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Characterization of the Unique Mechanism Mediating the Shear-dependent Binding of Soluble von Willebrand Factor to Platelets*

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We have studied the mechanism of interaction between soluble von Willebrand factor (vWF), labeled with fluorescein isothiocyanate (FITC), and platelets exposed to shear in a cone-and-plate viscometer. A flow cytometer calibrated with fluorescent bead standards was used to calculate the number of molecules associated with each platelet in suspension. To validate the methods and reagents used, binding of the same labeled vWF was assessed in the presence of ristocetin or α-thrombin and found to be saturable, with a narrow and symmetric distribution on >90% of the platelets. As expected, essentially all bound ligand interacted exclusively with platelet membrane glycoprotein (GP) Ibα in the presence of ristocetin and with GP IIb-IIIa after stimulation with α-thrombin. In contrast, only a minor proportion (<20%) of the platelets exposed to shear were found to bind vWF, with no evidence for saturation and markedly decreased interaction when the platelet count was below 100,000 μl. Moreover, shear-induced vWF binding was blocked equally effectively by selected monoclonal antibodies against either GP Ibα or GP IIb-IIIa or against the respective binding sites in vWF. Thus, both receptors are involved in the process, possibly through initial transient interactions mediated by GP Ibα that lead to platelet activation and subsequent irreversible binding supported by GP IIb-IIIa. While the levels of shear stress theoretically applied to platelets in these experiments are above those thought to occur in the normal circulation, our findings demonstrate a unique vWF binding mechanism that is not mimicked by other known modulators and correlates with platelet aggregation. Similar processes may occur in response to lower shear stress when platelets are exposed to thrombogenic surfaces and agonists generated at sites of vascular injury during thrombus formation.

The role of von Willebrand factor (vWF)[1] in platelet thrombus formation, particularly under conditions of high shear, is well established and supported by experimental evidence as well as clinical observations (1). It is known that platelets have two distinct binding sites for vWF, glycoprotein (GP) Ibα in the GP Ib-IX-V complex and the integrin $\alpha_{IIb}\beta_3$ (GP IIb-IIIa complex) (2). Binding of soluble normal vWF to GP Ibα in the absence of flow requires the presence of exogenous modulators, like ristocetin (3) or botrocetin (4), while interaction with GP IIb-IIIa can only occur after platelet activation (5). In addition to vWF, the GP IIb-IIIa receptor can also bind fibrinogen, fibronectin (6), and vitronectin (7), but the respective role of these proteins in thrombogenesis is still a topic for investigation.

Exposure of platelets to levels of shear higher than thought to occur in the normal circulation results in aggregation if soluble vWF is present (8); no other adhesive ligand can support this process (9). Shear-induced platelet aggregation may represent an important pathophysiological function of vWF, but the underlying mechanism is still poorly understood. It has been proposed that vWF binds to GP Ibα under the effect of shear, causing an increase in intracytoplasmic calcium ion levels and, consequently, GP IIb-IIIa activation; vWF interaction with activated GP IIb-IIIa would then mediate aggregation (10, 11). There is, however, no evidence that shear can promote the binding of vWF to GP Ibα; rather, there is only the indirect observation that anti-GP Ibα antibodies capable of inhibiting vWF binding mediated by other modulators can block shear-induced aggregation (9, 12). Thus, it is not known whether the characteristics of vWF binding to GP Ibα induced by shear are comparable to those defined in the presence of exogenous modulators like ristocetin or botrocetin.

We have performed studies to clarify the process of vWF interaction with platelets under shear. Our results demonstrate the existence of a unique mechanism that involves both GP Ibα and GP IIb-IIIa in supporting vWF binding and is not mimicked by the effect of exogenous modulators nor platelet agonists inducing activation. The observed binding appears to correlate well with the occurrence of aggregation.

EXPERIMENTAL PROCEDURES

Preparation of Washed Platelets—Blood was drawn from an antecubital vein of normal donors with a 19-gauge needle into plastic syringes and immediately transferred into polypropylene tubes containing trisodium citrate, pH 7.5, as anticoagulant (final concentration 0.013 M) and the ADP scavenger, apyrase (Sigma), to prevent platelet activation (final concentration 10 units/ml). All human subjects who participated

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[1] The abbreviations used are: vWF, von Willebrand factor; GP, platelet membrane glycoprotein; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate.

in these studies were aware of the experimental nature of the research and gave their informed consent in accordance with the Declaration of Helsinki. Blood was divided into 1-ml aliquots in plastic microcentrifuge tubes and centrifuged at 12,000 revolutions/min for 15 s; the resulting supernatant plasma was removed and replaced with an equivalent volume of divalent cation-free HEPES-Tyrode buffer (10 mM HEPES, 140 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 10 mM $NaHCO_3$, and 5 mM dextrose), pH 6.5 (2). The sedimented cells (including platelets and leukocytes on top of the erythrocyte cushion) were resuspended and centrifuged again at 12,000 revolutions/min for 15 s; the procedure of removing the supernatant fluid, containing progressively decreasing residual amounts of plasma proteins, and replacing it with fresh buffer was repeated a total of four times. After the last centrifugation, the cell pellet was resuspended in modified HEPES-Tyrode buffer, pH 7.4, containing 1.25 mM $CaCl_2$ and 6.25 mg/ml of bovine serum albumin (BSA, Sigma), and a platelet-rich suspension was obtained by centrifuging the sample at 1,500 revolutions/min for 1 min. The platelet count in the final suspension was then adjusted to 240,000/μl using the same modified HEPES-Tyrode buffer with $CaCl_2$ and BSA. These platelets were not significantly activated by the washing procedure since the expression of P-selectin, detected by flow cytometric analysis using the monoclonal antibody S-12 labeled with fluorescein isothiocyanate (FITC, Sigma), was not increased as compared to that measured in platelet-rich plasma (13) (the antibody S-12 was obtained as a generous gift from Dr. Rodger P. McEver of the Oklahoma Medical Research Foundation, Oklahoma City, OK). Moreover, the functional reactivity of the washed platelets was well preserved since there was no response to the addition of either ristocetin (1.25 mg/ml, Sigma) or ADP (10 μM, Sigma) alone but prompt aggregation occurred when vWF or fibrinogen, respectively, were also added.

Labeling of Ligands with Fluorescein Isothiocyanate—Plasma vWF was purified from cryoprecipitate and characterized as described previously (2). The purified protein supported both ristocetin-induced and shear-induced aggregation of washed platelets in a manner similar to that seen with platelet-rich plasma. Purified vWF was labeled with FITC according to the method described for labeling IgG, with slight modifications (14, 15). Briefly, after adjusting the pH of the purified vWF solution to 9.5 with 5% sodium carbonate, a 10 mg/ml solution of FITC in dimethyl sulfoxide (Me_2SO, Sigma) was added to achieve a final fluorescein/protein (F/P) weight ratio of 1:60 and the mixture was incubated for 10 min at room temperature (22–25 °C). The FITC-labeled protein was separated from free FITC by gel permeation chromatography on a Sephadex G-25 PD-10 column (Pharmacia Biotech Inc.) equilibrated with HEPES buffer (10 mM HEPES and 140 mM NaCl), pH 7.4. The concentration of FITC-labeled vWF was calculated by spectrophotometric analysis (14), according to the formula: vWF (mg/ml) = $[A_{280} - (0.35 \times A_{495})]/0.7$; where 0.7 is the extinction coefficient for purified vWF. The concentration of fluorescein was calculated from the A_{495} with the extinction coefficient of 200 (14). The molar F/P ratio was then calculated on the basis of the known molecular mass of vWF and fluorescein. The validity of the calculated concentration of vWF was verified by protein assay with the micro BCA reagent (Pierce); the results obtained with the two methods were in excellent agreement. The effects of FITC labeling on the functional properties of vWF were evaluated in preliminary experiments. Thus, an F/P molar ratio >5 caused a marked reduction in the ristocetin-induced platelet binding of vWF, presumably due to the modification of lysine residues in the GP Ibα recognition site located in the A1 domain of the molecule; on the other hand, an F/P molar ratio <1 resulted in unfavorable signal/noise ratio. Consequently, all experiments for these studies were performed with FITC-labeled vWF having an F/P molar ratio between 1 and 3.

