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Platelet Size Distribution Measurements as Indicators of Shear Stress–Induced Platelet Aggregation

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Abstract—The mechanisms underlying shear stress–induced platelet aggregation (SIPA) were investigated by measuring changes in the platelet size distributions resulting from the exposure of human platelet-rich plasma (PRP) to well-defined shear stresses in a modified viscometer. Exposure of PRP to a shear stress of 100 dyne/cm^2 for 1 min at 37°C resulted in the loss of single platelets, an overall shift in the distribution to larger particle sizes, and the generation of platelet fragments. Treatment of PRP prior to shearing with a monoclonal antibody directed against platelet glycoprotein (GP) IIb-IIIa (integrin $\alpha_{IIb}\beta_3$) at a concentration that completely inhibited ADP-induced platelet aggregation also inhibited SIPA. Furthermore, incubation of PRP with a recombinant fragment of von Willebrand factor (vWF) that abolishes ristocetin-induced platelet agglutination significantly inhibited but did not eliminate SIPA. Pretreatment of PRP with the tetrapeptides RGDS or RGDV, which constitute the GP IIb-IIIa peptide recognition sequences on fibrinogen and vWF, almost completely blocked platelet aggregation at 100 dyne/cm^2, whereas the negative control peptide RGES had no discernible effect. Finally, incubation of PRP with a monoclonal antibody directed against the platelet vitronectin receptor (integrin $\alpha_v\beta_3$) did not affect SIPA. These results indicate that both GP IIb-IIIa and GP Ib, the latter through its interaction with vWF, are required for SIPA at 100 dyne/cm^2; that the interaction of GP IIb-IIIa with its adhesive ligands under shear stress can be inhibited by RGD-containing peptides; and that the vitronectin receptor on platelets, which shares the same β_3 subunit as GP IIb-IIIa, plays no role in SIPA. On the basis of these results, the assessment of platelet size distributions provides a sensitive and quantitative measurement for the study of SIPA.

Keywords—Shear stress, Viscometer, Platelet aggregation, Integrins

INTRODUCTION

Platelet adhesion and aggregate (thrombus) formation constitute central events in normal hemostasis and, in association with the coagulation pathway, serve to arrest blood loss at sites of vascular injury (18,21). Both the initial events of platelet attachment and the subsequent thrombus formation have been shown to depend strongly on the local flow conditions (1,2,11,23,25). Studies of platelet adhesion to the subendothelial matrix at shear rates encountered in the microvasculature (2,600 sec^{-1}) have demonstrated that the initial attachment of platelets is mediated by the binding of von Willebrand factor (vWF) to platelet glycoprotein Ib (GP Ib) (4,12,20,23,24). Subsequent work revealed that platelet GP IIb-IIIa ($\alpha_{IIb}\beta_3$) is also involved in the adhesion process, although the ligand responsible for the attachment has not been completely specified (22). Platelet spreading, leading to irreversible adhesion, and aggregate formation also require the interaction of GP Ib with vWF and GP IIb-IIIa with either fibrinogen, vWF, or possibly vitronectin, depending on the magnitude of the shear rate (22). This latter observation contrasts with the traditional notion that fibrinogen is the primary ligand mediating platelet aggregation through the GP IIb-IIIa receptor, but is supported by work showing that vWF can substitute for fibrinogen in aggregometry studies using washed normal platelets (19) or platelet-rich plasma (PRP) from afibrinogenemic patients (6).

In addition to the conventional agonists known to induce platelet aggregation, shear forces (stresses) can also cause aggregate formation (9,13–15), a phenomenon that may contribute to thrombotic events under shear conditions potentially present in the vicinity of stenosed blood vessels or inadequately designed prosthetic heart valves. Recent work by Moake and his colleagues (13–15) has demonstrated that exposure of platelets to physiological shear stresses (1–50 dyne/cm^2) causes reversible platelet aggregation in the absence of exogenous agonists; larger shear stresses ($\geq$100 dyne/cm^2) induce irreversible platelet aggregate formation, a process that is apparently mediated by the interaction of vWf with both the GP Ib and GP IIb-IIIa platelet receptors (9) and that occurs in an aspirin-independent manner (5). The binding of vWF to GP Ib induced by arterial shear stresses has also been reported to activate protein kinase C independently of diacylglycerol formation (10), although the mechanism of action is not understood.

