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Flow Cytometric Analysis of the Platelet Surface Area and Surface Density of Glycoprotein IIb-IIIa of Unactivated Human Platelets of Various Sizes

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Large (L) *activated* platelets exhibit greater aggregability and express more activated glycoprotein IIb-IIIa (GPIIb-IIIa) per cell and per unit surface area than small (S) activated platelets. We studied the binding of CD61 monoclonal antibody to GPIIb-IIIa on *resting* platelets and developed a new method for determining platelet surface area by flow cytometry. Using this method, we found that resting L platelets contain two times more GPIIb-IIIa *per cell* than S platelets but the *same* amount of GPIIb-IIIa *per unit surface area.* The data suggest that the greater aggregability of L platelets is likely to be due to increased activation and/or expression of GPIIb-IIIa rather than to elevated density of unactivated GPIIb-IIIa on resting L platelets.

Platelet aggregation plays a key role in haemostasis and thrombosis. This fundamental reaction is mediated by the binding of fibrinogen to the trans-membrane αIIbβ3 integrin, the platelet GPIIb-IIIa complex (1-5). GPIIb-IIIa complexes do not bind fibrinogen on resting platelets. Activation of platelets by various agonists, regardless of the initiating stimulus, induces a common event - conformational changes resulting in the conversion of unactivated GPIIb-IIIa into activated GPIIb-IIIa complexes which serve as fibrinogen receptors (1-5). The binding of fibrinogen to these receptors on adjoining platelets results in the cross-linking of platelets into an aggregate (6).

Human platelets are markedly heterogeneous in size and manifest size-dependent functional heterogeneity. L platelets are more sensitive to aggregating agents, more rapidly recruited into aggregates in vitro (7-9) and more effective in arresting bleeding than S ones (7,10). Using flow cytometry and FITC-PAC1, a monoclonal antibody specific for activated GPIIb-IIIa, Frojmovic et al. demonstrated that after stimulation, L platelets contain 3-4 times more *activated GPIIb-IIIa per cell* than S platelets (11). In addition, L platelets expressed 2-3 times more *activated GPIIb-IIIa per unit SA* than S platelets, as determined by direct microscopic SA measurements of L and S platelets isolated by a cell sorter (11). Flow cytometric analysis of *resting* platelets showed that L platelets contained more *unactivated GPIIb-IIIa per cell* than S platelets (12-15). However, there are no data on the *densities* of GPIIb-IIIa on the platelet surface in these subpopulations since SA of S and L platelets have not been determined.

Here we study the distribution of GPIIb-IIIa on resting S, M and L platelets using FITC-

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Abbreviations: GPIIb-IIIa: glycoprotein IIb-IIIa; PRP: platelet-rich plasma; S, M, L, T: small, medium, large, total platelets, respectively; FS: forward scatter; SS: side scatter; FITC: fluorescein isothiocyanate; FL: fluorescence of platelet-bound FITC-antibody; SA, V: surface area and volume of platelets, respectively; D: density of GPIIb-IIIa on the platelet surface (amount of GPIIb-IIIa per unit SA).

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CD61 monoclonal antibody and flow cytometry. To assay the amount of GPIIb-IIIa per unit SA, we developed a method for determining platelet SA from flow cytometric data. Using this approach, we demonstrated that although the amount of GPIIb-IIIa per resting cell was approximately twofold greater on L than on S platelets, the amount of GPIIb-IIIa per unit SA was the same. These results indicate that the increased density of fibrinogen receptor and the enhanced aggregability of activated L platelets is unlikely to be due to the greater density of GPIIb-IIIa on resting L platelets.

METHODS

Monoclonal antibody. To detect the GPIIb-IIIa complex on platelets, we used FITC-CD61 (clone BL-E6, FITC:protein = 5), an IgG1 mouse antihuman monoclonal antibody that binds to the GPIIIa (β3) subunit of the complex (Caltag, CA). Although CD61 also binds to the β3 subunit of the αVβ3 vitronectin receptor, this interaction should not prevent identification of GPIIb-IIIa since human platelets contain very small amounts of αVβ3 receptor (50-100 αVβ3 copies vs 45,000 GPIIb-IIIa copies per cell) (16). Mouse FITC-IgG1 (Caltag) was used as isotype control. Ca^{2+}-free HEPES-Tyrode buffer (137 mM NaCI, 2.7 mM KCI, 3.3 mM NaH_2PO_4, 12 mM $NaHCO_3$, 2 mM $MgCI_2$, 5.5 mM dextrose, 10 mg/ml bovine serum albumin (Fraction V), 20 mM HEPES, pH 7.4) was freshly prepared, filtered through a 0.22 μm filter and used for the dilution of CD61 and IgG1.

