Published May 15, 1993

Activated Platelets Form Protected Zones of Adhesion on Fibrinogen and Fibronectin-coated Surfaces

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Abstract. Leukocytes form zones of close apposition when they adhere to ligand-coated surfaces. Because plasma proteins are excluded from these contact zones, we have termed them protected zones of adhesion. To determine whether platelets form similar protected zones of adhesion, gel-filtered platelets stimulated with thrombin or ADP were allowed to adhere to fibrinogen- or fibronectin-coated surfaces. The protein-coated surfaces with platelets attached were stained with either fluorochrome-conjugated goat anti-human fibrinogen or anti-human fibronectin antibodies, or with rhodamine-conjugated polyethylene glycol polymers. Fluorescence microscopy revealed that $F(ab')_2$ anti-fibrinogen (100 kD) did not penetrate into the contact zones between stimulated platelets and the underlying fibrinogen-coated surface, while Fab antifibrinogen (50 kD) and 10 kD polyethylene glycol readily penetrated and stained the substrate beneath the platelets. Thrombin- or ADP-stimulated platelets also formed protected zones of adhesion on fibronectin-coated surfaces. $F(ab')_2$ anti-fibronectin and 10 kD polyethylene glycol were excluded from these adhesion zones, indicating that they are much less permeable than those formed by platelets on fibrinogen-coated surfaces. The permeability properties of protected zones of adhesion formed by stimulated platelets on surfaces coated with both fibrinogen and fibronectin were similar to the zones of adhesion formed on fibronectin alone. mAb 7E3, directed against the $\alpha_{IIb}\beta_3$ integrin blocked the formation of protected adhesion zones between thrombin-stimulated platelets and fibrinogen or fibronectin coated surfaces. mAb C13 is directed against the $\alpha_5\beta_1$ integrin on platelets. Stimulated platelets treated with this mAb formed protected zones of adhesion on surfaces coated with fibronectin. These protected zones were impermeable to $F(ab')_2$ antifibronectin but were permeable to 10 kD polyethylene glycol. These results show that activated platelets form protected zones of adhesion and that the size of molecules excluded from these zones depends upon the composition of the matrix proteins to which the platelets adhere. They also show that formation of protected zones of adhesion by platelets requires $\alpha_{IIb}\beta_3$ integrins while the permeability properties of these zones of adhesion are regulated by both $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_1$ integrins.

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THE formation of a platelet (Plt)[1] plug at the site of vascular injury can be divided into three steps: first, adhesion of resting Plts to the subendothelial matrix. Second, stimulation of Plts, which promotes Plt spreading and recruitment of additional Plts, to form an aggregate referred to as a Plt plug. Third, simultaneous secretion of various mediators from the Plts. These mediators stabilize the plug by promoting the recruitment of more Plts into the plug and by facilitating the formation of a fibrin clot (Fitzgerald and Phillips, 1989; Leung and Nachman, 1986).

Adhesion of Plts to extracellular matrices and Plt–Plt aggregation are mediated by specific Plt receptors. For example, upon activation, Plt integrin $\alpha_{IIb}\beta_3$ mediates attachment and spreading of Plts on matrices containing fibrin/fibrinogen (Peerschke, 1985), von Willebrand factor (Timmons et al., 1984) and fibronectin (Plow et al., 1985). Plt receptors other than $\alpha_{IIb}\beta_3$ also participate in Plt adhesion. For example, the glycoprotein Ib-IX complex mediates adhesion of Plts to von Willebrand factor (Roth, 1991), and several members of the β_1 integrin superfamily serve as receptors for collagen (Kunicki et al., 1988), fibronectin (Piotrowicz et al., 1988) and laminin (Sonnenberg et al., 1988) in the subendothelial matrix.

1. *Abbreviations used in this paper*: Fg, fibrinogen; PEG, polyethylene glycol; Plt, platelet; PZA, protected zones of adhesion.

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Published May 15, 1993

Plt aggregation is mediated by several receptors. The bridging of dimeric fibrinogen (Fg) molecules between $\alpha_{IIb}\beta_3$ integrins on adjacent Plts is required for Plt aggregation (Bennett et al., 1982). Although fibrinogen is the major protein involved in Plt aggregation, other proteins such as von Willebrand factor (Turitoo et al., 1984), vitronectin (Thiagarjan and Kelly, 1988), thrombospondin (Leung, 1984), and fibronectin (Dixit et al., 1985) also are thought to be involved in this process.

