

## Efficiency of Platelet Adhesion to Fibrinogen Depends on both Cell Activation and Flow

Arnaud Bonnefoy,\* Qingde Liu,<sup>†</sup> Chantal Legrand,\* and Mony M. Frojmovic<sup>†</sup>

\*Unité 353 INSERM, Institut d'Hématologie, Université Paris VII, Hôpital St. Louis, Paris, France, and <sup>†</sup>Department of Physiology, McGill University, Montreal, Quebec H3G 1Y6, Canada

**ABSTRACT** The kinetics of adhesion of platelets to fibrinogen (Fg) immobilized on polystyrene latex beads (Fg-beads) was determined in suspensions undergoing Couette flow at well-defined homogeneous shear rates. The efficiency of platelet adhesion to Fg-beads was compared for ADP-activated versus “resting” platelets. The effects of the shear rate (100–2000  $s^{-1}$ ), Fg density on the beads (24–2882  $Fg/\mu m^2$ ), the concentration of ADP used to activate the platelets, and the presence of soluble fibrinogen were assessed. “Resting” platelets did not specifically adhere to Fg-beads at levels detectable with our methodology. The apparent efficiency of platelet adhesion to Fg-beads readily correlated with the proportion of platelets “quantally” activated by doses of ADP, i.e., only ADP-activated platelets appeared to adhere to Fg-beads, with a maximal adhesion efficiency of 6–10% at shear rates of 100–300  $s^{-1}$ , decreasing with increasing shear rates up to 2000  $s^{-1}$ . The adhesion efficiency was found to decrease by only threefold when decreasing the density of Fg at the surface of the beads by 100-fold, with only moderate decreases in the presence of physiologic concentrations of soluble Fg. These adhesive interactions were also compared using activated GPIIb/IIIa-coated beads. Our studies provide novel model particles for studying platelet adhesion relevant to hemostasis and thrombosis, and show how the state of activation of the platelet and the local flow conditions regulate Fg-dependent adhesion.

### INTRODUCTION

Fibrinogen (Fg) can mediate the adhesion of platelets to surfaces and to other platelets (aggregation). Platelet aggregation mediated in flowing suspensions by soluble Fg requires the activation of platelets, such that resting (inactivated) platelets do not aggregate with Fg (reviewed in Peerschke, 1985). The Fg receptor on the platelet membrane is the glycoprotein IIb and IIIa complex (GPIIb/IIIa) (Marguerie et al., 1979; Bennett et al., 1982). GPIIb/IIIa can exist in either resting or activated conformational/functional states (Plow and Ginsberg, 1989). Resting GPIIb/IIIa does not bind fibrinogen in solution, and only when platelets are activated, by a platelet agonist such as ADP or thrombin, is the GPIIb/IIIa transformed into the activated state, and then able to bind soluble Fg (Plow and Marguerie, 1980; Bennett and Vilaire, 1979). It has been shown that the platelet GPIIb/IIIa-bound Fg, by its dimeric structure, cross-links to another GPIIb/IIIa on the membrane of a second platelet, and induces platelet aggregation (Peerschke, 1985; Hawiger, 1995; Liu et al., 1997).

Platelet GPIIb/IIIa is also the receptor that mediates platelet adhesion to surface-adsorbed Fg. In contrast to platelet aggregation in solution, it has been suggested that platelet adhesion to surface-immobilized Fg does not require the preactivation of the platelets. Thus, resting platelets appear to adhere to Fg-coated surfaces (Gartner et al., 1993; Pol-

anowska-Grabowska et al., 1999; Savage et al., 1995; Zaidi et al., 1996; Shiba et al., 1991). The adhesion of activated platelets to surface-adsorbed Fg has received less attention than that of resting platelets. Although available evidence suggests that in the absence of flow, activated platelets adhere to surface-immobilized Fg faster and more extensively than do resting platelets (Gartner et al., 1993; Savage et al., 1995), no quantitative comparisons have been reported. There has not been a study that directly compares the adhesion of resting versus activated platelets to surface-immobilized Fg in flowing suspensions.

By analogy with platelet aggregation (Xia and Frojmovic, 1994), adhesion is determined by the collision frequency of platelets to the surface, and the capture (adhesion) efficiency of those collisions (van de Ven, 1989). The collision frequency is a function of the shear rate, while the adhesion efficiency, which can also be defined as the fraction of collisions that results in the adhesion of platelets to the Fg-coated surface, is determined by the adhesive properties of both the surface and the platelet membrane, as well as the shear rate/stress. Therefore, at a given shear rate, the adhesion efficiency is a characteristic description of the interaction between the platelet membrane and the Fg-coated surface. Direct observation of particle deposition onto solid surfaces is best done with the impinging jet technique, described for uncoated colloidal spheres (Dabros and van de Ven, 1983; Varennes and van de Ven, 1988), polymer-coated spheres (Polverari and van de Ven, 1995), bacteria (Xia et al., 1989), and red blood cells (Xia et al., 1993). A variety of flow chambers, including the parallel plate chamber, the rotating disk, and the radial flow chamber (Xia et al., 1993) allow controlled hydrodynamics, but generally serve to measure kinetics of detachment of particles. Adhe-

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Address reprint requests to Dr. M. M. Frojmovic, Dept. of Physiology, McGill University, 3655 Drummond, #1137, Montreal, Quebec H3G 1Y6, Canada. Tel.: 514-398-4326; Fax: 514-398-7452; E-mail: mony@med.mcgill.ca.

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sion rates are complicated by the need for red blood cells to enhance transport of platelets to the wall, where chemical contributions also come into play (Goldsmith et al., 1995) though some modeling for effects of red cells on leukocyte-endothelial interactions has been reported (Munn et al., 1996). It is only in the small area near the stagnation point in the impinging jet technique where the boundary layer is relatively thin and uniform, and the experimental data subject to theoretical treatment of adhesion kinetics. These are generally very tedious, time-consuming experiments, requiring large particle volumes (>5–10 ml). Qualitatively similar results have been observed for the dependence of capture efficiencies on shear rate for particles colliding in suspension in simple shear flow compared to capture onto immobilized particles evaluated by the impinging jet technique (Petlicki and van de Ven, 1992). We have, therefore, used the microcouette, which generates well-defined homogeneous shear rates, to study the coaggregation of platelets with Fg-coated polystyrene beads, as previously used in studies of platelet-to-platelet aggregation (Xia and Frojmovic, 1994).

## MATERIALS AND METHODS

### Materials

Human Fg depleted of vWF and fibronectin was purchased from Enzyme Research Laboratories Inc. (South Bend, IN); fluorescein isothiocyanate-labeled-Fg (FITC-Fg) was prepared as previously described (Xia et al., 1996); peptide Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) was from Calbiochem Corporation (La Jolla, CA); Ro 44-9883, a non-peptide analog of the Arg-Gly-Asp (RGD) peptide, was a generous gift from Dr. T. Weller (F. Hoffmann-La Roche Ltd., Basel, Switzerland); Dr. T. Kraus (Schering Co., Berlin, Germany) generously provided ZK 36 374, a stable prostacyclin analog. The FITC-labeled 4A5, a monoclonal antibody directed against the  $\gamma$  chain carboxyl terminus domain of the Fg (comprising the AGDV residue) was kindly provided by Dr. G. Matsueda (Princeton University, NJ). Polystyrene latex beads (4.5  $\mu\text{m}$  diameter) were from Polysciences Inc. (Warrington, PA), and surfactant-free aldehyde/sulfate polystyrene latex beads (4.5  $\mu\text{m}$  diameter) were purchased from Interfacial Dynamics Corporation (Portland, OR).