Fibrinogen was purified from frozen plasma by glycine precipitation and characterized as previously reported (16). It was labeled with FITC as described above for vWF, except that the pH of the labeling mixture was kept at 8.5 to avoid denaturation of the molecule and the F/P weight ratio was 1:10. A final molar F/P ratio between 1 and 3 was found compatible with preservation of FITC-labeled fibrinogen function. The extinction coefficient (A_{280}) used to calculate the fibrinogen concentration was 1.5. Bovine serum albumin was labeled with FITC as described above for vWF; the extinction coefficient (A_{280}) used to calculate its concentration was 0.5.

Measurement of Shear-induced Platelet Aggregation and Binding of FITC-labeled vWF—Mixtures containing washed platelets and purified vWF, as well as other reagents as described, were exposed to shear in a computer-controlled modified cone-and-plate viscometer (9). The final concentration of $CaCl_2$ and BSA in these mixtures was 1 mM and 5 mg/ml, respectively. The shear rate value during the experiment was 10,800 s^{-1}, unless otherwise indicated and was applied for 6 min,

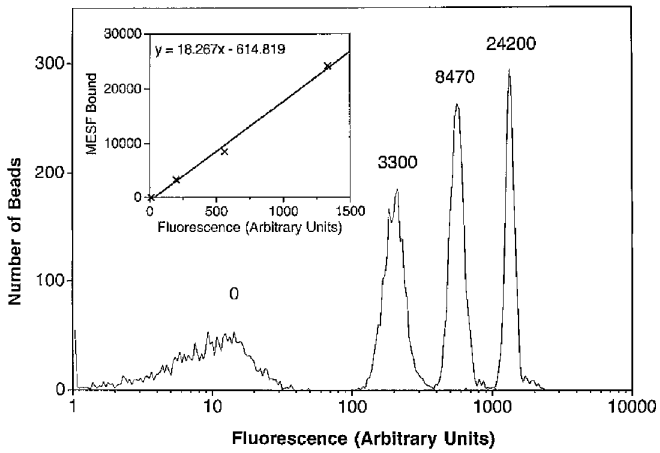

FIG. 1. **Flow cytometer calibration using standard fluorescent beads.** These measurements were performed using a gain setting identical to that used for the evaluation of platelet-bound fluorescence. One drop of each bead standard was mixed with 200 μl of modified HEPES-Tyrode buffer, pH 7.4, and the mixture was kept on ice, protected from light, until tested. The distribution of measured fluorescence is shown for each standard identified with the stated number of MESF bound/bead; the correlation between the two parameters is shown in the *inset*.

unless otherwise specified, at room temperature (22–25 °C). The instrument used provides continuous recording of the extent of platelet aggregation, calculated by measuring laser light transmittance through the suspension and applying the Lambert-Beer's equation (9). The validity of these measurements was verified in selected cases by counting single platelets in suspension before and after shear with an electronic particle counter (System 9000 Diff model, Serono Baker Diagnostics, Allentown, PA). After the application of shear, platelets were diluted five times in modified HEPES-Tyrode buffer, pH 7.4, and stored on ice with protection from light for determination of bound vWF (or other ligand) by flow cytometry. Fluorescence was measured at 530 nm using the FL-1 detector of a FACScan instrument (Becton-Dickinson, San Jose, CA) equipped with a 488 nm argon laser for excitation. The flow rate during analysis was controlled at <500 cell/s. Light scatter and fluorescence data were obtained at a fixed gain setting in the logarithmic mode. The data were initially processed on a computer (Hewlett-Packard, Palo Alto, CA) equipped with Lysis II software (Becton-Dickinson). For further analysis, the fluorescence intensity of each particle analyzed, stored as channel number (0–255) in a list-mode file, was converted to a DOS text file using appropriate software developed and made available by Perran McDaniel (Cytometry Associates, San Diego, CA). Conversion to the DOS text format allowed us to create mean curves from the data of multiple experiments and subtract one curve from another (for example, antibody inhibitable binding from total binding) to determine shear-induced binding. Conversion of measured fluorescence intensity to number of vWF molecules bound/platelet was based on a calibration curve of standard beads carrying a known number of molecules of equivalent soluble fluorochrome, or MESF (Quantum 24, Flow Cytometry Standards Corporation, St. Juan, Puerto Rico) analyzed with the same gain setting used for measuring platelet fluorescence. The fluorescence intensity distribution of the bead standards was narrow and symmetric, allowing use of the median as a representative value for each distribution (Fig. 1). Regression analysis showed that the measured fluorescence intensity could be converted to number of MESF with the following equation (Fig. 1): number of MESF = 18.267 (fluorescence intensity) − 614.819. Then, the number of MESF on the platelet surface was converted to number of vWF subunit molecules based on the known molar F/P ratio of the FITC-labeled vWF, assuming that one MESF corresponds to one molecule of FITC bound to protein. The standard bead calibration curves were done at approximately 2-month intervals during the period of data collection, and the results did not differ significantly. The flow cytometer laser was calibrated on two occasions during this period using caliBRITE beads and the autoCOMP software (Becton Dickinson) showing good stability of the power and beam parameters.

Measurement of FITC-labeled vWF Binding to Platelets Induced by Ristocetin and α-Thrombin—These assays were performed using platelet suspensions and labeled ligand prepared as described for the measurement of shear-induced binding and according to a method previously reported in detail (2). In brief, washed platelets in a volume to give a

final count in the experimental mixture of 100,000/μl were mixed with ristocetin (1.25 mg/ml, final concentration) and the desired amount of FITC-labeled vWF, or stimulated with α-thrombin (0.65 NIH unit/ml in the activation mixture) for 15 min at room temperature followed by a 10-fold excess (unit/unit) of recombinant hirudin (a generous gift from Iketon, Milan, Italy) for at least 5 min and then the desired amount of FITC-labeled vWF. α-Thrombin was the generous gift of Dr. John W. Fenton, II, Wadsworth Center for Laboratory and Research, and Departments of Physiology and Biochemistry, Albany Medical College, Union University, Albany, NY. The mixture of platelets and vWF was incubated for 15 min at room temperature (22–25 °C) and then processed as described above for the measurement of shear-induced vWF binding using the flow cytometer. Although the platelet binding of vWF mediated by ristocetin or induced by α-thrombin is not fully reversible, thus not at equilibrium, apparent B_{max} and k_d values were calculated by Scatchard-type analysis with the computer-assisted program Ligand (17) for the purpose of comparison with data obtained with ^{125}I-labeled vWF and reported in the literature (2).

