embedded in the resin Lowicryl K4M. The sections were incubated with a polyclonal rabbit antibody to GP IIb and bound IgG located with an affinity purified anti-rabbit IgG adsorbed to 10 nm gold particles. Staining associated with internal membrane systems is highlighted (arrow heads). Bar = 0.2 μm.
mucocutaneous tissues. Clinically, his symptoms resemble a mild form of Glanzmann’s thrombasthenia. However, detailed investigations of his platelets have shown distinct differences from the classic type I or type II subgroups. First of all, crossed immunoelectrophoresis (CIE) analysis showed a total platelet GP IIb-IIIa content of 40% to 50% of the normal platelet level. This amount of GP IIb-IIIa would suggest that (A.P.) is a heterozygote for Glanzmann’s thrombasthenia. Several studies have shown that obligate heterozygotes possess platelets with intermediate levels of GP IIb-IIIa complexes. However, such heterozygotes generally do not bleed abnormally and their bleeding times and platelet function testing are typically indistinguishable from controls. Patient (A.P.) has a prolonged bleeding time and a platelet aggregation defect. However, the aggregation abnormality is unusual in that it is partial and varies in degree according to the conditions and the agonist. For example, the defect with ADP was more marked in citrated-PRP than with washed platelet suspensions. All definitions of the thrombasthenia phenotype emphasize the lack of platelet aggregation to all physiologic agonists. This usually means an absence of the light transmission change signifying macroscopic platelet aggregation in the aggregometer. Notwithstanding, Caen et al. noted in their original report of 15 cases of thrombasthenia, that small aggregates could be observed by light microscopy when the platelets of some patients were stirred with collagen. Others have reported similar findings following the stimulation of platelets from certain patients with thrombin. Patient (A.P.) differs from these in that residual macroscopic platelet aggregation was readily detectable in the platelet aggregometer.

Surface-labeling (125I) procedures followed by single- or two-dimensional SDS-PAGE first showed that (A.P.) platelets were markedly deficient in the surface pool of GP IIb-IIIa complexes. When 125I-labeled MoAbs were used in a direct binding assay, the estimated mean number of surface-expressed GP IIb-IIIa complexes, 18% to 20% of that of a typical control donor, was slightly greater than that previously obtained for the platelets of two type II patients whose platelets failed to aggregate with ADP but whose PRP supported a modified clot retraction. The difference between the number of surface GP IIb-IIIa complexes recognized by MoAb binding (18% to 20%) and the total platelet content of GP IIb-IIIa complexes as estimated by CIE (40% to 50%), suggested a selective deficiency of surface-located GP IIb-IIIa complexes in (A.P.) platelets. This hypothesis was supported by the fact that thrombin stimulation induced a further 20,000 binding sites for AP-2, a complex-dependent anti-GP IIb-IIIa MoAb, on (A.P.) platelets. This increase was the same as that observed on normal platelets. Such a result could be explained by (1) thrombin-induced changes in the accessibility of surface GP IIb-IIIa molecules to AP-2 or (2) an increase in the total number of GP IIb-IIIa complexes present on the surface of the activated platelets. Because a similar percentage increase in binding was obtained with antibodies to two other distinct epitopes on GP IIb-IIIa complexes, Tab (anti-GP IIb) and SZ 21 (anti-GP IIIa), thrombin-induced changes in the conformation of the complexes appear unlikely to account for the findings. Furthermore, the ability of thrombin to activate (A.P.) platelets was established by a normal secretion response. Whereas both flow cytometry and electron microscopy showed considerable heterogeneity in the level of residual GP IIb-IIIa complexes expressed on the surface of individual platelets, both procedures showed that these were distributed throughout the total platelet population, independently of platelet size. Therefore, we speculate that there is an abnormal processing and/or compartmentalization of GP IIb-IIIa complexes in (A.P.) platelets.

Previous studies with MoAbs to GP IIb-IIIa complexes have pointed to significant increases in their binding to thrombin-stimulated normal platelets. Our studies, with three different antibodies, showed about a 50% increase. However, the anti-GP IIIa antibody (SZ 21) gave absolute values, with both unstimulated and stimulated platelets, that were about half those of the other two antibodies. We have no explanation for this, but note that a similar finding has been previously reported for an alloantibody to GP IIIa, anti-Pen3. Some epitopes on GP IIIa appear more accessible to antibodies than others. Overwhelming evidence for an intracellular pool of GP IIb-IIIa complexes in platelets has been obtained from immunofluorescence experiments and from immunocytochemical studies combined with electron microscopy. Such studies show GP IIb-IIIa complexes in the membranes of the surface-connected canalicular system (SCCS), in membranes surrounding vacuole-like bodies, and in the membranes of α-granules. Gogstad et al. directly identified GP IIb-IIIa complexes in detergent-soluble extracts of membranes obtained from a crude α-granule preparation. One current theory is that following platelet stimulation with thrombin, centralized α-granules join together to form giant secretory vesicles and that, during secretion, the membrane systems of these vesicles fuse with membranes of the SCCS. The morphologic changes continue with the externalization of many of these membrane systems. It is in this way that internal pools of GP IIb-IIIa become accessible to MoAbs. Therefore, the results of the binding of the anti-GP IIb-IIIa MoAbs to (A.P.) platelets after thrombin stimulation suggested that these internal pools were normally present. Immunocytochemical studies performed on thin sections of (A.P.) platelets confirmed that this was the case. Furthermore, flow cytometry established that the thrombin-induced increase of GP IIb-IIIa complexes was seen throughout the total population of (A.P.) platelets and that this occurred under physiologic conditions, ie, when thrombin was added to whole blood. Finally, immunogold staining established that the complexes were evenly distributed on the surface of the thrombin-activated platelets.