### Washed platelets (WP)

WP were prepared from platelet-rich plasma (PRP) by the "single centrifuging and dilution procedure" described by Goldsmith et al. (1994). Briefly, blood was taken from the antecubital vein of healthy volunteers not taking any medication, and directly added into 3.8% sodium citrate (1:9 vol/vol blood), followed by centrifugation at  $150 \times g$  for 15 min. PRP was acidified to pH 6.5 with 0.1% citric acid and ZK 36 374 was added to 50 nM, followed by centrifugation at  $800 \times g$  for 15 min. The platelet pellets were redispersed and resuspended in  $\text{Ca}^{2+}$ -free Tyrode's-albumin buffer (Tyrode's, glucose 5.6 mM, bovine serum albumin (BSA) fraction V 0.35%) and kept at 37°C. No aggregation occurred in the absence of exogenously added activators (Goldsmith et al., 1994; Frojmovic et al., 1997).

### Coating of latex beads with Fg (Fg-beads)

The protocol was based on a previously published method (Liu et al., 1998). Polystyrene latex beads were incubated in phosphate-buffered saline

(PBS) buffer at  $\sim 0.5\%$  solids for 5 min at room temperature followed by centrifuging at  $10,000 \times g$  for 30 s. The washing procedure was repeated two more times, and the bead pellets were resuspended in PBS. Fg was added to the above bead suspension at 500 nM and incubated at room temperature for 30 min. Then BSA was added to a final concentration of 10 mg/ml and incubated for another 20 min to block the sites unoccupied by Fg. The beads were then pelleted at  $10,000 \times g$  for 30 s, resuspended in PBS with 10 mg/ml BSA, and re-incubated for 20 min. Finally, the beads were centrifuged and resuspended in distilled and deionized water and kept under 4°C. The antibody-binding and platelet adhesion properties of these beads were the same as those suspended in physiological buffers. Beads coated only with BSA (BSA-beads) following the same procedure served as controls. We determined the number of Fg bound to the beads by incorporating FITC-Fg to the Fg (FITC-Fg/Fg ratio of 1:10) and measuring the bound fluorescence on a FACScan flow cytometer (Becton-Dickinson Canada, Mississauga, Ontario). Knowing the average FITC/Fg ratio (F/P), we calculated the number of Fg molecules bound to the beads. The beads thus coated have  $183,034 \pm 11,740$  Fg molecules per bead, or  $2882 \pm 185$  Fg molecules per  $\mu\text{m}^2$  surface area. This number represents the maximal saturating density we obtained in our coating conditions (100%), as determined by saturating assays in which the concentration of Fg and the incubation time were varied (not shown). We also prepared latex beads coated with Fg at a density of  $250 \pm 9/\mu\text{m}^2$  ( $\sim 9\%$  of saturation), and  $24 \pm 1/\mu\text{m}^2$  ( $\sim 1\%$ ), by incubating the beads with 10 nM and 1 nM of Fg, respectively, instead of 500 nM, for 30 min at room temperature.

### Preparation of GRGDSP-activated GPIIb/IIIa-beads

Aldehyde/sulfate latex beads were washed three times and incubated with 110 nM GPIIb/IIIa and 1 mM GRGDSP in 6.7 mM Hepes, pH 6.5, for 120 min (rotating) at RT. Beads were then pelleted ( $10,000 \times g$ , 30 s), washed three times, and incubated overnight (rotating) at 4°C in PBS pH 6.5, containing 6.7 mM Hepes and 2.5% BSA to block sites unoccupied by GPIIb/IIIa. Beads were then washed three times and stored at 4°C in PBS, 61  $\mu\text{M}$  Hepes, and 0.1% BSA, pH 7.4. A negligible number of doublets formed during the storage. The beads obtained had  $\sim 30,000$ – $35,000$  GPIIb/IIIa in an "activated" state, as measured by FITC-Fg binding at saturating concentrations (not shown), which correspond to a surface density of  $\sim 476$ – $555$  "activated" GPIIb/IIIa per  $\mu\text{m}^2$ .

### Flow device

Shear experiments were conducted in a microcouette as previously described (Xia and Frojmovic, 1994). Briefly, the device is composed of two concentric plexiglass cylinders of radius of 7.0 mm (inner,  $R_1$ ) and 7.3 mm (outer,  $R_2$ ), with a gap  $h = 0.3$  mm. The inner cylinder, driven by a high-precision step motor, rotates at a desired angular velocity ( $\omega$ ), with respect to the stationary outer cylinder, to yield a simple shear rate ranging from 1 to  $2200 \text{ s}^{-1}$ , determined from  $G = R_1 \cdot \omega/h$ . Suspensions of 400  $\mu\text{l}$  were loaded in the gap between the two cylinders and subjected to shear. Shear was stopped at consecutive times for subsampling (20  $\mu\text{l}$ ), after discarding a dead volume of 10  $\mu\text{l}$ .

### Adhesion of platelets to Fg-beads

Beads and WP were mixed in THA buffer (Tyrode's supplemented with 5 mM Hepes and 0.015 mM BSA) containing 1 mM  $\text{Ca}^{2+}$  and 1 mM  $\text{Mg}^{2+}$ . The final concentrations of the beads and the platelets were 15,000 and 5000 per  $\mu\text{l}$ , respectively, and the total volume was 400  $\mu\text{l}$ . Ten  $\mu\text{M}$  ADP was used to activate platelets, while 50 nM ZK 36 374, a stable prostacyclin analog, was used to keep platelets in the inactivated state. When specified, the concentration of beads, platelets, and/or ADP were modified

from the above. When adhesion of platelet to Fg-beads was conducted in the presence of soluble Fg, platelets and soluble Fg were allowed to incubate for 2 min, before the addition of Fg-beads. The suspensions were mixed gently with a pipette and then immediately transferred to the microcouette and sheared at variable shear rate from  $100 \text{ s}^{-1}$  to  $2000 \text{ s}^{-1}$  at room temperature. At given times,  $20\text{-}\mu\text{l}$  subsamples were taken and fixed with  $100 \mu\text{l}$  of 0.8% glutaraldehyde. The fixed samples were then diluted to  $200\text{--}500$  particles/ $\mu\text{l}$  and counted on the FACScan on high flow rate ( $1 \mu\text{l/s}$ ) for 20 s. Due to different light scatter profiles, the beads and the platelets appear as distinct populations on the dot plot of side light scattering versus forward light scattering, as previously reported (Liu et al., 1998). When a platelet adhered to a bead, the forward light scattering of the platelet-bead aggregate increased to remove this adhering platelet from the forward/side light scattering profile containing the single platelets. Therefore, by counting the number of free (unaggregated) platelets in a unit volume of suspension, the adhesion of platelets to beads due to coaggregation can be quantified from the change in platelet concentration, with time expressed as the fraction of platelets present at time  $t$  after coaggregation,

$$PA_t = (1 - N_t/N_0) \quad (1)$$

where  $N_t$  and  $N_0$  are the concentrations of the free (not adhered) platelets in the suspension at time  $t$  and time 0, respectively.

