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REVIEW ARTICLE

Flow Cytometry: A Clinical Test of Platelet Function

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SINCE PLATELETS were first identified in 1881,[1] there has been continuous and recently accelerating progress in our basic understanding of platelet function.[2,3] Despite this great progress and the fact that platelets are so readily accessible for study compared with noncirculating human cells, there is a remarkable paucity of clinically useful tests of platelet function. Tests of platelet function have included measurements of the ability of platelets to adhere, aggregate, and/or activate. The only standard clinical tests of platelet function are the bleeding time and platelet aggregometry, both of which have major limitations.[4-6] Other tests used to study platelet function in clinical research settings include plasma assays for platelet factor 4, β-thromboglobulin, and soluble P-selectin,[7-9] plasma and urine assays for thromboxane A_2 metabolites,[10,11] and the Wu and Hoak method for the detection of circulating platelet aggregates.[12]

Platelet hyperreactivity and/or circulating activated platelets have been reported to be associated with many common clinical settings, including unstable angina,[10] acute myocardial infarction,[13] angioplasty,[14] cardiopulmonary bypass,[15] stroke,[16] transient ischemic attacks,[12] diabetes mellitus,[17] cigarette smoking,[18] hyperlipoproteinemia,[19] emotional stress,[20] strenuous exercise,[21] and blood bank storage of platelets for transfusion.[22] However, in many of these clinical settings, the role of platelet function testing is controversial, in part because the methods used to detect platelet function (eg, platelet aggregation and radioimmunoassays of plasma β-thromboglobulin and/or platelet factor 4) have such significant methodologic problems.[5-8]

Recently, there have been an increasing number of published studies that have attempted to use whole blood flow cytometry[23] as a platelet function test in clinical settings. Although there are many advantages to this method, methodologic and practical issues remain. This review will focus on the current and potential role of flow cytometry as a clinical test of platelet function.

PRINCIPLES OF FLOW CYTOMETRY

Flow cytometry rapidly measures the specific characteristics of a large number of individual cells. Before flow cytometric analysis, cells in suspension are fluorescently labeled, typically with a fluorescently conjugated monoclonal antibody (MoAb). In the flow cytometer, the suspended cells pass through a flow chamber and, at a rate of 1,000 to 10,000 cells per minute, through the focused beam of a laser. After fluorescent activation of the fluorophore at the excitation wavelength, a detector processes the emitted fluorescence and light scattering properties of each cell. (Givan[24] provides a very readable overview of the principles of flow cytometry.)

Clinical studies that use flow cytometric assays of washed platelets or platelet-rich plasma[25,26] are, like other assays of platelet function, potentially susceptible to artifactual in vitro platelet activation as a result of the obligatory separation procedures. The introduction of whole blood flow cytometry by Shattil et al[23] was therefore a major step towards the application of flow cytometry to clinical settings.

In the absence of an added exogenous platelet agonist, whole blood flow cytometry determines the activation state of circulating platelets, as judged by the binding of an activation-dependent MoAb. In addition to this assessment of platelet function in vivo, inclusion of an exogenous agonist in the assay enables analysis of the reactivity of circulating platelets in vitro. In the latter application, whole blood flow cytometry is a physiologic assay of platelet function in that an agonist results in a specific functional response by the platelets, ie, a change in the surface expression of a physiologic receptor (or other antigen or bound ligand), as determined by a change in the binding of an MoAb.

A typical schema of sample preparation for whole blood flow cytometry is shown in Table 1. The anticoagulant is usually buffered sodium citrate. The purpose of the dilution is to minimize the formation of platelet aggregates (see below). The biotinylated test MoAb should be added in a saturating concentration. Fixation is typically with a final concentration of 1% paraformaldehyde. The biotinylated test MoAb can be added after fixation, provided that fixation does not interfere with antibody binding (Michelson et al[27] and see below). The fluorescein isothiocyanate (FITC)-con-

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Table 1. Typical Schema of Sample Preparation for Whole Blood Flow Cytometry

Blood
↓
Anticoagulant
↓
Dilution
↓
Biotinylated test MoAb
↓
Agonist or buffer
↓
Fixation
↓
FITC-conjugated MoAb (platelet identifier)
↓
Phycoerythrin-streptavidin
↓
Dilution

jugated platelet identifier MoAb (eg, glycoprotein [GP] Ib-, IIb-, or IIIa-specific) should be added at a near saturating concentration. If a directly phycoerythrin-conjugated (rather than a biotinylated) test MoAb is used, the addition of phycoerythrin-streptavidin is not required.

Samples are then analyzed in a flow cytometer. After identification of platelets by both fluorescein positivity and their characteristic light scatter, binding of the biotinylated or phycoerythrin-conjugated test MoAb is determined by analyzing 5,000 to 10,000 individual platelets for phycoerythrin fluorescence. Background binding obtained from parallel samples with biotinylated or phycoerythrin-conjugated isotypic species-specific Ig is subtracted from each test sample. The assay can also be performed with a biotinylated or phycoerythrin-conjugated MoAb as the platelet identifier and an FITC-conjugated MoAb as the test antibody.

