Biochemical and Biophysical Research Communications **273,** 565–570 (2000)
doi:10.1006/bbrc.2000.2977, available online at http://www.idealibrary.com on IDEAL®

Quantification of Platelet Activation Status by Analyzing P-Selectin Expression

Valery Leytin,[1] Meera Mody, John W. Semple, Bernadette Garvey, and John Freedman
Department of Transfusion Medicine and Department of Laboratory Medicine and Pathobiology, St. Michael's Hospital and University of Toronto, Toronto, Ontario, Canada

Received May 7, 2000

Platelet activation status (PAS) is used for characterizing quality and function of platelets in various experimental and clinical settings. In this study, we created a set of platelet populations differing in PAS, using stimulation of platelets with thrombin in a wide range of concentrations, and analyzed a number of flow cytometric parameters, which characterize PAS by measuring P-selectin (CD62) expression. We found that PAS of a platelet population depends significantly on the specific parameters used for detecting CD62 expression and can differ several fold. We revealed the parameters which are more sensitive for distinguishing the differences between populations with similar low and similar high PAS. Selection of valid and sensitive flow cytometric parameters for PAS evaluation and distinguishing the differences between platelet populations with similar PAS can serve for diagnosis of platelet-associated disorders and monitoring their course and therapeutic interventions. © 2000 Academic Press

Key Words: **flow cytometry; P-selectin; CD62; platelet activation status; thrombin.**

Flow cytometric assays of P-selectin (CD62) expression on the platelet surface have been widely employed to characterize platelet activation in various experimental and clinical conditions (1–8). However, it is still unclear and deserves an additional consideration which flow cytometric parameters of CD62 expression should be used for sensitive standardized evaluation of platelet activation in specific situations with different levels of platelet activation.

Previously, we have performed the theoretical consideration of a number flow cytometric parameters to characterize CD62 expression. These parameters include: (a) the percentage CD62-positive platelets ($\%^+$), which reflects the proportion (quantity) of activated cells in the total population, but not the level of platelet activation, (b) the mean channel fluorescence of CD62-positive (MCF^+) and total (MCF^Σ) platelet populations, which characterize the mean level of platelet activation in respective populations, but not the quantity of activated cells, and (c) indices of platelet activation of CD62-positive (IPA^+) and total (IPA^Σ) cells, which reflect integrated amounts of CD62 expressed in these populations and characterize both quantity and 'quality' of activated cells. It was shown that IPA^+ can be defined as the product of MCF^+ and $\%^+$, whereas IPA^Σ is determined exclusively by MCF^Σ and does not depend on $\%^+$ (9).

For diagnostics of platelet-associated disorders as well as for monitoring their clinical course and effects of therapeutic interventions, it is important to determine the most sensitive flow cytometric parameter(s) for evaluating platelet activation status (PAS) and for distinguishing the differences between populations with similar PAS. In the present study, we created a set of platelet populations differing in their PAS by the treatment of whole blood samples with the platelet agonist human α-thrombin in a wide range of concentrations. We then employed $\%^+$, MCF^+, IPA^+, MCF^Σ parameters, which characterize different aspects of CD62 expression, for quantification of PAS in the various thrombin-treated platelet populations. Two main results have been obtained. First, the evaluation of PAS in a tested platelet population depends on the specific parameter used for characterizing CD62 expression and can differ up to 3.5- to 7-fold. Second, different parameters have different sensitivity for revealing the differences between populations with similar low or similar high PAS; this allows selection of more sensitive and practical flow cytometric parameter(s) depending on the level of activation of analyzed platelet populations.

[1] To whom correspondence should be addressed at the Department of Transfusion Medicine, Shuter Room 2003, St. Michael's Hospital, 30 Bond Street, Toronto, Ontario M5B 1W8, Canada. Fax: +1 416 864 5294. E-mail: valley@idirect.com.

0006-291X/00 $35.00

METHODS

Preparation of whole blood samples for flow cytometry was described previously (9–11). Briefly, blood from normal volunteers was collected into an acid-citrate-dextrose anticoagulant and diluted 2.5-fold with buffer (phosphate-buffered saline–HEPES–bovine serum albumin, pH 7.4), containing GPRP peptide to prevent thrombin-induced fibrin polymerization and platelet aggregation. Fifty μl aliquots were incubated for 10 min at 37°C with human α-thrombin to yield final thrombin concentrations of 0, 0.005, 0.01, 0.025, 0.05, 0.10, 0.25, 0.50 and 1.00 NIH U/ml. The samples were fixed with paraformaldehyde, diluted with the buffer and stained by dual-color technique with phycoerythrin-conjugated anti-CD62 and fluorescein isothiocyanate-conjugated anti-CD41 monoclonal antibodies.