### Aggregation of GPIIbIIIa-beads by receptor-bound Fg

GPIIbIIIa-beads ( $7000/\mu\text{l}$ ) were incubated for 30 min at RT, with varying concentrations of Fg, in THA buffer containing 1 mM  $\text{Ca}^{2+}$ , 1 mM  $\text{Mg}^{2+}$ , 10 mg/ml BSA, and 0.05% (v/v) Tween 20 to reach percentages of receptor occupancy ranging from 0.2% to 50%. The concentrations of Fg chosen to conduct the assays were determined by referring to isotherm experiments of FITC-Fg binding on GPIIbIIIa-beads (results are not presented). We also used FITC-Fg to control the percentage of receptor occupancy during the aggregation assays. Beads were then sheared in the microcouette at a shear rate of  $300 \text{ s}^{-1}$  and subsamples were taken and processed as described above.

### Determination of adhesion efficiency of platelets on Fg-beads

The two-body collision capture efficiency in the coaggregation of platelets and Fg-beads is defined as the fraction of all shear-induced collisions that results in the formation of platelet-bead doublets, i.e., the ratio of the rate of the measured initial aggregate formation ( $dN_t/dt$ ) to the frequency of two-body collision in suspension. The total two-body collision frequency ( $J$ ) between the platelets and the beads per unit volume of suspension is given by the Smoluchowski equation for smooth spherical, rigid particles (Smoluchowski, 1917):

$$J = \frac{4}{3} G(a_b + a_p)^3 N_b \quad (2)$$

where  $G$  is the shear rate,  $a_b$  ( $2.25 \mu\text{m}$ ) and  $a_p$  ( $1.13 \mu\text{m}$ ) (Wong et al., 1989) are the equivalent spherical radius of the beads and the platelets, and  $N_b$  is the concentration of the beads (number per unit volume). The adhesion (capture) efficiency,  $\alpha$ , can then be expressed as

$$\alpha = \frac{dN_t/dt|_{t \rightarrow 0}}{J} \quad (3)$$

where  $dN_t/dt|_{t \rightarrow 0}$  is the experimentally determined initial rate of platelet removal into platelet-bead doublet formation (see Eq. 5).

At time 0, there were on average 15% of all bead particles present as doublets with no significant higher multiplets. This effect was accounted for by assuming that the equivalent spherical radius of the bead doublets corresponds to the combined volume of the two spherical singlets, i.e., the equivalent spherical radius of a doublet is  $\sqrt[3]{2} = 1.26$  times that of the singlet (Petlicki and van de Ven, 1992), and taking into consideration the concentrations of the singlet beads (85% of total) and doublet beads (15% of total), as expressed by Eq. 4:

$$\frac{dN_t}{dt} \Big|_{t \rightarrow 0} = \frac{4}{3} \alpha G [(a_{bs} + a_p)^3 \times 0.85 + (1.26a_{bs} + a_p)^3 \times 0.15] N_b \quad (4)$$

where  $a_{bs}$  and  $1.26a_{bs}$  are the radius and the equivalent spherical radius of the singlets and the doublets of the beads, respectively. Homotypic platelet-platelet aggregation was negligible for both activated and resting platelets, as confirmed by phase-contrast microscopy (Zeiss,  $500\times$  magnification). Less than 1% of platelets were involved in forming platelet-to-platelet aggregates in suspension or on the surface of a polystyrene bead, after 30 s of shearing, and this, whatever the shear rate used and the condition of activation tested. Furthermore, we did not detect disappearance of platelets or beads from the samples due to adhesion to the walls of the microcouette during shear. Though there was significant homotypic aggregation between Fg-beads at longer times, this aggregation between the Fg-beads within 10 s was very low ( $<2\%$ ). Therefore the homotypic Fg-bead aggregation does not have a significant effect on the determination of the adhesion efficiencies, which are derived from the rates of adhesion and collision frequencies when  $t \rightarrow 0$ .

The initial rate of adhesion can be obtained experimentally by finding the best fit of  $PA$  values at time  $t$  to the equation

$$PA = PA_{\max}(1 - e^{-t/B}) \quad (5)$$

where the initial rate of adhesion is  $PA_{\max}/B$ . Hence,  $\alpha$  values can be calculated from Eq. 4.

### Determination of adhesion efficiency of GPIIbIIIa-beads on Fg-beads

The equations above were used, replacing the mean radius of platelets ( $a_p = 1.13 \mu\text{m}$ ) by the radius of GPIIbIIIa-beads ( $2.25 \mu\text{m}$ ).

### Determination of adhesion efficiency of GPIIbIIIa-beads with varying receptor occupancy by Fg

The total two-body collision frequency ( $J$ ) between two GPIIbIIIa-beads per unit volume of suspension is given by the Smoluchowski equation for smooth spherical, rigid particles:

$$J = \frac{16}{3} G a^3 C^2, \quad (6)$$

where  $G$  is the shear rate,  $a$  ( $2.25 \mu\text{m}$ ) is the equivalent spherical radius of the beads, and  $C$  is the concentration of the beads. The adhesion efficiency can then be expressed as

$$\alpha = \frac{dPA/dt|_{t \rightarrow 0}}{J} \quad (7)$$

where  $dPA/dt|_{t \rightarrow 0}$  is the experimentally determined initial rate of platelet removal into bead-doublet formation (see Eq. 5).

During the 30 min preincubation of GPIIb/IIIa-beads with soluble Fg, doublet formation occurred to represent ~15% of the total number of particles at time 0 (with no significant higher multipliers). The pre-formation of doublets and the consecutive modification of the particle concentration at  $t = 0$  were accounted to form Eq. 8:

$$\left. \frac{dPA}{dt} \right|_{t=0} = \frac{16}{3} \alpha G [a^3 \times 0.85 + (1.26a)^3 \times 0.15] \times \frac{C_i}{1.15} \quad (8)$$

where  $a$  and  $1.26a$  are the radius and the equivalent spherical radius of the singlets and the doublets of the beads, respectively,  $C_i$  is the concentration of beads (singlets) before the preincubation with Fg ( $7000/\mu\text{l}$ ), and  $C_i/1.15$  is the concentration of particles (singlets plus doublets) at  $t = 0$ , following incubation with Fg associated with ~15% aggregation.

The initial rate of adhesion was obtained experimentally by finding the best fit of  $PA$  values at time  $t$  to Eq. 5, and  $\alpha$  values were calculated from Eq. 8.

### Measurement of the percentage of platelet activated in response to increasing doses of ADP

Platelets ( $20,000/\mu\text{l}$ ) activated by increasing doses of ADP ( $0.1$ – $100 \mu\text{M}$ ) were incubated for 30 min at  $37^\circ\text{C}$  with  $1.2 \mu\text{M}$  FITC-Fg (diluted twofold with unlabeled Fg to minimize surface self-quenching, as described by Xia et al., 1996). Platelet-bound fluorescence ( $Fl$ ) was thereafter measured in the FACScan flow cytometer. The method used is based on the previously published observation that at high ADP concentration most of the platelets have  $Fl$  values greater than a critical threshold value,  $Fl_c$ , but decreasing fraction of these platelets remained above this threshold with decreasing ADP, reflecting a bimodal distribution of “resting” platelets and “maximally activated” platelets (Frojmovic et al., 1994). For this type of sub-population analysis, we report the fraction of maximally activated platelets ( $Fl > Fl_c$ ) as a function of varying ADP concentration.  $Fl_c$  was experimentally selected such that >95% of all platelets had  $Fl$  values  $< Fl_c$  in the absence of ADP and in the presence of 1 mM GRGDSP.

