Biochemical and Biophysical Research Communications **269,** 85–90 (2000)
doi:10.1006/bbrc.2000.2255, available online at http://www.idealibrary.com on IDEAL®

Flow Cytometric Parameters for Characterizing Platelet Activation by Measuring P-Selectin (CD62) Expression: Theoretical Consideration and Evaluation in Thrombin-Treated Platelet Populations

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Received January 14, 2000

Two flow cytometric parameters are generally used to quantify platelet activation as measured by P-selectin (CD62) expression: percentage and mean channel fluorescence of CD62-positive platelets (%$^+$ and MCF$^+$, respectively). We describe a method for calculation of indices of platelet activation for positive (IPA$^+$) and total (IPA$^\Sigma$) platelets, which reflect integrated amounts of CD62 expressed in these populations; IPA$^+$ is calculated as the product of %$^+$ and MCF$^+$, whereas IPA$^\Sigma$ is exclusively determined by mean fluorescence of the total platelet population (MCF$^\Sigma$) and does not depend on %$^+$. We use these parameters to characterize human platelet activation in whole blood samples treated with varying human α-thrombin concentrations, mimicking the variations in platelet activation in a number of clinical settings. Multiparameter analysis of CD62 expression may be useful for selective diagnosis of disorders with systemic or localized platelet activation and for monitoring the clinical course of the disease and effect of therapeutic interventions.

Key Words: **flow cytometric parameters; P-selectin (CD62) expression; platelet activation; human α-thrombin.**

CD62 (P-selectin, GMP-140, PADGEM) is a platelet α-granule protein which is expressed on the surface of the platelet following platelet activation (1–3). Flow cytometric assays of the binding of fluorochrome-labeled antibody to CD62 on the platelet surface have been widely employed to characterize platelet activation in various experimental and clinical conditions (4–7). Two CD62-binding parameters are generally used for determining platelet activation, i.e., (a) the percentage CD62-positive platelets in the total platelet population (%$^+$), and (b) the mean fluorescence of CD62-positive cells expressed in arbitrary units of mean channel fluorescence (MCF$^+$). In most reports platelet activation is indicated using one of these two measurements. Each of these parameters, however, reflects a different specific aspect of platelet activation. The %$^+$ characterizes the proportion of activated cells in the total platelet population without registering the activation level of individual cells; this allows a quantitative, but not a "qualitative," judgment of activated cells in the total population. In contrast, MCF$^+$ characterizes the mean epitope density of CD62 molecules on the platelet surface, providing a parameter of activation of an average platelet, i.e., reflects the quality of activation of individual platelets, but not their quantity.

It is still unclear which parameter(s) should be used to characterize platelet activation in the total platelet population. In some clinical and laboratory situations it may be important to analyze not only the total platelet population, but also the subpopulation of activated cells, since it is the latter which determines the adhesion and aggregation of platelets on damaged vessel wall, as well as platelet–platelet and platelet–leukocyte interactions in the circulation.

In the present study, we attempted to establish the parameters of flow cytometric analysis which best characterize platelet activation in the subpopulation of CD62-positive platelets and in the total platelet population. To determine an integrated level of CD62 expression in these populations, we performed a theoretical consideration of two additional CD62-binding parameters which we define as the index of platelet activation (IPA) of positive (IPA$^+$) and total (IPA$^\Sigma$) platelets. This analysis indicates that IPA$^+$ is deter-

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0006-291X/00 $35.00

mined as the product of $\%^+$ and MCF^+. In contrast, IPA^Σ is exclusively determined by the mean channel fluorescence of total platelets (MCF^Σ) and does not depend on $\%^+$.

We analyzed activation of human platelets following stimulation of whole blood with the potent platelet agonist human α-thrombin in a wide range of concentrations. This allowed us to create a set of platelet populations differing in their activation status, resembling the variation in platelet activation seen in various clinical situations, and to measure the changes of $\%^+$, MCF^+, IPA^+ and MCF^Σ parameters in these populations.