Microscopic analyses of Plt plugs and aggregates show that Plts are closely opposed to each other and to underlying matrix proteins (Zucker and Nachmias, 1985). While there are no data describing the ability of plasma proteins to permeate into the spaces between Plts and extracellular matrices or other Plts, Coller (1990) reported that Plt-rich thrombi are particularly resistant to thrombolysis. This raises the possibility that thrombolytic agents have limited activity against such thrombi because they cannot gain access to the contact regions between aggregated Plts or between Plts and extracellular matrix proteins.

Several investigators have reported that neutrophils (Campbell et al., 1982; Sandhaus, 1987; Rice and Weiss, 1990; Weitz et al., 1987; Wright et al., 1988) and macrophages (Wright and Silverstein, 1984; Heiple et al., 1990) have the capacity to form a tight seal between their membranes and ligand-coated surfaces. Electron microscopic (Wright and Silverstein, 1984), and reflection interference contrast microscopic (Heiple et al., 1990) studies indicate that this seal surrounds a space(s) between the macrophages and the underlying ligand-coated surface. The tightness of the seal has been characterized by its capacity to exclude soluble extracellular proteins from the interface between the cell and the substrate. There is evidence that these protected zones of adhesion play a significant role in the effector functions of cells. For example, chemoattractant-stimulated neutrophils form such protected zones as they adhere to Fg-coated surfaces or as they migrate through filters coated with Fg (Weitz et al., 1987; Wright et al., 1988). Elastase secreted by the neutrophils into these zones is protected from inactivation by proteinase inhibitors present in the surrounding medium (Weitz et al., 1987). Such findings have led us (Weitz et al., 1987; Wright et al., 1988; Wright and Silverstein, 1984; Heiple et al., 1990) and others (Campbell et al., 1982; Sandhaus, 1987; Rice and Weiss, 1990) to suggest that the protected zones of adhesion formed between leukocytes and extracellular matrices serve as sites at which secretory products function without interference by inhibitory substances present in plasma.

In this paper, we report that activated Plts form protected extracellular zones of adhesion when they adhere to Fg- and fibronectin-coated surfaces. Plts adhering to Fg- or fibrin-coated surfaces form seals that exclude molecules of 100 kD, but are permeable to molecules of 50 kD. In contrast, Plts adhering to surfaces coated with either fibronectin alone or with a mixture of fibronectin and Fg form tighter seals that exclude molecules greater than 8 kD. Thus, the permeability properties of protected zones of adhesion formed by Plts vary depending upon the composition of the matrix to which the Plts adhere. In addition, we report that the Plt integrin $\alpha_{IIb}\beta_3$ mediates the formation of protected zones of adhesion, while both $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_1$ integrins regulate the permeability properties of such contact zones.

Materials and Methods

Reagents

Human Fg (grade L; Kabi AB, Stockholm) was depleted of plasminogen by lysine Sepharose 4B affinity chromatography in the presence of aprotinin (100 Kallikrein inhibition U/ml). It migrated as three bands of unequal intensity on reduced SDS PAGE, corresponding to the Aα,Bβ and γ chains. Human plasma derived fibronectin was a kind gift of Dr. B. Horowitz, the New York Blood Center (New York, NY). ADP was obtained from Sigma Chemical Co. (St. Louis, MO). Anti $\alpha_{IIb}\beta_3$ antibody (7E3) was a kind gift of Barry Coller (State University of NY at Stony Brook, Stony Brook, NY) and anti-$\alpha_5\beta_1$ (CP13) was obtained from Oncogene Sciences Inc. (Manhasset, NY).