### Data analysis

Data are expressed as mean  $\pm$  SE (standard error of the mean). To fit the nonlinear equation  $\%PA = PA_{\max} (1 - e^{-t/B}) \times 100$  to our data, we used a nonlinear regression curve-fitter software (Sigma Plot, Jandel Scientific Software, San Rafael, CA), as previously described (Xia and Frojmovic, 1994).

## RESULTS

### Efficiency of adhesion of activated or resting platelets to Fg-beads

Fig. 1 A shows the time course of adhesion of activated platelets to Fg-beads from the measured coaggregation of platelets with Fg-beads. The adhesion kinetics were exponential with a halftime of ~20–30 s, plateauing by ~2 min, with initial rates and extents increasing continuously with increasing shear rates from  $100 \text{ s}^{-1}$  to  $2000 \text{ s}^{-1}$ . The activated platelets did not significantly adhere to BSA-beads at any of the shear rates tested. The adhesion efficiency of activated platelets (Fig. 1 B) decreased by 67%, from 5.8 to 1.9%, as the shear rate increased from  $100 \text{ s}^{-1}$  to  $1000 \text{ s}^{-1}$ , with the largest drop in efficiency occurring

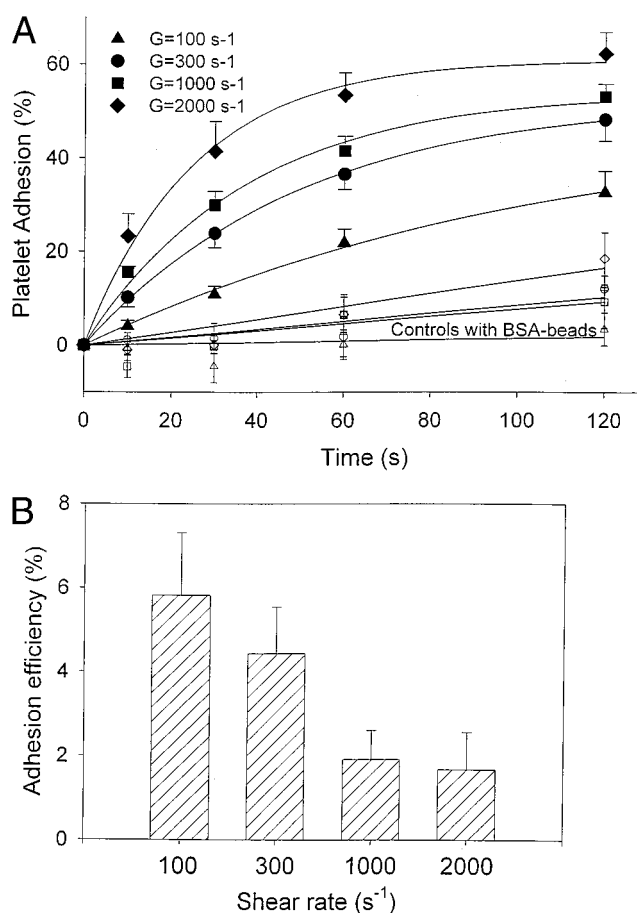


FIGURE 1 (A) Adhesion of activated washed platelets to Fg immobilized on polystyrene beads. Platelets ( $30,000/\mu\text{l}$ ) and polystyrene beads ( $15,000/\mu\text{l}$ ) coated with Fg or BSA, were sheared in a microcouette as described in Methods. Platelets were either activated with  $10 \mu\text{M}$  ADP or kept resting with  $50 \text{ nM}$  ZK 36 374, a stable prostacyclin analog. Samples were taken at times indicated, and free platelet concentrations were determined on a FACScan flow cytometer. Adhesion was calculated from the free platelet concentrations as described in Methods. Mean  $\pm$  SE of at least three experiments. (B) Adhesion efficiencies. Data in (A) were used to calculate the adhesion efficiencies, as detailed in Methods. Mean  $\pm$  SE of at least three experiments.

between  $300 \text{ s}^{-1}$  and  $1000 \text{ s}^{-1}$ . Thereafter, the efficiency remained effectively constant.

The adhesion of resting platelets to Fg-beads was, on average, comparable to their adhesion to BSA-beads, suggesting that resting platelets do not specifically adhere to Fg-beads at levels detectable with our methodology. In fact, we did obtain variations in resting-platelet adhesion to Fg beads. In a few experiments (4 of 22 experiments), the platelets prepared as “resting” cells using  $50 \text{ nM}$  ZK 36 374 did adhere to Fg-beads (but not to BSA-beads), with an apparent adhesion efficiency ranging from 0.2 to 2.7% according to the shear rate. However, this adhesion occurred sporadically and was rarely observed when resting platelets were used within one hour after taking blood from the

donor. It is likely that even in the presence of ZK 36 374, some partial activation persisted in these samples.

The time course of adhesion of resting platelets, or platelets activated by 10  $\mu\text{M}$  ADP, was studied as a function of the density of the Fg immobilized on the polystyrene beads (Fig. 2, A and B). We used beads coated with Fg at surface densities of 100% ( $2882 \pm 185 \text{ Fg}/\mu\text{m}^2$ ), 9% ( $250 \pm 9/\mu\text{m}^2$ ), and 1% ( $24 \pm 1/\mu\text{m}^2$ ). Adhesion of resting platelets to Fg-beads was not distinguishable from the negative control (adhesion to BSA-beads), either at 100  $\text{s}^{-1}$  or 2000  $\text{s}^{-1}$  (not shown). Adhesion efficiency of activated platelets at 100  $\text{s}^{-1}$  was reduced by 62% when surface density decreased from 100% to 9%, but only a further 11% reduction for surface density decrease from 9% to 1%. At 2000  $\text{s}^{-1}$ , adhesion efficiency of activated platelets was similarly reduced by 45% when Fg density decreased from 100% to 9%, and by a further 36% for reductions from 9% to 1% of Fg density.

Washed platelets, activated with concentrations of ADP ranging from 0.1 to 100  $\mu\text{M}$ , were sheared together with

Fg-beads (100% of surface density), at 2000  $\text{s}^{-1}$  (Fig. 3 A). The log-dose-response curve of adhesion efficiency for varying ADP concentration is shown in Fig. 3 B. Platelet adhesion efficiency was low with  $[\text{ADP}] < 1 \mu\text{M}$  ( $< 0.7 \pm 0.2\%$ ), while it was near-maximal at 10  $\mu\text{M}$  ADP ( $2.7 \pm 0.3\%$ ), and maximal at 100  $\mu\text{M}$  ADP ( $3.1 \pm 0.6\%$ ). The ADP concentration necessary to reach one-half of maximal adhesion efficiency was 3  $\mu\text{M}$ . No adhesion occurred when washed platelets and beads were sheared without ADP.

The fraction of washed platelets “quantally” transformed from the “resting” into the “activated” state by varying ADP concentrations was evaluated by specific FITC-Fg binding to its activated GPIIb/IIIa receptor. Thus, the percentage of cells with fluorescence ( $Fl$ ) above a critical fluorescence threshold ( $Fl_c$ ) corresponding to maximally activated platelets was determined by flow cytometry as described in the Methods. The cross-plot comparing the platelet adhesion efficiency against the fraction of cells with  $Fl > Fl_c$  (Fig. 4) demonstrates a linear correlation between these two variables (dependency  $r^2 = 0.97$ ).