Although calculations similar to that for IPA^+ have been described by others (4, 7, 8), we believe that this is the first report of comparative study of $\%^+$, MCF^+, IPA^+, and MCF^Σ as parameters for evaluation of platelet activation.

METHODS

Materials. Phycoerythrin (PE)-conjugated monoclonal antibody to CD62 (clone AC1.2) was purchased from Becton Dickinson (San Jose, CA) and fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-CD41 (clone P2) was purchased from Immunotech Coulter (Westbrook, ME). Ca^{2+} and Mg^{2+}-free phosphate-buffered saline, pH 7.4 (PBS) was obtained from GIBCO Life Technologies (Grand Island, NY) and HEPES, bovine serum albumin (BSA) and glycyl-L-prolyl-L-arginyl-L-proline (GPRP) were from Sigma Chemical Co. (St. Louis, MO). Human α-thrombin (FIBRINDEX, Ortho Diagnostic Systems Inc., Raritan, NJ) was dissolved in PBS-10 mM HEPES-1% BSA buffer, pH 7.4 and stored in aliquots at −70°C.

Preparation of whole blood samples for flow cytometry. Blood was obtained from normal volunteers who had taken no medications for at least two weeks. To minimize platelet activation during blood collection, we used a 20-gauge butterfly needle with light tourniquet and discarded the first 2 ml of blood (9). Blood was collected into a 10 ml vacutainer tube containing 1.5 ml of acid-citrate-dextrose anticoagulant (ACD Solution A; Becton Dickinson, Franklin Lakes, NJ), resulting in a final pH of 6.5. Within 1–2 min of drawing, 300 μl of whole blood was added to 300 μl of PBS-HEPES-BSA buffer containing 150 μl of 25 mM GPRP, to dilute the blood and to prevent thrombin-induced fibrin polymerization and platelet aggregation (10). 50 μl aliquots of diluted blood were then added to 12 × 75 mm polystyrene tubes containing 50 μl of thrombin diluted in PBS-HEPES-BSA to yield final thrombin concentrations of 0, 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, and 1 NIH U/ml (1 U/ml = 10 nM of human α-thrombin). The samples were incubated undisturbed for 10 min at 37°C to prevent platelet aggregation, fixed with an equal volume of 1% paraformaldehyde in PBS for 30 min at 22°C and diluted with 1 ml of PBS-HEPES-BSA. Five μl of anti-CD62-PE and 5 μl anti-CD41-FITC were added at saturating concentrations to 50 μl of fixed blood sample and, following incubation for 30 min at 22°C in the dark, the samples were analysed by flow cytometry.

Flow cytometric analysis. Flow cytometric evaluation of platelets were done as described previously (11, 12); 10,000 dual-color labeled platelet events were acquired on a FACScan flow cytometer (Becton Dickinson), equipped with 15 mW argon ion laser and LYSYS II software. The flow cytometer settings were optimized for the acquisition of platelets by logarithmic signal amplification in all four detectors (forward and side light scatter channels and fluorescence channels FL1 for FITC and FL2 for PE). A threshold on FL1 was set high enough so that only platelet CD41-positive events were acquired. A gate was set around the single intact platelets as defined by forward and 90° side light scatter characteristics and positivity for CD41 platelet marker. Gating for activated (CD62-positive), nonactivated (CD62-negative) and total platelets was performed on one-parameter fluorescence histograms as shown in Fig. 1.

Analysis of results. Thrombin-induced platelet activation was evaluated by the expression of CD62 using a number of CD62-binding parameters. The percentage of cells expressing or not expressing CD62 ($\%^+$ and $\%^-$ respectively) and the mean channel fluorescence of CD62-positive (MCF^+), -negative (MCF^-) and total (MCF^Σ) platelet populations were obtained from the software list mode data. Fractions of CD62 positive (f^+) and negative (f^-) cells were calculated as follows: $f^+ = \%^+/100$ and $f^- = \%^-/100$. Indices of platelet activation for positive (IPA^+), negative (IPA^-), and total (IPA^Σ) platelets were calculated from Eqs. [1b], [2], and [3a], respectively, as described below. Data are expressed as means ± SD.