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The present studies were undertaken to assess the utility of platelet size distribution measurements as markers of shear-induced platelet aggregation (SIPA). Monoclonal antibodies directed against the platelet integrin receptors $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$, RGDX peptides, and a recombinant fragment of vWF that competitively inhibits vWF binding to GP Ib were used to explore further the mechanisms by which fluid forces induce platelet aggregate formation.

METHODS

Materials

The anticoagulant used in these studies was buffered sodium citrate (100 mM sodium citrate, 20 mM citric acid [pH 5.6]). RGDS (lot 31H08931) and RGES (lot 51H08271) were obtained from Sigma Chemical Co. (St. Louis, MO), and RGDV (lot 018958) was purchased from Peninsula Labs (Belmont, CA). The final concentrations of RGDS and RGES were 1 mM, and that of RGDV was 0.5 mM. Stock paraformaldehyde solution was prepared as a 4% solution in phosphate-buffered saline (pH 7.4) and used at a final concentration of 0.5%. The recombinant vWF fragment (VCL) was a generous gift of Dr. Leonard Garfinkel (Bio-Technology General, Rehovot, Israel) (8). At the concentration used in these studies (50 μg/ml), VCL completely abolished ristocetin-induced platelet agglutination.

Platelet Isolation

Venous blood was collected from normal, consenting, adult volunteers who had abstained from aspirin use for at least 1 week and whose platelets exhibited normal aggregation when challenged with 10 μM ADP. Nine parts blood were collected into 1 part anticoagulant, PRP was prepared by centrifugation at 135 × g for 20 min. Autologous platelet-poor plasma, prepared by centrifugation of blood at 1,000 × g for 20 min, was used to adjust platelet counts in the PRP to $2 \times 10^5/\mu l$ to $4 \times 10^5/\mu l$. Platelet counts were measured with a Coulter Counter (Model ZM, Coulter Electronics, Hialeah, FL) using a 70 μm aperture. PRP was incubated with the various peptides, antibodies, or VCL for at least 20 min at 37°C prior to shearing in the viscometer.

Monoclonal Antibodies

The complex-specific anti–GP IIb-IIIa ($\alpha_{IIb}\beta_3$) monoclonal antibody 7E3 was a generous gift of Dr. Barry Coller (SUNY, Stony Brook, NY), and the anti-vitronectin receptor ($\alpha_v\beta_3$) monoclonal antibody LM-609 was a gift of Dr. David Cheresh (Research Institute of Scripps Clinic, La Jolla, CA). Both antibodies were used at a concentration of 10 μg/ml. As measured in an aggregometer (Model 1020B, Payton Scientific, Buffalo, NY), 7E3 completely inhibited platelet aggregation induced by 10 μM ADP, whereas LM-609 had no effect.

Modified Viscometer Used to Study Shear-Induced Platelet Aggregation

The modified viscometer (Model TS-8, Kaltec Scientific, Novi, MI) is a coaxial cylinder viscometer consisting of a stainless steel cup (diameter 4 cm) fitted with a polymeric insert; the bob (diameter 3.8976 cm), also stainless steel, was fashioned with a cone angle of 0.91°. The average shear stress (dynes/cm^2) in the two regions of the viscometer depends on the dimensions of the bob and cup, and they were thus designed such that a uniform and equivalent shear stress would be exerted on the fluid in both regions of the modified viscometer. This system allows for the examination of an extensive range of shear stresses as well as the use of relatively small volumes of PRP (~1.8 ml).

Prior to each experiment, the cup and bob were rinsed extensively with deionized, distilled water, carefully dried, and equilibrated to 37°C using heating tape. PRP at 37°C was pipetted into the cup, and the bob was lowered such that air bubbles were removed and the platelet suspension was displaced into the entire gap region. After exposing the PRP to the desired shear stress for 1 min, the bob was raised, and 1 ml of the platelet suspension was added to paraformaldehyde (0.5% final concentration) for fixation, unless otherwise stated. This process did not alter the size distribution of the platelet suspension relative to an unfixed control, nor were any differences between fixed and unfixed platelets detectable as judged by the Coomassie blue staining patterns of the solubilized platelet proteins electrophoresed through exponential gradient polyacrylamide gels.