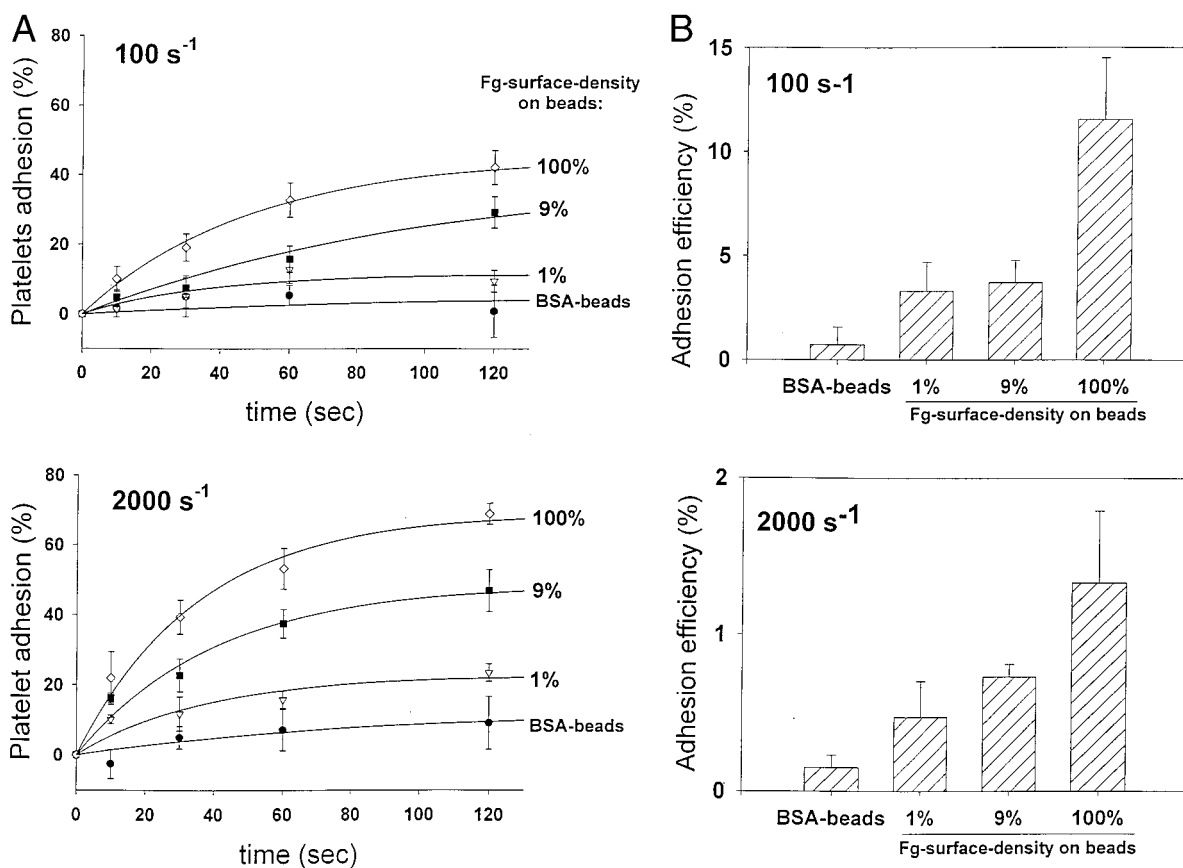


FIGURE 2 (A) Adhesion of activated washed platelets to polystyrene beads of varied Fg surface coverage density. Platelets ( $5000/\mu\text{l}$ ) and polystyrene beads ( $15,000/\mu\text{l}$ ) coated with Fg, with a surface density of 100% ( $2882 \text{ Fg}/\mu\text{m}^2$ ), 9% or 1%, or coated with BSA, were sheared at 100  $\text{s}^{-1}$  or 2000  $\text{s}^{-1}$ , in a microcouette as described in Methods. Platelets were activated with 10  $\mu\text{M}$  ADP. Subsamples were taken at times indicated, and free platelet concentrations were determined on a FACScan flow cytometer. Adhesion was calculated from the free platelet concentrations as described in Methods. Mean  $\pm$  SE of at least three experiments. (B) Efficiency of platelet adhesion to Fg immobilized on polystyrene beads with varying surface density. Adhesion efficiencies were calculated from results shown in (A) as described in Methods. Mean  $\pm$  SE of at least three experiments.

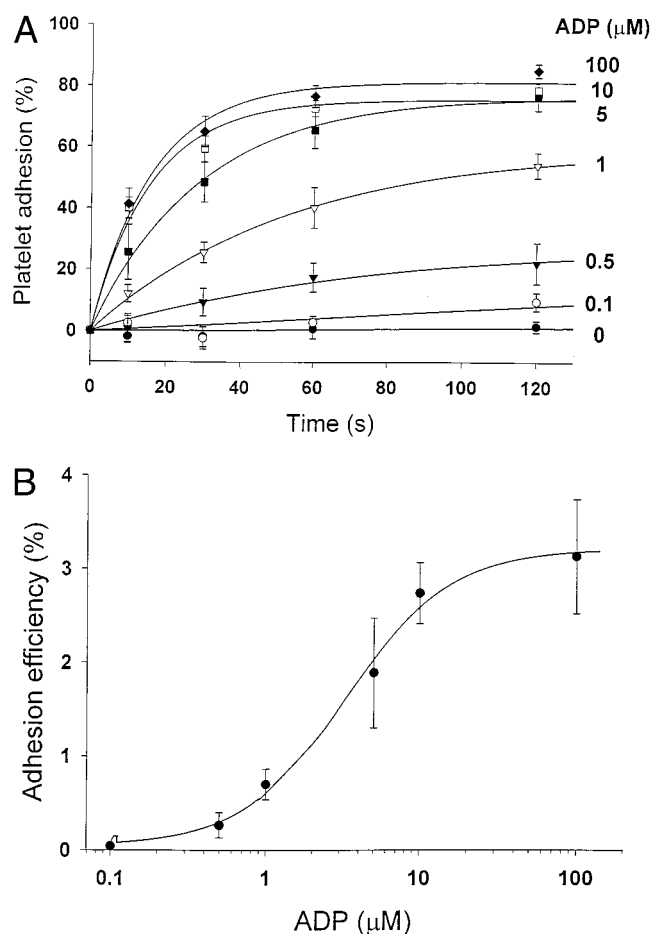


FIGURE 3 (A) Adhesion of washed platelets to Fg immobilized on polystyrene beads with varying ADP concentration, at a shear rate of  $2000\text{s}^{-1}$ . Platelets ( $5000/\mu\text{l}$ ) and Fg-beads ( $15,000/\mu\text{l}$ ) were sheared in a microcouette as described in Methods. Washed platelets were activated with an ADP concentration ranging from 0 to  $100\ \mu\text{M}$ . Subsamples were taken at times indicated, and free platelet concentrations were determined on a FACScan flow cytometer. Adhesion was calculated from the free platelet concentrations as described in Methods. Mean  $\pm$  SE of at least three experiments. (B) Log-dose-response analysis for efficiency of platelet adhesion to Fg immobilized on polystyrene beads for varying ADP concentration. Adhesion efficiencies were calculated from results shown in (A) as described in Methods. Mean  $\pm$  SE of three experiments.