RESULTS

Theoretical Consideration of CD62-Binding Parameters

The index of platelet activation (IPA) for any given platelet population should reflect the integrated level of platelet activation marker (e.g., CD62) in the entire population, measured as the total binding of activation-dependent antibody to the surface of all platelets in the population. IPA for the subpopulation of CD62-positive platelets ($IPÅ^+$) can be expressed as

$$IPÅ^+ = MCF^+ \times N^+, \qquad [1a]$$

where MCF^+ is the mean fluorescence per cell of platelet-bound anti-CD62 in the subpopulation of CD62-positive cells and N^+ is the number of CD62-positive cells analyzed.

To obtain $IPÅ^+$ independently of absolute number of cells analyzed (IPA^+), Eq. [1a] can be transformed by division to the total number of CD62-positive and -negative cells in a sample (N^Σ)

$$IPA^+ = MCF^+ \times f^+, \qquad [1b]$$

where $f^+ = N^+/N^\Sigma = \%^+/100$, i.e., the fraction of CD62-positive cells in the total population.

IPA for the subpopulation of CD62-negative cells (IPA^-) is

$$IPA^- = MCF^- \times (1 - f^+), \qquad [2]$$

where MCF^- is the mean fluorescence of the subpopulation of negative cells, and $f^- = 1 - f^+$, i.e., the fraction of CD62-negative cells in the total population.

IPA for the total platelet population (IPA^Σ), consisting of CD62-positive and -negative cells is

