Detection of Activated Platelets in Whole Blood Using Activation-Dependent Monoclonal Antibodies and Flow Cytometry

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Platelets may become activated in a number of clinical disorders and participate in thrombus formation. We developed a direct test for activated platelets in whole blood using flow cytometry. Whole blood was incubated with either fibrinogen-PAC1, a monoclonal antibody specific for the fibrinogen receptor on activated platelets, or fibrinogen-S12, an antibody specific for an α-granule membrane protein that associates with the platelet surface during secretion. Platelet-bound antibodies were detected with streptavidin conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Platelets were differentiated from the larger erythrocytes and WBCs by their light-scatter profile. Alternatively, platelets could be identified with FITC-APC1, an antibody specific for platelet membrane glycoprotein Ib, and analyzed further for PAC1 or S12 binding with PE-streptavidin. No centrifugation or washing steps were required. With gel-filtered platelets, there was a direct correlation between ADP-induced fibrinogen-PAC1 binding and fibrinogen-S12 binding in a competitive manner.5 Moreover, PAC1 binding is an epitope on the IIb-IIIa complex critical for fibrinogen receptor expression and cause secretion by stimulating arachidonic acid metabolism and thromboxane A2 synthesis.9,11

Recently, several murine monoclonal antibodies have been described that recognize antigenic determinants on the platelet surface, but only after platelet activation.9,12,13 For example, PAC1 is an IgM antibody that binds only to the activated form of the IIb–IIIa complex.9 It appears to recognize an epitope on the IIb–IIIa complex critical for fibrinogen binding. PAC1 and fibrinogen inhibit each other’s binding in a competitive manner.9,15 Moreover, PAC1 binding is competitively inhibited by the tetrapeptide, Arg-Gly-Asp-Ser, which is present in the α chain of fibrinogen and functions as a cell attachment site for fibrinogen and other adhesive macromolecules.16 PAC1 has been useful in identifying the intracellular mediators responsible for fibrinogen receptor expression in activated platelets.9,12 Two other "activation-dependent" antibodies, S12 and KC4, recognize a 140-kd α-granule membrane protein that becomes associated with the platelet surface during secretion.2,13,15,16 Radiolabeled S12 has been used to detect activated platelets when activated platelets were mixed with unstimulated platelets. In whole blood, unstimulated platelets demonstrated no PAC1- or S12-specific fluorescence, indicating that they did not bind these antibodies. On stimulation with agonists, however, the platelets demonstrated a dose-dependent increase in fluorescence similar to that observed for platelets in plasma or buffer. Low concentrations of ADP and epinephrine, which induce fibrinogen receptors but little secretion, stimulated near-maximal PAC1 binding but little S12 binding. On the other hand, a concentration of phorbol myristate acetate (TPA) that evokes full platelet aggregation and secretion induced maximal PAC1 and S12 binding. Activated platelets could also be analyzed in whole blood samples that had been fixed with paraformaldehyde. These studies demonstrate that activated platelets can be reliably detected in whole blood using activation-dependent monoclonal antibodies and flow cytometry. This technique may be useful to assess the degree of platelet activation and the efficacy of antiplatelet therapy in clinical disorders.

Changes occur at the platelet surface during cell activation. First, agonists bind to their membrane receptors and initiate the activation process. Then, for platelets to aggregate, heterodimer complexes of the membrane glycoproteins IIb and IIIa must be converted into competent receptors for fibrinogen.1 During platelet secretion, granule membranes fuse with the plasma membrane or with membranes of the surface-connected open canilicular system.2 The metabolic reactions responsible for these surface changes are only partly understood. In response to some agonists, such as thrombin, the exposure of fibrinogen receptors and the secretory response can occur as a result of agonist-induced phosphoinositide hydrolysis.4 On the other hand, weaker agonists such as epinephrine can stimulate fibrinogen receptor expression independent of phosphoinositide hydrolysis and cause secretion by stimulating arachidonic acid metabolism and thromboxane A2 synthesis.9,11

