Analysis of Human Platelet Glycoproteins IIb-IIIa and Glanzmann’s Thrombasthenia in Whole Blood by Flow Cytometry

By Lisa K. Jennings, Richard A. Ashmun, Winfred C. Wang, and Michael E. Dockter

Antibodies that bind to human platelet membrane glycoproteins IIb and IIIa were used to develop methods for analyzing platelet membrane components by flow cytometry. Platelets were tentatively identified by their low-intensity light scatter profiles in whole blood or platelet-rich plasma preparations. Identification of this cell population as platelets was verified by using platelet-specific antibodies and fluorescein-conjugated antimmunoglobulin. Two-parameter analysis of light scatter versus fluorescence intensity identified >98% of the cells in the “platelet” light scatter profile as platelets due to their acquired fluorescence. Both platelet-rich plasma and whole blood were used to study platelet membrane glycoproteins IIb and IIIa on a single cell basis in an unwashed system.

FLOW cytometry is a technique that is being increasingly used to analyze individual components in a homogeneous or heterogeneous population of cells. Analysis by flow cytometry has been used successfully to quantitate cellular DNA and ploidy frequency distributions of cells, particularly those residing in the bone marrow, and has been increasingly used for analysis of ligand-receptor interactions (for review, see references 1 and 2). Flow cytometry may provide one of the best overall methods for analysis of receptor proteins as this technique permits rapid and sensitive analysis of cell surface proteins on a single cell basis.

We have developed a method for analyzing platelet surface components in platelet-rich plasma and whole blood using flow cytometry. Platelet membrane glycoproteins IIb and IIIa were detected on the platelet surface by using as probes monoclonal or heterologous antibodies specific for these glycoproteins. The level of glycoproteins IIb and IIIa on platelets from normal donors was compared to that of platelets obtained from Glanzmann’s thrombasthenic individuals. Platelets from individuals with Glanzmann’s thrombasthenia and heterozygote carriers for this disorder were easily distinguished by their relative fluorescence intensity from platelets of normal donors. During our studies, we also observed that all cells within the platelet population displayed decreased amounts of glycoproteins IIb and IIIa (characteristic of this inherited platelet disorder), thus confirming that this is a homogeneous defect. Platelet analysis by flow cytometry provides an alternative rapid diagnostic procedure for Glanzmann’s thrombasthenia as well as for the detection of other functional defects caused by an altered expression of platelet surface components.

MATERIALS AND METHODS

Chemicals and Materials

Acrylamide, n,n’-methylene bis-acrylamide, ammonium persulfate, bromophenol blue, sodium dodecyl sulfate (SDS), Coomassie brilliant blue, and molecular weight standards were obtained from Bio-Rad (Richmond, Calif.). Lactoperoxidase was a generous gift from Dr. M. Morrison (St. Jude Children’s Research Hospital, Memphis, Tenn.). Na[125]I was purchased from New England Nuclear (Boston, Mass.). All other chemicals were purchased from Sigma Chemical Co (St. Louis, Mo.) and were of reagent grade. RP Trimax x-ray film was purchased from Eastman Kodak Company (Rochester, NY).

Platelet Donors

RG, a 17-year-old male, had bruised extremities at birth and a subsequent history of gingival bleeding. Glanzmann’s thrombasthenia was diagnosed at age 1½ years. Laboratory evaluation showed bleeding time >15 minutes; clot retraction 21%; a platelet count of 193 x 10⁹/L; and platelet aggregation absent with ADP, epinephrine, thrombin, and collagen. Parent SJ has no history of a bleeding disorder.

BL, a 5-year-old male, has had easy bruising since age 9 months. Glanzmann’s thrombasthenia was diagnosed at age 2 years. Laboratory evaluation showed bleeding time 9.5 minutes; a platelet count of 280 x 10⁹/L; and no aggregation to ADP, collagen, or epinephrine. He is the offspring of a consanguineous sibling relationship. Parent DL has no history of a bleeding disorder.

Patient EA, a 34-year-old female, has been described in an earlier publication. Laboratory evaluations have shown a prolonged bleeding time; normal platelet count and morphology; impaired clot retraction; and aggregation absent with ADP, thrombin, collagen, or epinephrine.