Preparation of platelet-rich plasma. Venous blood was obtained from healthy donors who had taken no medications for at least two weeks. To minimize platelet activation during blood collection, we used a 19-gauge butterfly needle with a light tourniquet and discarded the first 2 ml of blood. Blood was collected into 1/9th volume of 3.8% sodium citrate and PRP was obtained by centrifugation at 160g for 8 min. 15 PRP samples were analysed.

Incubation of platelets with CD61 antibody. Immediately after isolation, 5 μl aliquots of PRP (1-1.5$\times 10^6$ platelets) were added to 10×75 mm polystyrene tubes containing 40 μl of HEPES-Tyrode buffer and 5 μl of a saturating concentration (20 μg/ml) of FITC-CD61 or FITC-IgG1. The mixtures were incubated at room temperature for 30-40 min without stirring, diluted with 450 μl of HEPES-Tyrode buffer and analysed on a flow cytometer without any washing steps.

Flow cytometry. The samples were analysed on an EPICS Profile II flow cytometer (Coulter Corp., Hialeah, FL) equipped with a 14 mW argon ion laser, wavelength of 488 nm. A neutral density filter was placed in front of the FS detector. Fluorescein (green) FL and SS were detected using 530 nm and 488 nm band pass filters. All signals were amplified by using a three decade logarithmic amplifier and were displayed on a 256-channel scale. 20,000-25,000 events were analysed for each PRP sample.

Determination of SA and D from flow cytometric data. For any platelet population consisting of large numbers of cells, the mean density of GPIIb-IIIa on the platelet surface (D°_m, μm^{-2}) can be expressed as:

$$D^\circ_m = FL_m/SA^\circ_m \quad [1]$$

where FL_m is the mean GPIIb-IIIa-associated fluorescence measured by flow cytometry in arbitrary units at saturating concentrations of FITC-CD61 in the incubation mixture, and SA°_m is the mean platelet surface area (μm^2).

To determine D°_m values for S, M, L and T platelets, FL_m and SA°_m values for these platelet populations have to be estimated. FL_m values for S, M, L and T platelets may be obtained from the respective FL histograms.

For SA°_m calculations, we used FS as an index of platelet size. Holme et al. (17) showed that the log of the mean resistive platelet volume, as measured by the Coulter Counter, and the log of the mean FS, as measured by flow cytometry, are related by the linear regression:

$$\log_{10} V^\circ_m = \log_{10}\alpha + \beta \log_{10} FS_m \quad [2a]$$

Thus, the mean platelet volume (V°_m, μm^3) is a power function of platelet FS_m (arbitrary units):

$$V^\circ_m = \alpha FS^\beta_m \quad [2b]$$

where the constant α is the mean volume of particles (μm^3) with the minimal FS ($FS_m = 1$), and the constant β is the degree of the power function, independent of platelet FS_m (size), over a wide range of FS_m (ratio of maximal FS_m to minimal $FS_m \approx 4.5$). The regression, defined by Eq. 2a, was obtained for T platelets from normal donors and from patients with thrombocytopenia, thus covering a wide range of size distribution (17).

Since the shape of unactivated platelets is independent of their size (9, 11, 18), the equation