Monoclonal Antibodies—All the murine monoclonal antibodies used in these experiments were obtained and characterized as described previously. They were purified using protein A (Sigma) chromatography according to published procedures (18). LJ-Ib1 (IgG1) reacts with the amino-terminal 45-kDa domain of GP Ibα containing the vWF-binding site (19–21) and inhibits completely the vWF-GP Ib interaction under all experimental conditions tested (22); in particular, this antibody blocks shear-induced platelet aggregation (9). Of note, LJ-Ib1 has no effect on α-thrombin binding to platelets (23). LJ-Ib10 (IgG2a) reacts with the 45-kDa amino-terminal domain of GP Ibα between residues Ala^{238} and Arg^{293} (19) and inhibits α-thrombin but not vWF binding to GP Ib (23–25). LJ-CP8 (IgG1) reacts with the GP IIb-IIIa complex and blocks the activation-dependent binding of soluble ligands to this receptor (16, 26) as well as platelet aggregation and thrombus formation under all experimental conditions tested (26), including shear-induced aggregation (9). LJ-P5 (IgG1) is directed against the GP IIb-IIIa complex and, particularly as a monovalent Fab fragment, inhibits selectively the binding of soluble vWF but not fibrinogen to activated platelets (27); it also inhibits platelet thrombus formation on denuded subendothelium under high flow conditions (26). LJ-P9 (IgG2b) is also directed against the GP IIb-IIIa complex and has high affinity comparable to that of LJ-P5; it inhibits both vWF and fibrinogen binding to activated platelets (27). LJ-152B/6 (IgG1) reacts with the vWF site that includes the Arg-Gly-Asp sequence recognized by GP IIb-IIIa, but has no cross-reactivity with other ligands of this receptor containing the same sequence (28). This antibody selectively inhibits vWF binding to GP IIb-IIIa as well as platelet aggregation and thrombus formation under high shear conditions (9, 26). NMC-4 (IgG1) reacts with the A1 domain of vWF including the disulfide bond between residues Cys^{509} and Cys^{695} (29, 30) and blocks the interaction between vWF and GP Ib under all the experimental conditions tested; it also inhibits shear-induced platelet aggregation (11, 31). LJ-229 (IgG1) reacts with a COOH-terminal domain of vWF present in the dimeric fragment II (residues 1366–2050 of the mature vWF subunit) generated by *Staphylococcus aureus* V8 protease (32, 33); it has no known inhibitory activity on vWF function. LJ-C3 (IgG1) reacts with the NH_2-terminal domain of vWF corresponding to residues 1–272 of the mature subunit; it inhibits the interaction of vWF with coagulation factor VIII (34) but has no demonstrable effects on vWF-platelet interactions. Monovalent Fab fragments of all these antibodies were prepared according to methods previously described (35). Briefly, IgG was incubated for 4.5 h at 37 °C with 4–8% (w/w) mercury-papain (Sigma) preactivated by cysteine; the optimal concentration of enzyme was determined for each antibody in preliminary trials by monitoring IgG digestion with polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. After digestion, Fc fragment was removed by passage through a protein A column and purified Fab was concentrated by Centriprep (Amicon, Beverly, MA). It should be noted that all IgG against vWF interfered with shear-induced aggregation and binding, including those (LJ-229 and LJ-C3) not expected to block the interaction with platelet receptors. This may be due to cross-linking effects between divalent antibody and multivalent vWF multimers since monovalent Fab fragments exhibited appropriate selectivity (*i.e.* the two control antibodies had no inhibitory effect). In contrast, IgG and Fab fragments of anti-GP Ibα and anti-GP IIb-IIIa antibodies gave consistently similar results.

RESULTS

Flow Cytometric Measurement of the Binding of FITC-labeled vWF to Platelets in the Presence of Ristocetin or after Stimulation with α-Thrombin—These experiments were performed to validate the methodology and reagents used for the study of shear-induced vWF binding to platelets. In agreement with data reported in the literature and obtained with ^{125}I-labeled vWF (2), saturable binding of FITC-labeled vWF was observed both in the presence of ristocetin or after stimulation with α-thrombin (Fig. 2). Scatchard-type analysis of the corresponding isotherms yielded values for apparent B_{max} (ristocetin, 42125 ± 6511 vWF subunit molecules/platelet; α-thrombin, 39425 ± 11092) and k_d (ristocetin, 5.3 ± 2.3 μg/ml; α-thrombin, 10.8 ± 2.2 μg/ml) consistent with those published previously (2). Moreover, the effect of specific monoclonal antibodies on

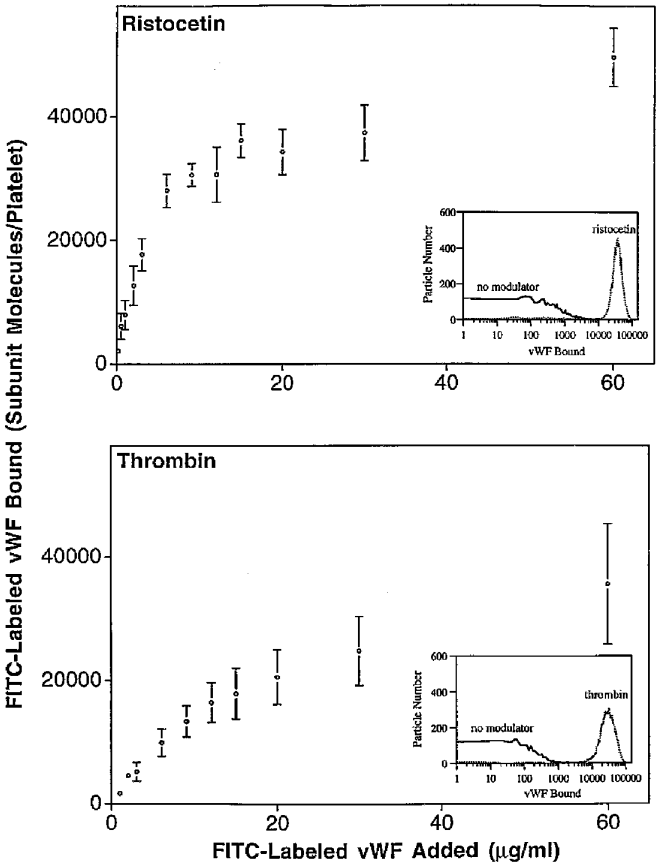

FIG. 2. **Binding of FITC-labeled vWF to platelets in the presence of ristocetin or after activation with α-thrombin.** For ristocetin-mediated binding (*upper panel*), 40 μl of 2.4 × 10^5 platelets/μl washed platelet suspension in modified HEPES-Tyrode buffer, pH 7.4, containing 6.25 mg/ml BSA and 1.25 mM Ca^{2+}, was mixed with 35 μl of the same buffer, 20 μl of FITC-labeled vWF in HEPES buffer, pH 7.4, at the concentration necessary to achieve the desired final concentration in the mixture, and 5 μl of a 25 mg/ml solution of ristocetin in HEPES buffer; or, for binding induced by α-thrombin (*lower panel*), 40 μl of the platelet suspension was mixed with 35 μl of modified HEPES-Tyrode buffer and 2.5 μl of a 20 NIH units/ml solution of α-thrombin in HEPES buffer followed, after a 15-min incubation at room temperature (22–25 °C), by 2.5 μl of a 200 unit/ml solution of hirudin in HEPES buffer followed, after 5 min, by 20 μl of FITC-labeled vWF solution at the concentration necessary to achieve the desired final concentration in the mixture. At this point, the mixtures were incubated at room temperature for 15 min, then diluted with 400 μl of modified HEPES-Tyrode solution (but with no BSA nor cation), and stored on ice with protection from light until tested in the flow cytometer. The calculated number of vWF subunit molecules bound/platelet (median of the distribution shown in the *inset*) is shown as a function of added FITC-labeled vWF concentrations, demonstrating near saturation of binding in both instances. The *insets* in each panel display the actual fluorescence distribution of the population of platelets analyzed in the flow cytometer, which is narrow and symmetric in both cases. *Error bars* indicate the standard error calculated from three (ristocetin) and five (thrombin) different experiments.