Antiplatelet Antibodies

An antiplatelet membrane glycoprotein IIb/IIIa monoclonal antibody (GB18) was used as a specific probe for human platelets. The preparation and characterization of this monoclonal antibody was described previously. Heterologous Protein A affinity-purified rabbit antihuman platelet glycoprotein IIb-IIIa antibody was kindly provided by Dr. M. Morrison.

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From the Department of Medicine, The University of Tennessee, and the Departments of Biochemistry and Hematology/Oncology, St. Jude Children’s Research Hospital, Memphis.

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Address reprint requests to Dr Lisa K. Jennings, Division of Hematology/Oncology, Department of Medicine, University of Tennessee, Memphis, 956 Court Ave. Rm H316, Memphis, TN 38163.

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provided by Dr David R. Phillips, Gladstone Foundation Laboratories (San Francisco, Calif). This antibody was demonstrated to be specific for glycoproteins IIb and IIa by immunoprecipitation of solubilized platelet membranes and by the Western blot procedure using solubilized membranes or solubilized washed platelets electrobotted onto nitrocellulose paper.

Fluorescein-conjugated (FITC) goat antirabbit and rabbit antimouse immunoglobulin were obtained from Sigma Chemical Co and had fluorescein-to-protein ratios of 4.7 and 4.2, respectively.

Preparation of Platelets

Venous blood from normal adult donors was collected by venipuncture through a 19-gauge needle and mixed with 0.16 volume of a solution containing 0.085 mol/L sodium citrate, 0.111 mol/L dextrose, and 0.071 mol/L citric acid (ACD). Donors took no medication for 2 weeks prior to blood collection.

The blood was centrifuged at 160 x g for 20 minutes, and the platelet-rich plasma removed. The platelets were separated from the plasma and washed twice in CGB buffer containing 0.12 mol/L sodium chloride, 0.013 mol/L trisodium citrate, and 0.03 mol/L dextrose, pH 7.0. The platelets were washed a final time in TS buffer containing 0.154 mol/L sodium chloride, 0.01 mol/L Tris, and 0.0001 mol/L leupeptin, pH 7.4. The platelets were suspended in TS buffer for surface labeling. All procedures were conducted at room temperature.

Iodination of Platelet Surface Proteins

Platelets were radiolabeled with 125I by lactoperoxidase-catalyzed iodination using a previously published procedure. Washed platelets were solubilized in 2% SDS (w/v) containing 2% 2-mercaptoethanol (v/v) incubated at 100°C for 10 minutes to ensure solubilization. Protein (10 to 50 μg) was electrophoresed through slab gels according to the method of Laemmli by using a 5% to 20% exponential gradient of acrylamide in the resolving gel and 3% acrylamide in the stacking gel. Protein was stained with Coomassie brilliant blue. For determination of the distribution of radioactivity, the gels of radiolabeled samples were subjected to autoradiography using a GS 300 Transmittance/Reflectance scanning densitometer interfaced with an Apple Iic computer for analysis (Hoefer Scientific Instruments, San Francisco, Calif).

Preparation of Samples for Flow Cytometry

Platelet-rich plasma preparation. Platelet-rich plasma (PRP) from freshly drawn blood was collected by low-speed centrifugation at 800 x g and then diluted 1:1 (v/v) with CaTS buffer (0.154 mol/L sodium chloride, 0.01 mol/L Tris, and 0.0005 mol/L calcium chloride, pH 7.4) containing 50 μmol/L prostacyclin (PGI2). To 200 μL PRP-CaTS, an antihuman platelet membrane glycoprotein monoclonal antibody (2 μg) was added and incubated for 30 minutes at room temperature (22°C). The addition of two micrograms of antibody was shown to be saturating for the number of platelets in the sample. FITC-conjugated rabbit antiamouse IgG (10 μg) was added and incubated for 10 minutes.

Preparation of whole human blood. Fresh human blood was diluted 1:1 (v/v) with CaTS buffer containing 50 μM PGI2. Within 1 hour of venipuncture, the antiglycoprotein IIb and IIa antibody (10 μg) was added to 400 μL of blood-CaTS and incubated for 30 minutes at room temperature. FITC-goat antirabbit IgG (40 μg) was added and after 10 minutes, the cells were analyzed by flow cyt fluorometry. Control experiments for both PRP and whole blood studies were treated in an identical manner except that buffer or a species-specific IgG (2 μg) was added instead of the antiplatelet antibody.