$$IPA^\Sigma = MCF^\Sigma \times f^\Sigma = MCF^\Sigma, \qquad [3a]$$

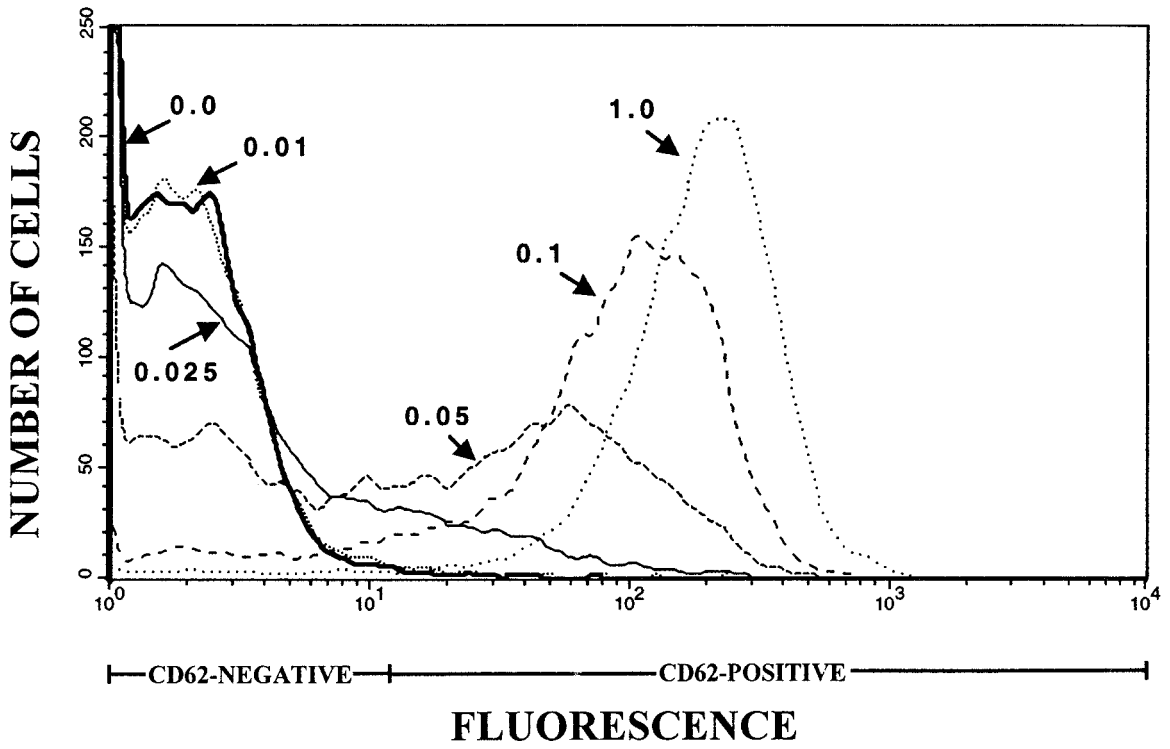

FIG. 1. CD62 expression in thrombin-induced human platelets. Whole blood samples were activated by α-thrombin and stained by phycoerythrin-conjugated anti-CD62 antibody; the final concentrations of thrombin (U/ml) are indicated by the arrows. Gating of nonactivated (CD62-negative) and activated (CD62-positive) platelet subpopulations are shown; total platelet population was gated as negative + positive platelets. The histograms demonstrate that both the proportion and platelet-bound fluorescence of activated platelets increase with increasing thrombin concentration.

where MCF^{Σ} is the mean fluorescence of total platelets and the $f^{\Sigma} = 1$, i.e., the sum of the fractions of positive and negative cells in the total population.

IPA^{Σ} which depends on the absolute number of cells in the total population ($IPÅ^{\Sigma}$) is

$$IPÅ^{\Sigma} = MCF^{\Sigma} \times N^{\Sigma}. \quad [3b]$$

IPA^{+}, IPA^{-}, and IPA^{Σ} reflect the integrated anti-CD62 fluorescence in the subpopulations of activated and nonactivated platelets and in the total platelet population, respectively and are expressed in arbitrary units (mean channel fluorescence number). IPA^{+} and IPA^{-} are determined by the product of mean fluorescence and the fractions of positive and negative cells, whereas IPA^{Σ} is determined exclusively by mean fluorescence of the total platelet population and does not depend on the percentage of CD62-positive cells.

Thrombin-Induced Changes of CD62-Binding Parameters

Platelet populations differing in activation status were prepared by incubation with thrombin concentrations ranging from 0.005 to 1 U/ml, as shown in Figs. 1 and 2, and Table 1. The dose-dependent changes in $\%^{+}$ and f^{+} parameters fitted a sigmoidal dose-response pattern with a high correlation ($R^2 = 0.999$) and 50% response was reached at $EC_{50} = 0.044$ U/ml thrombin (Figs. 2A and 2E). When treated by 0, 0.005 and 0.01 U/ml thrombin, 98–99% of platelets were not activated and had a low surface density of CD62 ($MCF^{-} \approx 2$); at these doses of thrombin, although CD62-positive platelets accounted for only 1–2% of the cells, they had ≈ 10-fold higher levels of CD62 expression ($MCF^{+} = 18$–26; Table 1a). At high thrombin concentrations (0.25–1 U/ml), the $\%^{+}$ and f^{+} parameters reached the top plateau of the curve ($\%^{+} = 98$–99%; $f^{+} = 0.98$–0.99; Figs. 2A and 2E), but MCF^{Σ}, MCF^{+} and IPA^{+} did not reach the plateau level (Figs. 2A, 2B, and 2F, and Table 1d).