To make our model even more relevant to physiologic conditions, where platelets are normally surrounded by high concentrations of soluble Fg ( $\sim 9\ \mu\text{M}$ ) (Furie and Furie, 1988), we repeated our measurements of platelet adhesion to Fg-coated beads in the presence of soluble Fg. Platelets activated with  $10\ \mu\text{M}$  ADP were incubated for 2 min with 1.1, 3.2, or  $9\ \mu\text{M}$  of soluble Fg before the addition of Fg-coated beads (100% of surface density). Samples were then immediately sheared in the microcouette at  $2000\ \text{s}^{-1}$ . As shown in Fig. 5 A, the presence of soluble Fg did not interfere with the capacity for maximal platelet adhesion to immobilized Fg ( $PA_{\text{max}} \sim 80\%$ ), which was reached after 120 s of shear, even with  $9\ \mu\text{M}$  of soluble Fg. However, the

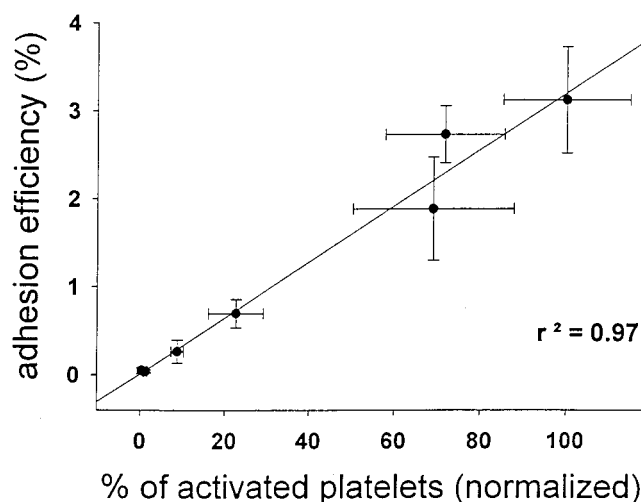


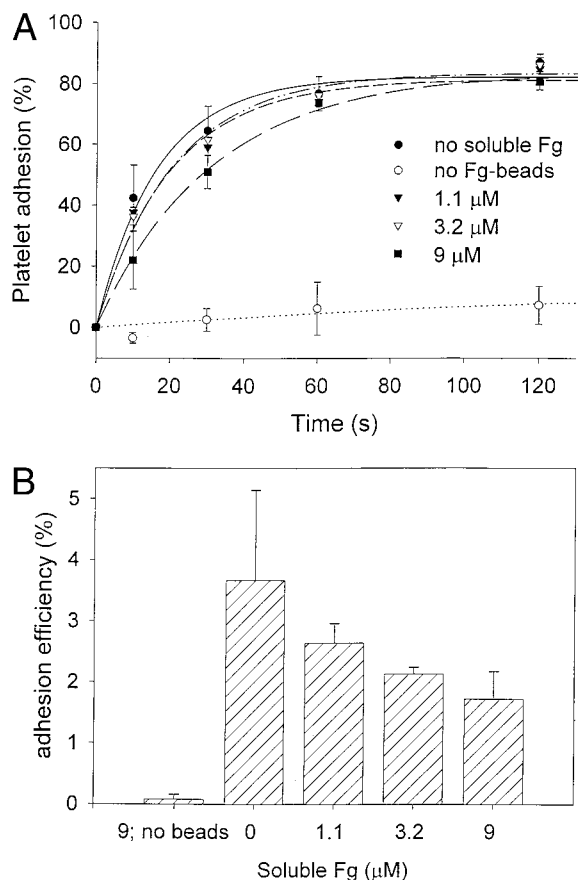
FIGURE 4 Correlation between the efficiency of platelet adhesion to Fg immobilized on polystyrene beads and the percentage of activated platelets. Adhesion efficiencies reported on the graph are those presented in Fig. 3 B and correspond to ADP concentrations ranging from 0 to  $100\ \mu\text{M}$ . The normalized percentages of platelets activated by the same ADP concentrations were determined as described in Methods (100% = 58% in fact), by measuring FITC-Fg binding on the platelets of the same donors. Results are expressed as means  $\pm$  bidirectional SE;  $n = 3$  for each set of experiments.

analysis of adhesion efficiencies (Fig. 5 B) revealed that soluble Fg could partially compete with immobilized Fg for the binding of activated platelets, but to a moderate extent, because adhesion efficiency only decreased by 28, 42, and 53% in the presence of 1.1, 3.2, and  $9\ \mu\text{M}$  soluble Fg, respectively.

#### Aggregation of GPIIbIIIa beads with varying Fg-receptor occupancy and adhesion of GPIIbIIIa-beads to Fg-beads

We next compared capture efficiencies determined above for adhesion of ADP-activated platelets via activated GPIIbIIIa receptors to Fg-coated spheres with the efficiencies for GPIIbIIIa-coated spheres adhering to these Fg-beads. We also compared the effect of Fg receptor occupancy on efficiencies of homotypic aggregation mediated by receptor-bound Fg between GPIIbIIIa-beads. Aggregation assays of GRGDSP-activated GPIIbIIIa-beads by receptor-bound Fg at varying percentages of occupancy were performed at  $300\ \text{s}^{-1}$  (Fig. 6). The percentage of receptor occupancy was determined by the use of FITC-Fg, as described in the Methods. The adhesion efficiency increased with percent of receptor occupancy and reached a plateau value of 30–35% for receptor occupancy from 5 to 50%. Half of the maximal adhesion efficiency occurred at only  $\sim 0.4\%$  of occupancy ( $\sim 2.1\ \text{Fg}/\mu\text{m}^2$ ).

When GPIIbIIIa-beads were sheared together with Fg-beads (saturated) at  $300\ \text{s}^{-1}$ , coaggregation of these beads occurred with an efficiency of  $10.9 \pm 3.2\%$ , which was

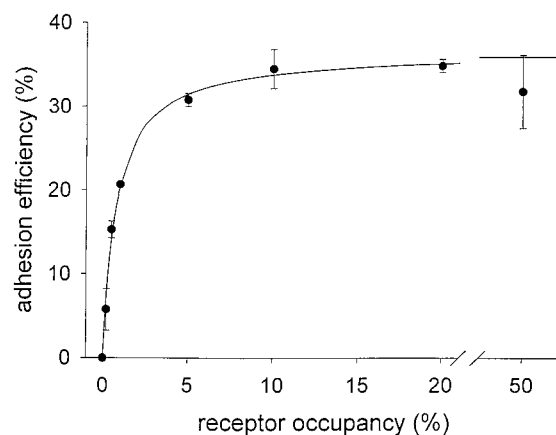


**FIGURE 5** (A) Adhesion of washed platelets to Fg immobilized on polystyrene beads in the presence of soluble Fg. Platelets ( $5000/\mu\text{l}$ ), activated with  $10 \mu\text{M}$  ADP, were preincubated for 2 min with 1.1, 3.2, or  $9 \mu\text{M}$  of soluble Fg before adding Fg-beads ( $15,000/\mu\text{l}$ ). Samples were then immediately loaded in a microcouette and sheared at  $2000 \text{ s}^{-1}$ , as described in Methods. Subsamples were taken at times indicated, and free platelet concentrations were determined on a FACScan flow cytometer. Adhesion was calculated from the free platelet concentrations as described in Methods. Mean  $\pm$  SE of three experiments. (B) Efficiency of platelet adhesion to Fg immobilized on polystyrene beads in the presence of varying concentration of soluble Fg. Adhesion efficiencies were calculated from results shown in Fig. 2 A, as described in Methods. Mean  $\pm$  SE of three experiments.

completely inhibited by the GPIIb/IIIa antagonist Ro 44-9883 at  $1 \mu\text{M}$  (Fig. 7).