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their routine clinical use. For example, extreme care must be taken during blood sample collection and processing to prevent the in vitro release of platelet factor 4 and β-thromboglobulin.20,21

The purpose of the present study was to develop a sensitive and specific direct assay for activated platelets in whole blood. To accomplish this, we used the activation-specific monoclonal antibodies, PAC1 and S12. The binding of these antibodies to platelets was detected using the sensitive technique of flow cytometry, which is capable of detecting and quantitating antigens on the surface of individual platelets.23-26

METHODS

Preparation of Monoclonal Antibodies

PAC1, an IgM murine monoclonal antibody specific for the activated form of the platelet membrane glycoprotein IIb–IIIa complex, and A2a, an IgG antibody specific for the IIb–IIIa complex on resting or activated platelets, were purified as described previously.27 API, an IgG antibody specific for membrane glycoprotein IIb,27 was kindly provided by Dr Thomas Kunicki, Blood Center of Southeastern Wisconsin, and purified by sequential precipitation of non-IgG proteins using rivanol and caprylic acid (T. Kunicki, personal communication). S12, an IgG antibody specific for a 140-kd α-granule membrane protein,28,29 was generously provided in pure form by Dr Rodger McEver, University of Texas at San Antonio. Antibody preparations were pure as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Prior to their use in flow cytometry, these antibodies were conjugated either with biotin or with fluorescein isothiocyanate (FITC).

Antibodies were biotinylated by incubating 500 μg of antibody in 500 μL of phosphate-buffered saline (PBS), pH 7.4, for 2 hours at room temperature with 50 μL of 3.2 mmol/L of N-hydroxy succinimide biotin ester in dimethyl sulfoxide and 50 μL of 1 mol/L of NaHCO3. The reaction was stopped with 50 μL of 1 mol/L of NH4Cl for 10 minutes, and the mixture was passed over a Sephadex G-25 column equilibrated with PBS. The biotinylated protein appeared in the void volume and was stored at 4°C until use. Antibodies were conjugated to FITC by first adjusting the pH of the antibody solution (1 mg/mL) to 8.5 to 9 with one-tenth vol of 1 mol/L of NaHCO3. Then, 1 mg of FITC-Celite (Calbiochem, San Diego) was added per milligram of antibody and the mixture was incubated for 10 minutes at room temperature with intermittent shaking. The sample was applied to a Sephadex G-25 column equilibrated with PBS, and FITC-conjugated antibody was recovered in the void volume. The fluorescein/protein molar ratio ranged between 3 and 4 for API and S12 and 12 and 15 for PAC1. Biotin- and FITC-conjugated antibodies appeared to function normally in that their binding to platelets was saturable at antibody concentrations reported for the unconjugated species.30-32 In some experiments, PAC1 was radiolabeled with 111In to compare the binding of the radiolabeled form of the antibody33 with that of the biotinylated form.

Preparation of Whole Blood Samples for Flow Cytometric Analysis

Blood was obtained from normal volunteers who had taken no medications for at least 10 days. In an attempt to minimize platelet activation during blood collection, the donor was supine, and blood was obtained from an antecubital vein through a 19-gauge butterfly needle with either a light tourniquet or no tourniquet. After the first 2 mL of blood were discarded, 4.5 mL of blood was collected into a plastic syringe containing 0.5 mL of 3.8% sodium citrate. In some experiments, the platelets were then fixed with 1% paraformaldehyde as described by George and colleagues.34 Within 1 minute of the collection of fresh blood or 45 minutes after platelet fixation, 5-μL aliquots of the blood (containing 0.75 to 1.0 x 108 platelets) were added to 12 x 75-mm polystyrene tubes containing 50 μL of an isotonic HEPES buffer35 and 5 μL of a saturating concentration of biotin-PAC1 (30 μg/mL) or biotin-S12 (10 μg/mL). In some experiments, platelets were activated by including 5 μL of epinephrine, ADP, or phorbol myristate acetate (PMA) in the tubes. The samples were incubated at room temperature for 15 minutes without stirring. As described in detail below, platelets were analyzed by flow cytometry using either a single fluorochrome (the "one-color" method) or two fluorochromes (the "two-color" method). For the one-color method, after the incubation with the antibodies, a saturating concentration of FITC-streptavidin (5 μL of a 1:10 dilution; Amersham, Arlington Heights, IL) was added, and the tubes were incubated for an additional 15 minutes. The samples were then diluted with 500 μL of the isotonic buffer and analyzed. No washing steps were required. For the two-color method, one fluorescent reagent, FITC-AP1, was used to label and identify all the platelets while a second fluorescent reagent, phycoerythrin (PE)-streptavidin (Becton Dickinson, Mountain View, CA) was used to detect biotin-PAC1 or biotin-S12 bound to activated platelets. Specifically, after 15 minutes of incubation with the biotinylated antibody, 15 μL of PE-streptavidin and 2 μL FITC-AP1 was added for 15 minutes, and the sample was diluted and analyzed. In some studies, platelet-rich plasma (PRP) or gel-filtered platelets were prepared4 and then examined by flow cytometry as described above for whole blood.