Platelet Size Distributions

To measure platelet size distributions, a 6.7 μl aliquot of the sheared or control platelet suspension was mixed with 20 ml of Isoton II (Curtis Matheson, Atlanta, GA) and the platelet concentration and size distribution (Channelyzer, Model 256, Coulter Electronics, Hialeah, FL) were determined simultaneously. The size distribution histogram, collected in 256 channels corresponding to particle diameters ranging from 1.33 to 4.94 μm, was transferred to a computer and stored for later analysis. Two different counting techniques were employed in this study. In the first, platelet size distributions were collected until 2,000 particles were collected in one of the 256 channels. Because of the reduced number of particles recognizable by the Channelyzer in sheared platelet suspensions, however, the time required for 2,000 particles to accumulate in a given channel was considerably longer than that required for the unsheared suspensions. Thus, distributions were

also collected by counting the sheared or unsheared platelet suspensions for identical lengths of time (identical volumes of fluid); this technique reflects the actual size distribution remaining in the sheared platelet suspensions and the consequent reduction in particle number resulting from aggregate formation. Platelet aggregation was quantified as the percent loss in single platelets, *i.e.*, % aggregation $= (1 - N/N_o) \times 100$, where N_o and N represent the number of single particles before and after shearing, respectively.

Statistical Analysis

A two-sample Student's t-test assuming unequal variances was used to compare the amounts of aggregation from control and treated PRP. The figures indicate representative results from a minimum of three independent experiments.

RESULTS

The platelet size distribution resulting from exposure of PRP to a shear stress of 100 dyne/cm^2 was measured and compared with that of the unsheared control, as shown in Fig. 1. A significant reduction (>50%) in the number of particles present in the peak channel was observed, suggestive of the formation of platelet microaggregates.

To evaluate the role of the platelet receptor GP IIb-IIIa in SIPA, the anti–GP IIb-IIIa monoclonal antibody 7E3, which recognizes both inactive and activated GP IIb-IIIa and completely inhibits ADP-induced platelet aggregation, was incubated with PRP prior to shearing. As shown in Fig. 2, 7E3 significantly inhibited the aggregation of platelets caused by a shear stress of 100 dyne/cm^2.

Because platelet GP IIb-IIIa binds to its ligands through

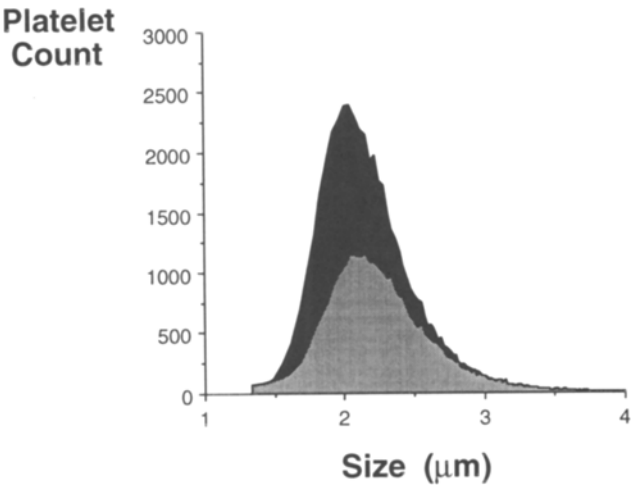

FIGURE 1. Representative size distributions of unsheared platelets (black) and of those exposed to a shear stress of 100 dyne/cm^2 (shaded). The size distributions were collected for 45 sec (see Methods).