## DISCUSSION

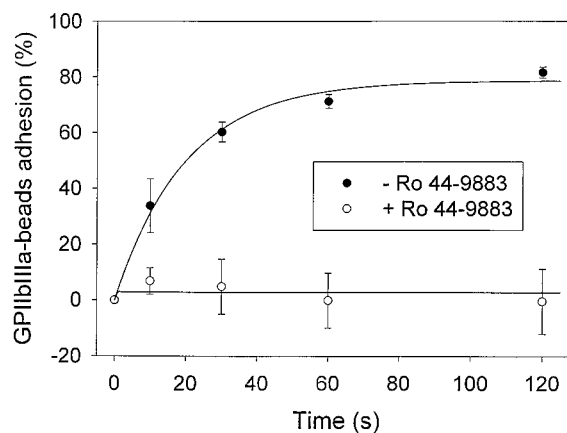
Platelets activated with ADP readily coaggregated with polystyrene beads containing surface-immobilized fibrinogen (Fg), in contrast to “resting” platelets, which generally did not show any detectable coaggregation. In a few cases, “resting” platelets prepared from whole blood “protected” with stable  $\text{PGI}_2$  analog (ZK 36 374), did show measurable coaggregation, but when averaging results for all the platelet preparations not treated with ADP, the efficiency of apparent coaggregation was at least 5–10-fold lower than that



**FIGURE 6** Aggregation of GPIIb/IIIa beads with varying Fg-receptor occupancy. GPIIb/IIIa-beads ( $10,000/\mu\text{l}$ ) were sheared at  $300 \text{ s}^{-1}$  after a preincubation with increasing concentration of Fg to reach increasing GPIIb/IIIa receptor occupancy by Fg (see Methods). Capture efficiencies were calculated from aggregation curves as described in Methods. Mean  $\pm$  SE of three experiments.

measured for ADP-activated platelets. In fact, we clearly demonstrated that ADP activation converted platelets unreactive with Fg-beads to activated platelets readily coaggregating with these same Fg-beads.

Our studies of the adhesion efficiencies of resting and activated platelets to immobilized Fg are distinct from all previous studies of platelet adhesion from whole blood onto Fg-coated planar surfaces. Resting platelets in whole blood evaluated at shear rates ranging from  $250$  to  $1500 \text{ s}^{-1}$  have been reported to adhere to immobilized Fg in a parallel-plate flow chamber system (Savage et al., 1996; Zaidi et al., 1996; Endenburg et al., 1996). However, these latter flow studies require red blood cells to drive platelets to the



**FIGURE 7** Adhesion of GPIIb/IIIa-beads to Fg-beads. GPIIb/IIIa-beads ( $10,000/\mu\text{l}$ ) were sheared at  $300 \text{ s}^{-1}$  with Fg-beads ( $10,000/\mu\text{l}$ ) (saturated), in presence or absence of  $1 \mu\text{M}$  Ro 44-9883. Adhesion was calculated from the free GPIIb/IIIa-beads concentrations as described in Methods. Mean  $\pm$  SE of three experiments.

surface, with collision frequencies not theoretically nor experimentally calculated. Moreover, although physical modeling of red cell effects on transport of other blood cells to a surface have been reported (Munn et al., 1996; Goldsmith et al., 1995), chemical contributions from sheared red blood cells, e.g., ADP release, also come into play. Our results suggest that a platelet adheres to surface-bound Fg only if it is already preactivated in suspension. In studies using perfusion chamber models, where 15 ml of freshly anti-coagulated whole blood are usually flowed onto surface-immobilized ligands for 5 min, if only 0.5–1% of the total platelet population were spontaneously activated, likely below usual experimental detectability, it would still represent  $\sim 1500$ – $3000$  activated platelets per  $\mu\text{l}$ . This concentration could readily be associated with measurable platelet adhesion on the coverslip because the actual fraction of platelets captured was not reported in any of the above studies.

The adhesion of activated platelets to polystyrene beads coated with Fg was completely dependent on platelet GPIIb/IIIa receptors since it was abolished by Ro 44-9883, a nonpeptide analog of RGD, which is markedly selective for GPIIb/IIIa versus  $\alpha_v\beta_3$  (Alig et al., 1992) (results not shown). We assessed the effects of different parameters on this adhesion: the shear rate, the density of Fg at the surface of the beads, the accessibility of the  $\gamma$  chain carboxyl terminus on Fg mediating the adhesion, the concentration of ADP used to activate the platelets, and the presence of soluble fibrinogen.

A 10-fold increase in shear rate ( $G$ ) from  $100\text{ s}^{-1}$  to  $1000\text{ s}^{-1}$  caused a threefold decrease in adhesion efficiency ( $\alpha$ ) for the capture of activated platelets by beads saturated with Fg ( $2882\text{ Fg}/\mu\text{m}^2$ ) ( $5.8 \pm 0.6$  and  $1.9 \pm 0.2\%$ , respectively), with no further decrease from  $1000\text{ s}^{-1}$  to  $2000\text{ s}^{-1}$  ( $1.9 \pm 0.2\%$  and  $1.7 \pm 0.3\%$ , respectively, Fig. 1 *B*). Remarkably, we previously reported a very similar dependence of  $\alpha$  on  $G$  for homotypic aggregation of activated platelets (Xia and Frojmovic, 1994), consistent with similar shear-dependent on/off rates for surface versus receptor-bound Fg “capturing” activated GPIIb/IIIa receptors on platelets.

The Fg is horizontally elongated when adsorbed on our beads (unpublished observations from electronmicroscopic studies with J. Jerome at Wake Forest University), as reported by others for various kinds of surfaces (Marchant et al., 1997; Taatjes et al., 1997). We measured the accessibility of the  $\gamma$  chain carboxyl terminus of the Fg coated on the beads (residues AGDV at  $\gamma$  408–411), present in two copies in the molecule, and which is known to play an exclusive role in the binding of Fg to GPIIb/IIIa (Farrell et al., 1992; Liu et al., 1997, 1998). We used the FITC-labeled 4A5, a monoclonal antibody directed against the  $\gamma$  chain carboxyl terminus domain of the Fg (comprising the AGDV residue). We found a stoichiometry 4A5/Fg of  $\sim 1.55$  when the surface density was 1% or 9%, and  $\sim 0.70$  with 100% of surface density. It is therefore likely that, on average, there is at least 1 AGDV site freely accessible per Fg over the entire range of 1–100% surface coverage, which can par-

ticipate in cross-bridging to activated GPIIb/IIIa receptors. We observed a relatively minor decrease ( $\sim 3$ -fold) in  $\alpha$  for a 100-fold drop in percentage of surface coverage. Furthermore, there was no major change in  $\alpha$  from 10% to 1% for coaggregation of Fg-beads with activated platelets (Fig. 2 *B*) seen at  $100\text{ s}^{-1}$  or  $2000\text{ s}^{-1}$ . This suggests that there is a broad range, down to 1% surface coverage, over which  $\alpha$  is still very significant. Thus, for homotypic aggregation of activated platelets, we similarly observed only a 2–3-fold decrease in  $\alpha$  for only 1% occupancy of all activated GPIIb/IIIa receptors on the activated platelets by Fg, corresponding to  $\sim 20\text{ Fg}/\mu\text{m}^2$ , compared to  $24\text{ Fg}/\mu\text{m}^2$  for 1% of maximal coverage of our Fg-beads. It is therefore predicted that  $< 20\text{ Fg}/\mu\text{m}^2$  can support adhesion of activated platelets found within thrombi or plaques containing Fg associated with thrombosis and atherosclerosis (Bini and Kudryk, 1995).

