

Evaluation of Platelet Glycoprotein Ib by Fluorescence Flow Cytometry

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Platelet glycoprotein Ib (GpIb), a receptor for von Willebrand's factor (vWF), was studied by way of fluorescence flow cytometry. Using a sandwich staining technique, GpIb was identified by a monoclonal antibody (6D1) directed against an epitope close to the vWF binding site. Platelets from normal individuals were symmetrically distributed with respect to GpIb content. Treatment of washed platelets with plasmin resulted in progressive loss of GpIb as measured by fluorescence flow cytometry and by loss of agglutination response when combined with ristocetin in

the presence of vWF. In mixing experiments with GpIb-deficient and normal platelets, it was possible to detect a subpopulation of deficient cells comprising 2% of the total population. Streptokinase treatment of platelet-rich plasma caused loss of the agglutination response to ristocetin and the emergence of a population of GpIb-deficient platelets. Fluorescence flow cytometry appears to be an important new technique by which to study platelet surface receptors.

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NORMAL PLATELET FUNCTION depends upon the presence of specific platelet surface membrane receptors. Receptors for fibrinogen,^{1,2} von Willebrand's factor (vWF),^{3,4} calcium,⁵ thrombin,⁶ and various prostaglandins⁷ have been identified and, in some instances, isolated and partially characterized.⁸ Current evidence suggests that the platelet surface receptor for vWF is a 170,000-mol wt glycoprotein called glycoprotein Ib (GpIb).^{9,10} Antibodies directed against GpIb prevent vWF binding to the platelet surface and inhibit vWF-dependent ristocetin-induced platelet agglutination.³

Most studies of platelet surface receptor function are performed by radioligand binding techniques. These methods are particularly useful for determining receptor number and affinity but do not permit identification of subpopulations because they depend upon averaged measurements derived from large numbers of cells. Cell surface analysis by flow cytometry has permitted investigators to examine surface proteins of individual cells.¹¹ Large numbers of cells can be studied quickly and for more than one property of each cell quantitated, facilitating the identification of subpopulations. For example, this method has permitted classification of T lymphocytes into subpopulations identified by unique surface marker proteins.¹²

We have developed a method using fluorescence flow cytometry to directly assess the platelet surface receptor GpIb. GpIb-vWF interactions are necessary during primary hemostasis as evidenced by the excessive bleeding that occurs in patients with von Willebrand's disease¹³ or the Bernard-Soulier syndrome.¹⁴ Platelet dysfunction may also occur during therapeutic or pathologic states of fibrinolysis due to plasmin-induced degradation of GpIb.

Castillo et al¹⁵ noted decreased ristocetin-induced agglutination of platelets from patients with advanced liver disease and evidence of systemic fibrinolysis. Studies by Ordinas et al¹⁶ indicate that platelets from similarly ill patients are deficient in surface glycoproteins from the group I complex. In addition, it has been demonstrated that platelets stored for infusion lose GpIb.¹⁷ This latter effect may be due to the action of a calcium-dependent protease present within platelets which is active against GpIb.¹⁸

In previous studies we have attempted to better define the effects of plasmin on platelets.¹⁹ We have shown that plasmin treatment of washed human platelets released a fragment of GpIb into the surrounding medium which can be detected by immunoassay, and that GpIb proteolysis is associated with loss of vWF-dependent ristocetin-induced platelet agglutina-

tion. Other surface membrane proteins are relatively preserved. Using these observations, we now present a reliable method by which to directly examine platelet-associated GpIb.

MATERIALS AND METHODS

Ristocetin was purchased from Bio-Data Corp (Horsham, Pa); streptokinase from Pharmacia Laboratories (Piscataway, NJ), fluorescein-conjugated goat anti-mouse Ig from Cooperbiomedical Inc (Malvern, Pa); and aprotinin from Sigma Chemical Corp (St Louis). Plasmin (KABI through Helena Laboratories, Beaumont, Tex), 1 caseinolytic unit (CU)/40 μ L, was dissolved in 10 mmol/L Tris-0.15 mol/L NaCl, pH 7.4, and stored in aliquots at -40° C. CUs were defined according to Sgouris et al²⁰; 1 CU equals 1.14 Committee on Thrombolytic Agents (CTA) units.²¹ A mouse monoclonal IgG antibody (6D1) directed against human platelet GpIb was kindly provided by Dr Barry Coller, SUNY at Stony Brook (NY). All other chemicals were reagent grade.