Flow cytometric analysis was done on a FACScan flow cytometer. Gating for activated (CD62-positive), nonactivated (CD62-negative) and total platelets was performed on one-parameter fluorescence histograms. Three parameters were obtained from the list mode data following gating: $\%^+$—percentage of CD62-positive platelets, MCF^+—mean channel fluorescence of CD62-positive platelets, MCF^Σ—mean channel fluorescence of total platelets. Indices of platelet activation of CD62-positive and total platelets (IPA^+ and IPA^Σ, respectively) were defined from equations: $IPA^+ = MCF^+ \times \%^+/100$ and $IPA^\Sigma = MCF^\Sigma$ as described previously (9).

Normalized values of $\%^+$, MCF^+, IPA^+ and MCF^Σ parameters were expressed as the percentage of the maximal value of the given parameters for platelets treated with 1 U/ml thrombin (designated as 100%).

Platelet activation status (PAS) of thrombin-treated platelet populations was calculated in four ways as normalized values of $\%^+$, MCF^+, IPA^+ and MCF^Σ parameters ($PAS_{\%+}$, PAS_{MCF+}, PAS_{IPA+} and $PAS_{MCF\Sigma}$, respectively) after the subtraction of background values of parameters at 0 U/ml thrombin ($\%^+ = 1.03 \pm 0.50$, $MCF^+ = 8.09 \pm 0.68$, $IPA^+ = 0.08 \pm 0.03$ and $MCF^\Sigma = 0.99 \pm 0.11$) and was expressed in the scale of 0 to 100%. $PAS_{IPA\Sigma} = PAS_{MCF\Sigma}$, since $IPA^\Sigma = MCF^\Sigma$ (9).

Differences between platelet populations with similar PAS were determined using original unnormalized values of $\%^+$, MCF^+, IPA^+ and MCF^Σ parameters without the subtraction of background. This allowed calculation of the differences between untreated (0 U/ml) platelet populations and populations treated with the lowest (0.005 U/ml) thrombin concentration and relevant for platelet activation in experimental and clinical conditions where there is background platelet activation due to the processing of the samples.

Statistical analysis. Data were expressed as means ± SD and analyzed by Student's *t*-test to determine the significance of the differences between means (*P*-values).

RESULTS

PAS of Thrombin-Treated Platelet Populations Measured by $\%^+$, MCF^+, IPA^+, and MCF^Σ Parameters

We characterized PAS in whole blood samples treated with human α-thrombin concentrations in the range of 0.005 to 1 U/ml using four parameters of CD62 expression ($\%^+$, MCF^+, IPA^+ and MCF^Σ), in order to compare different methods of PAS evaluation. Three of these parameters (MCF^+, IPA^+ and MCF^Σ) are expressed in the same units as the mean channel fluorescence numbers and can be compared directly without transformation. To compare the parameters expressed in different units (e.g., $\%^+$ vs MCF^Σ or $\%^+$ vs IPA^+), the measured values of these parameters have been normalized. Normalization allows a score for each parameter in relation to the maximal value of the parameter (assigned 100%) at 1 U/ml thrombin.

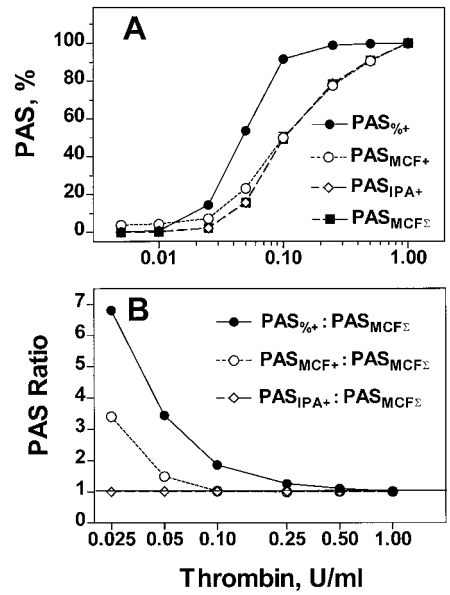

FIG. 1. (A) Platelet activation status (PAS) of thrombin-treated platelet populations as measured by different flow cytometric parameters of CD62 expression. For each thrombin concentration, $PAS_{\%+}$, PAS_{MCF+}, PAS_{IPA+} and $PAS_{MCF\Sigma}$ values were calculated as normalized values of $\%^+$, MCF^+, IPA^+ and MCF^Σ parameters, respectively (see Methods). (B) Comparison of PAS measured by $\%^+$, MCF^+ and IPA^+ with that measured by MCF^Σ. For each thrombin concentration, PAS ratios were calculated in relation to the respective $PAS_{MCF\Sigma}$ value; horizontal line shows the ratio of 1.