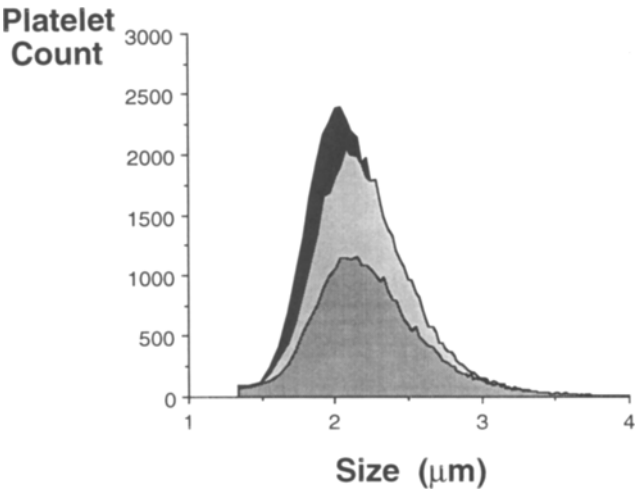

FIGURE 2. Size distributions of unsheared platelets (black) and of those exposed to a shear stress of 100 dyne/cm^2 in the presence (lightly shaded) or absence (medium shaded) of the anti–GP IIb-IIIa monoclonal antibody 7E3.

an RGDX recognition sequence, we examined whether the tetrapeptides RGDS and RGDV, which block platelet aggregation measured in the aggregometer, could also inhibit SIPA. As a control, RGES, which has no effect on platelet aggregation, was also used. Figure 3 illustrates that incubation of PRP with 1 mM RGDS or 0.5 mM RGDV prior to shearing at 100 dyne/cm^2 significantly blocked platelet aggregation, whereas 1 mM RGES had a negligible effect.

To assess what role, if any, vitronectin might play in SIPA, PRP was incubated with the anti-vitronectin receptor monoclonal antibody LM-609 prior to shearing. As seen in Fig. 4, blocking the platelet vitronectin receptor ($\alpha_v\beta_3$) did not inhibit SIPA, implying that aggregation induced by a shear stress of 100 dyne/cm^2 is not mediated by this integrin receptor.

The role of vWF in SIPA was determined by incubating PRP with a recombinant fragment of vWF prior to shearing. This fragment, termed VCL, did not inhibit platelet aggregation when challenged with 10 μM ADP in the aggregometer, but did abolish ristocetin-induced platelet agglutination. As shown in Fig. 5, VCL also strongly inhibited, but did not eliminate, aggregation induced by a shear stress of 100 dyne/cm^2.

Size distributions of sheared platelet suspensions were also collected by continuously measuring the distribution until 2,000 particles had accumulated in one of the 256 channels. Figure 6a shows the platelet size distribution obtained with this technique after exposure of PRP to a shear stress of 100 dyne/cm^2. The size distribution is shifted to larger particle sizes compared with the unsheared control, an increase that was significantly diminished when 7E3 was preincubated with PRP (Fig. 6b), in agreement with previous results. Additionally, some