Flow cytometry. A Coulter EPICS V flow cytometer (Coulter Electronics, Inc) equipped with a Spectra Physics 164-05 argon ion laser adjusted to deliver 800 milliwatts at 488 nm was used to analyze platelets. Cells in suspension were analyzed at a flow rate of approximately 1,000 cells/sec. Forward-angle light scatter (1σ to 1.9σ) was converted from linear to logarithmic scale by using a log amplifier of the instrument. Fluorescein fluorescence emission was detected after passage through a 510 nm long pass interference filter to exclude scattered laser light. The light scatter and green fluorescence signals from each cell were collected from 50,000 cells and stored and analyzed by the Coulter MDADS minicomputer analysis system as two-parameter 64 x 64 channel histograms. Routinely, the fluorescence signals measured were gated on a light-scattering profile that excluded contributions from debris. Data (log light scatter x log green fluorescence signal x cell number) were transferred to a Data General MV8000 computer and accompanying Techronics 4662 plotter for subsequent graphics.

RESULTS

Antibody binding to platelet membrane glycoproteins was assessed by fluorescence microscopy. Using standard fluorescein filter combinations for fluorescence microscopy, platelets plus antiplatelet glycoprotein IIb9 monoclonal antibody and FITC-rabbit antiamouse IgG fluoresce strongly over background (platelets plus FITC-rabbit antiamouse IgG alone). The labeling pattern of the antibody was uniform over the surface of the platelet as would be expected for a labeled major membrane component that is evenly dispersed (data not shown). A similar labeling pattern was observed when the platelets were reacted with heterologous antiglycoprotein IIb and IIa antibody and FITC-goat antirabbit antibody.

To identify platelets on the basis of their characteristic cell size, unlabeled platelets in plasma were subjected to flow cytometric analysis on the Coulter EPICS V cytometer. Fifty thousand cells were analyzed simultaneously for size by low-angle log light scatter measurements. The platelets had a broad distribution of light-scattering signal which is indicative of size heterogeneity.

Once light scatter parameters for platelets were established, unlabeled and antibody-labeled cells in platelet-rich plasma were analyzed by flow cyt fluorimetry. Figure 1 illustrates the fluorescence of the platelet population labeled with both the antiglycoprotein IIb9 and the species-specific FITC-conjugated antibody. Very little green fluorescence was associated with the platelets when only the FITC-conjugated rabbit antiamouse antibody was added to the platelet sample indicating that nonspecific binding of this antibody was minimal. Experiments where preimmune mouse IgG was added rather than buffer as the control showed minimal nonspecific binding that did not alter the results. The mean fluorescence associated with the platelets in Fig 2 was 13 times that observed in the control experiment. Integration of the fluorescence signal determined that >98% of the cells analyzed as platelets were positive for bound antibody.

Figures 2A and 2B are two-parameter histograms from
Fig 1. Log green fluorescence intensity of antiglycoprotein IIbβ-treated platelets (---) and platelets treated only with FITC-rabbit antimouse antibody (....). x-axis, log green fluorescence; y-axis, number of cells.

Fig 2. Two-parameter analysis of flow cytofluorimetry of platelets in PRP after addition of 2.5 µg of MoAb18 and FITC-rabbit antimouse IgG (Panel A). The control (Panel B) is of platelets treated in an identical manner except MoAb18 was omitted from the incubation medium. x-axis, log green fluorescence; y-axis, log light scatter; z-axis, number of cells.

Fig 3. Data in Fig 2, Panel A, replotted as the peak (+) and mean (O) channel of fluorescence for each light scatter channel.