Using this normalization approach, we expressed PAS for each thrombin-treated platelet population as the normalized values of $\%^+$, MCF^+, IPA^+ and MCF^Σ parameters ($PAS_{\%+}$, PAS_{MCF+}, PAS_{IPA+} and $PAS_{MCF\Sigma}$ values, respectively), using $PAS_{MCF\Sigma}$ as the reference PAS index. Figure 1A shows that for all thrombin doses which induce platelet activation (≥0.025 U/ml), $PAS_{\%+}$ values are higher than PAS_{MCF+}, PAS_{IPA+}, and $PAS_{MCF\Sigma}$ values. For platelets treated with 0.025 U/ml thrombin, $PAS_{\%+}$ equals 14.4% of the maximal value, which is 6.9-fold higher than the reference $PAS_{MCF\Sigma}$ value, whereas PAS_{MCF+} is only 3.4-fold higher (Table 1). With increasing thrombin concentration $PAS_{\%+}$:$PAS_{MCF\Sigma}$ and PAS_{MCF+}:$PAS_{MCF\Sigma}$ ratios gradually decrease, reaching a ratio of 1 (Fig. 1B, Table 1). PAS_{IPA+} do not differ significantly from the reference $PAS_{MCF\Sigma}$ value for platelet populations treated with all active (≥0.025 U/ml) thrombin concentrations (Fig. 1B, Table 1).

These data show that depending on the specific parameter employed to characterize CD62 expression, quite different impressions of the PAS can be obtained of the same platelet population. PAS values measured by the different parameters can vary several-fold; these differences are particular large for platelets activated by low (0.025 U/ml) and medium (0.050 U/ml) thrombin concentrations.

TABLE 1

Evaluation of Platelet Activation Status in Thrombin-Treated Platelet Populations Based on the Values of Different Parameters of CD62 Expression

Thrombin, U/ml	PAS	% of maximum	PAS Ratio
0.025	$PAS_{\%+}$	14.4 ± 2.8**	6.86
	PAS_{MCF+}	7.2 ± 2.7*	3.43
	PAS_{IPA+}	2.1 ± 0.3^{ns}	1.00
	$PAS_{MCF\Sigma}$	2.1 ± 0.2	1.00
0.05	$PAS_{\%+}$	53.7 ± 3.5***	3.51
	PAS_{MCF+}	23.2 ± 3.8*	1.49
	PAS_{IPA+}	15.9 ± 1.7^{ns}	1.02
	$PAS_{MCF\Sigma}$	15.6 ± 1.8	1.00
0.10	$PAS_{\%+}$	91.6 ± 1.2***	1.87
	PAS_{MCF+}	50.0 ± 4.9^{ns}	1.02
	PAS_{IPA+}	49.5 ± 4.1^{ns}	1.01
	$PAS_{MCF\Sigma}$	49.1 ± 4.1	1.00

Note. PAS of thrombin-treated platelet populations were calculated in four different ways as the normalized values of $\%^+$, MCF^+, IPA^+ and MCF^Σ parameters after the subtraction of respective background values at 0 U/ml of thrombin and expressed as the percentage of the maximal value of the given parameter assigning a value of 100% to 1 U/ml thrombin. Presented are means ± SD (N = 3). *P*-values vs $PAS_{MCF\Sigma}$ are shown: ***$P \le 0.001$; *$P \le 0.0.5$; $^{ns}P > 0.05$ (not significant). PAS ratio is the ratio of the given PAS to the reference $PAS_{MCF\Sigma} = PAS_{IPA\Sigma}$ (see Methods).

Differences in Platelet Activation between Platelet Populations with Similar PAS

In experimental and clinical situations it may be important to evaluate differences in platelet activation between populations with similar PAS. We compared the sensitivities of various flow cytometric parameters for revealing these differences. To perform the comparison we determined the values of $\%^+$, MCF^+, IPA^+ and MCF^Σ parameters for the platelet population activated by a given thrombin concentration (V_n) and that activated by the adjacent next higher concentration (V_{n+1}). The statistical significance of the differences between V_{n+1} and V_n for each parameter was calculated using *t*-test (*P*-values; Fig. 2) as well as the ratios (R) between these values ($R = V_{n+1}/V_n$; Fig. 3).