Flow Cytometric Analysis

Blood samples were analyzed in a Becton Dickinson FACStar flow cytometer. The instrument was equipped with a 5 W argon laser and operated at 200 mW power at a wavelength of 488 nm. Fluorescein fluorescence was detected using a 530/30 band pass filter, and PE fluorescence was detected with a 585/42 filter. The instrument was calibrated for fluorescence and light scatter daily using 2-μm Calibre beads (Becton Dickinson). Blood samples were passed through the laser beam through a 70-μm nozzle at a flow rate of 10,000 blood cells per second. Light scatter and fluorescence data were obtained with gain settings in the logarithmic mode, and the data were analyzed on a Hewlett-Packard Consorit 30 H-P 217 computer (Palio Alto, CA).

For one-color analysis, the platelets were distinguished from erythrocytes and WBCs on the basis of their forward- and side-light scatter profile (described in the Results section). Debris or "machine noise" demonstrated a scatter profile distinctly smaller than that of platelets and was excluded from the analysis by setting the appropriate forward-scatter threshold. A gate was set around the platelets, and 10,000 cells were analyzed for FITC fluorescence to quantitate the amount of platelet-bound PAC1 or S12. Antibody binding was expressed either as the mean fluorescence intensity or as the percentage of platelets positive for antibody. Antibody-positive cells were defined as those platelets with a fluorescence intensity >99.9% to 99.5% of unstimulated platelets that had been prepared in the presence of 1 μmol/L of PGI2.

For two-color analysis, a fluorescence threshold was set to analyze only those blood cells that had bound FITC-AP1. Because erythrocytes and WBCs do not bind this platelet-specific antibody, they were effectively excluded from the analysis. Platelet-bound biotin-PAC1 or S12 was then determined by analyzing 10,000 platelets for PE-streptavidin fluorescence.
ACTIVATED PLATELETS AND FLOW CYTOMETRY

RESULTS

Detection of Activated Gel-Filtered Platelets With Monoclonal Antibodies

To explore the feasibility of using activation-dependent monoclonal antibodies to detect activated platelets by flow cytometry, initial studies were performed with gel-filtered platelets. For these studies, the one-color method of flow cytometric analysis, described in the Materials and Methods section, was used. The binding of biotin-PAC1 as assessed by flow cytometry was compared with the binding of 125I-PAC1 determined in a standard filtration binding assay. Gel-filtered platelets were incubated with PGI2 to prevent platelet activation or were stimulated with concentrations of ADP ranging from 0.01 to 5 μmol/L. Whether biotin-PAC1 binding was expressed as mean fluorescence intensity or as the percentage of platelets binding PAC1, there was a high correlation between binding detected by flow cytometry and that detected with 125I-PAC1 (r = .99; P < .001) (Fig 1). An advantage of flow cytometry over conventional binding assays is its ability to detect heterogeneity in platelet responsiveness to agonists. When platelets were incubated with biotin-PAC1 in the presence of 0.4 μmol/L of ADP, only a portion of the platelets demonstrated fluorescence above baseline (Fig 2). With a higher concentration of ADP (4 μmol/L), an increasing percentage of the platelets demonstrated positive fluorescence, although these cells varied greatly in the extent to which they had bound PAC1. At an even higher concentration of ADP (40 μmol/L), most platelets had bound maximal or near-maximal amounts of PAC1.