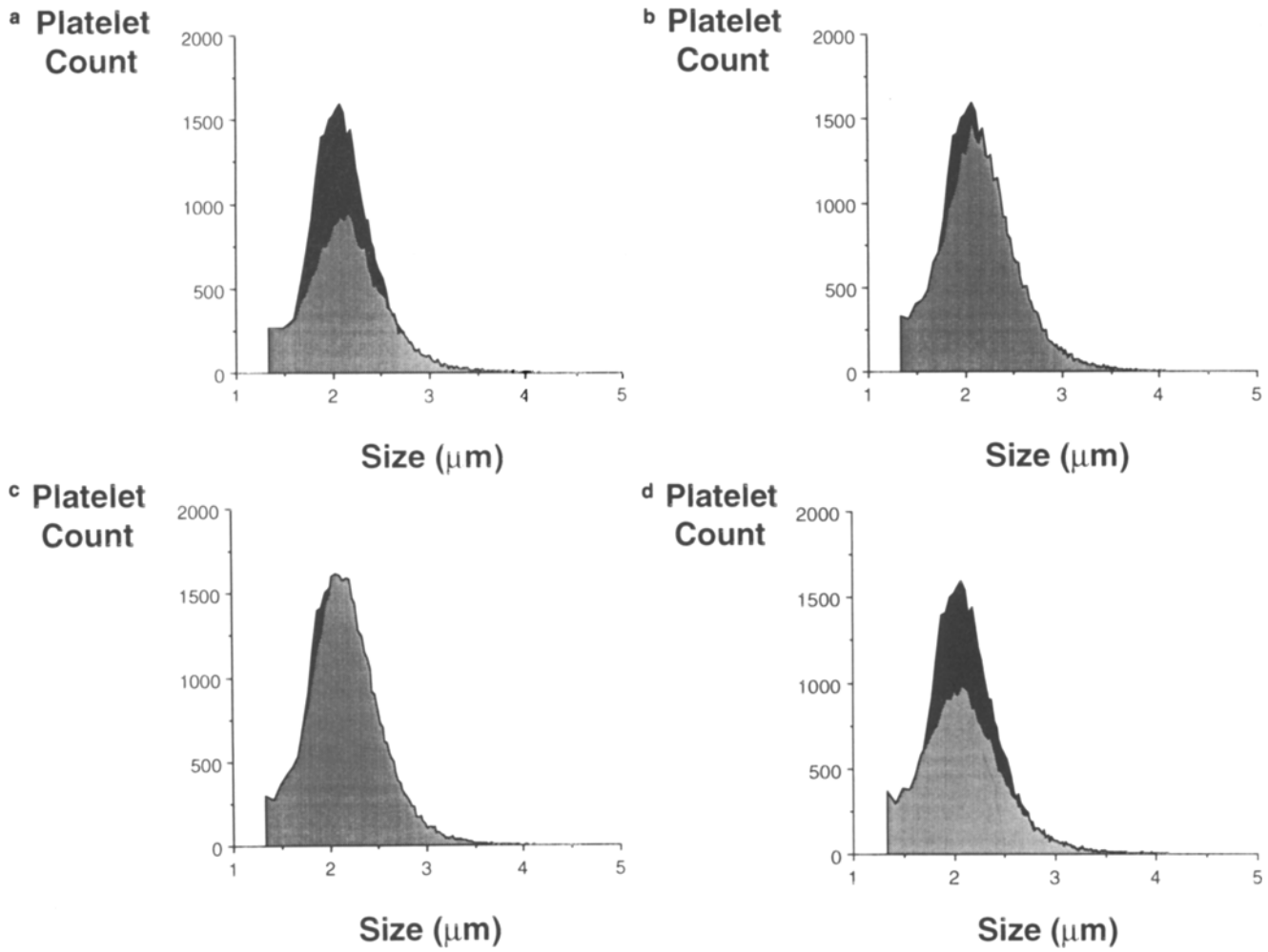

FIGURE 3. Size distributions of unsheared platelets (black) and of those exposed to a shear stress of 100 dyne/cm^2 (shaded) in the presence of saline (a), 1 mM RGDS (b), 0.5 mM RGDV (c), and 1 mM RGES (d).

platelet fragmentation occurred during the exposure to 100 dyne/cm^2, as suggested by the increase in the number of particles smaller than ~1.7 μm (see also Figs. 4 and 5).

DISCUSSION

In the present studies we have examined SIPA by measuring changes in the platelet size distribution generated during exposure of PRP to uniform shear stresses in a modified Couette/cone-and-plate viscometer. The results of this work demonstrate that size distributions of sheared and unsheared platelet suspensions differ considerably. Shear stress levels of 100 dyne/cm^2 induced shifts in platelet distributions both to larger particle sizes, through microaggregate formation, and to smaller sizes, through platelet fragmentation. Such fragmentation at this and higher shear levels has been observed by other investigators (3) and presumably arises from mechanical destruction of the platelet membrane by the shear forces. Our

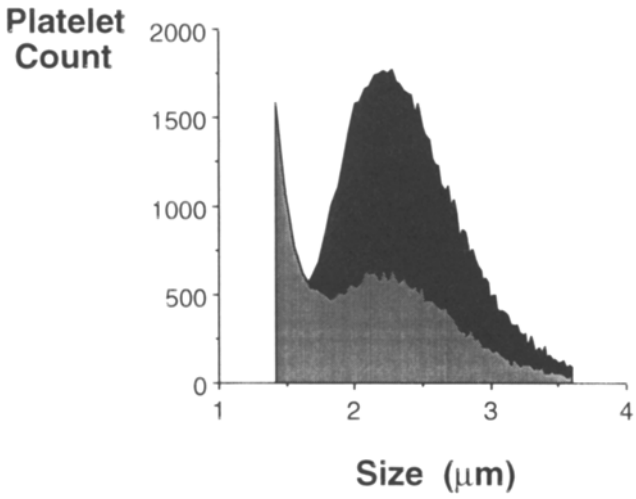

FIGURE 4. Size distributions of unsheared platelets (black) and of those exposed to a shear stress of 100 dyne/cm^2 in the presence (shaded) of the anti-vitronectin receptor monoclonal antibody LM-609.