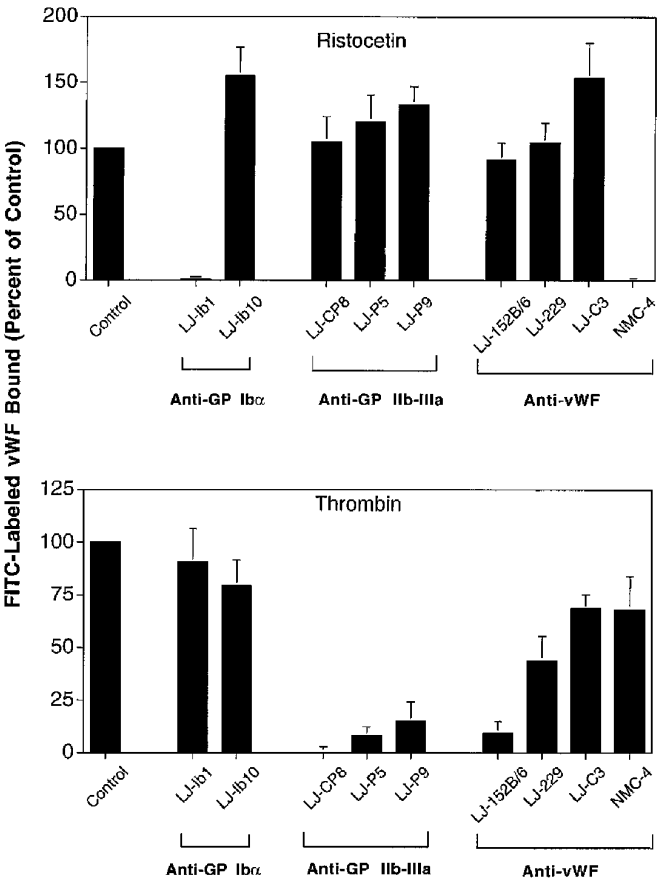

FIG. 3. **Effect of monoclonal antibodies on ristocetin and thrombin-induced vWF binding to platelets.** These assays were performed as described in the legend to Fig. 2, except that a fixed concentration (15 μg/ml) of FITC-labeled vWF was used, and 10 μl of the appropriate monoclonal antibody solution (purified IgG or Fab) was added to platelets, replacing an equivalent volume of buffer, before adding the ligand (in the case of experiments with thrombin, antibodies were added during the first activation step). Anti GP Ibα IgG were used at a final concentration of 150 μg/ml; anti-GP IIb-IIIa IgG at 50 μg/ml; anti vWF Fab at 500 μg/ml. The number of vWF molecules bound/platelet in the presence of antibodies is expressed as percent of the value measured in control mixtures without antibody. Data are the mean ± S.E. of four to eight experiments. *Upper panel*, ristocetin-mediated binding; note that antibody NMC-4 inhibits completely. *Lower panel*, binding induced by activation with α-thrombin.

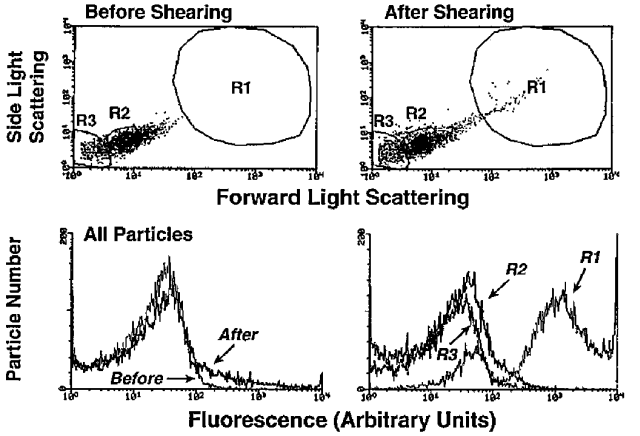

FIG. 4. **Flow cytometric analysis of shear-induced vWF binding to platelets.** The *two upper panels* show the distribution of forward and side light scattering (representing essentially particle size and granularity) measured in a mixture of platelets (400 μl of the suspension described in the legend to Fig. 3; final count $1.92 \times 10^5/\mu$l) and FITC-vWF (100 μl of a solution in HEPES buffer; final concentration 15 μg/ml) before and after exposure to shear (10,800 dynes/cm^2 for 6 min at 24 °C). After rotation in the cone-and-plate viscometer (or incubation without agitation at the same temperature for the samples not exposed to shear), 100 μl of the suspension was mixed with 400 μl of HEPES-Tyrode buffer, pH 7.4 (with no BSA nor divalent cation), and kept on ice protected from light until analyzed in the flow cytometer. Particles both larger (*R1*) and smaller (*R3*) than single platelets (*R2*) appeared after exposure to shear, representing aggregated platelets and platelet-derived microparticles, respectively. The *two lower panels* show the fluorescence distribution in the same experimental mixtures. Analysis of the whole population of particles in suspension without gating for size (*lower left*) demonstrated a small number of highly fluorescent elements appearing in the sample exposed to shear as compared to the distribution seen before exposure to shear. After gating for size (*lower right*), it became apparent that the fluorescent particles seen after exposure to shear correspond to aggregated platelets (*R1*).

the interaction between FITC-labeled vWF and platelets was also compatible with previously published results (2). Thus, the binding modulated by ristocetin was mediated by GP Ib (inhibited by LJ-Ib1 and NMC-4) and independent of vWF interaction with GP IIb-IIIa (not inhibited by LJ-CP8, LJ-P5, LJ-P9, and LJ-152B/6), and the opposite was true of the binding induced by α-thrombin stimulation (Fig. 3). In these experiments, identical results were obtained using either intact IgG or Fab fragments.

Shear-induced Platelet Aggregation and vWF Binding—The effect of applying shear to washed platelets in the presence of FITC-labeled vWF (15 μg/ml) was evaluated by flow cytometric analysis (Fig. 4). After shearing, there was a clear change in the size and granularity of particles in suspension, as judged by side and forward light scattering, compatible with the occurrence of platelet aggregation (appearance of larger particles, indicated as *R1*) and activation (appearance of smaller particles, indicated as *R3*, corresponding to microparticles released from stimulated platelets). Only particles with the size of aggregated platelets showed a change in the distribution of fluorescence intensity and increased fluorescence, whereas particles with the size of single platelets (indicated as *R2*) showed no such increase (Fig. 4). Because of the assumption that the primary vWF-binding site on platelets is GP Ibα, we reasoned that we could use antibodies directed against GP IIb-IIIa to prevent shear-induced platelet aggregation, thus avoiding nonspecific vWF trapping in aggregates and allowing demonstration of specific binding to single platelets. Indeed, in the presence of the anti-GP IIb-IIIa antibody LJ-CP 8 aggregation was reduced >80%, activation-dependent release of microparticles was unchanged, and a fraction (<20%) of the single platelet population became fluorescent with bound FITC-vWF (Fig. 5). These results suggest that, in the absence of inhibitory antibodies against GP IIb-IIIa, all the platelets that bind vWF become part of aggregates. In fact, examination by epifluorescence microscopy confirmed that single platelets but no aggregates were positive for bound FITC-labeled vWF in the presence of the anti-GP IIb-IIIa antibody, whereas only aggregates were positive in the absence of the antibody (data not shown here). The antibody LJ-Ib1, which is known to inhibit vWF binding to GP Ib, inhibited the shear-induced vWF interaction with single platelets as well as the release of microparticles (Fig. 5). In agreement with previously published results (9), this antibody completely inhibited shear-induced aggregation even in the absence of GP IIb-IIIa blockade (not shown). The specificity of shear-dependent vWF binding to single platelets was demonstrated by an experiment in which FITC-labeled fibrinogen (Fig. 6) or FITC-labeled BSA were added to platelets exposed to shear in the presence of nonlabeled vWF (the latter was necessary to support shear-induced aggregation). Both fluorescent ligands were incorporated into the platelet aggregates (although less than vWF), consistent with nonspecific trapping; however, minimal binding to single platelets was