Washed human platelets. Blood was collected by venipuncture into 1 mL acid-citrate-dextrose (ACD) per 5 mL blood. ACD is citric acid 7.3 g, sodium citrate-2H₂O 22.0 g, and glucose 24.5 g in 1,000 mL distilled water. After mixing, the blood was centrifuged at 200 g for ten minutes at room temperature, and the upper two thirds of the platelet-rich plasma (PRP) was collected. The pH of the PRP was adjusted to 6.5 by further addition of ACD. The platelets were then pelleted by centrifugation at 3,200 g for 15 minutes at room temperature. The platelet pellet was resuspended in washing buffer (10 mmol/L Tris, 0.15 mol/L NaCl, 20 mmol/L EDTA, pH 7.4) and washed an additional four times. The platelets were finally suspended in the same buffer at a concentration of 4×10^5 per microliter. Formaldehyde-treated platelets were prepared as pre-

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viously described.²² Platelets were formalin-treated in either PRP or washing buffer.

Effect of plasmin and streptokinase on platelet GpIb and ristocetin-induced vWF-dependent platelet agglutination. Streptokinase, 25,000 IU/mL, was added to PRP and the mixture was incubated at room temperature. Control PRP was handled in a similar fashion, but streptokinase was not added. Following a 60-min incubation period, 1,000 kallikrein inhibitor units (KIU)/mL aprotinin was added to stop plasmin action. The samples were then either formaldehyde-treated in preparation for cytometric analysis, or used to study ristocetin-induced platelet agglutination.

Plasmin treatment of washed platelets was conducted as previously described.¹⁹ Each incubation was stopped by the addition of an equal volume of 2% formaldehyde. Following fixation, the platelets were prepared for both cytometric analysis and platelet agglutination. For the agglutination studies, the fixed platelets were washed three times by centrifugation in washing buffer (3,200 g for 15 minutes at room temperature) and then suspended in platelet-poor plasma from the same donor. The final concentration of platelets was 2.0×10^5 per microliter.

Agglutination was measured by a standard nephelometric technique.²³ Agglutination was initiated by adding 50 μ L of a 10 mg/mL solution (final concentration, 1.25 mg/mL) of ristocetin to each PRP sample while the sample was stirred at 37 °C in a Payton Dual Channel Aggregometer (Buffalo). The slope of the steepest segment of the reaction curve was determined.

Fluorescence flow cytometry. Formalin-treated platelets were washed three times in Tris-saline (10 mmol/L Tris, 0.15 mol/L NaCl, pH 7.4) and diluted to 50,000 platelets per microliter. They were then incubated with 6D1 (suspended in the same buffer) for 30 minutes at 4 °C, again washed three times by centrifugation (3,200 g for 15 minutes at 4 °C) and resuspended in Tris-buffer. The treated platelets were then incubated with the fluorescein-labeled goat anti-rabbit antibody for 20 minutes at 4 °C, washed three times by centrifugation, and resuspended at a concentration of 50,000 platelets per microliter. A dose-response curve with 6D1 was generated using varying dilutions of 6D1 ranging from 88 μ g/mL to 44 ng/mL (final concentration), and a 350 ng/mL dilution was selected for use in subsequent studies. The second antibody was used at a saturating concentration. Studies using the irrelevant antibody OX6 in place of 6D1 were done as described above (OX6 was the kind gift of Dr Bruce Woda, University of Massachusetts Medical Center).