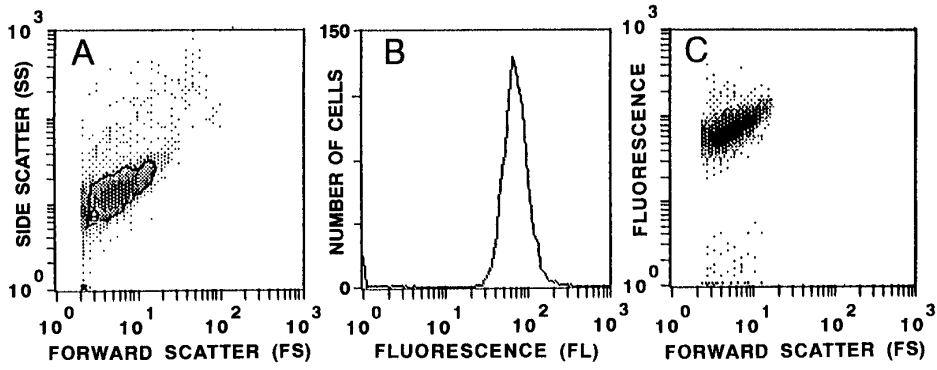

FIG. 1. Flow cytometric analysis of anti-GPIIb-IIIa FITC-CD61 antibody binding to resting total platelets. 5 μl of human PRP were incubated with a saturating concentration (13 nM) of FITC-CD61, diluted and analysed on a flow cytometer (see Methods). (A) Detection of T platelets from two-parameter SS versus FS dot plot. Platelets were gated by the platelet specific contour plot. A lower FS threshold was set to separate platelets from cell debris and electronic noise. 10,000-15,000 events were accumulated within the gated area. (B) One-parameter FL histogram of T platelets gated from (A). FL profile has a log normal distribution. (C) FL versus FS dot plot gated from (A). The plot shows a direct relationship between log GPIIb-IIIa-bound fluorescence and logFS (size) for T platelets.

$$SA_m^\circ = k(V_m^\circ)^{2/3} \quad [3]$$

is correct for any platelet size. Here SA_m° is the mean platelet SA (μm^2), and k is the size-independent platelet shape constant.

Then, the relation between surface area and forward scatter of platelets is

$$SA_m = FS_m^b \quad [4]$$

where $SA_m = SA_m^\circ/k\alpha^{2/3}$ is the mean platelet SA in arbitrary units; the constant $k\alpha^{2/3}$ is the particle SA (μm^2) with $FS_m = 1$; the constant $b = 2\beta/3$ does not depend on FS_m of platelets.

Combining Eqs. 1 and 4, we obtain

$$D_m = FL_m/FS_m^b \quad [5a]$$

where $D_m = D_m^\circ k\alpha^{2/3}$ is the mean density of GPIIb-IIIa on platelet surface in arbitrary units.

By writing Eq. 5a as

$$\log_{10}FL_m = \log_{10}D_m + b\log_{10}FS_m \quad [5b]$$

we obtain the linear regression with the Y-intercept $\log_{10}D_m$ and the slope b.

Thus, by determining the slope b from the linear regression Eq. 5b for the S + M + L population, the values SA_m and D_m for S, M, L and T platelets can then be calculated from Eqs. 4 and 5a, respectively.

RESULTS

To determine GPIIb-IIIa on the surface of resting platelets, we used FITC-CD61 monoclonal antibody that binds to the GPIIIa subunit of the complex. Platelets in PRP were identified and gated from specific SS vs FS dot plots (Fig. 1A). Platelet-bound FL has a log normal distribution (Fig. 1B) specific for platelets (15, 17). The population of T platelets, gated from SS-FS plot, contains 93.5 $\pm$ 2.9% of CD61 positive cells (Fig. 1B) which is significantly greater than the percent of positive cells gated from one-parameter FS histograms (83.5 $\pm$ 6.3%, n=15, P $<$

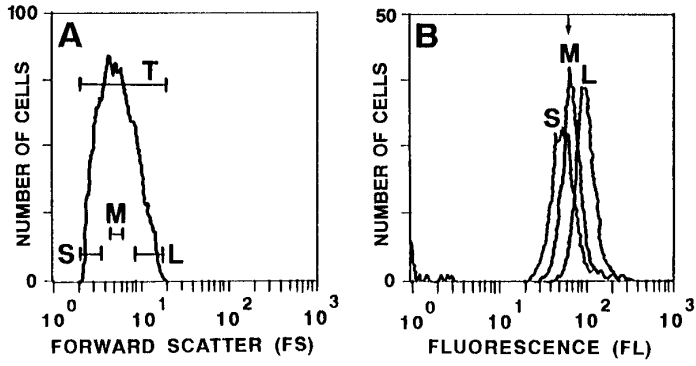

FIG. 2. (A) Analytical separation of FS histogram for T platelets into S, M, L subpopulations. The FS histogram for T platelets was gated from Fig. 1A. S, M, L subpopulations were gated to obtain approximately 20% of forward scattering particles in each subpopulation. (B) FL histograms for S, M, L platelets gated from (A). The maximal binding of FITC-CD61 was observed for all groups of platelets at 13 nM CD61. The arrow indicates the FL peak for T platelets.