Figure 2 shows that none of the four parameters reveal the differences between untreated platelet population and population treated by the lowest subthreshold thrombin concentration (0 vs 0.005 U/ml; $P = 0.16–0.69$) as well as between populations treated by two adjacent subthreshold concentrations (0.005 vs 0.01 U/ml; $P = 0.11–0.85$). For platelets treated by 0.01 vs 0.025 U/ml thrombin, however, three parameters, $\%^+$, IPA^+ and MCF^Σ, are sensitive for distinguishing the differences ($\%^+$, $P = 0.0017$; IPA^+, $P = 0.0004$; MCF^Σ, $P = 0.0011$), but MCF^+ is insensitive ($P = 0.26$). For platelets treated with 0.05–0.25 U/ml thrombin, where the differences between PAS values of adjacent populations are high (Fig. 1A), all four parameters reveal the differences between adjacent populations with high significance ($P = 0.0001–0.0006$ for $\%^+$, IPA^+ and MCF^Σ; $P = 0.0011–0.0027$ for MCF^+). However, for populations treated with 0.25 vs 0.50 U/ml thrombin, MCF^+, IPA^+ and MCF^Σ parameters are much more sensitive ($P = 0.005–0.006$) than the $\%^+$ parameter ($P = 0.02$). For platelets activated by 0.50–1.00 U/ml thrombin, MCF^+, IPA^+ and MCF^Σ parameters showed the differences in platelet activation ($P = 0.003–0.004$), whereas $\%^+$ values did not differ significantly ($P = 0.36$), since $\%^+$ is the only parameter which reaches saturation at these concentrations (Fig. 1A).

In contrast to the *P*-value statistical approach, R-values show how many times the given parameter

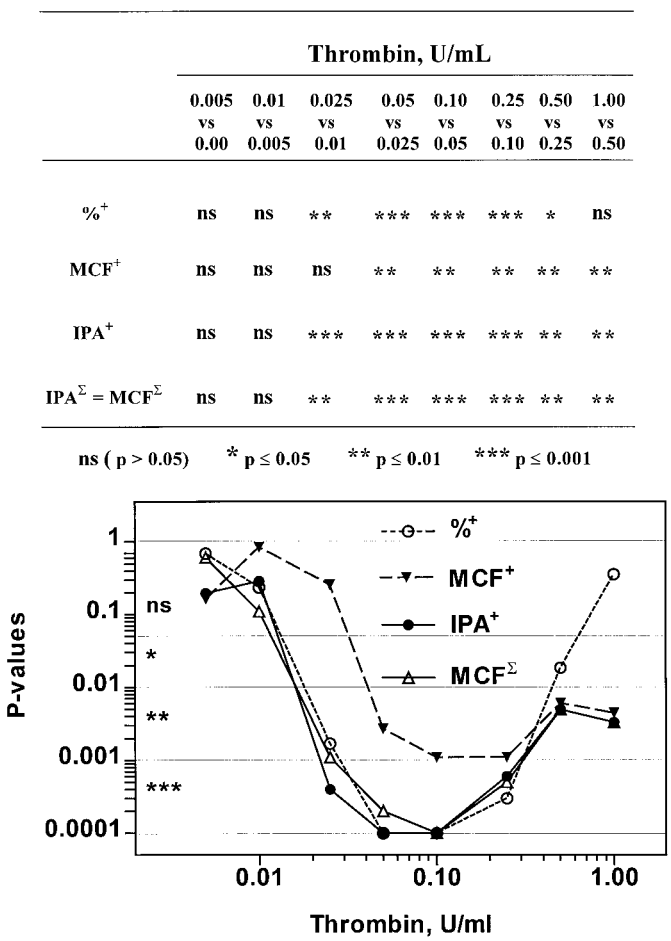

FIG. 2. Statistical significance of the differences between the mean values of flow cytometric parameters for platelet populations treated by 'adjacent' higher and lower thrombin concentrations. *P*-values were calculated as indicated in the text, N = 3. The upper panel shows that *P*-values are not significant (ns, $P > 0.05$) (a) for all parameters: for platelets treated by 0.005 vs 0.00 and 0.01 vs 0.005 U/ml thrombin; (b) for MCF^+: for platelets treated by 0.025 vs 0.01 U/ml thrombin; and (c) for $\%^+$: for platelets treated by 1.00 vs 0.50 U/ml thrombin. *P*-values are significant for all other pairs of platelet populations. At the lower panel, *P*-values are plotted vs the higher thrombin concentration in each pair; horizontal lines show the borders between different zones of *P*-values: ***$P \le 0.001$; **$P \le 0.01$; *$P \le 0.05$; ns ($P > 0.05$).