Protein-coated Surfaces

Individual wells on glass microslides (Carlson Scientific, Peotone, IL) were coated with either Fg or fibronectin by adding 20 μl of a solution (250 μg/ml) in PBS containing calcium and magnesium to each well for either 1-4 h [for Fg] or 8 h [for fibronectin] in a humidified chamber at room temperature. The amount of protein adhering per well was assayed directly on the wells using the Micro BCA protein assay reagent kit (Pierce Chemical Co., Rockford, IL). Wells coated with either Fg or fibronectin contained ~3.0 and 3.5 μg per well, respectively. Glass coated with both Fg and fibronectin was prepared by adding 20 μl of a solution containing 125 μg/ml of Fg and 125 μg/ml fibronectin in PBS containing calcium and magnesium to each well for up to 8 h. Under these conditions, each well was coated with ~4 μg of protein per well. Protein-coated microslides were washed three times with PBS and used immediately for experiments.

Collection of Platelets

Plts were collected as described (Silverstein and Nachman, 1987) by gel filtration of Plt-rich plasma at 22-24°C through Sepharose 2B (Pharmacia Fine Chemicals, Piscataway, NJ), equilibrated with Hepes-buffered Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 3.3 mM Na_2PO_4, 5.5 mM glucose and 10 mM Hepes, pH 7.4). Fractions rich in Plts were pooled, diluted to ~1-2 × 10^8 Plts/ml with Tyrode's buffer containing 1 mg/ml human serum albumin and sufficient divalent cations to bring the final $CaCl_2$ and $MgCl_2$ concentrations to 1 and 0.5 mM, respectively. Plts were used for experiments within 60 min after isolation.

Platelet Adhesion and the Formation of Protected Zones of Adhesion

Thrombin-stimulated Plts were prepared by incubating resting Plts with 1 NIH unit/ml of thrombin (a gift from Dr. John Fenton; New York State Department of Health, Albany, NY) for 5 min at room temperature and then adding PPACK (Calbiochem Corp., La Jolla, CA; 10^{-5} M final concentration) to inactivate the thrombin. ADP-stimulated Plts were prepared by incubating Plts with 1 μM ADP for 5 min at room temperature. Stimulated Plts or untreated Plts were then allowed to adhere to protein-coated glass microslides at room temperature. After a 10-min incubation, the microslides were washed five times in Tyrode's buffer and fixed at room temperature for 15 min with 3.7% formaldehyde. The Plts were washed again with Tyrode's buffer and incubated with various fluorescein or rhodamine conjugated probes or antibodies at room temperature for 60 min. The preparation then was washed with Tyrode's buffer and immediately observed by phase and fluorescent microscopy at a magnification of 400. The number of adherent Plts per mm^2 was determined using phase microscopy; four random fields were counted in each of at least two separate wells. Photomicrographs were taken with either Kodak EktaKrome Professional color slide film (ASA 800/1600) or with Tri-Pan X black and white film.

Fluorescein- and Rhodamine-conjugated Probes

Fluorescein-conjugated F(ab')$_2$ fragments of goat anti-human Fg and fluorescein-conjugated F(ab')$_2$ fragments of goat anti-human fibronectin were purchased from Organon Teknika (Rockville, MD). Rhodamine-conjugated F(ab')$_2$ and Fab fragments of mouse mAb 4A5 (Matsueda et al., 1988), directed against an epitope at the carboxy terminus of the γ chain of human Fg, were prepared by purifying the antibody from ascites fluid using Affi-gel DEAE Blue (BioRad Labs, Richmond, CA) (Matsueda and Bernatowicz, 1988) and then digesting the antibody with papain (Parharm,