For specific methodologic protocols of whole blood flow cytometric assays of platelet function, the reader is referred to Michelson and Shattil.[28]

MoAbs

MoAbs can be used in the flow cytometric assay to measure the expression of any platelet surface antigen. However, there has been particular interest in the use of MoAbs that are activation-dependent, ie, antibodies that bind only to activated platelets but not to resting platelets (Table 2). The two most widely studied types of activation-dependent MoAbs are those directed against conformational changes in the GPIIb-IIIa complex and those directed against granule membrane proteins (Table 2).

The GPIIb-IIIa complex (CD41/61) is a receptor for fibrinogen, von Willebrand factor, fibronectin, and vitronectin that is essential for platelet aggregation.[46] Whereas most MoAbs directed against the GPIIb-IIIa complex bind to resting platelets, MoAb PAC1 is directed against the fibrinogen binding site exposed by a conformational change in the GPIIb-IIIa complex of activated platelets.[29] Thus, PAC1 only binds to activated platelets, not to resting platelets. Whereas native PAC1 is an IgM, a recombinant F_{ab} fragment of PAC1 produced in a baculovirus expression system also binds to platelets in an activation-dependent manner.[47] Other GPIIb-IIIa–specific activation-dependent MoAbs are directed against either ligand-induced conformational changes in the GPIIb-IIIa complex (ligand-induced binding sites [LIBS])[30-32] or receptor-induced conformational changes in the bound ligand (fibrinogen; receptor-induced binding sites [RIBS][33-35]; Table 2). Rather than GPIIb-IIIa–specific MoAbs, fluorescein-conjugated fibrinogen can also be used in flow cytometric assays to detect the activated form of the platelet surface GPIIb-IIIa complex.[48,49]

The most widely studied type of activation-dependent MoAbs directed against granule membrane proteins are those directed against P-selectin (also known as CD62P,[50] and previously referred to as GMP-140[36] or PADGEM protein[51]). P-selectin mediates adhesion of activated platelets to neutrophils and monocytes.[37,52,53] P-selectin is a component of the α granule membrane of resting platelets that is only expressed on the platelet surface membrane after α granule secretion.[36,51,54] Therefore a P-selectin–specific MoAb only binds to degranulated platelets and not to resting platelets. However, a recent report[55] showed that in vivo circulating degranulated platelets rapidly lose their surface P-selectin, but continue to circulate and function. This report[55] therefore suggests that platelet surface P-selectin is not an ideal marker for the detection of circulating degranulated platelets, unless (1) the blood sample is drawn immediately distal to the site of platelet activation, (2) the blood sample is drawn within 5 minutes of the activating stimulus, or (3) there is continuous

Table 2. Activation-Dependent MoAbs, ie, Antibodies That Bind to Activated But Not Resting Platelets

Activation-Dependent Surface Change	Prototypic Antibodies	References
Changes in GPIIb-IIIa		
Activation-induced conformational change in GPIIb-IIIa resulting in exposure of the fibrinogen binding site	PAC1	29
Ligand-induced conformational change in GPIIb-IIIa	PM 1.1; LIBS1; LIBS6	30-32
Receptor-induced conformational change in bound ligand (fibrinogen)	2G5; 9F9; F26	33-35
Exposure of granule membrane proteins		
P-selectin (α-granules)	S12; AC1.2	36, 37
CD63 (lysosomes)	CLB-gran/12	38
LAMP-1 (lysosomes)	H5G11	39
Binding of secreted platelet proteins		
Thrombospondin	P8; TSP-1	40, 41
Multimerin	JS-1	42, 43
Development of a procoagulant surface		
Factor Va binding	V237	44
Factor VIII binding	1B3	45

Modified with permission of Oxford University Press from Michelson and Shattil.[28]

Table 3. Advantages of Whole Blood Flow Cytometry for the Study of Platelet Activation

(1) Platelet activation can be studied in the more physiological milieu of whole blood.
(2) Minimal manipulation of samples prevents artifactual in vitro platelet activation and potential loss of platelet subpopulations.
(3) Both the activation state of circulating platelets and the reactivity of circulating platelets can be determined.
(4) Activation-dependent changes in multiple surface receptors can be detected.
(5) New MoAbs directed against novel functional epitopes can easily be incorporated into the assay.
(6) High degree of sensitivity for the detection of platelet subpopulations.
(7) Only ~2 μL of blood required.
(8) The platelets of patients with profound thrombocytopenia can be accurately analyzed.
(9) Platelet activation by thrombin can be directly measured in whole blood through the use of the peptide Gly-Pro-Arg-Pro.
(10) No radioactivity.

activation of platelets. The length of time that other activation-dependent surface markers remain expressed on the platelet surface in vivo has not yet been determined.