As seen in Table 1, calculation of IPA^{+}, IPA^{-}, and IPA^{Σ} indices allows determination of the CD62 balance for platelet populations activated by different thrombin concentrations; i.e., the contribution of subpopulations of positive and negative cells to the integrated amount of CD62 expressed in the total platelet population can be seen. As might be expected for a large number of cells analyzed (10,000), for all thrombin concentrations $IPA^{+} + IPA^{-} = IPA^{\Sigma}$. For untreated platelet populations and populations treated by subthreshold (0.005 and 0.01 U/ml) thrombin concentra-

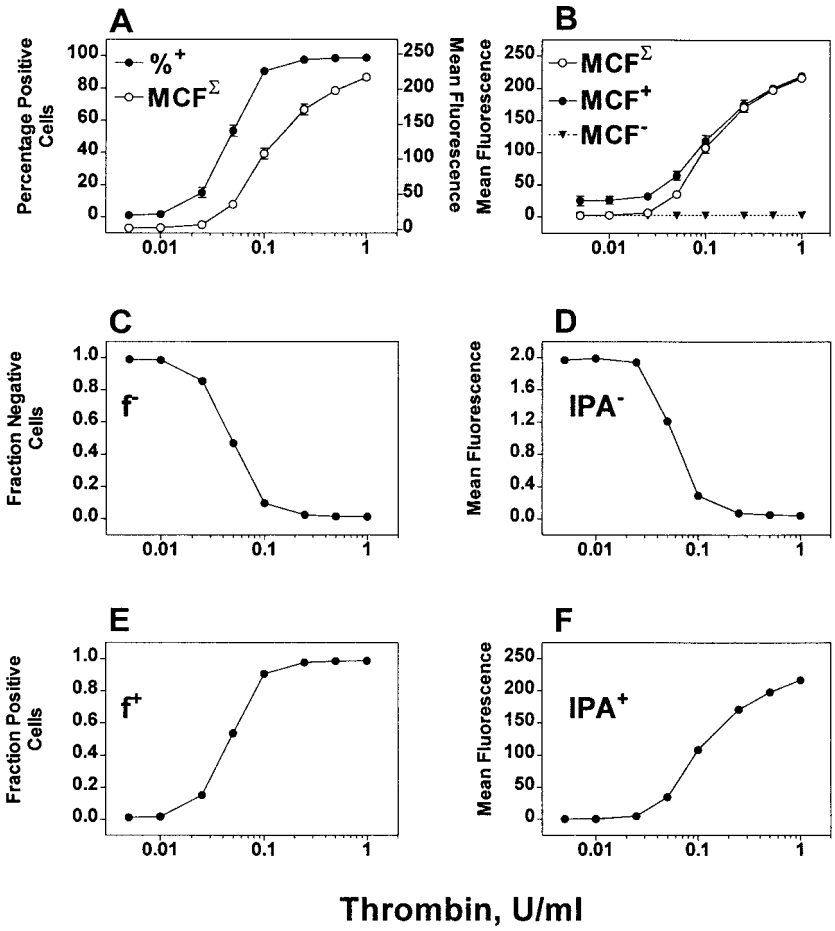

FIG. 2. Thrombin-induced changes of CD62-binding parameters for positive, negative, and total platelet populations. The parameters were plotted (A, B, D, F) or calculated (C, E) from Table 1; $f^- = \%^-/100$; $f^+ = \%^+/100$. Mean fluorescence is expressed as channel numbers. Means ± SD (N = 3) are presented.

tions, the total index of platelet activation is very low (IPA^Σ = 2.15–2.77; Table 1a) and is mainly determined by the negative cells; the positive cells express only 8–16% of CD62 of the total population (IPA^- = 1.97–1.99 vs IPA^+ = 0.18–0.44; $P < 0.001$). Platelets activated by low (0.025 U/m) thrombin contain 15% CD62-positive cells, with $MCF^+ = 32$ and 85% CD62-negative platelets with $MCF^- = 2.3$ (Table 1b); this relatively small fraction of positive cells expressed >70% of the total amount of CD62 ($IPA^+ = 4.8$ vs $IPA^\Sigma = 6.7$; $P = 0.03$; Table 1b). Platelets exposed to medium (0.05–0.1 U/ml) and high (0.25–1 U/ml) thrombin concentrations expressed 17–50 and 80–100 fold more CD62 than untreated platelets respectively (IPA^Σ values; Table 1a, c, d); they contain from 53 to 99% CD62-positive cells, which essentially completely determine the amount of CD62 expressed in the entire population ($IPA^+ = IPA^\Sigma$), since at 0.05 U/ml thrombin $f^+ \approx 0.5$ and $MCF^+ \approx 2 \times MCF^\Sigma$ and at 0.1–1 U/ml thrombin $f^+ \approx 1$ and $MCF^+ \approx MCF^\Sigma$ (Table 1c, d; Eqs. [1b] and [3a]).