The physiological relevance of the above results was tested with respect to the high concentration of soluble Fg present in blood ( $\sim 9\text{ }\mu\text{M}$ ). We observed only a 30–50% decrease in  $\alpha$  when ADP-activated platelets were preincubated 2 min with 1.1, 3.2, or  $9\text{ }\mu\text{M}$  of soluble Fg, before the addition and subsequent flow of these Fg-beads in the continued presence of these elevated Fg concentrations, as compared to  $\alpha$  in the absence of soluble Fg (Fig. 5 *B*). This incubation of platelets with 1–9  $\mu\text{M}$  soluble Fg would leave  $\sim 10$ – $50\%$  of unoccupied GPIIb/IIIa receptors (free of bound Fg) after two minutes, as calculated from previous studies on the kinetics of Fg binding to activated platelets (Xia and Frojmovic, 1994). This is clearly sufficient for largely maintaining the coaggregation with Fg-beads. Moreover, our observations regarding efficient capture of activated platelets by Fg on surfaces are highly relevant to physiologic situations where activation and capture may occur in  $< 1\text{ s}$ , with only a small fraction of receptors occupied by Fg in this time, and minor direct competition by the high concentrations of Fg ( $9\text{ }\mu\text{M}$ ).

The adhesion efficiency of platelets to Fg-beads increased with the concentration of ADP used to activate the platelets (Fig. 3 *B*), and correlated with the proportion of platelets quantally activated by the increasing doses of ADP (Fig. 4), as previously described by Frojmovic et al., 1994. No detectable adhesion occurred without ADP, and in these studies, even without ZK 36 374. This latter observation means that spontaneous activation following any reversible adhesion of initially inactivated platelets to Fg-coated beads, which might be blocked by ZK 36 374 when it is present, does not seem to occur. These results emphasize the need for platelet activation for adhesion to Fg-coated beads, and reinforce the idea that when we sporadically found measurable adhesion of resting platelets, this was due to the presence of spontaneously activated platelets in the preparation.

Adhesion of activated platelets to immobilized Fg has received less attention than the adhesion of resting platelets, probably due to the argument that in the real situation of thrombosis or hemostasis in vivo, there is probably not



**TABLE 1** Capture efficiencies of activated platelets (P\*) by receptor- and surface-immobilized fibrinogen

| Fg density<br>(Fg/ $\mu\text{m}^2$ ) | Capture efficiencies (%) for    |                                 |                  |                             |
|--------------------------------------|---------------------------------|---------------------------------|------------------|-----------------------------|
|                                      | Homotypic aggregation of        |                                 | Coaggregation of |                             |
|                                      | P*-Fg <sub>s</sub> <sup>†</sup> | GPIIbIIIa-beads-Fg <sub>s</sub> | P* to Fg-beads   | GPIIbIIIa-beads to Fg-beads |
| ~2500                                | 25.0 ± 1.3                      | —                               | 8.3 ± 2.2        | 10.9 ± 3.2                  |
| ~250                                 | 18.2 ± 6.1                      | 34.5 ± 2.3 <sup>‡</sup>         | 3.7 ± 1.0        | —                           |

Capture efficiencies ( $\alpha$ ) represent mean  $\pm$  SD values selected for the indicated average Fg surface densities and were determined at shear rates of 100–300/s.

<sup>†</sup>Fg<sub>s</sub> = soluble Fg at  $\mu\text{M}$  range corresponding to ~20–50% occupancy of all GPIIbIIIa receptors (note: a plateau in  $\alpha$  was observed over ~20–80% range (Xia and Frojmovic, 1994) and 5–50% range (Fig. 6), respectively, for P\* and GPIIbIIIa\*-beads homotypic aggregation).

<sup>‡</sup>Note that at ~1 Fg/ $\mu\text{m}^2$ ,  $\alpha$  was 6–10%.

enough time for the platelets to become activated before they can adhere to the exposed subendothelial matrix or to the already adherent platelets (Luscher and Weber, 1993; Ruggeri, 1994). However, though the platelets must pass the site of vascular damage very rapidly (~100 ms) (Born and Richardson, 1980), platelet activation is also equally rapid (Sage et al., 1989; Raha et al., 1993; Gear, 1994; Frojmovic et al., 1991). ADP opens calcium channels in the platelet membrane within 20 ms (Sage et al., 1989). Other biochemical changes typical of platelet activation, such as the synthesis and release of IP<sub>3</sub> and IP<sub>4</sub>, and phosphorylation of some proteins, take place as fast as one can measure, within a fraction of a second (Raha et al., 1993; Gear, 1994). In addition, platelets have been observed to roll on stimulated venular endothelium (Frenette et al., 1995). This could also be a mechanism in the damaged artery system that could serve to slow down the platelets, thus allowing longer time for the passing platelets to become activated. Our studies suggest that it may be activated, rather than “resting” platelets, that play a central role in both hemostasis and thrombosis, with efficiency being shear rate-dependent. Such activation could readily occur at sites of damage containing local concentrations of activators such ADP or thrombin.

The optimal capture efficiencies ( $\alpha$ ) for aggregation between ADP-activated platelets, at 20–80% receptor occupancy by Fg, were ~20–30% at shear rates of 300 s<sup>-1</sup> (Xia and Frojmovic, 1994), comparable to ~5–12% for such activated platelets to adhere to beads maximally coated by Fg. These are remarkably similar results for receptor-bound and for surface-bound Fg at similar Fg surface densities (see Table 1). The ~2–3-fold greater efficiency for capture of activated platelets (P\*) by receptor-bound Fg on P\* than surface-bound Fg may be more related to the organization of the surface Fg than geometric difference between platelets and beads, as previously theoretically suggested (Tandon and Diamond, 1997). Indeed, smooth-surfaced beads containing Fg coaggregated with beads bearing activated GPIIbIIIa receptors with a similar  $\alpha$  as for coaggregation with P\* (~10% in both cases) (Table 1). Again, similar  $\alpha$  values were seen at 10 times lower Fg densities (~250 Fg/ $\mu\text{m}^2$ ) for homotypic aggregation of receptor-bound Fg

on P\* or GPIIbIIIa-beads, but surface-bound Fg mediating adhesion to P\* showed up to ~10 times lower efficiencies at these lower Fg densities. Moreover, receptor-bound Fg is so efficient that on the pure GPIIbIIIa-beads, homotypic aggregation was still robust (~6–10% efficiency) at Fg densities as low as ~1 Fg/ $\mu\text{m}^2$  (Fig. 6; Table 1).

The actual modeling of our observations and differences in terms of receptor and Fg density on platelets and/or beads, as well as affinities and avidities, remains to be done, as recently reported for platelet aggregation in flow (Tandon and Diamond, 1997). Further studies of the mechanisms underlying these differences are expected to be relevant to Fg-dependent adhesion and aggregation, including events occurring via Fg and fibrin present in pathologic arterial walls (Hatton et al., 1989).

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