In addition to MoAbs that bind only to activated platelets (Table 2), some investigators have used MoAbs that bind to resting platelets but have increased binding to activated platelets, eg, GPIV (CD36)-specific MoAbs.[56,57]

The GPIb-IX-V complex (CD42) is a receptor for von Willebrand factor that is critical for platelet adhesion to damaged blood vessel walls.[58] In contrast to activation-dependent MoAbs (Table 2) and MoAbs that bind to resting platelets but have increased binding to activated platelets, the binding of GPIb-IX-V–specific MoAbs to activated platelets is markedly decreased compared with resting platelets.[59-62] The activation-induced decrease in the platelet surface expression of the GPIb-IX-V complex appears to be the result of a translocation of GPIb-IX-V complexes to the membranes of the surface-connected canalicular system,[62,63] except for the thrombin-induced proteolysis of platelet surface GPV.[62] The activation-dependent decrease in the binding of MoAbs to the platelet GPIb-IX-V complex may be a sensitive marker of platelet activation in vivo. For example, as determined by whole blood flow cytometry, strenuous exercise in sedentary subjects, but not physically active subjects, results in both platelet activation and platelet hyperreactivity.[57] However, these changes were more readily detected with MoAbs directed against GPIb and, to a lesser extent, GPIV than with MoAbs directed against the GPIIb-IIIa complex and P-selectin.[57] These exercise-induced changes in platelet surface glycoproteins may have been the result of plasmin generation, catecholamine release, and/or shear stress from a hyperdynamic circulation. This study[57] also illustrates the importance of analyzing activation-dependent changes with a panel of MoAbs directed against different platelet surface antigens.

ADVANTAGES OF FLOW CYTOMETRY

Other tests used to study platelet function in clinical settings have limitations. Platelet aggregometry may show whether a particular clinical condition results in changes in platelet reactivity, but cannot determine whether the condition directly activates platelets.[5] In contrast, plasma assays of β-thromboglobulin,[7,8] platelet factor 4,[7,8] and soluble P-selectin,[9] as well as plasma and urinary assays of thromboxane A_2 metabolites,[10,11] may indirectly determine that a clinical condition activates platelets, but cannot measure changes in platelet reactivity associated with the condition. None of these assays can measure the extent of activation of individual platelets or detect distinct subpopulations of platelets. Platelet aggregation studies are semiquantitative and subject to standardization problems.[5,6] As a result of the plasma separation procedures required, radioimmunoassays of plasma β-thromboglobulin and platelet factor 4 concentrations are particularly vulnerable to artifactual in vitro platelet activation.[7,8] Furthermore, soluble P-selectin in plasma may be of endothelial origin.[9]

Whole blood flow cytometric assays of platelet activation have none of these limitations. The advantages of whole blood flow cytometry are summarized in Table 3. Platelets are directly analyzed in their physiologic milieu of whole blood (including red blood cells and white blood cells, both of which affect platelet activation[64,65]). The minimal manipulation of the samples prevents artifactual in vitro activation and potential loss of platelet subpopulations.[23,61,66,67] Both the activation state of circulating platelets and the reactivity of circulating platelets can be determined. The flow cytometric method permits the detection of a spectrum of specific activation-dependent modifications in the platelet surface membrane. Furthermore, as new MoAbs directed against novel functional epitopes are developed, they can easily be incorporated into the assay. A subpopulation of as few as 1% partially activated platelets can be detected by whole blood flow cytometry.[57] Only minuscule volumes (~2 μL) of blood are required,[23,61] making whole blood flow cytometry particularly advantageous for neonatal studies.[68] The platelets of patients with profound thrombocytopenia can also be accurately analyzed. Unlike radioimmunoassays for plasma β-thromboglobulin and platelet factor 4, flow cytometric assays do not involve radioactivity.

Platelet activation by thrombin, one of the most physiologically important platelet activators,[69-71] can be directly measured in whole blood through the use of the synthetic tetrapeptide glycyl-L-prolyl-L-arginyl-L-proline (GPRP).[57,61,67] In the absence of GPRP, the addition of thrombin to whole blood results in a fibrin clot, thereby precluding the use of thrombin as an agonist in the whole blood assay. Furthermore, thrombin is a potent inducer of platelet-to-platelet aggregation, which precludes analysis by flow cytometry of activation-dependent changes in individual platelets. However, the addition to whole blood of GPRP together with thrombin inhibits both fibrin polymerization and platelet-to-platelet aggregation, without affecting thrombin-induced platelet activation.[57,61,67] An alternative to the use of thrombin and GPRP in the whole blood flow cytometric assay is the use of a thrombin receptor activating peptide (TRAP; a peptide fragment of the tethered ligand receptor for thrombin[72]). Without the need for GPRP, TRAP directly activates platelets in whole blood without resulting in a fibrin clot.[62] How-

Table 4. Methods of Sample Preparation for Whole Blood Flow Cytometry That Minimize the Formation of Platelet Aggregates