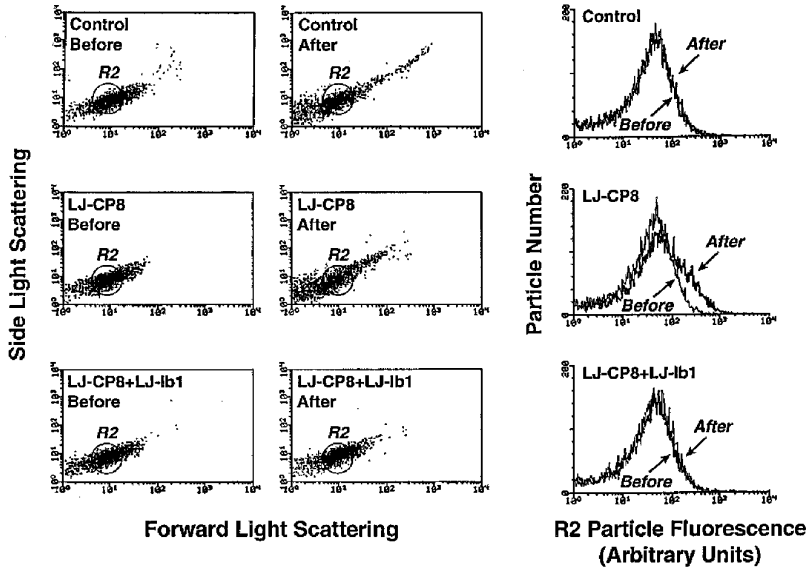

FIG. 5. **Flow cytometric analysis of FITC-vWF binding to platelets exposed to shear.** These experiments were conducted in a manner similar to that described in the legend to Fig. 4, exposing platelets to shear in the presence of FITC-labeled vWF (400 μl of platelet suspension and 100 μl of vWF solution; $1.92 \times 10^5/\mu$l and 15 μg/ml, respectively) with or without the addition of the monoclonal antibodies LJ-CP8 (50 μg/ml) or LJ-Ib1 (150 μg/ml), added in a volume of 10 to 20 μl replacing HEPES buffer in the FITC-vWF solution; all indicated concentrations are final. The *six panels* on the *left* show the distribution of particles in suspension with respect to side and forward light scattering, indicating the gate chosen for the analysis of single platelets (*R2*); the *three panels* on the *right* show the fluorescence distribution of the R2 population (single platelets). All different samples gave similar results when not exposed to shear (*Before*). After exposure to shear, aggregated platelets appeared in the absence of antibody LJ-CP8 but the fluorescence distribution of the single platelet population did not change (*Control*, *upper row*). In the presence of the anti-GP IIb-IIIa antibody LJ-CP8, aggregated platelets essentially disappeared, and the fluorescence of a limited portion of the single platelet population increased (*middle row*). The latter change was abolished when the anti-GP Ibα antibody LJ-Ib1 was added to LJ-CP8 (*lower row*).

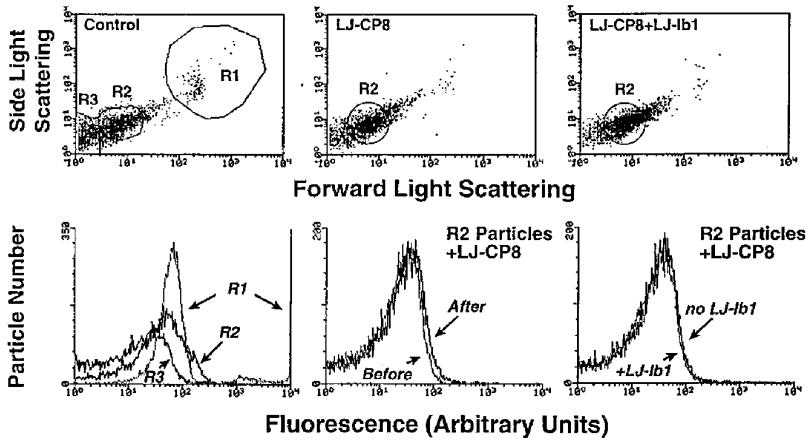

FIG. 6. **Flow cytometric analysis of FITC-fibrinogen binding to platelets exposed to shear.** These control experiments were performed as described in the legend to Fig. 5, except that nonlabeled vWF was used in the mixtures, and FITC-labeled fibrinogen was added at the concentration of 18 μg/ml (to give the same molar concentration as vWF subunit). The *panels* in the *upper row* show the distribution of particles in suspension with respect to side and forward light scattering, indicating the gates chosen for the analysis of particles of different size set exactly as for the experiments presented in Fig. 5; *left panel*, control experiment; *middle panel*, in the presence of the anti-GP IIb-IIIa antibody, LJ-CP8 (50 μg/ml); *right panel*, in the presence of antibody LJ-CP8 and the anti-GP Ibα antibody, LJ-Ib1 (150 μg/ml). Note that aggregation occurs due to the presence of vWF (R1 particles on the left) and is markedly reduced in the presence of the antibodies. The *panels* in the *bottom row* show the fluorescence distribution after exposure to shear in the three gated populations (*left*); the comparison between fluorescence distribution before and after exposure to shear in the R2 (single platelet) population in the presence of the antibody LJ-CP8 (*middle*); and the effect of the antibody LJ-Ib1 added to LJ-CP8 on the fluorescence distribution in the R2 (single platelet) population exposed to shear (*right*). Note that the population of aggregates (*R1*) contains some highly fluorescent particles, but much less so than in the experiment performed with FITC-labeled vWF (compare with Fig. 4). Note also that the R2 population shows a slight increase in fluorescence after shear but this is not affected by the addition of antibody LJ-Ib1 (compare with the results shown in Fig. 5).

observed when aggregation was blocked by addition of the antibody LJ-CP8.

Characteristics of Shear-induced vWF Binding to Platelets—In view of the data presented above, all experiments aimed at characterizing the interaction between vWF and single platelets under shear were performed in the presence of the anti-GP IIb-IIIa antibody LJ-CP8 to prevent aggregation. It should be carefully noted, however, that although binding was measured in the presence of this antibody, GP IIb-IIIa was eventually shown to be required to support the interaction (see below). In contrast to treatment with ristocetin or stimulation with α-thrombin, conditions that result in the binding to >90% of the platelets in suspension of >10,000 molecules of vWF subunit interacting with GP Ib or GP IIb-IIIa, respectively (Fig. 2), <10% of the platelets exposed to shear bound >10,000 molecules of vWF subunit even after addition of 60 μg/ml of vWF in solution (Fig. 7). In order to obtain the distribution of fluorescence on the positive single platelet population, experi-

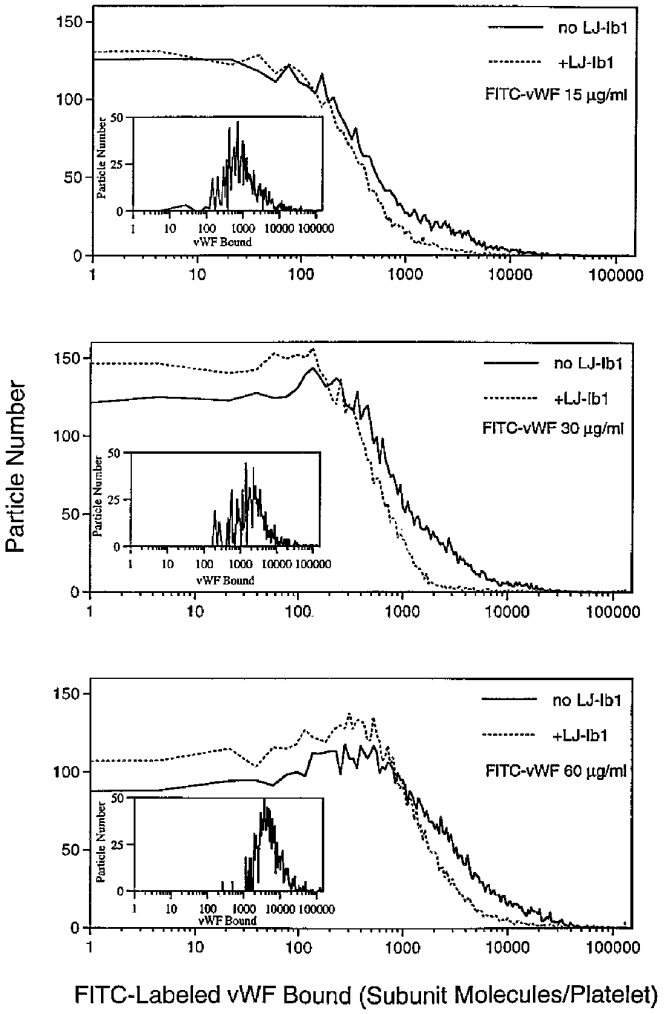

FIG. 7. **Flow cytometric analysis of the dose-dependent binding of FITC-labeled vWF to platelets exposed to shear.** All experiments presented here were performed as described in the legend to Fig. 5, in the presence of antibody LJ-CP8 (50 μg/ml) to block aggregation, with or without addition of antibody LJ-Ib1 (150 μg/ml) to block GP Ibα function (as indicated by two different lines) and in the presence of the indicated concentrations of FITC-labeled vWF. The figure shows the distribution of particles in the R2 population (single platelets as shown in Fig. 5) as a function of the calculated number of vWF molecules bound (see "Experimental Procedures" for the method used for calculation). The *insets* show the distribution obtained after subtracting the values of bound vWF measured in the presence of the anti-GP Ibα antibody, LJ-Ib1, from those measured in its absence; thus, the observed distribution corresponds to bound vWF that can be inhibited by the anti-GP Ibα antibody. Each curve represents the mean from four different experiments.