The labeled platelets were analyzed on a Becton Dickinson (Mountain View, Calif) FACS 440. The instrument was operated using an argon ion laser at 488 nm and 300 mW. Fluorescein fluorescence was detected through a 530+/-30 nm band-pass filter. Logarithmic amplification was used for the fluorescence signals. Linear amplification was used for the size (low-angle light scatter) signals. Fluorescence calibration was done using glutaraldehyde-fixed chicken erythrocytes as standards. Size calibration was done using 2- μ m plastic beads supplied by Becton Dickinson. A fluorescence histogram was obtained for 10,000 platelets whose size fell within ten channels of the 2- μ m peak. Thus, only those platelets whose size was very near to 2 μ m were being measured. This insures that debris and erythrocytes were excluded. In control studies, the proportion of particles in the suspension buffer falling within this size range was <1% of the number of platelets. The fluorescence histograms were analyzed on a Becton Dickinson Consort 40 computer. The channel-weighted mean fluorescence intensity for the 10,000 platelets was determined and is reported here. The formula used for mean fluorescence intensity was

$$\text{Mean}_{\log} = [\text{sum over all } n (n \times P_n)] / \text{total number of platelets},$$

where n = channel number and P_n = number of platelets in channel n . These means were obtained on a logarithmic scale. When neces-

sary, conversion was made to linear measure based on the fact that 30 channels represented a doubling of fluorescence intensity. Thus, the formula used was

$$\text{Mean}_{\text{linear}} = 2[(\text{Mean}_{\log} - \text{Mean}_{\text{control}})/30],$$

where $\text{mean}_{\text{control}} = \text{mean}_{\log}$ of platelets treated with second antibody alone.

For mixing experiments the shape of the fluorescence histogram to the right and left of channel number 44 was compared. This cutoff point was chosen because channels 0 through 44 never contain more than 5% of the total number of platelets in a control sample.

Platelets could be fixed in PRP or after washing. Specific fluorescence of platelets fixed in PRP was about 20% higher than when fixed after washing. For this reason, individual comparisons were only made between paired samples of identically treated specimens.

RESULTS

Studies were conducted to characterize the effect of anti-GpIb (6D1) concentration on mean fluorescence and to determine the amount of fluorescence produced by nonspecific binding of the second fluorescein-labeled antibody. Figure 1 demonstrates the dose-response curve generated by analyzing aliquots of platelets incubated with various dilutions of 6D1 at a constant concentration of second antibody. Minimal nonspecific binding of the second antibody was observed.

To further demonstrate the specificity of 6D1 binding to GpIb we examined platelets in which 6D1 had been replaced by an equal amount of an irrelevant first antibody. This antibody, OX6, is a mouse monoclonal antibody of the same subclass as 6D1 and is directed against the rat Ia antigen. Mean linear fluorescence of platelets labeled with OX6 and the fluorescein-labeled second antibody averaged 15% (range, 12% to 18%) of that obtained with 6D1 as the first antibody and was equal to the mean linear fluorescence obtained when only the second antibody was used.

To characterize the distribution of platelets with respect to surface content of GpIb, we analyzed specimens obtained from healthy persons taking no medication. From each individual we observed a symmetrical distribution of fluorescently labeled platelets, indicating that the most platelets in the circulation of normal individuals contain a similar

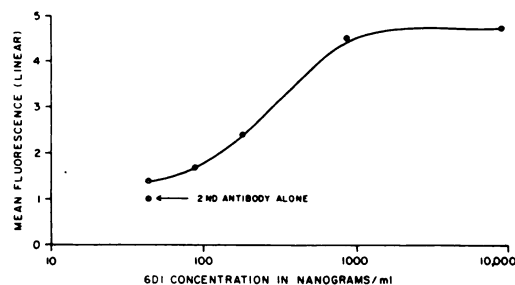


Fig 1. Effect of 6D1 concentration on mean fluorescence. Formalin-treated platelets were incubated with various dilutions of 6D1 and a constant concentration of fluorescein-labeled second antibody. Platelets incubated with the second antibody alone demonstrated minimal fluorescence. Units for fluorescence are arbitrary. The means for each point represent the averages of separate determinations of 10,000 platelets from each of two individuals.

amount of GpIb (Fig 2). In six normal adults, the coefficient of variation describing the fluorescence histograms averaged $18.4\% \pm 2.2\%$ (SD). No differences in the shape of the distribution curves were noted between men and women.