Most important:
Smooth, easy flow from blood draw.
Discard the first 2 mL of blood.
Polypropylene (or siliconized glass) tubes or syringes.
Immediate mixture with anticoagulant.
No washing, centrifugation, gel filtration, vortexing, or stirring steps.
Mix gently after addition of agonist, then incubate undisturbed.
Reduce the platelet count by dilution of the samples.
Other measures:
Prepare reagents in advance and avoid delays in procedure.
To collect blood, use a light tourniquet and a needle of not less than 21 gauge.
Fixation.
Optional:
If thrombin is the agonist, inclusion in the assay of the peptide GPRP.

ever, TRAP may not reflect all aspects of thrombin-induced platelet activation, because the tethered ligand receptor may not be the only platelet receptor for thrombin.[73]

DISADVANTAGES OF FLOW CYTOMETRY

There are disadvantages to flow cytometry. First, flow cytometers are expensive instruments to purchase and maintain. Second, for a clinical assay, sample preparation is quite complicated and requires a dedicated operator, although the future development of automated systems and more user-friendly software should simplify the assay. Third, to avoid ex vivo platelet activation, blood samples should be processed within approximately 45 minutes of drawing.[23] (For some MoAbs, this problem can be circumvented by immediate fixation; see Methodologic Issues: Fixation below.) Fourth, flow cytometry only measures the function of circulating platelets, whereas plasma assays of β-thromboglobulin and platelet factor 4 and plasma and urinary assays of thromboxane A_2 metabolites also reflect platelet activation at the blood vessel wall and recently cleared platelets. Thus, if the activated platelets are rapidly cleared or are adherent to blood vessel walls or to extracorporeal circuits, flow cytometry may not detect evidence of platelet activation. For example, during cardiopulmonary bypass, flow cytometry shows only modest evidence of circulating activated platelets,[34,74] whereas radioimmunoassays of plasma β-thromboglobulin and platelet factor 4 consistently provide evidence of marked activation of platelets.[15,75]

METHODOLOGICAL ISSUES

Blood drawing. In our hands, collection of blood into a sodium citrate Vacutainer (Becton Dickinson, Rutherford, NJ) does not result in platelet activation.[74] However, each laboratory should determine whether their method of collection, including the drawing of samples through angioplasty and other catheters, results in artifactual in vitro platelet activation, as determined by the binding of activation-dependent MoAbs.

Minimizing platelet aggregates. Platelet aggregates can be measured by flow cytometry.[76] However, if the platelets are aggregated, the amount of antigen per platelet cannot be determined by flow cytometry. This is because flow cytometry measures the amount of fluorescence per individual particle, irrespective of whether the particle is a single platelet or an aggregate of an unknown number of platelets. Platelet aggregates can be minimized in the preparation of platelets for whole blood flow cytometry by a combination of the methods shown in Table 4. Each sample should be monitored for evidence of platelet aggregation (smearing of the platelets into the upper right quadrant of the log forward light scatter versus log orthogonal light scatter histogram).

Fixation. Fixation is very advantageous in a clinical setting in which there may not be immediate access to a flow cytometer. Fixation prevents subsequent artifactual in vitro platelet activation. The antibody labeling before fixation method described above results in no significant differences in fluorescence intensity between samples analyzed immediately and samples analyzed within 24 hours of fixation.[23] A fixation before antibody labeling method also results in no significant differences in fluorescence intensity between samples analyzed immediately and samples analyzed within 24 hours of antibody tagging.[74] However, fixation is an important variable to be controlled for, especially in a fixation before antibody labeling method, because the binding of activation-dependent MoAbs to fixed platelets is often decreased compared with unfixed platelets.[27] Furthermore, the binding of some antibodies further decreases after fixation in a time-dependent manner (Massie and Michelson, unpublished observations). The optimal fixation method for each new MoAb must therefore be defined by each laboratory.

An argument in favor of immediate fixation is that activation-dependent changes are time-dependent, at least in vitro. For example, the platelet surface expression of the GPIb-IX-V complex decreases within 30 seconds of platelet activation, reaching a nadir at approximately 5 minutes, but over the next approximately 45 minutes the platelet surface expression of the GPIb-IX-V complex returns to normal.[62,77] The activation-dependent increase in the platelet surface expression of the GPIIb-IIIa complex is also reversible with time,[78] although the activation-dependent increase in platelet surface P-selectin is not.[77,78]

Choice of MoAbs. Because different epitopes reflect different aspects of platelet activation, it is preferable to use a panel of MoAbs (see above). Because it may vary in different clinical settings, the appropriate panel can only be determined by studies in each clinical setting. In any event, use of a panel of MoAbs may distinguish specific activation profiles that will expand our understanding of pathologic platelet-platelet, platelet-leukocyte, and platelet-endothelial cell interactions.