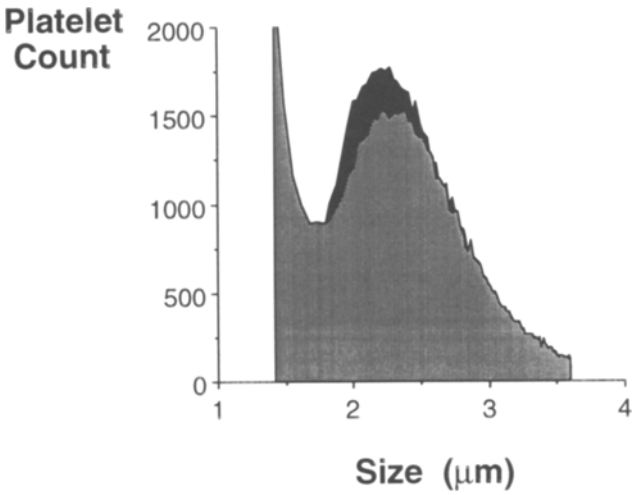

FIGURE 5. Size distributions of unsheared platelets (black) and of those exposed to a shear stress of 100 dyne/cm^2 (shaded) in the presence of 50 μg/ml VCL, a recombinant fragment of vWF that abolishes ristocetin-induced platelet agglutination.

methodology, however, does not allow for the determination of the mechanism by which the fragments were generated, *i.e.*, whether they represent pieces of the platelet membrane remaining after cell lysis or are microparticles formed as a result of platelet activation. Because such microparticles have been shown to exhibit enhanced procoagulant activity (26), it may be possible to discriminate between the two possibilities using measurements of prothrombinase activity. A recent study (7) showing that platelet microparticle formation requires intact and functional GP IIb-IIIa receptors, however, suggests that the small particles detected in these studies represent platelet fragments rather than true microparticles, because they were observed in sheared suspensions that had previously been incubated with RGDS or 7E3. In the aforementioned study, no microparticle formation was observed with platelets treated with RGDS or those congenitally deficient in GP IIb-IIIa.

The possibility that the platelet vitronectin receptor plays a role in SIPA was investigated by treating PRP with the anti-vitronectin receptor antibody LM-609 prior to exposure to shear stress. Blockade of this platelet receptor had no statistically significant effect on SIPA (see Table 1), a result that is not surprising in light of the fact that there are far fewer (several hundred) copies of this receptor on the platelet membrane than the number of GP IIb-IIIa (~40,000 copies per platelet) (16) and GP Ib-IX (~25,000 copies per platelet) (17) complexes.

The monoclonal antibody 7E3, directed against GP IIb-IIIa, and the recombinant vWF fragment VCL significantly reduced platelet aggregation induced by a shear stress of 100 dyne/cm^2 (see Table 1). Thus, a role for both of these receptors in SIPA is suggested. This finding is consistent with the observations of other investigators (9,13,15), who reported that interactions of vWF with both the GP IIb-IIIa and the GP Ib platelet receptors were necessary for the formation of stable platelet aggregates, and those of Chow *et al.* (5), who demonstrated an absolute necessity for vWF binding to GP Ib for SIPA to occur. The latter investigators also provided evidence that vWF binding to GP Ib initiates a transmembrane Ca^{2+} flux and that ADP is required for aggregate formation. Nevertheless, the mechanism(s) by which shear forces cause these receptors to become competent to bind their ligands remains unclear. One hypothesis is that physical forces can alter the conformation of the platelet receptors, thereby exposing their otherwise cryptic binding region to the