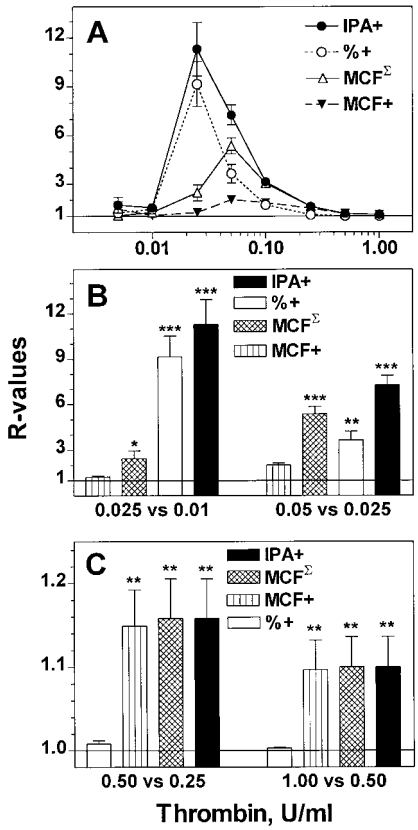

FIG. 3. The ratio between the values of flow cytometric parameters for platelet populations treated by adjacent thrombin concentrations. (A) R-values were calculated as indicated in the text ($R = V_{n+1}/V_n$) and plotted vs the higher thrombin concentration in each pair. *P*-statistics for R-values was calculated vs MCF^+ (B) and vs $\%^+$ (C): ***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$; N = 3. Horizontal lines indicate R = 1, i.e., equal values of parameters for adjacent thrombin concentrations. Note the different scale on the ordinates (A, B) vs (C).

changes for platelet populations activated by adjacent thrombin concentrations. As shown in Figs. 3A and 3B, for platelets activated by subthreshold (0.01 U/ml) vs low (0.025 U/ml) doses of thrombin, the highest increase (9- to 11-fold) was for the IPA^+ and $\%^+$ parameters ($R_{IPA+} = 11.3 \pm 1.6$; $R_{\%+} = 9.2 \pm 1.4$; $P = 0.16$) and the lowest for the MCF^+ parameter ($R_{MCF+} = 1.2 \pm 0.1$). For platelets activated by 0.025 vs 0.05 U/ml thrombin, again the highest increase was characteristically for IPA^+ ($R_{IPA+} = 7.3 \pm 0.6$) and the lowest for MCF^+ ($R_{MCF+} = 2.0 \pm 0.1$); MCF^{Σ} and $\%^+$ parameters displayed intermediate increased values ($R_{MCF\Sigma} = 5.4 \pm 0.5$; $R_{\%+} = 3.6 \pm 0.6$). With high (0.25–1.0 U/ml) thrombin concentrations (Fig. 3C), MCF^+, IPA^+ and MCF^{Σ} parameters increased only 10–16% (R = 1.10–1.16), but the R_{MCF+}, R_{IPA+} and $R_{MCF\Sigma}$ values were significantly higher than $R_{\%+}$ values ($P = 0.01$).

These data indicate that the IPA^+ and MCF^{Σ} parameters show the differences in platelet activation between platelet populations with similar PAS for all thrombin concentrations, including subthreshold (0.01 U/ml) vs low (0.025 U/ml) and two highest (0.50 vs 1.00 U/ml) concentrations.

The lowest R_{MCF+} values for platelets activated by 0.01–0.05 U/ml thrombin (Fig. 3B) and the lowest $R_{\%+}$ values for platelets activated by 0.25–1.00 U/ml thrombin (Fig. 3C) correlated with the highest *P*-values for the respective parameters at these thrombin concentrations (Fig. 2).

Comparison between IPA^+ and IPA^{Σ} Parameters in the Subthreshold and Low Levels of Platelet Activation

For untreated platelet populations (0 U/ml) and populations treated by subthreshold (0.005 and 0.01 U/ml) and low (0.025 U/ml) thrombin concentrations, IPA^{Σ} values are significantly higher than IPA^+ values (Table 2a, columns 2 and 3). However, these higher IPA^{Σ} values reflect the differences in the background fluorescence level of ungated total platelet population ($IPA^{\Sigma} = 0.99 \pm 0.11$) in comparison to the gated population of CD62-positive cells ($IPA^+ = 0.08 \pm 0.03$; $P = 0.0002$). After the subtraction of respective background levels, IPA^+ and IPA^{Σ} values do not differ significantly (Table 2a, columns 4 and 5). For platelet populations treated by medium (0.05–0.10 U/ml) and high (0.25–1.00 U/ml) thrombin, $IPA^+ = IPA^{\Sigma}$ (Table 2b). These data show that after the subtraction of background, IPA^{Σ} equals IPA^+ for all thrombin concentrations.