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1983) to yield Fab fragments. Thiol-free papain was used to generate the $F(ab')_2$ fragment of 4A5 (Goding, 1985). Rhodamine conjugates of these fragments were prepared as described (Harlow and Lane, 1988) to give rhodamine: fragment ratios of 0.89 and 1.24 for $F(ab')_2$ and Fab, respectively. Antibodies and their fragments were brought to a concentration of 1 mg/ml in PBS and then diluted 1:20 for staining protein-coated surfaces. Rhodamine-conjugated polyethylene glycol (PEG) was prepared from PEG of different mol wt (3.5, 5, 8, and 10 kD; from Sigma Chemical Co.). Solid PEG (0.02 mmol) was dissolved in 0.5 ml dimethylformamide. Solid rhodamine B (115 mg, 0.22 mmol), N-hydroxysuccinimide (25 mg, 0.22 mmol) and 4-dimethylaminopyridine (28 mg, 0.22 mmol) were added sequentially. Solid BOP reagent (97 mg, 0.22 mmol) (Advanced Chemtech Inc., Louisville, KY) was added to this viscous suspension at room temperature. After stirring for 20 h, 9.5 ml 0.1 M aqueous HCl was added and rapidly mixed into the resulting dark red viscous solution. The dark colored insoluble material which precipitated was separated by centrifugation at 1,000 rpm (500 *g*). Three ml of the supernatant was applied to a freshly prepared Bio-Gel P-2 (1.5 × 16.5 cm) column equilibrated with 0.1 M aqueous HCl. The product was eluted from the column with 0.1 M HCl. Visual observation indicated separation of rhodamine-conjugated PEG from unreacted rhodamine and its byproducts. The first set of red fractions (6–8 ml, total vol) contained the rhodamine-conjugated product. Rhodamine content of conjugates was estimated from absorbance at 560 nm based upon an observed extinction coefficient of 79,400 $M^{-1} cm^{-1}$ for rhodamine B in 0.1 M HCl. The rhodamine-conjugated products were stable in solution for at least 8 mo when stored at 4°C, as judged by SDS PAGE and gel filtration experiments.

Results

Stimulated Platelets Form Protected Zones of Adhesion when They Adhere to Fibrinogen-coated Surfaces

Freshly isolated human Plts were stimulated in suspension with either thrombin or ADP and allowed to adhere to Fg-coated surfaces as described in Materials and Methods. As shown in Fig. 1 (*A* and *B*), both ADP- and thrombin-stimulated Plts readily adhered to and spread on Fg-coated surfaces. Incubation of the Plt-containing wells with fluorescein-conjugated $F(ab')_2$ fragments of goat anti-human Fg, stained the Fg-coated surface except in areas directly underneath the thrombin-stimulated Plts (Fig. 1 *E*). A similar staining pattern was observed using rhodamine-conjugated $F(ab')_2$ fragments of monoclonal 4A5 anti-Fg antibody (Fig. 1 *F*). Because each of these conjugated antibodies recognizes a different epitope on Fg, the lack of staining underneath Plts is not a function of the antibody preparation used. The unstained area beneath thrombin- or ADP-stimulated Plts appeared as a black area on fluorescence microscopy. A dense spot of fluorescent staining was observed in the center of thrombin-stimulated Plts (Fig. 1 *E*). It is likely that these stained deposits on the Plt surfaces represent Fg that was secreted by the Plts onto their apical membranes after thrombin stimulation because: (*a*) this spot was generally not seen in Plts stimulated with concentrations of ADP that do not stimulate Plt degranulation (Kroll and Schafer, 1989, and unpublished data); and (*b*) these stained deposits were removed when thrombin-stimulated Plts were treated with detergent before the addition of anti-Fg antibodies (see below).

The absence of fluorescent $F(ab')_2$ staining underneath the ADP- or thrombin-stimulated Plts could result from exclusion of the probe from the Plt-Fg contact zone or from Fg degradation by the Plts, thereby rendering the extracellular Fg unreactive to anti-Fg antibodies. To differentiate between these two possibilities, thrombin-stimulated Plts were first allowed to adhere to Fg-coated surfaces for 15 min, fixed with formaldehyde, and then treated for an additional 10 min with a 0.1% SDS solution to permeabilize the plasma membranes of the Plts. The Plts were then washed with buffer. Under these conditions, fluorescein-conjugated $F(ab')_2$ fragments of goat anti-human Fg stained both underneath and around the Plts (not shown), indicating that the Fg underneath the Plts was intact and remained immunoreactive. In addition, no dense spot of fluorescent staining was observed after SDS treatment of thrombin-stimulated Plts (not shown), confirming that this stained material can be removed from Plt surfaces by detergent. These results suggest that the unstained areas (black in the color photographs) are created because these fluorescent probes do not gain access into the zones of close contact between Plts and the underlying Fg-coated surface. The shape of each unstained area corresponds in outline to the perimeter of a Plt and delineates a protected zone of adhesion beneath a single Plt.