MoAbs are preferable to polyclonal antibodies in whole blood flow cytometry, because they (1) can more reliably saturate all specific epitopes and (2) result in less nonspecific binding. Platelet-specific MoAbs are now available from a number of commercial sources and can often be purchased already conjugated to FITC, biotin, phycoerythrin, or a tandem conjugate (eg, phycoerythrin-Cy5 or phycoerythrin-

Texas Red). Alternatively, antibodies can be FITC-conjugated by the method of Rinderknecht[79] or (easier and more rapidly) by a kit method, eg, QuickTag FITC conjugation kit (Boehringer Mannheim, Indianapolis, IN). Antibodies can be biotinylated as described by Shattil et al[23] or by following the biotin manufacturer's directions. The use of antibodies that are directly conjugated with FITC, biotin, phycoerythrin, or tandem conjugates eliminates the requirement for the addition of secondary antibodies, thereby avoiding time-consuming washing procedures that, in unfixed samples, may result in artifactual in vitro activation of platelets. Furthermore, the use of secondary antibodies is likely to result in increased background fluorescence and decreased sensitivity of the assay.

F_{ab} fragments of MoAbs can be used to avoid (1) the F_c-induced platelet activation that has been reported with some MoAbs[80] and (2) nonspecific binding. However, the use of F_{ab} fragments is usually unnecessary, provided (1) that the absence of antibody-induced binding of other activation-dependent MoAbs is shown in control samples or the problem is avoided by fixation before test antibody binding; and (2) that the nonspecific binding obtained from parallel samples with isotypic species-specific Ig is subtracted from the binding of the test antibody or Igs are used as blocking reagents.

The saturating concentration of each antibody for platelet binding must be specifically determined by each laboratory. This concentration is typically between 1 and 20 μg/mL. In addition, when two MoAbs are used in the same assay (as is standard in whole blood flow cytometry), it is necessary to determine that they do not interfere with each other for platelet binding.

Platelets can be detected in whole blood by light scatter only. However, under certain experimental conditions, some of the particles falling within the light scatter gate for platelets may not bind any platelet-specific MoAb. It is therefore recommended that a two-color/two-antibody technique be used for whole blood flow cytometry: one MoAb (eg, GPIb-, GPIIb-, or GPIIIa-specific, typically FITC-conjugated) to identify a particle as a platelet and another MoAb (typically biotinylated or phycoerythrin-conjugated) to quantify the expression of the glycoprotein of interest.

Expression of antibody binding. Antibody binding can be expressed as mean particle fluorescence intensity or as the percentage of platelets staining positive for a particular antibody (based on an analysis marker placed to the right of the negative control fluorescence histogram). There are advantages and disadvantages to both methods and therefore, ideally, the data should be expressed both ways. The percentage of positive platelets method is simpler. Furthermore, unlike the mean fluorescence intensity method, the percentage of positive platelets method is independent of variations in signal amplification (eg, as a result of changes in PMT voltage or gain), because the isotypic control signal increases in proportion with the test sample. The percentage of positive platelets method may detect subpopulations of platelets arising from a local in vivo insult. However, it is very important to realize that antibody-positive platelets may have very little antigen expressed on their surface. For example, in a given clinical setting, the data may be reported as 20% circulating activated platelets, based on P-selectin positivity. However, if each P-selectin–positive platelet expresses only 10% of maximal platelet surface P-selectin, then the overall average increase in platelet surface P-selectin is only 2%. If the goal is to determine the total amount of platelet surface antigen, mean fluorescence intensity is therefore the preferred method of data presentation. For activation-dependent antibodies, inclusion of a control sample maximally activated by thrombin, TRAP, or phorbol myristate acetate assists in the quantification of the amount of surface antigen per platelet. Antibody binding to platelets is recorded in log mode (mean channel number), but the data should not be statistically compared before conversion to linear mode. The activation-dependent decrease in platelet surface GPIb-IX-V[59-62] should be quantified by mean fluorescence intensity rather than by the percentage of positive platelets method, because the decrease in platelet surface GPIb-IX-V on each platelet is usually insufficient to result in a negative platelet.

Although standard flow cytometry does not result in a measure of the absolute number of binding sites, Shattil et al[23] and Johnston et al[81] used MoAbs double-labeled with ^{125}I and biotin to show a direct linear relationship between the number of antibody binding sites per platelet as determined by ^{125}I-labeled and (after incubation with phycoerythrin-streptavidin) fluorescent-labeled antibody. Once this relationship is known for a given MoAb, it is possible to use subsequent batches of the biotinylated or FITC-conjugated antibody for binding site quantitation, provided that the molar ratio of fluorescein to antibody is known.

Commercial kits (eg, Quantum 26; Flow Cytometry Standards Corp, San Juan, Puerto Rico) containing a set of calibrated fluorescent standards can be used to determine molecules of equivalent soluble fluorochrome (MESF). Use of these standards allows (1) quantitation of the fluorescence intensity of samples in terms of MESF, (2) determination of the fluorescence threshold of the instrument, (3) determination of the linearity and stability of the instrument, and, most importantly, (4) data comparison over time and between different instruments and laboratories.[82] Furthermore, flow cytometric methods are now available for the absolute quantitation of the number of antibodies bound per cell (eg, Quantum Simply Cellular Microbeads Kit; Flow Cytometry Standards Corp) but have not yet been reported for platelets. The lower limit of detection of antibody binding by flow cytometry is approximately 500 antibody molecules per platelet.