To see if flow cytometry could detect minor degrees of platelet activation, platelets were activated with 20 μmol/L of ADP and epinephrine and labeled with biotin-PAC1. These platelets were then mixed with various amounts of unactivated platelets that had been labeled with PAC1 in the presence of PGI2. Even when the final platelet mixture contained as few as 0.8% activated platelets, these could still be detected and differentiated from a control sample containing only unactivated platelets (Fig 3).

Fibrinogen is a competitive inhibitor of PAC1 binding to platelets. Because this is a potential problem with whole blood, in which plasma fibrinogen is present in large amounts, biotin-PAC1 binding to platelets was compared in plasma and buffer. When a saturating concentration of PAC1 was added simultaneously with an agonist such as ADP or phorbol ester, there was no difference in PAC1 binding between PRP and gel-filtered platelets over a range of agonist concentrations (not shown). This may be due to

Fig 1. Relationship between biotin-PAC1 binding and 125I-PAC1 binding to ADP-stimulated platelets. Gel-filtered platelets were incubated either with 1 μmol/L PGI2 to prevent platelet activation, or stimulated for 1 minute with various concentrations of ADP ranging from 0.01 to 5.0 μmol/L. They were then incubated with 125I-PAC1 (18 μg/mL), and the amount of PAC1 bound to the platelets was determined. In parallel, the platelets were incubated with biotin-PAC1, and PAC1 binding was quantitated by flow cytometry. Biotin-PAC1 binding was expressed either as the mean fluorescence intensity of stimulated platelets relative to PGI2-treated platelets (left panel) or as the percentage of cells exhibiting positive fluorescence (right panel). Data represent the mean of two experiments.

Fig 2. Identification of subpopulations of activated platelets by flow cytometry. Gel-filtered platelets were incubated for 15 minutes with biotin-PAC1 in the presence of no ADP (A), 0.4 μmol/L of ADP (B), 4 μmol/L of ADP (C) or 40 μmol/L of ADP (D). Then FITC-streptavidin was added, and the cells were analyzed for PAC1 binding by flow cytometry.
the greater apparent affinity of PAC1 ($K_d = 5$ nmol/L) than fibrinogen ($K_d = 250$ nmol/L) for the fibrinogen receptor. Another potential confounding effect of fibrinogen in whole blood assays is that the binding of this ligand is initially reversible but becomes progressively irreversible over the course of 60 minutes. Indeed, when biotin-PAC1 was added to PRP after stimulation of the platelets with an agonist, there was a time-dependent reduction in the mean fluorescence intensity of PAC1-positive cells, suggesting that fibrinogen had become irreversibly bound to a portion of the fibrinogen receptors (Fig 4). Despite this, there was no reduction in the percentage of platelets binding PAC1. This suggests that with sustained platelet activation, fibrinogen had become irreversibly bound to a fraction of the fibrinogen receptors on each platelet (Fig 4).

**Detection of Activated Platelets in Whole Blood With Monoclonal Antibodies**

*Flow cytometric analysis of whole blood using the one-color method.* Having demonstrated the feasibility of using PAC1 to detect activated platelets after gel filtration, we asked whether similar studies could be carried out in whole blood. To be successful, whole blood analysis would have to include all platelets but exclude all erythrocytes and WBCs. Two different methods were evaluated to accomplish this. In the one-color method, platelets were identified on the basis of their light-scatter profile (Fig 5A). The forward- and side-scatter profiles of platelets could be clearly separated from the larger erythrocytes and WBCs, and a gate could be set around the platelets for analysis. The light-scatter profile of platelets was not affected by the presence of platelet agonists. Studies with gel-filtered platelets and washed erythrocytes confirmed their separation by this method. Approximately 0.5% of gel-filtered platelets appeared outside the platelet gate, whereas no more than 0.3% of erythrocytes appeared inside this gate. Thus, assuming a ratio of 20:1 of erythrocytes to platelets in whole blood, at most only 6% of the particles in the platelet gate could have been accounted for by erythrocytes. This agrees well with the observation that ~95% of the cells from whole blood appearing within the platelet gate bound biotin-AP1 or biotin-A2A9, antibodies specific for platelet membrane glycoproteins Ib and IIb–IIIa, respectively.