We also examined the effects of temperature on the formation of protected zones of adhesion by Plts and of formaldehyde fixation on their appearance. As shown in Fig. 1, thrombin- (Fig. 1 *C*) and ADP- (Fig. 1 *G*) stimulated Plts formed protected zones of adhesion that excluded fluorescent probes in the absence of formaldehyde fixation. The protected zones of adhesion observed when Plts were first fixed and then incubated with $F(ab')_2$ were approximately the same size as those observed when Plts were fixed after incubation with $F(ab')_2$. Allowing ADP- or thrombin-stimulated Plts to adhere to Fg-coated surfaces at 37°C vs 22°C did not affect the size of protected zones of adhesion formed by Plts (not shown).

Stimulation of Plts increased the proportion of adherent Plts that formed protected zones of adhesion (Fig. 2). Greater than 95% of thrombin-stimulated, and 86% of ADP-stimulated Plts formed protected zones of adhesion. In contrast, only 26% of unstimulated Plts formed zones of contact between the Plts and Fg-coated surfaces that excluded fluorescein-conjugated $F(ab')_2$ anti-human Fg.

Platelet Integrin $\alpha_{IIb}\beta_3$ Is Required for the Formation of Protected Zones of Adhesion Between Platelets and Fibrinogen-coated Surfaces

Because binding of activated Plts to soluble or surface-bound Fg is primarily mediated by the integrin $\alpha_{IIb}\beta_3$ (Bennett et al., 1982), we evaluated the role of this integrin in the formation of protected zones of adhesion. Thrombin-stimulated Plts were incubated with 10 μg/ml of mAb 7E3 (directed against the Fg-binding site of integrin $\alpha_{IIb}\beta_3$ [Coller et al., 1983]) for 10 min at room temperature, and the mixture then was added to surfaces coated with Fg. Under these conditions (Fig. 1, *D* and *H*), <15% of the Plts adhered to the surface. Furthermore, <5% of the few Plts that did adhere formed protected zones of adhesion. Similar results were obtained using ADP-stimulated Plts (not shown). These results substantiate earlier observations (Peerschke, 1985) that the integrin $\alpha_{IIb}\beta_3$ is primarily responsible for Plt adhesion to Fg-coated surfaces, and indicate that this integrin plays an essential role in the formation of protected zones of adhesion by Plts adherent to Fg-coated surfaces.

Platelets Form Protected Zones of Adhesion when They Adhere to Fibronectin-coated Surfaces

Plts readily adhere to matrices containing fibronectin. Therefore, we examined whether untreated or stimulated Plts form protected zones of adhesion when they adhere to fibro-