Platelet aggregation is mediated through the binding of fibrinogen or other adhesive proteins to GP IIb-IIIa receptors. Initial results showed that the residual complexes of (A.P.) platelets were capable of binding 125I-fibrinogen in CIE and when washed platelets were stimulated with ADP or thrombin (C. Legrand, unpublished data, April 1987) an increased binding was seen with thrombin-stimulated plate-
lets. We have chosen to illustrate the binding of fibrinogen to platelets stimulated with ADP in whole blood. Under these conditions, changes in the distribution of membrane glycoprotein receptors following blood sampling would be minimal. A reduced fibrinogen-binding was observed and is the logical explanation for the platelet aggregation abnormality. Similar findings were obtained for (A.P.) platelets stimulated with ADP in PRP. Previous results obtained for patients with type II thrombasthenia, which do not aggregate when stimulated, and type I heterozygotes which do aggregate, suggest that the level of surface-localized GP IIb-IIIa complexes in (A.P.) platelets is close to the threshold amount required for ADP-induced platelet aggregation to occur. Studies with 125I-labeled Fab fragments showed that part at least of the α-granule pool of fibrinogen in (A.P.) platelets is expressed on the platelet surface following platelet activation by thrombin. This is further indirect evidence that the residual GP IIb-IIIa complexes are functional. However, notwithstanding this expression, and the appearance on the surface of the internal pool of GP IIb-IIIa complexes, thrombin-induced platelet aggregation remained slow and of reduced intensity. This result requires further study but may mean that the newly expressed pools of GP IIb-IIIa and adhesive proteins are relatively inefficient in promoting platelet-platelet contact.

It has recently been shown that both GP IIb and GP IIIa are glycosylated by complex-type oligosaccharide chains that are rich in mannose. GP IIb derives from a precursor, pro-GP IIb, in which the α- (heavy chain) is joined to the β-subunit (light chain) by a peptide bond. Western blotting failed to locate increased amounts of pro-GP IIb in (A.P.) platelets, therefore newly synthesized GP IIb was presumably being normally processed. The formation of a complex between pro-GP IIb and GP IIIa appears critical for the intracellular processing of the complex and its exposure at the platelet surface. CIE and MoAb binding confirmed that complex formation had occurred in (A.P.) platelets. Complex formation occurs before the synthesis of the oligosaccharide chains that are added in the golgi apparatus. An absent glycosylation of GP IIb (and/or GP IIIa) would be accompanied by reductions in the mass of the glycoproteins and their altered migration during SDS-PAGE. Studies using 125I-labeled glycoproteins showed that this was not the case in our patient. However, we cannot as yet exclude a modified glycosylation of GP IIb and/or IIIa. The low levels of surface-orientated GP IIb-IIIa in (A.P.) platelets mean that metabolic labeling of megakaryocytes will probably be required to investigate this hypothesis. An altered glycosylation is one factor that could determine the compartmentalization of GP IIb-IIIa complexes within the membrane systems of forming platelets.

Recent advances have shown that GP IIb-IIIa complexes (αIβIIb) belong to the cytoadhesion class of integrins. GP IIb is specific for the megakaryocytic lineage while GP IIIa is also present in the vitronectin receptor associated with the α, subunit. Although it is the major cytoadhesion of endothelial and other cells, the vitronectin receptor is present in platelets in very small amounts. We do not know if the vitronectin receptor is normally present in (A.P.) platelets. However, the reactivity of (A.P.) platelets with complex-specific monoclonals such as AP-2, and also with Tab (anti-GP IIb), establish that our observations relate to residual GP IIb-IIIa complexes and not increased levels of the vitronectin receptor.

Thrombasthenic variants have been described with a reduced platelet content of GP IIb-IIIa. One example is the Paris variant-Lariboisière I. Here, approximately 50% levels of GP IIb-IIIa are associated with a presumed defect in the transmission of the message through which the fibrinogen binding sites are exposed following platelet stimulation. This variant resembles (A.P.) in that platelet fibrinogen levels were normal, or near normal, and that the GP IIb-IIIa complexes bound fibrinogen in an in vitro test system. However, platelets from Paris variant-Lariboisière I bound no fibrinogen when stimulated and the distribution of GP IIb-IIIa complexes within the platelet was normal. Other variants to be studied have no platelet fibrinogen, and residual GP IIb-IIIa complexes that are unable to bind fibrinogen when platelets are stimulated. Patient (A.P.) is a unique case and differs from the above variants in that the residual complexes were able to bind fibrinogen when his platelets were activated.

In summary, patient (A.P.) has a bleeding syndrome that is associated with a partial platelet aggregation defect and a reduced content of GP IIb-IIIa complexes that have an altered distribution within the platelet. Although the exact nature of the genetic lesion(s) giving rise to this unique combination of defects remains to be defined, their elucidation may throw some light on the mechanism(s) whereby membrane glycoproteins are directed toward different membrane systems within the cell. The inheritance of the defect is also intriguing. Preliminary studies suggest that the patient may have inherited a platelet size abnormality from his father and a GP IIb-IIIa deficiency from his mother. Recent studies have shown that molecular defects within either the GP IIb or GP IIIa genes can give rise to the thrombasthenia lesion. Patient (A.P.) may represent a new type of genetic defect. Not least, he raises important questions concerning the precise definition of the Glanzmann's thrombasthenia phenotype.

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A defect of platelet aggregation associated with an abnormal distribution of glycoprotein IIb-IIIa complexes within the platelet: the cause of a lifelong bleeding disorder

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