*Fig 3.* Detection of activated platelets when mixed with unstimulated platelets. Gel-filtered platelets were stimulated with a combination of 20 μmol/L of ADP and epinephrine and then incubated with biotin-PAC1. The sample contained an average of 85% PAC1-positive platelets. Small but increasing amounts of the sample were then added to a sample of unstimulated, PGI2-treated platelets, and the final mixtures were analyzed for PAC1 binding by flow cytometry. The scale on the ordinate ranges from 0% to 10% positive platelets. All mixtures containing stimulated platelets showed more PAC1-positive cells than did the PGI2 control ($P \leq .01$). Data represent the mean ± SEM of four experiments.

*Fig 4.* Effect of duration of platelet activation on subsequent binding of biotin-PAC1 to platelets in plasma. Platelet-rich plasma was incubated with 0.2 μmol/L of phorbol myristate acetate (TPA). At the indicated times, biotin-PAC1 was added to the sample, and PAC1 was binding determined by one-color flow cytometry. The percentage of PAC1-positive cells was not affected by the prolonged incubation of activated platelets with fibrinogen, whereas the amount of PAC1 bound per cell (mean fluorescence intensity) did decrease with time.

By flow cytometry, the fluorescence of these platelets was compared with that of platelets incubated with PGI2 to prevent activation. Resting platelets demonstrated minimal fluorescence, similar to control platelets that had not been incubated with antibody. In contrast, platelets stimulated with ADP and epinephrine demonstrated a marked increase in fluorescence.

*Flow cytometric analysis of whole blood using the two-color method.* Although significant overlap of the platelets with erythrocytes was not a problem in the one-color studies, such overlap may conceivably exist when a different flow cytometer is used or if blood cell light-scatter profiles are abnormal in disease states. Therefore, we evaluated a different approach in which a fluorescent platelet-specific marker, FITC-AP1, was used to identify the platelets in whole blood. Whole blood was incubated first with biotin-PAC1 and then with PE-streptavidin and FITC-AP1. Having bound FITC-AP1, the platelets now exhibited a strong green fluorescence. The machine threshold was then set to exclude from analysis cells that did not exhibit this degree of
green fluorescence (erythrocytes and WBCs), and the platelets were analyzed for biotin-PAC1 binding with PE-streptavidin. Preliminary studies showed that the binding of PAC1 or S12 to platelets did not affect the binding of API, nor did API interfere with the binding of the other antibodies. The light-scatter profile for API-positive platelets in whole blood showed that ~95% of the cells were in an area expected for single platelets, whereas 5% of the cells demonstrated increased forward- and side-light scatter (Fig 6A).

Separate studies using isolated WBCs and leukocyte-specific antibodies demonstrated that this latter area also contained granulocytes and monocytes (not shown). Thus, since API does not bind to WBCs, this minor population of API-positive cells probably represents platelets associated with WBCs. It is unlikely that this population represents platelet aggregates since we found no increase in the percentage of platelets in this scatter area when either platelets in whole blood or gel-filtered platelets were stimulated with agonists.

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**Fig 5.** Example of the one-color method of flow cytometric analysis of activated platelets. As described in the Materials and Methods Section, 5 μL of whole blood were incubated with biotin-PAC1 and with either 1 μmol/L of PGI2 to maintain the platelets in a resting state or with 20 μmol/L of ADP plus epinephrine to expose fibrinogen receptors. Then FITC-streptavidin was added, and the sample was analyzed by flow cytometry. The light scatter data are depicted over 4 log decades (A). The platelets appeared to be separated from erythrocytes and WBCs on the basis of their forward- and side-light scatter, and a gate was placed around the platelets for analysis. For each sample, the cytometer analyzed 10,000 cells within the platelet gate; a histogram of FITC ("green") fluorescence of these cells is shown (B).