DISCUSSION

Evaluation of platelet activation status (PAS) of platelet populations and detecting differences between populations with similar PAS may be important for diagnostics and monitoring therapy. In the present work, we created platelet populations differing in activation status. The levels of platelet activation in these populations resemble that in many clinically significant situations. Thus, platelet populations activated with subthreshold (0.005–0.01 U/ml) and low (0.025 U/ml) thrombin contain 1–2 and 14% CD62-positive cells, respectively, mimicking the relatively low (5–19%) levels of platelet activation in many clinical settings (4, 7, 8). Platelets activated by medium (0.05–0.10 U/ml) thrombin doses contain 50–92% CD62-positive cells, resembling the range of platelet activation (30–90%) seen during preparation and storage of platelet concentrates for transfusion (1, 12–18).

PAS is frequently intuitively identified with the values of the proportion ($\%^+$) or/and mean fluorescence (MCF^+) of positive cells but it is still unclear which parameter(s) should be used for PAS definition. The data obtained in this study show that depending on the specific parameter used for PAS evaluation, quite different impressions of the PAS can be obtained for the same platelet population. For example, for platelets

TABLE 2

Comparison of IPA^+ and MCF^Σ in Thrombin-Treated Platelet Populations without and with Subtraction of Background Values of the Parameters in the Absence of Thrombin

	Background is not subtracted		Background is subtracted	
Thrombin, U/ml	IPA^+	$IPA^\Sigma = MCF^\Sigma$	IPA^+	$IPA^\Sigma = MCF^\Sigma$
(a) 0	0.08 ± 0.03	0.99 ± 0.11***	0.00 ± 0.00	0.00 ± 0.00
0.005	0.14 ± 0.06	1.04 ± 0.11***	0.06 ± 0.04	0.05 ± 0.04
0.010	0.20 ± 0.06	1.28 ± 0.17***	0.12 ± 0.03	0.29 ± 0.29
0.025	2.21 ± 0.32	3.09 ± 0.33*	2.12 ± 0.27	2.11 ± 0.25
(b) 0.05	15.90 ± 0.27	16.50 ± 1.80	15.85 ± 1.74	15.62 ± 1.77
0.10	49.80 ± 3.60	49.80 ± 3.50	49.49 ± 4.06	49.14 ± 4.13
0.25	78.80 ± 3.50	78.80 ± 3.50	78.57 ± 2.98	78.39 ± 3.01
0.50	91.20 ± 1.50	91.20 ± 1.50	90.96 ± 2.97	90.88 ± 3.00
1.00	100.00 ± 1.90	100.00 ± 1.90	100.00 ± 1.87	100.00 ± 1.80

Note. Presented are means ± SD (N = 3). *P*-values of MCF^Σ vs IPA^+ were calculated for the data without and with subtracted background for each thrombin concentration: ***$P \leq 0.001$; *$P \leq 0.05$; the other *P*-values are not significant ($P > 0.05$).

treated by low (0.025 U/ml) thrombin, the $\%^+$ and MCF^+ give 3.4- to 6.9-fold higher PAS values than when MCF^Σ and IPA^+ are used; $PAS_{\%+} = 14.4\%$ (or 1:7 of the maximal value), whereas $PAS_{MCF\Sigma} = 2.1\%$ (or only 1:48 of the maximal value). For 0.10 U/ml thrombin, PAS measured as normalized $\%^+$ yields 92% of the maximal value and can be defined as high, whereas PAS of the same platelets measured as MCF^+, IPA^+ and MCF^Σ yield 49–50% and can be defined as medium (Table 1).

From the formal (theoretical) point of view $PAS_{MCF\Sigma}$ can be considered as the reference PAS index, since MCF^Σ is equivalent to IPA^Σ and reflects an integrated amount of CD62 expressed in the total population (9). $PAS_{\%+}$, which reflects only the quantity of activated platelets but not their quality, can be considered as having 'overestimated' in comparison with the reference $PAS_{MCF\Sigma}$ index. In experimental and clinical practice, however, the $\%^+$ parameter is much more frequently used than MCF^+ and MCF^Σ for evaluating PAS in platelet populations with low and medium levels of activation. In the light of data presented, the common usage of $\%^+$ probably reflects the higher sensitivity of $\%^+$ than MCF^+ and MCF^Σ for determining PAS in low- and medium-activated platelet populations.

The normalization procedure described here is likely already 'intuitively' used for PAS determination. When the $\%^+$ is used for evaluating PAS, the $\%^+$ value of a tested platelet population is compared mentally with the maximal theoretical percentage of positive cells in the total population, which is assumed to be 100%, and with the 'ideal' nonactivated platelet population, assumed to be 0%. In addition to PAS determination, the normalization of the flow cytometric parameters to the maximal thrombin response is a useful approach for standardization of experimental and clinical assays of platelet activation. Using 'thrombin-equivalent' units, it can allow comparison of activation by different agonists detected by different activation-dependent antibodies, standardization of results of different experiments, with different donors, in different institutions; it can be also used as a comparative method for expressing platelet activation and/or reactivity in clinical settings. A similar normalization approach has been used by others for MCF^+ parameter (19).