Fig 4. Log light scatter profile of whole human blood–containing platelets (peak 1) and erythrocytes (peak 2) and white blood cells (peak 3). x-axis, log light scatter; y-axis, number of cells.
containing platelets, erythrocytes, and white cells. Once the platelets were identified in whole blood, rabbit antiglycoprotein IIb and IIIa antibody and FITC-conjugated goat anti-rabbit immunoglobulin were added to whole blood as described in Materials and Methods, and the platelets were analyzed on the basis of light scatter and green fluorescence. Fifty thousand platelets were analyzed by collecting data based on log green fluorescence gated on light scatter profile for the platelet population. Figure 5A is the two-parameter histogram of whole blood after addition of antibodies with the corresponding plot of control cells (minus first antibody addition) in Panel B. Platelets were easily distinguishable in whole blood preparations and were specifically labeled by the antiglycoprotein IIb and IIIa antibody. The fluorescence intensity of the antibody-labeled cells was compared to the control group in Fig 6. The log green fluorescence of the antibody-treated platelets was approximately 4 to 5 times higher than that observed with the untreated or control platelet group (plus buffer or normal rabbit IgG instead of the antiglycoprotein IIb and IIIa antibody). Fluorescence of both the red cell and white cell populations appears increased in the antiglycoprotein IIb and IIIa antibody-treated whole blood. While a finite number of platelets were analyzed by collecting data gated on the light scatter profile for platelets, the number of red cells analyzed was different in the control and antibody-treated groups. In the experiment depicted, 56% more red cells were analyzed in the antibody-treated sample than in the corresponding control. Thus, what appears to be an apparent increase in RBC fluorescence is due to increased numbers of red cells being analyzed and not due to specific binding of the antiglycoprotein IIb and IIIa antibody. In contrast, the increased fluorescence observed in the white cell population is due to the detection of bound platelets to monocytes (manuscript in preparation) and is the

Table 1. The Amount of GPIIb-IIIa Determined by Scanning Densitometry of 125I-Labeled Platelet Proteins and by Flow Cytometry Analysis of Relative Fluorescence Intensity

<table>
<thead>
<tr>
<th>Individual Analyzed</th>
<th>GPIIb-IIIa Levels</th>
<th>Scanning Densitometry*</th>
<th>RFI†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>100%</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>Heterozygote SJ</td>
<td>73%</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>Heterozygote DL</td>
<td>61%</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>GT patient RG</td>
<td>6%</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>GT patient BL</td>
<td>33%</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>GT patient EA</td>
<td>2%</td>
<td>1.4</td>
<td></td>
</tr>
</tbody>
</table>

*Based on the integration of the scan peak corresponding to 125I-labeled platelet GPIIa relative to platelet actin.
†RFI = relative fluorescence intensity, determined by using the ratio of the mean channel of log green fluorescence intensities of the labeled cells treated with and without anti-GPIIb-IIIa antibody.
result of specific binding of the antiglycoprotein IIb and IIIa antibody to these adherent platelets.

This system was then extended to analyze glycoprotein IIb and IIIa content in Glanzmann’s thrombasthenic individuals. These values were compared to the levels of glycoproteins IIb and IIIa present in a Glanzmann’s thrombasthenic individual’s platelets determined by scanning densitometry of autoradiograms of gels of 125I-labeled surface proteins. Two parents of Glanzmann’s thrombasthenia patients were included in this study.

Figure 7 is a densitometry scan of radiolabeled platelet surface proteins separated on SDS polyacrylamide gels from patient RG, his parent (SJ), and a representative normal donor. Integration of the peak corresponding to radiolabeled glycoproteins IIb or IIIa of patient RG showed 5.7% of the normal amount of glycoproteins IIb and IIIa (Table 1). The parent had approximately 73% of the normal amount. Patients BL and EA had approximately 33% and 2%, respectively, while DL (parent of BL) had approximately 61% of the normal levels of glycoproteins IIb and IIIa (Table 1).

Platelets were analyzed in whole blood from these individuals by flow cytometric techniques. The histograms of log green fluorescence versus cell number for RG, SJ, and a normal donor are illustrated in Fig 8. Figure 9 is the two-parameter analysis of the same individuals. Individual EA had essentially no detectable levels of glycoprotein IIb and IIIa with a relative fluorescence intensity (RFI) 1.4 times that of untreated platelets, whereas platelets from RG, BL, SJ, and DL and a normal donor had a RFI of 1.7, 2.3, 3.0, and 4.0, respectively (Table 1). Relative fluorescence intensity was determined by using the mean channel of log green fluorescence. These determinations are comparable to glycoprotein IIb and IIIa levels quantitated by autoradiography in that there is a high correlation (r = 0.98) of these levels to the RFI values calculated for each individual (Fig 10). The plot of this data would be expected to intersect at a RFI value of 1.0. The intersection at a slightly higher RFI value is due to the fluorescence associated with nonspecific antibody binding and the inherent autofluorescence of the platelets.