DISCUSSION

A number of flow cytometric parameters (e.g., $\%^+$, MCF^+, and MCF^Σ) can be employed to evaluate platelet activation by measuring CD62 expression. However, it is not clear which parameter(s) should be used as an integral index of platelet activation of the total platelet population (IPA^Σ). Our theoretical consideration shows that IPA^Σ, which reflects the total amount of CD62 expressed in the entire population, is equivalent to MCF^Σ and does not depend on $\%^+$ (Eq. [3a]), indicating that MCF^Σ only (and not $\%^+$, $\%^+$ plus MCF^+ or $\%^+$ plus MCF^Σ) should be used as the valid index of platelet activation of the total population. While in Eq. [3a] IPA^Σ is expressed in arbitrary units, as a mean channel fluorescence number, CD62 expression can, using quantitative fluorescent beads, be expressed as antibody binding capacity or number of antibody molecules per platelet (11, 13–15); this can be multiplied by the absolute number of cells analyzed (N^Σ in Eq.

TABLE 1

Thrombin-Induced Changes of CD62-Binding Parameters for Positive, Negative, and Total Platelet Populations

Thrombin, U/ml	CD62-negative platelets			CD62-positive platelets			Total platelets
	$\%^-$	MCF^-	IPA^-	$\%^+$	MCF^+	IPA^+	$IPA^\Sigma = MCF^\Sigma$
(a) 0	99.1 ± 0.5	2.00 ± 0.21	1.98 ± 0.21	1.02 ± 0.49	17.8 ± 1.5	0.18 ± 0.07	2.15 ± 0.24
0.005	98.9 ± 0.4	1.99 ± 0.22	1.97 ± 0.21	1.17 ± 0.34	25.3 ± 7.4	0.30 ± 0.13	2.26 ± 0.24
0.010	98.4 ± 0.5	2.02 ± 0.22	1.99 ± 0.21	1.70 ± 0.57	26.4 ± 6.0	0.44 ± 0.13	2.77 ± 0.36
(b) 0.025	85.3 ± 2.9	2.28 ± 0.28	1.94 ± 0.19	15.1 ± 3.1	32.3 ± 5.1	4.79 ± 0.70	6.70 ± 0.71
(c) 0.05	46.9 ± 3.4	2.58 ± 0.27	1.21 ± 0.08	53.5 ± 3.5	64.7 ± 6.8	34.6 ± 3.8	35.8 ± 3.8
0.1	9.6 ± 1.1	3.02 ± 0.22	0.29 ± 0.02	90.6 ± 1.0	119 ± 8.7	108 ± 7.7	108 ± 7.6
(d) 0.25	2.3 ± 0.2	3.14 ± 0.08	0.07 ± 0.01	97.7 ± 0.2	175 ± 7.7	171 ± 7.5	171 ± 7.5
0.5	1.5 ± 0.3	3.32 ± 0.14	0.05 ± 0.01	98.5 ± 0.3	201 ± 3.4	198 ± 3.3	198 ± 3.3
1.0	1.2 ± 0.2	3.23 ± 0.33	0.04 ± 0.01	98.8 ± 0.4	220 ± 4.4	217 ± 4.1	217 ± 4.1

Note. Whole blood samples were treated with different thrombin concentrations and gated for CD62-positive, -negative and total platelets as shown in Fig. 1. The $\%^+$, $\%^-$, MCF^+, MCF^-, and MCF^Σ values were obtained from the flow cytometric list mode data following the gating; IPA^+ and IPA^- values were calculated from Eqs. [1b] and [2]; $IPA^\Sigma = MCF^\Sigma$ in accordance with Eq. [3a]. Means ± SD (N = 3) are presented.