Giant platelet syndromes. Because light scatter (especially forward light scatter) reflects platelet size, light scatter gates may need to be adjusted in giant platelet syndromes (eg, Bernard-Soulier syndrome, an inherited deficiency of GPIb-IX-V[83]). This adjustment may result in overlap of the light scatter of giant platelets with red and white blood cells. It is therefore essential to include in the assay a platelet-specific MoAb as a platelet identifier. For Bernard-Soulier syndrome platelets, this identifier antibody obviously cannot be GPIb-, GPIX-, or GPV-specific.

Calibration of the flow cytometer. To ensure day-to-day sample reproducibility, the flow cytometer should be calibrated daily using commercially available fluorescent beads. As per the instructions of the manufacturer of the flow cyto-

meter, daily confirmation of satisfactory electronics, fluidics, and alignment should also be performed. Because of spectral emission overlap, the proper electronic color compensation must be set for each combination of antibodies (fluorophores) per the instructions of the manufacturer of the flow cytometer and confirmed by each laboratory.

Detection of the activation-dependent decrease in the platelet surface expression of the GPIb-IX-V complex. Whole blood flow cytometric assays frequently use a GPIb-specific MoAb to identify platelets. Because GPIb is not present on any circulating blood cell except platelets,[84,85] the activation-induced decrease in the platelet surface expression of GPIb[59-61] generally does not result in fluorescence below the threshold used to distinguish platelets from other cells and debris.[23,57] Thus, no subpopulations of platelets are excluded. A method of avoiding the activation-induced decrease in binding of a GPIb-specific MoAb is to add a direct conjugate of the GPIb-specific antibody before addition of the agonist (Michelson and Barnard[86] and see below).

To specifically analyze the activation-induced decrease in the platelet surface expression of the GPIb-IX complex in whole blood, a GPIIb- or GPIIIa-specific MoAb can be used as the platelet-identifying reagent.[74]

There is an important methodologic point that needs to be emphasized with regard to the flow cytometric detection of the activation-induced decrease in the platelet surface expression of the GPIb-IX complex.[86] If a FITC-conjugated GPIb-IX–specific test MoAb is added before the platelet agonist (as in the typical schema shown in Table 1), the activation-induced redistribution of GPIb-IX to the surface-connected canalicular system[63] will not result in a significant decrease in platelet fluorescence, because a flow cytometer can detect FITC fluorescence irrespective of whether the conjugated antibody is on the surface or the interior of the platelet. Therefore, in flow cytometric assays, GPIb-IX–specific antibodies that are directly conjugated (eg, with FITC) must be added to the assay after the addition of the agonist. In contrast, GPIb-IX–specific antibodies that require an additional (indirect) detection reagent (eg, phycoerythrin-streptavidin[57,61] or FITC-conjugated polyclonal goat antimouse antibody[60]) can be added to the assay before or after the addition of thrombin, provided that the additional detection reagent is added to the assay after the addition of thrombin.[86]

POTENTIAL CLINICAL APPLICATIONS OF FLOW CYTOMETRY

Platelet hyperreactivity and/or circulating activated platelets. Platelets play an important role in the pathogenesis of coronary artery disease, including unstable angina and acute myocardial infarction.[13,87] Angiography[88] and angioscopy[89] during acute coronary ischemic syndromes frequently reveal intracoronary arterial thrombi that pathologic studies have found to be rich in platelets.[90] In large clinical trials, antiplatelet agents have been shown to reduce the incidence of cardiovascular ischemic events, supporting the concept of an association between platelet activation and cardiovascular ischemia.[91] Furthermore, some investigators have found biochemical markers of platelet activation to be elevated during episodes of acute cardiac ischemia,[10,92] which may identify patients who could benefit from additional antiplatelet therapy.[92] Whole blood flow cytometric studies have shown circulating activated platelets in patients with unstable angina and acute myocardial infarction.[93,94] Whole blood flow cytometric studies have also shown that platelet reactivity is increased in patients with stable coronary artery disease.[95] In addition, coronary angioplasty results in platelet activation, as evidenced by whole blood flow cytometric analysis of coronary sinus blood.[96,97] Flow cytometric analysis of platelet activation markers before angioplasty may help to predict an increased risk of acute ischemic events after angioplasty.[98]

The precise sensitivity and specificity of platelet flow cytometry in clinical settings remains unknown. This is because there is no accepted gold standard assay available for comparison. The sensitivity and specificity of the flow cytometric assay will therefore need to be determined by correlation with clinical parameters of disease in large numbers of patients. For example, in our center, ongoing clinical studies of patients with coronary artery disease are evaluating the sensitivity and specificity of whole blood flow cytometry for the following potential indications: (1) monitoring the clinical course of the disease, (2) identifying patients who could benefit from additional antiplatelet therapy, (3) monitoring the effects of therapeutic interventions (eg, antithrombotic agents), (4) predicting complications, and/or (5) identifying patients at risk for thrombosis.