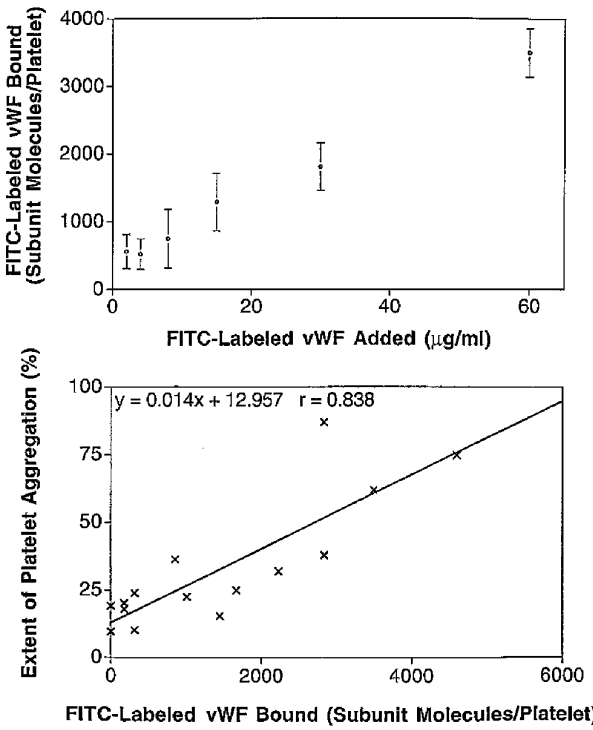

FIG. 8. **Dose-response analysis of shear-induced vWF binding to platelets and correlation with aggregation.** The *upper panel* shows the dose-response curve of specific (inhibited by anti-GP Ibα antibody) shear-induced vWF binding calculated from the median of the distribution observed for each experimental point (see Fig. 7). Each point is the mean ± S.E. of two to six different experiments. The *lower panel* shows the relationship between shear-induced vWF binding and platelet aggregation (based on single platelet count, *i.e.* the values shown represent the percent decrease in the number of single platelets in suspension, or 100 −% residual single platelets, after shear *versus* before shear). One to four experiments were performed for each point, and the mean is shown when more than one experiment was performed. Regression analysis demonstrated the positive correlation between the two parameters. Aggregation was measured in mixtures prepared as described for measuring binding, but without the monoclonal antibodies.

ments were performed where increasing amounts of FITC-labeled vWF were added to platelets either in the presence or absence of the function blocking anti-GP Ib antibody, LJ-Ib1 (as mentioned above, the anti-GP IIb-IIIa antibody LJ-CP8 was always present in these experiments). After shearing, the values of fluorescence measured in the presence of the antibody were subtracted from those measured in its absence, thus yielding a distribution corresponding to vWF molecules interacting with GP Ib (Fig. 7, *insets*); for the purpose of our present studies, only this was considered specific binding. Using this approach, we established a correlation between the concentration of vWF added in solution and the median number of vWF subunit molecules bound/positive platelet after shearing at 10,800 s^{-1}. Within the range of concentrations tested, including some well in excess of the physiological plasma levels of vWF, there was no tendency to saturation of binding (Fig. 8, *upper panel*). This result is in contrast to those shown in Fig. 2, obtained in the presence of ristocetin or after stimulation with α-thrombin. Nevertheless, a correlation could be established between the calculated number of vWF molecules bound and the extent of shear-induced aggregation (Fig. 8, *lower panel*).

The binding of vWF under shear was greatly influenced by the number of platelets in suspension and was minimal at a count below 50,000/μl (Fig. 9). This is in contrast to the fact that the binding mediated by ristocetin or induced by activation with α-thrombin is essentially the same with platelet count between 50,000 and 600,000/μl (not shown). Shear-induced aggregation was similarly influenced by the count of platelets in suspension and was diminished considerably at 50,000/μl (not shown).

The level of shear rate to which platelets were exposed was also a crucial determinant of vWF binding. A positive population of single platelets with bound vWF was clearly detectable after shearing at 10,800 s^{-1}, but binding was barely above background level after shearing at 7,200 s^{-1} for the same period of time (Fig. 10).

Another unique and important feature of vWF binding to platelets exposed to shear was highlighted by the use of well characterized monoclonal antibodies (Table I). As expected, antibodies known to interfere with the ristocetin-dependent vWF-GP Ib interaction, one against the receptor (LJ-Ib1) and the other against the corresponding binding domain in the

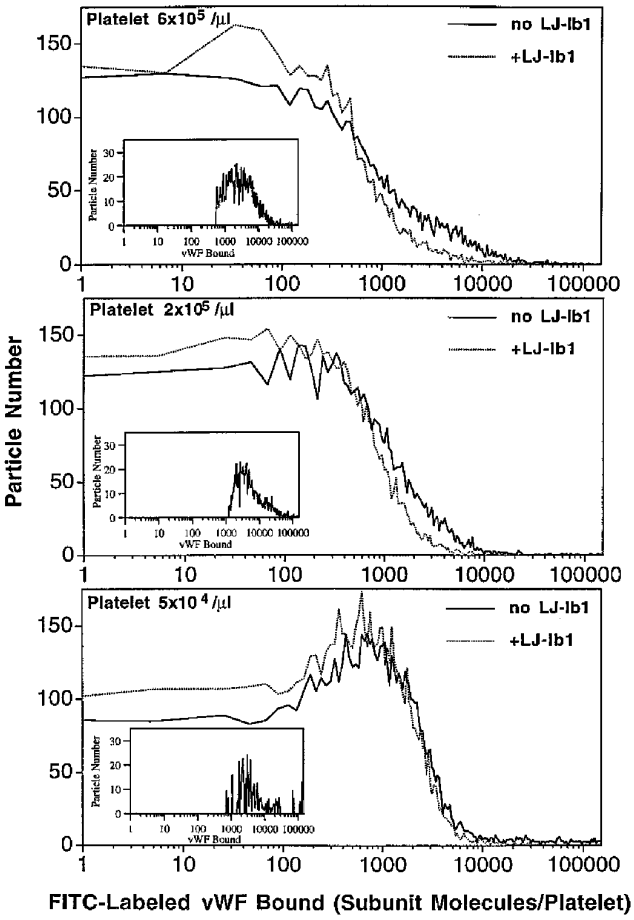

FIG. 9. **Effect of platelet count on shear-induced vWF binding.** These experiments were performed as described in the legend to Fig. 5, adjusting the count of platelets in suspension to obtain the indicated values, with a constant amount of FITC vWF present (15 μg/ml) as well as antibody LJ-CP 8 to inhibit aggregation, with or without the addition of antibody LJ-Ib1 to block GP Ibα function (shown by different lines). The figure shows the distribution of particles in the R2 population (single platelets as shown in Fig. 5) as a function of the calculated number of vWF molecules bound (see "Experimental Procedures" for the method used for calculation). The *insets* show the distribution obtained after subtracting the values of bound vWF measured in the presence of the anti-GP Ibα antibody, LJ-Ib1, from those measured in its absence; thus, the observed distribution corresponds to bound vWF that can be inhibited by the anti-GP Ibα antibody. Each curve represents the mean of three different experiments. Note the progressive decrease in binding with decreasing platelet count.