It is difficult to determine the differences between platelet populations with similar activation status. We modelled this situation by the treatment of platelets with various concentrations of thrombin and determined the differences between populations stimulated with adjacent agonist concentrations using $\%^+$, MCF^+, IPA^+ and MCF^Σ parameters. The data presented show that $\%^+$ and MCF^+ parameters have some limitations for revealing differences between platelet populations with similar PAS. $\%^+$ is highly sensitive for revealing the differences in platelet activation in the area of subthreshold (0.01 U/ml) and low (0.025 U/ml) thrombin concentrations but in the area of high (0.25–1.00 U/ml) thrombin, $\%^+$ is insensitive or much less sensitive than MCF^Σ, MCF^+ and IPA^+ parameters. In contrast to $\%^+$, MCF^+ fails to reveal the differences between subthreshold- and low-activated platelets, but is as sensitive as the IPA^+ and MCF^Σ parameters for highly activated platelets (Figs. 2 and 3).

Two other parameters, IPA^+ and $MCF^\Sigma = IPA^\Sigma$, can be considered as parameters with 'universal' sensitivity since they reveal the differences between platelet populations both in the area of subthreshold-low and high PAS range (Figs. 2 and 3). However, in comparison to MCF^Σ, IPA^+ has an advantage since it has significantly lower level of background fluorescence (Table 2a), as the result of cut off of CD62-negative population during the gating of CD62-positive cells; this can be important for more sensitive distinguishing of the differences between platelet populations with similar low PAS in experimental and clinical situa-

tions. Actually, for platelet populations treated by subthreshold versus low thrombin, the increase of IPA^+ values is significantly higher than that of MCF^Σ ($R_{IPA+} = 11.3 \pm 1.6$; $R_{MCF\Sigma} = 2.5 \pm 0.5$; $P = 0.0009$; Fig. 3B). For medium and high levels of platelet activation, on the other hand, IPA^+ and MCF^Σ indices are equivalent and interchangeable, since they give the same levels of CD62 expression (Table 2b), and both parameters are equally effective for revealing the differences in this PAS range (Figs. 3B and 3C). From the practical point of view, however, MCF^Σ is more convenient to use for these levels of platelet activation since it can be obtained directly from flow cytometric list mode, whereas IPA^+ should be calculated as a product of MCF^+ and $\%^+$.

In summary, PAS of agonist-treated platelet populations defined through the values of four flow cytometric parameters, $\%^+$, MCF^+, IPA^+ and MCF^Σ, differs several-fold. Two of these parameters, which reflect the integrated level of CD62 expression (IPA^+ and MCF^Σ), are 'universal', since they distinguish the differences between populations with similar low and similar high PAS. However, in the area of low PAS, IPA^+, but not MCF^Σ, probably should be used for increasing the sensitivity of assay. The $\%^+$ parameter is as sensitive as IPA^+ for revealing the differences between populations in the area of subthreshold-low PAS, but MCF^+ is insensitive in this area. Three parameters, MCF^+, IPA^+ and MCF^Σ, are equally sensitive for distinguishing the differences in the area of high PAS, in contrast to $\%^+$, which is insensitive and should be excluded. Selection of valid and sensitive flow cytometric parameters for detecting platelet activation can serve for the diagnosis of platelet-related disorders, monitoring the course of the disease and effects of antiplatelet therapy.

ACKNOWLEDGMENTS

This work was supported by the Bayer Blood Partnership Fund (Grant 2-77760-2-2601) and the Cara Phelan Trauma Centre, the Trauma Program and the Research Centre (Grant 2-77760-8142) of St. Michael's Hospital. The authors thank Miss Samantha Shakoor and Mr. David J. Allen for preparing the graphs.