In previous studies we have demonstrated that plasmin treatment of washed platelets causes degradation of GpIb and concomitant inhibition of platelet agglutination following addition of vWF and ristocetin.¹⁹ Therefore, washed platelets were incubated with plasmin prior to formalin treatment and immunostaining. As seen in Fig 3, a dose-dependent effect of plasmin on platelet surface GpIb content was clearly demonstrated. Mean fluorescence declined progressively, approaching 0 at 1 CU/mL of plasmin. Concurrently, ristocetin-induced vWF-dependent platelet agglutination declined following plasmin treatment. Agglutination was totally inhibited by incubation with 1 CU/mL of plasmin.

To determine the ability of this technique to identify subpopulations of GpIb-deficient platelets, we performed a series of mixing experiments with normal platelets and platelets made GpIb deficient by plasmin treatment. Even when we added only two GpIb-deficient platelets per 98 normal platelets, a 2% mixture, the shape of the fluorescence histogram was clearly different from that of the control. Figure 4 illustrates the changes that were observed when varying numbers of GpIb-deficient platelets were mixed with normal platelets.

In a final series of experiments, we added streptokinase to PRP and evaluated its effect on platelet GpIb content and ristocetin-induced platelet agglutination. Sixty minutes after the addition of 25,000 IU/mL of streptokinase, an amount adequate to ensure rapid plasminogen activation, ristocetin-induced platelet agglutination declined by 50%. Cytometric analysis of platelets recovered from the streptokinase-treated PRP revealed a bimodal distribution with respect to GpIb content (Fig 5). In approximately 5% to 10% of the total number of platelets studied, the amount of surface-bound GpIb was markedly decreased.

DISCUSSION

Using fluorescence flow cytometry, we have developed a sensitive and reproducible technique to detect platelet GpIb,

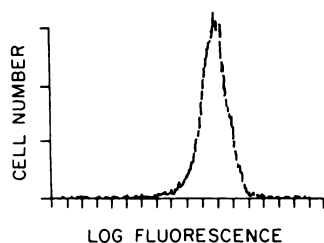


Fig 2. Immunofluorescence profile of normal human platelets stained for GpIb. Formalin-treated platelets incubated with 6D1 and a fluorescein-labeled second antibody were analyzed by fluorescence flow cytometry. The platelets are symmetrically distributed into one population. To obtain this histogram 10,000 platelets were analyzed. The scale units for log fluorescence intensity are arbitrary and the curve is generated by a computer directly linked to the flow cytometer.

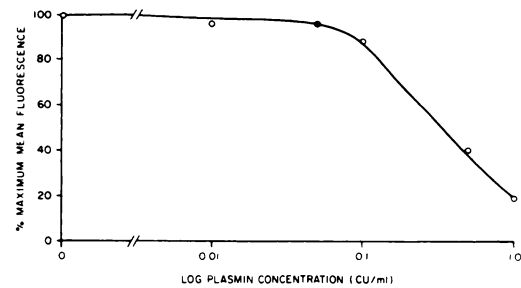


Fig 3. Plasmin effect of platelet-associated GpIb. Washed platelets were treated with various amounts of plasmin for one hour and then prepared for analysis of GpIb content by fluorescence flow cytometry. For each point, 10,000 platelets were analyzed.

a vWF receptor. To do so, we have used a monoclonal antibody that recognizes a site on GpIb that is close to, or includes, the vWF binding site. With this method we have demonstrated (1) the presence of GpIb in a constant amount on the surface of all platelets obtained from normal individuals, (2) loss of GpIb from the platelet surface following plasmin treatment of washed platelets, and (3) the emergence of a population of GpIb-deficient platelets following the addition of streptokinase to PRP. In addition, mixing experiments indicate that the technique is sensitive enough to identify a population of GpIb-deficient platelets amounting to only 2% of the total number present in a particular sample.