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**Fig 6.** Example of the two-color method of flow cytometric analysis of activated platelets. Whole blood was incubated with biotin-PAC1 and either PGI2 or ADP plus epinephrine. Then phycoerythrin-streptavidin and FITC-API were added, and the samples were analyzed by flow cytometry. Light-scatter profile of API-positive cells (A). These platelets exhibited two separate light-scatter populations. About 95% of the cells appeared to be single platelets, whereas 5% of the platelets exhibited greater light scatter. Once the platelets were identified by their API binding, biotin-PAC1 binding was quantitated by analyzing the platelets for phycoerythrin ("red") fluorescence (B).
When the API-positive cells were analyzed for biotin-PAC1 binding, activated platelets showed increased PE fluorescence (Fig 6B). There was no difference in the degree of PAC1 binding between platelets in the major and minor light-scatter profiles. On the other hand, platelet stimulation with epinephrine, ADP, or phorbol ester did not cause significant changes in API binding. In the present studies, the one-color and two-color methods of platelet analysis yielded similar results. Therefore, in the experiments presented below, these methods were used interchangeably.

Effect of platelet agonists on PAC1 and S12 binding. When whole blood was incubated with platelet agonists, the binding of biotin-PAC1 was dependent on the concentration of the agonist (Fig 7). Dose–response curves for epinephrine, ADP, and TPA in whole blood were essentially identical to the corresponding dose–response curves for platelets in plasma or buffer. Moreover, PAC1 binding in response to a submaximal concentration of ADP was augmented by the addition of a low concentration of epinephrine (Fig 7).

S12 is an antibody that recognizes a 140-kd α-granule membrane protein which fuses with the platelet plasma membrane during secretion. The binding of saturating concentrations of biotin-S12 was compared with the binding of biotin-PAC1 to platelets in whole blood. Platelets activated with ADP or ADP plus epinephrine demonstrated binding of both antibodies. At a given concentration of agonist, however, there was more PAC1 than S12 binding (Fig 8). This was expected because unstirred platelets stimulated with weak agonists undergo minimal secretion yet are capable of expressing most of their fibrinogen receptors. In contrast, platelets stimulated maximally with TPA bound maximal amounts of PAC1 and S12 (Fig 8).

Stability of platelets in whole blood. The above studies were carried out using whole blood that had been processed within 1 minute of venipuncture. If the testing of clinical samples is to become feasible, however, it is essential that platelets not become activated spontaneously before analysis. Therefore, we examined the stability of both resting and stimulated platelets during longer periods of incubation. Whole blood containing 0.38% sodium citrate was placed in a capped polypropylene tube. At various times up to 60 minutes aliquots of the blood were incubated with biotin-PAC1 or biotin S12 and then evaluated by flow cytometry. If the blood was allowed to stand for >10 minutes before the addition of antibody, there was a small but perceptible increase in the percentage of platelets binding either PAC1 or S12 (Fig 8). Once either antibody was added to resting or stimulated platelets, however, the platelets maintained their initial degree of platelet activation for up to 2 hours (not shown). Thus, if unfixed blood is to be analyzed and spontaneous platelet activation avoided, the blood specimen should be processed within 10 minutes and analyzed within 2 hours of collection.