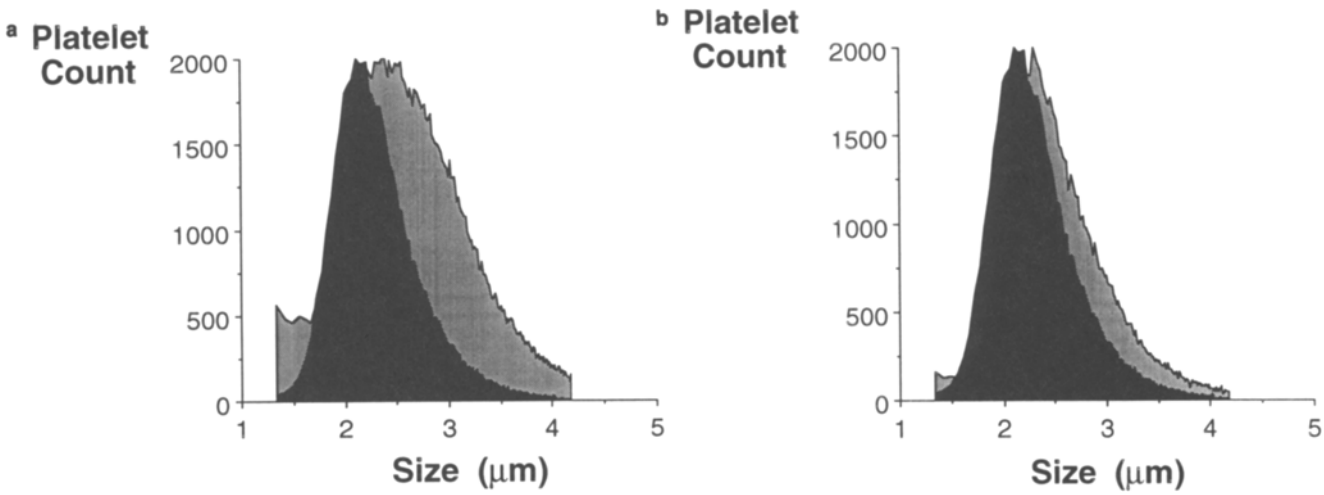

FIGURE 6. Size distributions of unsheared platelets (black) and of those exposed to a shear stress of 100 dyne/cm^2 (shaded) in the absence (a) or presence (b) of the anti-GP IIb-IIIa monoclonal antibody 7E3. Distributions were collected until 2,000 particles were detected in one of the 256 channels (see Methods).

TABLE 1. A comparison of the measured platelet aggregation induced by a shear stress of 100 dyne/cm^2 in the absence (saline) or presence of various peptides and monoclonal antibodies.

Treatment	% Aggregation	Significance*
Saline	32.9 ± 13.2 (n = 10)	-
RGDS	2.4 ± 6.4 (n = 8)	Yes
RGES	36.4 ± 18.4 (n = 8)	No
RGDV	3.8 ± 6.6 (n = 3)	Yes
7E3	1.8 ± 2.0 (n = 8)	Yes
LM609	45.5 ± 11.4 (n = 5)	No
VCL	11.8 ± 5.5 (n = 6)	Yes

*Significance between mean % aggregation was determined for $p < 0.001$.

available ligands. In essence, shear forces may act as a physical analog of ristocetin, enabling vWF to interact with GP Ib and initiate platelet aggregation. Alternatively, physical destruction of a small subset of the platelet population may result in the release of sufficient ADP to activate the remainder of the platelets. The latter possibility is supported by recent work demonstrating inhibition of SIPA by creatine phosphate/creatine phosphokinase, compounds that rapidly convert ADP to ATP and negate the former's effect on platelets (5). This mechanism may be particularly important *in vivo* because shear stresses may also damage erythrocytes and cause the release of their substantial stores of metabolic ADP. On the other hand, we have observed that VCL does not interfere with aggregation induced by 10 μM ADP, as measured in an aggregometer, although VCL clearly blocks aggregation induced by a shear stress of 100 dyne/cm^2. These data suggest that the internal secretion of ADP, which then leads to the activation of GP IIb-IIIa, requires the binding of intact vWF to GP Ib. Alternatively, the levels of ADP released by fragmented platelets may be too low to initiate the responses required for platelet aggregation.

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