The effect of streptokinase on platelets in plasma is of particular interest. Plasmin treatment of washed platelets causes a progressive loss of GpIb from the surface of all exposed platelets, while plasmin generated in PRP produces a distinct population of GpIb-deficient platelets. In each instance, ristocetin-dependent platelet agglutination is inhibited. The reason for this difference in effect is not clear. A number of plasma substrates, including alpha-2-antiplasmin, fibrinogen, fibrin, and factors V, VIII, and IX, may be competing with platelets for plasmin action.²³⁻²⁸ Perhaps following a longer incubation period, more GpIb would have been released. It is also possible that in plasma only a subset of platelets is susceptible to the effect of plasmin on GpIb. These might be partially activated platelets, as suggested by recent data indicating that thrombin stimulation of platelets enhances plasminogen binding.²⁹

Cell surface receptors are important mediators of cell responses to stimuli. Platelets have a number of surface receptors that appear to be physiologically important in mediating adhesion and aggregation.^{1-6,8-10,30,31} Precise characterization of these receptors with respect to number, functional state, and relative distribution among platelets may advance our understanding of the processes of normal coagulation and pathologic thrombosis. Flow cytometry can clearly add an important new dimension to such studies. We have focused this investigation on GpIb because of the availability of a well-characterized monoclonal antibody (6D1)³ that reacts with GpIb; however, it should not be difficult to apply these methods to the study of other platelet receptors.