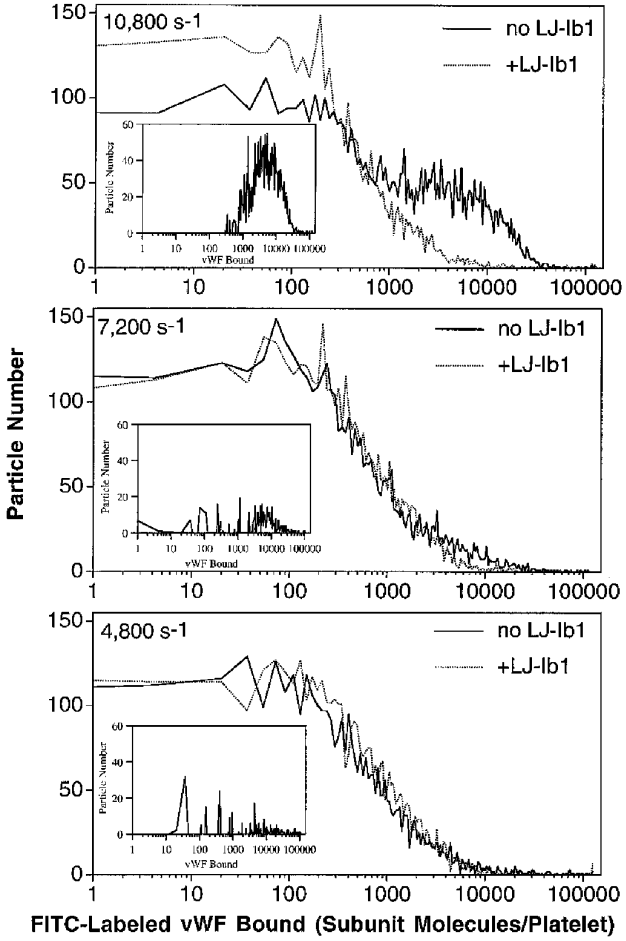

FIG. 10. **Effect of shear rate on vWF binding to platelets.** These experiments were performed as described in the legend to Fig. 5, with a constant amount of FITC vWF present (15 μg/ml) as well as antibody LJ-CP 8 to inhibit aggregation, with or without the addition of antibody LJ-Ib1 to block GP Ibα function (shown by different lines), but varying the rotational speed of the cone in the viscometer to achieve the indicated shear rate values. The figure shows the distribution of particles in the R2 population (single platelets as shown in Fig. 5) as a function of the calculated number of vWF molecules bound (see "Experimental Procedures" for the method used for calculation). The *insets* show the distribution obtained after subtracting the values of bound vWF measured in the presence of the anti-GP Ibα antibody LJ-Ib1 from those measured in its absence; thus, the observed distribution corresponds to bound vWF that can be inhibited by the anti-GP Ibα antibody. Each curve represents the mean of three different experiments. Note the progressive decrease in binding with the decreasing shear rate.

ligand (NMC-4), also inhibited shear-dependent binding. Control antibodies defined as noninhibitory in the assay modulated by ristocetin were also without effect on shear-dependent binding. The unexpected result was that two antibodies known to block the interaction of vWF with GP IIb-IIIa, one against the receptor (LJ-P5) and the other against the domain of vWF containing the Arg-Gly-Asp integrin recognition sequence (LJ-152B/6), consistently inhibited vWF binding to platelets exposed to shear (Table I). Thus, unlike ristocetin- or thrombin-induced binding, the stable interaction of vWF with platelets under shear appears to require ligand binding to both GP Ib and GP IIb-IIIa, either in a sequential or concurrent fashion. Of note, all antibodies known to inhibit the ristocetin-mediated vWF-GP Ib interaction also prevented shear-induced binding and aggregation; however, not all those interfering with vWF binding to GP IIb-IIIa on platelets stimulated by α-thrombin had the same effect on platelets exposed to shear, although they all inhibited aggregation. Like the latter group of antibodies, the synthetic peptide GRGDSP, containing the Arg-Gly-Asp integrin recognition sequence, was effective in blocking shear-induced aggregation but not vWF binding (not shown).

DISCUSSION

These studies provide experimental evidence that shear can modulate the binding of vWF to platelets eliciting a unique mechanism of interaction that requires two platelet receptors, the GP Ib-IX-V complex and the integrin $\alpha_{IIb}\beta_3$ (GP IIb-IIIa). While there is no other example of a dual receptor requirement for soluble vWF binding to platelets, our findings are reminiscent of conditions previously established for the adhesion of nonstimulated platelets to surface-bound vWF, a process that depends on both GP Ib and GP IIb-IIIa to become irreversible (36). There are obvious limitations in the methodology we have used to perform these studies, essentially dictated by the nature of the phenomena being evaluated. Since aggregation always occurs when platelets are exposed to high levels of shear in the presence of vWF, it was necessary to use a blocking anti-GP IIb-IIIa antibody in order to study vWF binding to

TABLE I
Shear-induced vWF binding: inhibition by monoclonal antibodies

The monoclonal antibodies against GP Ib and GP IIb-IIIa (all intact IgG) were tested at the final concentration of 150 and 50 μg/ml, respectively; the Fab fragments against vWF were at 500 μg/ml. The inhibition of shear-induced vWF binding was indicated as positive when the results obtained with a given antibody were essentially the same as seen in the presence of LJ-Ib1; negative, when the results were as seen in the absence of any antibody.

Antibodies tested		Inhibition of vWF binding
Anti-GP Ibα	LJ-Ib1	+
	LJ-Ib10	−
Anti-GP IIb-IIIa	LJ-P5	+
	LJ-P9	−
Anti-vWF	NMC-4	+
	LJ-229	−
	LJ-C3	−
	LJ-152B/6	+

single platelets. We cannot exclude the possibility that the extent of measured vWF binding was substantially less than would have otherwise been detected in the absence of antibody. Other factors that may have influenced the results are the need to stop shear in order to measure vWF binding and the need to manipulate platelets for flow cytometric analysis, making it impossible to measure transient interactions that may only occur under shear. Unfortunately, such concerns cannot be addressed satisfactorily with presently available technology. It is possible, therefore, that all the numerical estimates obtained with these experiments are an approximation by defect. Nevertheless, the important conclusion that, unlike in the presence of exogenous modulators, GP Ib by itself cannot support irreversible vWF binding to platelets exposed to shear, and yet is indispensable for such binding to occur, cannot have been influenced by the methodology used.

A proposed model for the sequence of events leading to shear-dependent vWF binding to platelets, and then aggregation, is presented in Fig. 11. The model considers that, under flow, vWF and GP Ibα interact through the respective recognition sites (20, 30) in a transient and reversible manner. When shear exceeds a certain level, GP IIb-IIIa is activated, interacts with the Arg-Gly-Asp (RGD) sequence in vWF (28) and the ligand becomes irreversibly bound to the platelet surface through both receptors. Elongated vWF multimers can then bridge multiple platelets and cause aggregation. Of interest is the fact, not documented here, that inhibitors of platelet activation, like prostaglandin E_1, can indeed prevent shear-induced vWF binding,[2] providing additional evidence in support of a dual receptor mechanisms where the function of one is linked to a response initiated by the other. This is also consistent with the observation that vWF interaction with GP Ib under shear leads to a transmembrane flux of calcium ions that precedes and is independent of an interaction with GP IIb-IIIa (10, 11). This hypothetical mechanism of shear-dependent vWF binding to platelets poses some key questions that our present results begin to address.