It may be necessary to study blood samples from patients hours or days after blood collection. Therefore, the effect of fixing whole blood with 1% paraformaldehyde was examined. Whole blood was fixed as described by George and colleagues, either within seconds of venipuncture or 15 minutes after activation with phorbol ester. Then biotin-PAC1 or S12 was added. Although fixation of blood before the addition of the antibodies was successful in preventing the small time-dependent spontaneous activation of platelets that had been observed with unstimulated and fixed platelets, this procedure resulted in a significant reduction in PAC1 and S12 binding to stimulated cells (Fig 10). In contrast, when the blood was sequentially stimulated with an agonist, incubated with the antibodies and then fixed, PAC1 and S12 binding was similar to that observed for binding to stimulated, unfixed platelets (Fig 10). Furthermore, the amount of antibody bound to platelets fixed after antibody addition was stable for at least 24 hours. Similar results with
0.38% sodium citrate was either processed immediately for flow cytometry or allowed to stand in a capped polypropylene tube at room temperature for periods of 0 up to 60 minutes. At the indicated times, aliquots of blood were incubated with saturating concentrations of biotin-PAC1 (A) or biotin-S12 (B), and antibody binding was determined by two-color flow cytometry. The scale on the ordinates range from 0% to only 2% positive platelets. The P values compare the data at each time point with that obtained at zero time on platelets incubated with PGI₂ (solid bars). The data represent the mean ± SEM of platelets from ten normal donors.

**DISCUSSION**

To function during hemostasis, platelets must become activated. Platelets may also become activated in certain pathologic conditions and participate in the process of thrombogenesis. In the present studies, we sought to develop a direct method to detect activated platelets in whole blood specimens immediately after venipuncture and without the need to centrifuge or wash the specimen. We took advantage of the availability of PAC1 and S12, two monoclonal antibodies that bind specifically to activated but not to resting platelets. PAC1 detects a change in the glycoprotein IIb-IIIa complex occurring during platelet activation that converts the complex into a competent fibrinogen receptor. S12 detects an α-granule membrane protein that becomes exposed on the platelet surface coincident with granule secretion. The binding of these antibodies to activated platelets was detected by flow cytometry. The antibodies were conjugated with biotin, and binding was detected with streptavidin conjugated either with fluorescein or PE. Platelets activated with agonists in vitro bound the fluorescently tagged antibodies and were distinguishable from resting platelets on the basis of their fluorescence intensity. The amount of platelet-bound antibody measured by flow cytometry correlated strongly with the amount of bound 125I-antibody measured in a standard filtration assay. Antibody binding to platelets in whole blood was dependent on the agonist concentration, and the dose-response curves were similar to those for platelets in plasma or buffer. Furthermore, mixing studies showed that if as little as 0.8% activated platelets was present in the platelet sample, they could be detected by flow cytometry. Thus, the method described appears to be a sensitive and direct one for the detection of activated platelets.

Precise analysis of platelets in whole blood requires that these cells be clearly differentiated from erythrocytes and WBCs by the flow cytometer. Two different protocols were established for this purpose. In the one-color method, platelets were identified by their light-scatter profile. Under the conditions used, there was minimal overlap between platelets and the larger blood cells so that only ~5% of the cells in the platelet scatter area could be accounted for by erythrocytes. Therefore, for platelet analysis, a gate was set around this scatter area, and antibody binding was determined by quantitating platelet fluorescence. When platelets were stimulated by an agonist but not stirred, the activated platelets exhibited the same forward- and side-scatter profiles as...
resting platelets. Although the one-color method was acceptable for the present studies, it has potential disadvantages. First, it may not be possible to so clearly differentiate platelets from erythrocytes on the basis of light scatter utilizing other flow cytometers. Second, blood in this study was obtained from normal volunteers. Platelets may show more light scatter overlap with erythrocytes in certain disease states. Third, we found that >5% of the platelets in whole blood demonstrated a light-scatter profile similar to that of WBCs, consistent with the report of Jennings and colleagues that platelets can be loosely associated with granulocytes or monocytes. This minor population of platelets would not be analyzed by the one-color method. Although the percentage of platelets apparently associated with WBCs did not change with platelet activation, this may not be the case in disease states. To overcome these potential drawbacks, we evaluated a different two-color method of analysis that did not depend on light scatter to identify platelets.