As determined by P-selectin, CD63, and the GPIIb-IIIa complex, flow cytometric studies of platelets stored in the blood bank before transfusion have provided direct evidence of a time-dependent platelet activation.[99-101] These changes correlated with modifications in platelet morphology (decrease in swirling), leakage of lactate dehydrogenase, and release of β-thromboglobulin.[99] It has been suggested that platelet surface P-selectin may be useful as a quality control measurement, based on correlations between the platelet surface expression of P-selectin in stored blood bank platelets and (1) posttransfusion platelet counts,[102] (2) platelet survival determined by In^{111},[100,103] and (3) clearance of P-selectin–positive platelets from the recipient.[100]

Other conditions in which whole blood flow cytometric measurement of platelet hyperreactivity and/or circulating activated platelets may prove to have a clinical role include pre-eclampsia,[104] peripheral vascular disease,[105] stroke,[106] and diabetes mellitus.[107]

Platelet hyporeactivity. Although there are currently few published studies in this area, whole blood flow cytometry may be useful in the clinical assessment of platelet hyporeactivity. For example, compared with adults, the platelets of very low birth weight preterm neonates are markedly hyporeactive to thrombin, ADP/epinephrine, and thromboxane A_2, as determined by flow cytometric detection of (1) the exposure of the fibrinogen binding site on the GPIIb-IIIa complex, (2) fibrinogen binding, (3) the increase in platelet surface P-selectin, and (4) the decrease in platelet surface GPIb.[108] This platelet hyporeactivity may contribute to the propensity of very low birth weight neonates to intraventricular hemorrhage.[109] Clinical studies to address this hypothesis are in progress.

Monitoring of treatment with GPIIb-IIIa antagonists. The chimeric F_{ab} fragment of MoAb 7E3 (c7E3 F_{ab}), a specific antagonist of the GPIIb-IIIa complex, is a potentially useful antithrombotic agent, eg, in the setting of angioplasty.[110,111] Flow cytometric methods have been developed to monitor this treatment.[112,113] Because in vivo administration of c7E3 F_{ab} inhibits the subsequent in vitro binding of biotinylated c7E3 F_{ab} to platelets, the decrease in flow cytometric detection of the biotinylated c7E3 F_{ab} correlates with the percentage of GPIIb-IIIa receptors occupied by the drug.[112] Alternatively, flow cytometry can be used to monitor the inhibition by c7E3 F_{ab} of ADP-induced fibrinogen binding to platelets.[113] These assays may also be useful in monitoring treatment with other GPIIb-IIIa antagonists.[113,114]

Diagnosis of inherited deficiencies of platelet surface glycoproteins. Flow cytometry provides a rapid and simple means for the diagnosis of the homozygous and heterozygous states of platelet membrane glycoprotein deficiencies, such as Bernard-Soulier syndrome and Glanzmann's thrombasthenia.[115,116] Furthermore, in Bernard-Soulier syndrome, whole blood flow cytometry allows analysis of platelets without attempting the technically difficult procedure of physically separating the giant platelets from similarly sized red and white blood cells. (See also Methodologic Issues: Giant Platelet Syndromes, above.)

In addition, a panel of activation-dependent MoAbs can be used to evaluate patients with defects in platelet aggregation,[30] secretion,[117] or procoagulant activity.[118]

Diagnosis of storage pool disease. Inherited dense granule storage pool deficiency, a relatively common cause of a mild hemorrhagic diathesis, cannot be reliably diagnosed by standard platelet aggregometry.[119] The conventional method to establish the diagnosis of storage pool disease is to label the platelets with the fluorescent dye mepacrine and then measure platelet fluorescence by microscopy.[120] This assay is based on the selective binding of mepacrine to adenine nucleotides in dense granules. The assay is not ideal for a clinical laboratory because it is subjective, tedious, and examines only a small number of platelets. In contrast, dense granule storage pool deficiency can be accurately diagnosed by a simple, rapid, one-step flow cytometric assay in a clinical laboratory.[121,122] The method shows good correlation with fluorescent light microscopic methods, but improves the detection of the mepacrine-loaded platelets by quantitatively measuring fluorescence on a large number of platelets (5,000).[121,122] Acquired dense granule storage pool deficiency, which occurs in myeloproliferative disorders and end-stage renal failure, can also be diagnosed by flow cytometry.[121,123]