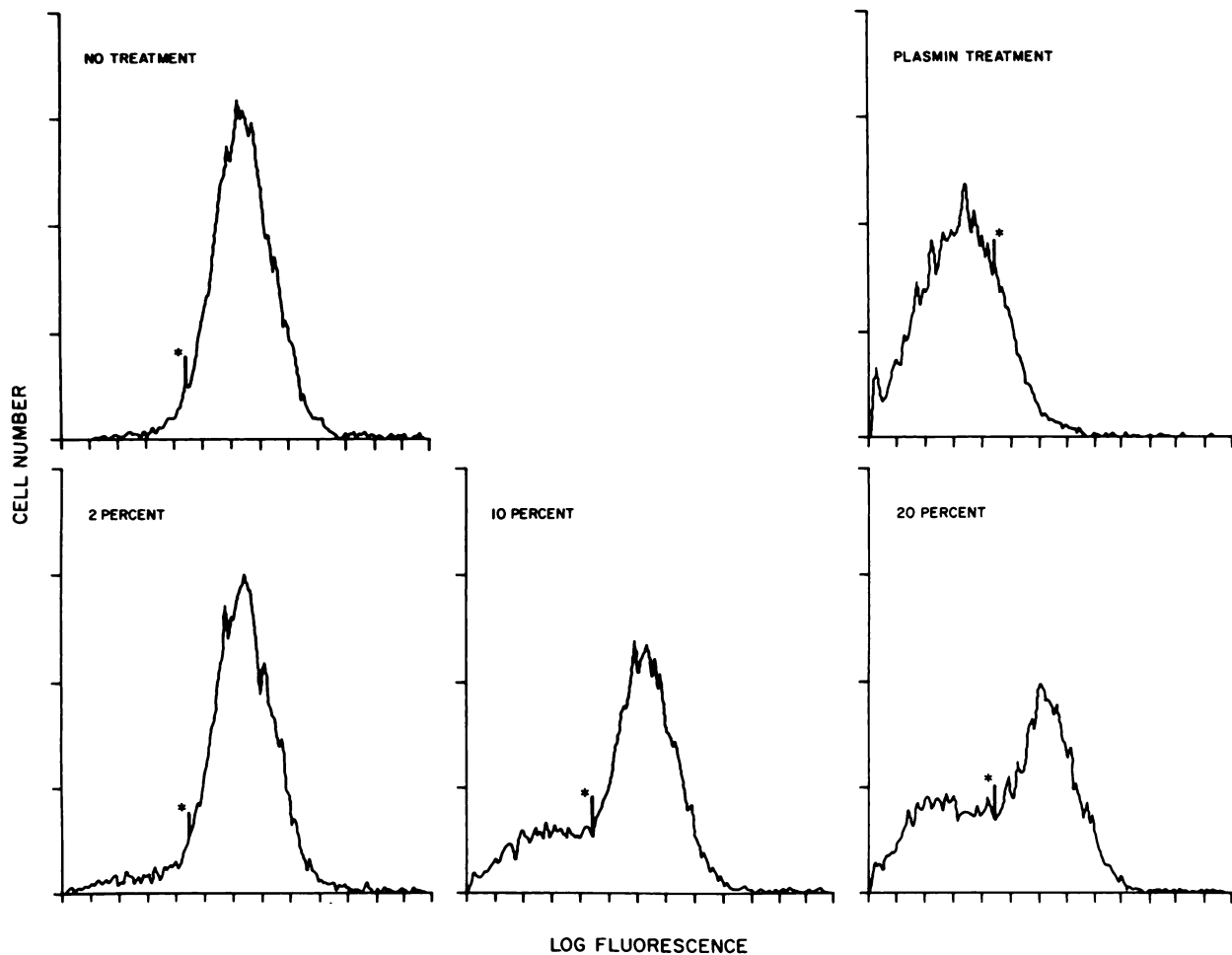


Fig 4. Fluorescence histograms of mixtures of GpIb-containing and GpIb-deficient platelets. GpIb-deficient platelets (incubated with 1 CU/mL plasmin for 60 minutes) and control platelets prepared for analysis of GpIb content were mixed in various proportions. The GpIb-deficient platelets in the samples studied (2%, 10% and 20%) are identified by the area under the curve between channels 0 and 44 (channel 44 is marked by *). Fewer than 5% of control platelets were found within this area. Each curve was obtained by analyzing 10,000 platelets.

Fixation of the platelets with formalin prior to staining made our technique simple and allows for the storage of specimens prior to analysis. Although GpIb reactivity persists after treatment with formaldehyde, it is unlikely that all platelet surface antigens will react with antibody following

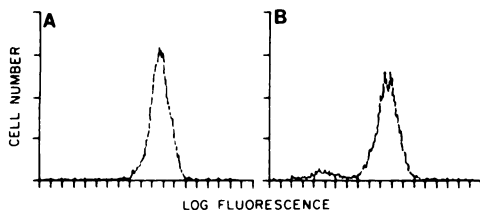


Fig 5. The effect of streptokinase on GpIb content of platelets in PRP. Streptokinase was added to PRP and allowed to incubate for one hour. Following formalin treatment, the platelets were prepared for GpIb analysis. Control platelets (A) are distributed symmetrically with respect to GpIb content but the streptokinase-treated platelets (B) demonstrate a bimodal distribution including a peak of GpIb-deficient platelets. This is a representative histogram derived from the analysis of 10,000 platelets.

fixation. This should not pose a major technical problem because viable platelets can be washed or gel-filtered and then incubated with appropriate antibodies prior to fixation.

Also of consideration is the anticoagulant used. In this study, ACD, followed by a buffer containing EDTA was adequate to inhibit the platelet calcium protease and preserve surface GpIb. If an absolute determination of GpIb is needed, or if a patient is suspected of having active plasmin in the circulation, EDTA and aprotinin should be added to the anticoagulant mixture.

Flow cytometry offers a number of advantages over more conventional binding assays for the study of cell surface receptors.¹¹ Because flow cytometry permits every cell in a defined population to be individually examined, small populations displaying distinct characteristics can be readily identified. Such populations could not be appreciated by conventional ligand binding assays. Also, because flow cytometry equipment is highly efficient, very small numbers of cells are required for analysis. This should permit direct examination of platelets from thrombocytopenic individuals

with disorders such as disseminated intravascular coagulation or thrombotic thrombocytopenia. Based on our studies, adaptation of fluorescence flow cytometry techniques to the study of platelets appears to be simple and of significant value. Such studies should shed new light on the pathogenesis of platelet-related bleeding disorders.

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