REFERENCES

1. Rinder, H. M., Snyder, E. L., Bonan, J. L., Napychank, P. A., Malkus, H., and Smith, B. R. (1993) Activation in stored platelet concentrates: correlation between membrane expression of P-selectin, glycoprotein IIb/IIIa, and β-thromboglobulin release. *Transfusion* **33,** 25–29.
2. Michelson, A. D. (1996) Flow cytometry: A clinical test of platelet function. *Blood* **87,** 4925–4936.
3. Dumont, L. J., VandenBroeke, T., and Ault, K. A. (1999) Platelet surface P-selectin measurements in platelet preparations: An international collaborative study. *Transfus. Med. Rev.* **13,** 31–42.
4. Zeller, J. A., Tschoepe, D., and Kessler, C. (1999) Circulating platelets show increased activation in patients with acute cerebral ischemia. *Thromb. Haemost.* **81,** 373–377.
5. Hjemdahl, P., Chronos, N. A., Wilson, D. J., Bouloux, P., and Goodall, A. H. (1994) Epinephrine sensitizes human platelets in vivo and in vitro as studied by fibrinogen binding and P-selectin expression. *Arterioscler. Thromb.* **14,** 77–84.
6. Shattil, S., Cunningham, M., and Hoxie, J. A. (1987) Detection of activated platelets in whole blood using activation-dependent monoclonal antibodies and flow cytometry. *Blood* **70,** 307–315.
7. Tschoepe, D., Schultheiss, H. P., Koralov, P., Schwippert, B., Dannehl, K., Neuwenhuis, H. K., Kehrel, B., Strauer, B., and Gries, F. A. (1993) Platelet membrane activation markers are predictive for increased risk of acute ischaemic events after PTCA. *Circulation* **88,** 37–42.
8. Reverter, J. C., Escolar, G., Sanz, C., Cases, A., Villamor, N., Niewenhuis, H. K., Lopes, J., and Ordinas, A. (1994) Platelet activation during hemodialysis measured through exposure of p-selectin: Analysis by flow cytometry and ultrastructural techniques. *J. Lab. Clin. Med.* **124,** 79–85.
9. Leytin, V., Mody, M., Semple, J. W., Garvey, B., and Freedman, J. (2000) Flow cytometric parameters for characterizing platelet activation by measuring P-selectin (CD62) expression: Theoretical consideration and evaluation in thrombin-treated platelet populations. *Biochem. Biophys. Res. Commun.* **269,** 85–90, doi: 10.1006/bbrc.2000.2255.
10. Kühne, T., Hornstein, A., Semple, J., Chan, W., Blanchette, V., and Freedman, J. (1995) Flow cytometric evaluation of platelet activation collected into EDTA vs. Diatube-H, a sodium citrate solution supplemented with theophylline, adenosine, and dipyridamole. *Am. J. Hematol.* **50,** 40–45.
11. Leytin, V., Shapiro, H., Novikov, I., and Radnay, J. (1996) Flow cytometric analysis of the platelet surface area and surface density of glycoprotein IIb-IIIa of unactivated human platelets of various sizes. *Biochem. Biophys. Res. Commun.* **226,** 94–100.
12. Triulzi, D. J., Kikler, T. S., and Braine, H. G. (1992) Detection and significance of alpha granule membrane protein 140 expression on platelets collected by apheresis. *Transfusion* **32,** 529–533.
13. Pedigo, M., Wun, T., and Paglieroni, T. (1993) Removal by white cell-reduction filters of activated platelets expressing CD62. *Transfusion* **33,** 930–935.
14. Divers, S. G., Kannan, K., and Stewart, R. M. (1995) Quantitation of CD62, soluble CD62, and lysosome-associated membrane proteins 1 and 2 for evaluation of the quality of stored platelet concentrates. *Transfusion* **35,** 292–297.
15. Holme, S., Sweeney, J. D., Sawyer, S., and Elfath, M. D. (1997) The expression of p-selectin during collection, processing, and storage of platelet concentrates: Relationship to loss of in vivo viability. *Transfusion* **37,** 12–17.
16. Metcalfe, P., Williamson, L. M., Reutelingsperger, C. P. M., Swaan, I., Ouwehand, W. H., and Goodall, A. H. (1997) Activation during preparation of therapeutic platelets affects deterioration during storage: A comparative flow cytometric study of different production methods. *Br. J. Haematol.* **98,** 86–95.
17. Wang, C., Mody, M., Herst, R., Sher, G., and Freedman, J. (1999) Flow cytometric analysis of platelet function in stored platelet concentrates. *Transfus. Sci.* **20,** 129–139.
18. Devine, D. V., Bradley, A. J., Maurer, E., Levin, E., Chahal, S., Serrano, K., and Gyongyossy-Issa, M. I. C. (1999) Effect of prestorage white cell reduction on platelet aggregate formation and the activation state of platelets and plasma enzyme systems. *Transfusion* **39,** 724–734.
19. Michelson, A. D., Ellis, P. A., Barnard, M. R., Matic, G. B., Viles, A. F., and Kestin, A. S. (1991) Downregulation of the platelet surface glycoprotein Ib-IX complex in whole blood stimulated by thrombin, adenosine diphosphate, or an in vivo wound. *Blood* **77,** 770–779.