Reticulated platelets. Thiazole orange is a fluorescent dye originally synthesized for erythrocyte reticulocyte analysis. Thiazole orange readily permeates live cell membranes and is characterized by a large fluorescence enhancement on binding to nucleic acids, particularly RNA. Whole blood flow cytometric methods have been developed for the identification of young platelets (ie, those containing mRNA) by their staining with thiazole orange.[124,125] Because of the analogy to reticulocytes, these thiazole orange-positive platelets have been termed reticulated platelets.[125,126] Thrombocytopenic patients whose bone marrow contained normal or increased numbers of megakaryocytes had significantly elevated proportions of circulating reticulated platelets.[124] In contrast, the proportion of reticulated platelets in thrombocytopenic patients with impaired platelet production (reduced bone marrow megakaryocytes) did not differ from normal controls and the absolute number of reticulated platelets was significantly lowered.[124] Thus, the reticulated platelet count represents recently released platelets and it has been used as a measure of the rate of thrombopoiesis, analogous to an erythrocyte reticulocyte count as a measure of erythropoiesis.[124,126] However, Ault et al[126] reported that, if the platelet count is less than 50,000/μL, a normal or decreased absolute level of reticulated platelets is not a reliable indicator of decreased thrombopoiesis, thereby limiting the clinical usefulness of this test. Nevertheless, measurement of reticulated platelets has been used as an aid in assessing bone marrow recovery after bone marrow transplantation.[127] Because thiazole orange also binds to ADP and ATP (contained in dense granules), important controls in the flow cytometric measurement of reticulated platelets are the demonstration that thiazole orange staining is (1) abolished by pretreatment of the sample with RNAase and (2) not abolished by pretreatment of the sample with thrombin.

Other potential clinical applications of flow cytometry. As determined by flow cytometry, in vitro activation of platelets by some agonists (eg, C5b-9 and the calcium ionophore A23187) results in platelet-derived microparticles (defined by low forward angle light scatter and binding of a platelet-specific MoAb) that are procoagulant (determined by binding of MoAbs to activated factor VIII or V).[44,45] These findings suggest that procoagulant platelet-derived microparticles may have an important role in the assembly of the tenase and prothrombinase components of the coagulation system in vivo. However, the clinical significance of procoagulant platelet-derived microparticles remains unclear.[34,118,128-131] One group of investigators has reported that, unlike normal sera and sera from patients with quinine- or quinidine-induced thrombocytopenia, sera from patients with heparin-induced thrombocytopenia generate procoagulant platelet-derived microparticles from normal platelets.[132]

Because of the minuscule volumes of blood required, whole blood flow cytometry can be used to analyze the shed blood that emerges from a standardized bleeding time wound.[34,61,74,133] The time-dependent increase in the platelet surface expression of P-selectin in this shed blood reflects in vivo activation of platelets.[34,61,74,133] Immediate fixation (before antibody incubation) is obligatory to observe these time-dependent changes. The assay can be used to show deficient platelet reactivity in response to an in vivo wound, eg, during cardiopulmonary bypass.[74]

Although platelet aggregation can be detected in whole blood by flow cytometry,[76,133] the detected aggregates are much smaller than the aggregates of standard nephelometric tests and a clinical role for the test has not been established.

By a combination of platelet-specific and leukocyte-specific MoAbs, heterotypic aggregates (either platelet/neutrophil or platelet/monocyte) can be determined by flow cytometry.[134] These heterotypic aggregates have been observed

during cardiopulmonary bypass,[135] but their clinical significance remains to be determined.

Flow cytometry can also be used to immunophenotype platelet HPA-1a,[136] detect maternal and fetal anti-HPA-1a antibodies,[137] measure platelet-associated IgG in immune thrombocytopenias[138] and alloimmunization,[139] and cross-match platelets, which may be useful for alloimmunized patients for whom HLA-compatible platelets are not readily available.[140]

Flow cytometry can be used to measure platelet calcium flux with Indo-1[141,142] and platelet F-actin content with NBD- or bodipy-phallicidin.[143-145]

Although not a platelet function assay, flow cytometry can be used to easily and precisely count very low numbers of platelets. A known number of fluorescently labeled microbeads is added to a given volume of diluted anticoagulated whole blood. After fluorescent labeling of the platelets, the platelet count can be calculated. This method is particularly advantageous for severely thrombocytopenic patients for whom standard Coulter counters are less accurate.

SUMMARY

Flow cytometry can now be used as an assay of platelet function in specific clinical settings, including the diagnosis of inherited deficiencies of platelet surface glycoproteins,[115,116] the diagnosis of storage pool disease,[121,122] and the monitoring of treatment with GPIIb-IIIa receptor antagonists.[112,113] However, the most important clinical role of flow cytometry may prove to be the measurement of platelet hyperreactivity and/or circulating activated platelets in a number of clinical settings, including angina pectoris and acute myocardial infarction,[93-95] angioplasty,[96-98] stroke,[106] peripheral vascular disease,[105] diabetes mellitus,[107] and pre-eclampsia.[104] Based on clinical studies of these diseases that are currently ongoing in our center and other laboratories, future applications of whole blood flow cytometric assays of platelet function might include monitoring the clinical course of these diseases, identifying patients who could benefit from additional antiplatelet therapy, monitoring the effects of therapeutic interventions (eg, antithrombotic agents), predicting complications, and/or identifying patients at risk for thrombotic disorders. In addition, flow cytometry may be useful in the quality control of stored platelet concentrates.[100,102,103] Although whole blood flow cytometry is a powerful new technique for the assessment of platelet function, there are some methodologic issues with regard to the execution of the assay, as outlined above. The whole blood flow cytometric assay must therefore be carefully standardized in future controlled clinical studies.

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