0.001). We used two-parameter SS-FS gating in this study to obtain the higher percent of positive cells.

Fig. 1C shows the size-dependent distribution of GPIIb-IIIa-bound fluorescence for resting T platelets. As reported (13-15), we observed a direct relationship between logFL and logFS. These data indicate that the amount of GPIIb-IIIa per resting cell increases with increasing platelet size.

To quantify the GPIIb-IIIa on platelets of different sizes, we analytically separated T platelets into S, M and L subpopulations. The gates for S, M, L platelets were set on the FS histogram of T platelets, to represent approximately 20% of the particles in each subpopulation (Fig. 2A). These gates were used to create FL histograms for S, M, L platelets (Fig. 2B). Mean FS and FL values (FS_m, FL_m) for S, M, L, T platelets were obtained from FS and FL histograms (Fig. 2) for each PRP, and means of the means ($FS_{\bar{m}}$, $FL_{\bar{m}}$) were calculated for 15 PRP samples. Table 1 shows that both $FS_{\bar{m}}$ and $FL_{\bar{m}}$ are significantly greater for L platelets than for M and S platelets ($P < 0.001$).

To determine the platelet surface area and density of GPIIb-IIIa on the surface of unactivated, different-sized platelets, we plotted $\log_{10}FL_m$ vs $\log_{10}FS_m$ data for S, M, L platelets from 15 PRP samples in accordance with Eq. 5b (Fig. 3) and analysed individual linear regressions for each subpopulation with the same size-independent b slope (see Methods). The significance of the difference between $\log_{10}D_{\bar{m}}$ values for S, M, L platelets was tested using a one-way ANACOVA model for $\log_{10}FL_m$ with $\log_{10}FS_m$ as a covariate (19). The analysis demonstrated that the $\log_{10}D_{\bar{m}}$ values for all three groups of platelets did not differ significantly ($P_{L:S:M} < 0.17$).

Therefore, the relationship between $\log_{10}FL_m$ and $\log_{10}FS_m$ values for S, M, L platelets was analysed in accordance with the common linear regression. Fig. 3 shows a strong positive correlation ($R = 0.96$, $P < 0.001$) between $\log_{10}FL_m$ and $\log_{10}FS_m$ for S + M + L platelets with the regression line:

$$\log_{10}FL_m = 1.52 + 0.47 \log_{10}FS_m \quad [5c]$$

where $b = 0.47$ is the size-independent slope of the regression line, and $D_m = 33.1$ ($\log_{10}D_m$

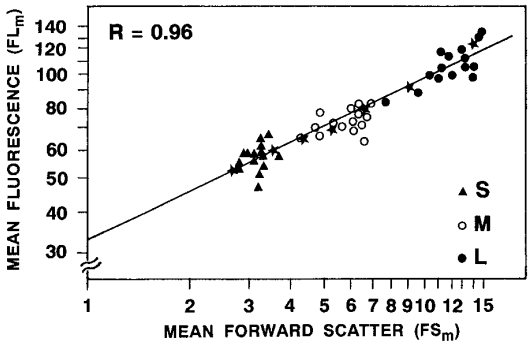

FIG. 3. Relationship between mean GPIIb-IIIa-associated fluorescence (log scale) and mean forward scatter (log scale) for S, M, L resting platelets. 15 PRP samples were incubated with FITC-CD61 and studied by flow cytometry. 10,000-15,000 events were accumulated within platelet gate for each sample. T platelets were divided into S, M, L subpopulations and FS and FL profiles analysed (see Fig. 2). Each of 45 points was plotted using FS_m and FL_m values (arbitrary units) for 2,000-3,000 cells. The linear regression, represented by the solid line $\log_{10}FL_m = 1.52 + 0.47\log_{10}FS_m$ with R = 0.96, was calculated using the regression procedure of SPSS 6.0 (19). For one PRP sample, T platelets were divided into 7 subpopulations (asterisks), each containing ~10% of platelets interspaced by ~5% of platelets. The data fit the linear regression $\log_{10}FL_m = 1.50 + 0.50\log_{10}FS_m$ with R = 0.99.

= 1.52) is the density of GPIIb-IIIa for the S + M + L population, which contains about 60% of T platelets (Fig. 2A).