In the two-color method, platelets were labeled with biotin-PAC1 or S12 and then identified with the platelet-specific antibody, FITC-AP1, which recognizes platelet membrane glycoprotein Ib. The cytometer threshold was set to analyze only FITC-AP1-positive cells, thereby eliminating erythrocytes and WBCs from the analysis. Biotin-PAC1 or S12 binding was then detected with PE-streptavidin, and activated platelets were identified by their PE fluorescence. When using this method to differentiate platelets from other blood cells, it is important to use an antibody to detect platelets whose binding does not change significantly with platelet activation. Although AP1 binding decreases during thrombin-induced platelet activation, we did not observe sufficient changes in AP1 binding with ADP, epinephrine, or phorbol ester to preclude its use as a platelet marker for two-color analysis.

Several variations of this two-color method could increase its range of usefulness for specific applications. For example, the activation-dependent antibodies can be directly labeled with FITC, and the AP1 could be labeled with biotin. Indeed, S12 has been simultaneously labeled with FITC and 125I to correlate directly the antibody binding observed in flow cytometric and radioisotopic assays. It should also be possible to label PAC1 with FITC and S12 with biotin and evaluate the binding of these two activation-dependent antibodies simultaneously.

Whole blood flow cytometry for the study of activated platelets has several advantages. First, only microliter volumes of blood are required, making it possible to study infants or individuals with thrombocytopenia. Second, as illustrated in Fig 5, platelet subpopulations that are heterogeneous in their platelet response to stimulation can be detected, which is not possible with methods that rely on 125I-antibody binding or on measurements of released platelet factor 4 or β-thromboglobulin. Third, the use of whole blood eliminates the need for centrifugation or washing. These procedures can result in platelet activation. No method of ex vivo analysis, however, will prevent activation of platelets that may take place during a traumatic or prolonged venipuncture. Thus, close attention must be paid to the details of venipuncture. In this study, we attempted to minimize activation by drawing blood from the antecubital vein with the subject supine, using little or no tourniquet, and discarding the initial 2 mL of blood.

The stability of platelets in vitro is a major variable in these types of studies. We found that spontaneous activation of platelets did not occur if PAC1 or S12 was added to the blood sample within 10 minutes of venipuncture. A small but detectable increase in PAC1 or S12 binding was observed, however, if blood was allowed to stand for longer periods of time before the addition of antibody. Therefore, if a direct assay for activated platelets is to gain wider use, it may be desirable to fix the blood sample after venipuncture. Fixation of the blood with 1% paraformaldehyde immediately after venipuncture prevents spontaneous platelet activation. If platelets were stimulated with an agonist and then fixed, however, antibody binding to the fixed platelets was significantly reduced as compared with unfixed platelets. This problem was circumvented by adding the paraformaldehyde after the antibody. The effects of platelet fixation for each antibody used must be carefully evaluated.

Whether the direct analysis of platelets will be useful in detecting and quantitating the degree of platelet activation in clinical disorders remains to be established. Based on studies with plasma platelet factor 4, β-thromboglobulin, and the plasma and urinary metabolites of thromboxane A2, it appears that platelet activation can accompany unstable angina, adult respiratory distress syndrome, cardiopulmonary bypass, and renal failure. In addition, it is conceivable that activated platelets circulate in other disorders, such as thrombotic thrombocytopenic purpura. Even if a fraction of the platelets activated in these conditions continued to circulate in the blood, they might be detectable using activation-dependent monoclonal antibodies. Indeed, George and co-workers, using 125I-S12, found activated platelets in 46% of patients with adult respiratory distress syndrome but in only 11% of patients undergoing cardiopulmonary bypass. Although this may indicate that the mechanism of platelet activation varies in different clinical disorders, the results may also be accounted for by the detection limits of the assay. The present studies suggest that assay sensitivity may be increased by using flow cytometry rather than conventional radioligand binding methods and by using a panel of activation-dependent monoclonal antibodies. Nonetheless, careful studies will be required to establish the sensitivity and specificity of the flow cytometry technique and its clinical value in detecting activated platelets in humans.

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Detection of activated platelets in whole blood using activation-dependent monoclonal antibodies and flow cytometry

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