Flow cytometric analysis also permitted us to determine the uniformity of this defect in all individuals tested. The decreased amount of glycoprotein IIb and IIIa was homogeneous within the entire population of analyzed cells confirming that Glanzmann’s thrombasthenia affects all platelets in circulation.

**DISCUSSION**

A variety of biochemical techniques have been used to study the structure of platelet membrane glycoproteins IIb and IIIa and to determine their function in platelet aggrega-
tion. Analysis of these membrane glycoproteins has been based primarily on the development of surface-labeling techniques. Alternative immunochemical methods such as radioimmunoassay (RIA) and crossed immunoelectrophoresis (CIE) have been used to identify and quantify specific platelet surface receptors. These methods provide ways of identifying the specific glycoprotein epitopes involved in mediating platelet functions. Studies by Nurden et al have demonstrated that analysis by the immunoblot procedure detected residual amounts of platelet glycoproteins IIb and IIIa which were not initially observed by other methods such as CIE or SDS polyacrylamide gel electrophoresis.

All of these techniques demand a relatively large homogeneous platelet population that requires manipulation through isolation and washing procedures before analysis. Such manipulations may alter the expression or accessibility of surface components and involve considerable preparation time. Recent studies have shown that the association of these glycoproteins is dependent on the buffer and incubation conditions employed. For example, prolonged incubation of platelets or isolated membranes in the presence of EDTA will cause formation of glycoprotein oligomers that cannot be dissociated readily.

We used antibodies that bind to membrane glycoproteins IIb and IIIa to develop methods for analyzing platelets in an unwashed preparation by flow cytometry and to determine if differences in glycoprotein expression could be detected by these methods. We were able to distinguish between Glanzmann's thrombasthenic individuals and carriers for the disorder. We also showed a high correlation between the RFI of the antibody-labeled platelets and the amount of glycoproteins IIb and IIIa analyzed by autoradiography of 125I-labeled platelet proteins.

Platelets in platelet-rich plasma and whole blood were identified by their low-angle log light scatter profile. Although in whole blood preparations there was some overlap in the light scatter distribution of erythrocytes and platelets, erythrocytes can be gated out for platelet analysis or specific probes for platelets can be used in conjunction with their light scatter characteristics. Therefore, flow cytometry is an excellent technique for analyzing platelet surface proteins on a single cell basis. While clinically diagnosed Glanzmann's thrombasthenia patients have variable amounts of glycoproteins IIb and IIIa on their platelet membrane surface, we found that all platelets from each of these individuals were affected equally and that this disorder is not due to distinct populations of circulating platelets. Evaluation of platelets at the single cell levels by flow cytometry would be particularly useful for analysis of acquired platelet disorders such as paroxysmal nocturnal hemoglobinuria (PNH) where distinct platelet populations may occur.

Other investigators have reported using flow cytometry to examine platelet-specific proteins. Adelman and coworkers examined glycoprotein IIb content in washed and formaldehyde-treated human platelets. Johnston and coworkers recently reported their use of flow cytometric techniques to study the effect of diamide and iodoacetamide on the expression of platelet glycoproteins and on platelet aggregation. Their data support our earlier findings that flow cytometry is an efficient method to examine platelet membrane components.

Flow cytometry is also suitable for clinical analysis of platelet surface defects. This technique eliminates the use of radiolabeling techniques and can be performed in conjunction with other surface marker studies. Flow cytometric analysis provides a reliable index of the relative quantity of glycoproteins IIb and IIIa on the platelet membrane. We have demonstrated with our studies of four individuals with various levels of these glycoproteins that the RFI values are comparable with the percentages of glycoproteins IIb and IIIa determined by autoradiography. This method for examining the expression of platelet surface antigens has clear advantages in that platelets may be studied with little or no manipulation and requires only μL quantities of material for analysis. Analyzing platelets in platelet-rich plasma or whole blood provides a milieu most like the in vivo situation. Therefore, the expression of specific epitopes in resting platelets versus activated platelets may be more rigorously and accurately examined by flow cytometric methods.

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