SA_m and D_m values for S, M, L platelets were calculated for each PRP in compliance with Eqs. 4 and 5a using FL_m and FS_m values obtained from FL and FS histograms (Fig. 2) and the regression coefficient b = 0.47 (Fig. 3, Eq. 5c). Table 1 shows $SA_{\bar{m}}$ and $D_{\bar{m}}$ values, means of the means for 15 PRP samples. The $SA_{\bar{m}}$ value for L platelets was 1.86 ± 0.12 times greater than that for S platelets and 1.39 ± 0.05 times greater than that for M platelets. The $D_{\bar{m}}$ values for S, M, L platelets did not differ significantly.

Since the data presented above was obtained by arbitrary division of T platelets into three S, M, L fractions, each containing ≈20% of platelets, we also tested a different way of separating into size-dependent fractions. Seven subpopulations of different platelet size, each consisting of about 10% of platelets, were gated from their FS intensities (as in Fig. 2) and data analysed in accordance with Eq. 5b. The $\log_{10}FL_m$ versus $\log_{10}FS_m$ plot fits the linear regression with a high correlation: R = 0.99 (Fig. 3, asterisks). D_m values for these independently sized fractions also did not differ significantly: 32.1, 31.6, 31.3, 30.0, 30.4, 30.4, 32.4 (for fractions of increasing platelet size, respectively); mean ± SD = 31.2 ± 1.0 (3.2%).

DISCUSSION

Frojmovic et al. demonstrated that L platelets expressed 3-4 times more activated GPIIb-IIIa per cell and 2-3 times more activated GPIIb-IIIa per unit SA than S platelets when maximally stimulated with ADP or phorbol myristate acetate (11). To determine the mean density of GPIIb-IIIa on the platelet surface, these authors sorted platelets on a flow cytometer and calculated the mean SA for L and S platelets from phase-contrast microscopic measurements of the thickness and diameter of individual platelets.

Two models may be proposed to explain the greater density of activated GPIIb-IIIa on the surface of activated L platelets: 1) resting L platelets contain more GPIIb-IIIa complexes per unit SA than S platelets; 2) after activation, L platelets expose more GPIIb-IIIa complexes from intracellular pools than S platelets and/or are more efficient in the transformation of unactivated surface GPIIb-IIIa complexes into the active conformation.

TABLE 1
Analysis of Fluorescence ($FL_{\bar{m}}$), Forward Scatter ($FS_{\bar{m}}$), Surface Area ($SA_{\bar{m}}$) and Density of GPIIb-IIIa ($D_{\bar{m}}$) for S, M, L and T Platelets

Platelets	n	$FL_{\bar{m}}$	$FS_{\bar{m}}$	$SA_{\bar{m}}$	$D_{\bar{m}}$
S	15	57.4 ± 5.0	3.17 ± 0.24	1.71 ± 0.06	33.6 ± 2.9
M	15	72.7 ± 6.0	5.91 ± 0.80	2.28 ± 0.15	31.9 ± 2.2
L	15	106.8 ± 13.6	12.07 ± 1.95	3.18 ± 0.25	33.5 ± 2.7
S + M + L	45	79.0 ± 22.7	7.05 ± 3.95	2.39 ± 0.63	33.0 ± 2.7
T	15	76.7 ± 7.2	6.04 ± 0.77	2.32 ± 0.13	33.0 ± 2.6

Note. Means of the means ($FL_{\bar{m}}$, $FS_{\bar{m}}$, $SA_{\bar{m}}$, $D_{\bar{m}}$) ± standard deviations for 15 PRP samples are reported. All values are in arbitrary units (see Methods). $FL_{\bar{m}}$ ± SD and $FS_{\bar{m}}$ ± SD were calculated from FL_m and FS_m values for each PRP plotted on Fig. 3. SA_m and D_m for each sample were calculated from Eqs. 4 and 5a using FS_m and FL_m values and the regression coefficient b = 0.47 from Fig. 3. $SA_{\bar{m}}$ ± SD and $D_{\bar{m}}$ ± SD were calculated from SA_m and D_m values. The one-way ANOVA procedure with appropriate contrasts was used to calculate the significance of differences between the values for S, M, L platelets (19). All the differences between $FL_{\bar{m}}$, $FS_{\bar{m}}$ and $SA_{\bar{m}}$ for S, M, L platelets are significant ($P < 0.001$). The differences between $D_{\bar{m}}$ values are not significant: L vs S ($P_{L:S} < 0.96$), L vs M ($P_{L:M} < 0.09$), and M vs S ($P_{M:S} < 0.10$).