[3b]) to obtain the absolute number of CD62 expressed in the total population.

Although MCF^Σ is generally considered as an index of mean CD62 expression in the total platelet population, Eq. [3a] shows that MCF^Σ also reflects an integrated level of CD62 expression in the total population. This parameter may therefore be useful to characterize the entire population of circulating platelets with a systemic (unlocalized) mechanism of platelet activation. However, in a number of clinical settings, e.g., at the site of mural thrombus formation, locally activated platelets may appear in the circulation and it may be of interest to evaluate just this fraction of activated cells. The present report shows that $\%^+$ (f^+), MCF^+ and IPA^+ parameters permit characterization of a small subset of activated platelets in the bulk population of nonactivated cells. In contrast to MCF^Σ, which reflects the mean level of platelet activation in the heterogeneous total population, MCF^+ reflects mean level of activation for the CD62-positive population only.

In some publications, indices similar to IPA^+ have been introduced and termed as "platelet activation$_{total}$" (4) or "binding index" (7, 8). In contrast to IPA^Σ, which does not depend on the fraction of positive cells (Eq. [3a]), IPA^+ is determined as the product of MCF^+ and f^+ (Eq. 1b) and reflects an integrated amount of CD62 expressed in the subpopulation of positive cells. Comparison of IPA^+ and IPA^Σ allows distinction of the contribution of only positive cells to CD62 expression in the total population. Since IPA^+ is the product of MCF^+ and f^+, the same IPA^+ value can be obtained by different combinations of MCF^+ and f^+; e.g., $IPA^+ = 1.5$ can be obtained either as the product of (a) $f^+ = 0.01$ and $MCF^+ = 150$, or (b) $f^+ = 0.3$ and $MCF^+ = 5$. In the first case, a very small proportion (1%) of activated cells has 30-fold higher mean level of activation than in the second case, which is characterized by a relatively high proportion (30%) of activated cells. The ability to distinguish between such situations by measuring MCF^+ and f^+ separately, may be useful for the specific evaluation and diagnosis of platelet-associated disorders; e.g., it is conceivable that only situation (a), but not (b), may be associated with the local mural thrombi formation.

Platelet populations activated by subthreshold (0.005 and 0.01 U/ml) and low (0.025 U/ml) thrombin concentrations contain 1–2 and 15% CD62-positive cells, respectively (Table 1a, b) and evaluation of platelet activation at these levels is probably the most interesting from the clinical point of view, since many in vivo settings are characterized by the relatively low (5–19%) activation levels (7, 16, 17). In other situations, e.g. during blood bank storage of platelet concentrates for transfusion, medium and high levels of platelet activation have been observed, as measured by 30–90% CD62-positive cells (4, 18); this is comparable to activation of platelets treated by medium (0.05–0.1 U/ml) thrombin doses (Table 1c).

In the present study, in addition to the common flow cytometric parameter ($\%^+$) employed to characterize activation in the total platelet population, we have also determined parameters which characterize the populations of CD62-positive (f^+, MCF^+, and IPA^+), CD62-negative (f^-, MCF^- and IPA^-) and total cells (MCF^Σ). All of these "unconventional" parameters have been obtained simply by specific gating of flow cytometric histograms and calculations. These expanded measurements may be useful for the selective and sensitive diagnosis of specific platelet-associated disorders with systemic or localized mechanisms of platelet activation and for monitoring the clinical course of the disease and effect of therapeutic interventions.

ACKNOWLEDGMENTS

The authors thank Miss Samantha Shakoor and Mr. David J. Allen for preparing the graphs and the Cara Phelan Trauma Centre, the Trauma Program and Health Sciences Research Grant 2-77760-8142 from St. Michael's Hospital for support of these studies.

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