One problem is to understand how shear modulates the vWF-GP Ibα interaction. Many have hypothesized, but without supporting experimental evidence, that shear forces cause a conformational change in either the ligand or the receptor, or both, thus allowing the binding to occur. Our data cannot formally exclude this possibility, but they do not favor it. First, it seems clear that vWF cannot bind irreversibly to GP Ib under

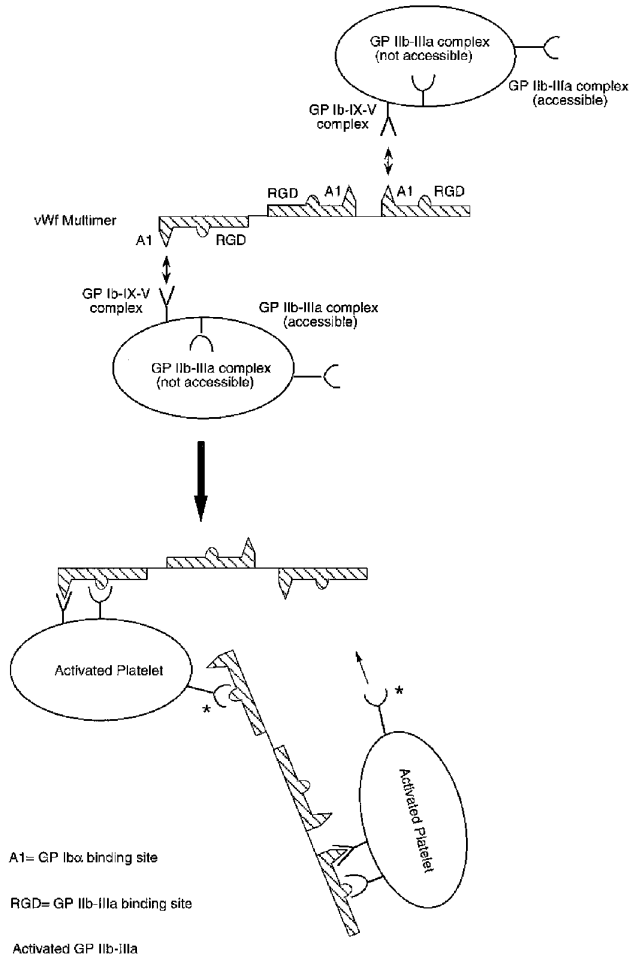

FIG. 11. **Schematic model of the mechanism of shear-induced vWF binding to platelets.** See "Discussion" for a justification of the hypothetical processes shown here. The *double-headed arrows* (figure on top) indicate the reversible interaction between A1 domain of vWF and GP Ibα. The *asterisks* (figure on bottom) indicate GP IIb-IIIa molecules after activation, when they can interact with soluble vWF. RGD indicates the Arg-Gly-Asp sequence recognized by GP IIb-IIIa.

shear, as it does in the presence of exogenous modulators, since GP IIb-IIIa function is also required for binding to become manifest. This may be because the off rate of the interaction is inherently high, as suggested by studies performed with surface-bound vWF to be reported elsewhere.[3] Those experiments demonstrate a GP Ib-dependent, transient platelet contact with immobilized vWF that occurs at any shear rate tested between 50 and 1,500 s^{-1}. Indeed, evidence for reversible GP Ib interaction with immobilized vWF can also be obtained without imposition of flow (36), clearly indicating that vWF binding to GP Ib in the absence of exogenous modulators is not necessarily or exclusively regulated by shear forces. Moreover, the characteristics of shear-induced binding shown here are not congruent with an interaction regulated by diffusion rate, since no saturation is apparent at concentrations greatly in excess of those required for saturation in the presence of modulators, and the extent of binding is markedly reduced when the platelet count is below a threshold level.

Another important consideration is that, at present, there is no clear understanding of what responses are evoked when platelets in suspension are exposed to a shear field. According to fluid dynamics theory, the surface of a body moving freely in

[2] S. Goto, D. R. Salomon, Y. Ikeda, and Z. M. Ruggeri, personal observation.

[3] B. Savage, E. Saldívar, and Z. M. Ruggeri, manuscript in preparation.

a streaming fluid is not subject to tangential forces imposed by the flow (37); thus, it is unlikely that platelets are subjected to a significant shear stress when moving freely with the liquid as single particles. We hypothesize that if more than one platelet is transiently interacting with a vWF multimer (Fig. 11), greater shear stress may be exerted on the larger, more irregular "particle." Increased shear stress on the membrane may combine with the consequences of proximity, established during the transient binding of several platelets to a vWF multimer, and lead to activation (38–41). Such a mechanism could favor the local availability of greater concentrations of released ADP, a synergistic agonist that has been shown to be required for shear-induced aggregation (42), and could also account for the greater efficiency of larger vWF multimers in mediating shear-induced activation and aggregation. Limiting factors in a process like this would be a low platelet count, in agreement with our experimental results, and the relatively low concentration of soluble large vWF multimers present, thus explaining why only a small proportion of platelets in suspension appear to bind vWF. These hypotheses will have to be tested by future experiments.

Our results indicate that the interaction of vWF with GP IIb-IIIa is absolutely required to achieve irreversible shear-induced binding to the platelet surface, but this cannot be prevented by blocking the pool of GP IIb-IIIa molecules accessible to inhibitory antibodies and peptides on the membrane of resting platelets. As a possible explanation of this observation, we suggest that vWF interacts with GP IIb-IIIa molecules, initially not accessible to antibodies, that become exposed on the platelet surface only after GP Ib-mediated activation (Fig. 11). The surface expression and functional integrity of a previously internal pool of GP IIb-IIIa molecules has been well documented after platelet stimulation (16). These newly exposed molecules may be more easily occupied by vWF already interacting with GP Ibα on the platelet surface than by competing antibodies or peptides in solution. If this concept is correct, it is not immediately apparent why an anti-GP IIb-IIIa antibody like LJ-P5 can inhibit shear-induced vWF binding when other antibodies with well established inhibitory activity on GP IIb-IIIa function (like LJ-CP8) cannot. The unique effect of LJ-P5 may be due to its epitope specificity, since this is the only antibody available to us that has been well documented to inhibit selectively soluble vWF binding to activated GP IIb-IIIa (27). This property, coupled with the high affinity of the antibody (and presumably fast on rate) may allow more effective blockade of newly exposed GP IIb-IIIa in competition with vWF. It is consistent with the proposed model that blocking the Arg-Gly-Asp sequence in vWF (as with the antibody LJ-152B/6) is effective in preventing a stable interaction with platelets under shear. It is also relevant that antibodies like LJ-CP8 and RGD peptides, that block GP IIb-IIIa function in most other situations but are not inhibitors of shear-dependent vWF binding, do nevertheless interfere with shear-induced aggregation. This implies that, once bound irreversibly to single platelets, a vWF multimer interacts with GP IIb-IIIa on other platelets that have also bound vWF and have become activated, thus mediating aggregation (Fig. 11). It follows that the pools of GP IIb-IIIa molecules that mediate shear-dependent vWF binding and aggregation are not necessarily the same, explaining why we could measure binding after blocking aggregation with an anti-GP IIb-IIIa antibody. At present, it is not known whether vWF binding to GP Ib becomes stable after GP IIb-IIIa is also engaged in the interaction (concurrent binding) or whether only GP IIb-IIIa supports vWF binding to platelets after activation mediated by the transient interactions with GP Ib (sequential binding).

In conclusion, our studies provide evidence for a unique mechanism of interaction between soluble vWF and platelets that is not mimicked by other known modulators of vWF binding to GP Ib. These findings are likely to be relevant for normal hemostasis as well as pathological thrombosis even though the levels of shear stress theoretically applied to platelets in these experiments are above those thought to occur in the normal circulation of man and other mammals, for which estimates of maximal shear rate vary from 800 to 2,000 s^{-1} (43). In fact, it is conceivable that similar processes occur even in response to lower shear when platelets are exposed to thrombogenic surfaces and agonists generated at sites of vascular injury during thrombus formation. For example, some key aspects of the mechanism described here, notably the sequential requirement for two distinct receptors, appear to be similar if not identical to those involved with platelet adhesion to surface-bound vWF even in the absence of flow (36). Further progress in this important area of platelet physiopathology should come from a better understanding of the nature of the vWF-GP Ib bond and the subsequent mechanisms of platelet activation.

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