To test these models, we studied the distribution of GPIIb-IIIa on resting platelets as a function of platelet size using flow cytometry and FITC-CD61 antibody. In agreement with the data of others (13-15), we found that for T platelet population, the amount of GPIIb-IIIa per resting cell increases with increasing platelet size (Fig. 1C). After analytical separation of S, M and L platelets according to their FS profiles (Fig. 2A), we showed than L platelets contain 1.86 ± 0.23 more unactivated GPIIb-IIIa per cell that S platelets, and 1.47 ± 0.10 more glycoprotein per cell than M platelets (Table 1, Fig. 2B).

To calculate the densities of GPIIb-IIIa on S, M, L platelets, the SA of these platelets had to be assayed. Here we developed a new method for determining platelet SA from flow cytometric data only. This method obviates the need for physical separation of T platelets into S, M, L fractions by density gradient centrifugation (20), counterflow centrifugation (9) or cell sorting (11) and determination of platelet SA by independent techniques such as resistive particle sizing (9, 20) or quantitative microscopy (11, 18).

Using this method, we found that the density of GPIIb-IIIa on the surface of resting platelets is the same for S, M, L subpopulations (Table 1). These data suggest that it is the enhanced activation and/or expression of GPIIb-IIIa, rather than the greater density of unactivated GPIIb-IIIa on resting L platelets that determines the increased density of fibrinogen receptor and the enhanced aggregation response of activated L platelets.

REFERENCES

1. Kieffer, N., and Phillips, D. R. (1990) *Annu. Rev. Cell. Biol.* **6,** 329–357.
2. Phillips, D. R., Charo, I. F., Parise, L. V., and Fitzgerald, L. A. (1988) *Blood* **71,** 831–843.
3. Ginsberg, M. H., Loftus, J. C., and Plow, E. F. (1988) *Thromb. Haemostas.* **59,** 1–6.
4. Plow, E. F., and Ginsberg, M. H. (1988) *Prog. Hemost. Thromb.* **9,** 117–156.
5. Coller, B. S. (1990) *N. Engl. J. Med.* **322,** 33–42.
6. Hawiger, J., Kloczewiak, M., Bednarek, M. A., and Timmons, S. (1989) *Biochemistry* **28,** 2909–2914.
7. Karpatkin, S. (1978) *Blood* **51,** 307–316.
8. Thompson, C. B., Jakubowski, J. A., Quinn, P. G., Deykin, D., Valeri, C. R. (1983) *J. Lab. Clin. Med.* **101,** 205–213.
9. Wong, T., Pedvis, L., and Frojmovic, M. M. (1989) *Thromb. Haemostas.* **62,** 733–741.
10. Thompson, C. B. (1985) *Br. Med. J.* **291,** 95–96.
11. Frojmovic, M., and Wong, T. (1991) *Biophys. J.* **59,** 828–837.

12. Johnston, G. I., Heptinstall, S., Robins, R. A., and Price, M. R. (1984) *Biochem. Biophys. Res. Commun.* **123,** 1091–1098.
13. Jennings, L., Ashmun, R., Wang, W., and Dockter, M. (1986) *Blood* **68,** 173–179.
14. Mitchelson, A. D. (1987) *J. Lab. Clin. Med.* **110,** 346–354.
15. Giles, H., Smith, R. E. A., and Martin, J. F. (1994) *Eur. J. Clin. Invest.* **24,** 69–72.
16. Coller, B., Cheresh, D., Asch, E., and Seligsohn, U. (1991) *Blood* **77,** 75–83.
17. Holme, S., Heaton, A., Konchuba, A., and Hartman, P. (1990) *J. Lab. Clin. Med.* **112,** 223–241.
18. Frojmovic, M., and Panjwani, R. (1976) *Biophys. J.* **16,** 1071–1089.
19. SPSS Base System Syntax Reference Guide, Release 6.0 (1993) SPSS Chicago.
20. Jung, S., Tanoue, K., and Yamazaki, H. (1985) *Thromb. Haemostas.* **53,** 188–194.