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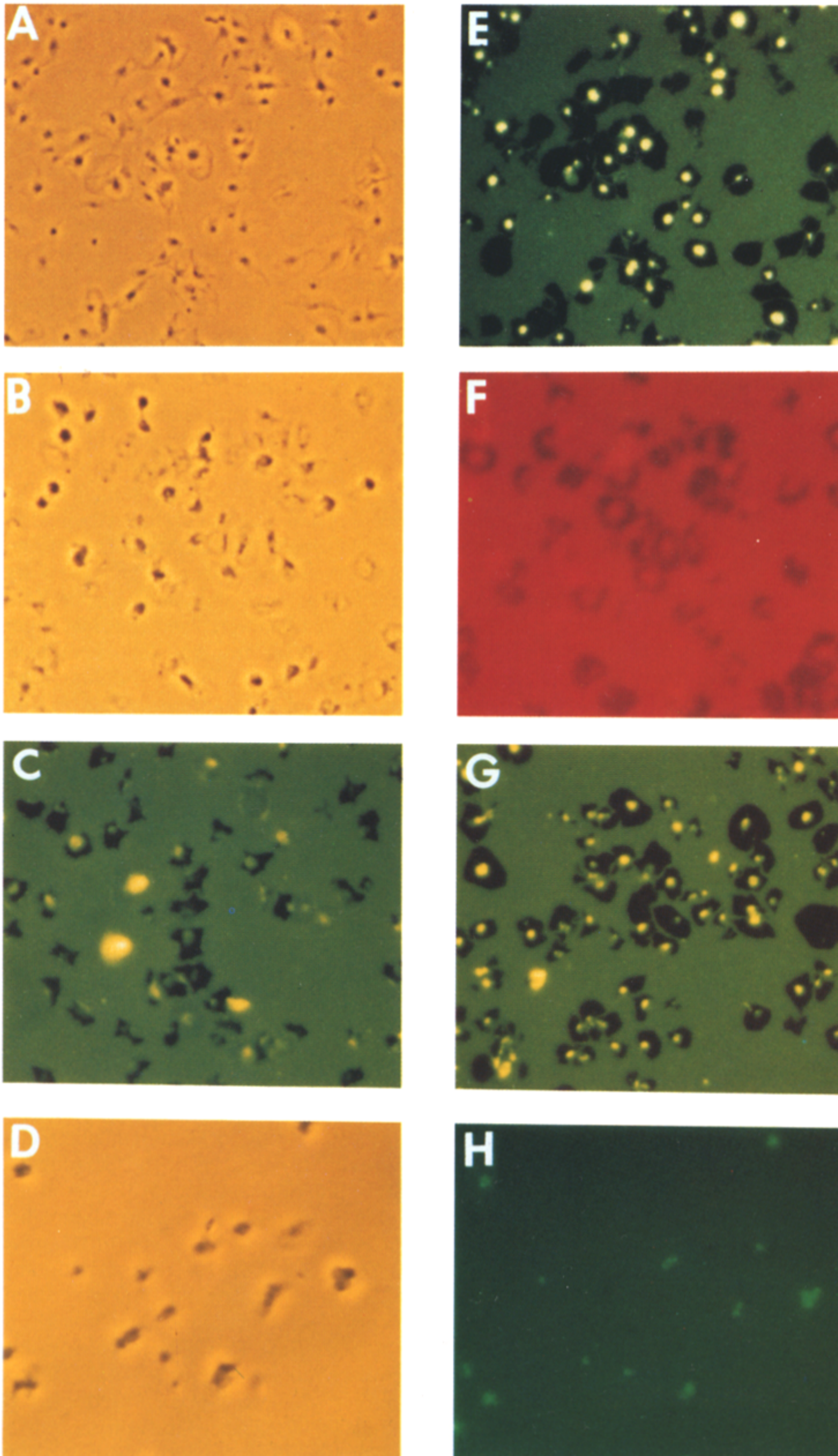

Figure 1. $F(ab')_2$ is excluded from zones of contact between stimulated Plts and Fg-coated surfaces: ADP- or thrombin-stimulated Plts were prepared and allowed to adhere to Fg-coated glass wells (∼3.0 μg/well) for 5 min as described under Materials and Methods. As indicated in the text, the fibrinogen on the surface of the wells was visualized by incubating the wells with either fluorescein or rhodamine-conjugated $F(ab')_2$ fragments of anti-human Fg and was observed under phase or fluorescence microscopy. *A* (phase) and *E* (fluorescence): adherent thrombin-stimulated Plts were fixed with formaldehyde and incubated with fluorescein-conjugated $F(ab')_2$ fragments of goat anti-human Fg for 30 min. *B* (phase) and *F* (fluorescence): adherent ADP-stimulated Plts were fixed with formaldehyde and incubated with rhodamine-conjugated $F(ab')_2$ fragments of 4A5 anti-human Fg for 30 min. *C* and *G*: adherent ADP-stimulated and thrombin-stimulated Plts, respectively, were first incubated with fluorescein-conjugated $F(ab')_2$ fragments of goat anti-human Fg for 30 min and then fixed with formaldehyde. *D* (phase) and *H* (fluorescence): thrombin-stimulated Plts that were incubated with monoclonal antibody 7E3 (10 μg/ml) for 15 min in suspension, allowed to adhere to Fg-coated surfaces, and then stained with fluorescein-conjugated $F(ab')_2$ fragments of goat anti-human Fg.

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nectin-coated surfaces. Plts were allowed to adhere to fibronectin-coated surfaces under conditions similar to those described above for Fg-coated surfaces. The capacity of Plts to exclude fluorescein-conjugated $F(ab')_2$ fragments of goat anti-human fibronectin from the Plt-fibronectin contact region was monitored by fluorescence microscopy. Untreated Plts (Fig. 3, *A* and *D*), ADP-stimulated Plts (not shown), and thrombin-stimulated Plts (Fig. 3, *B* and *E*) formed protected zones of adhesion as measured by the exclusion of fluorescein-conjugated $F(ab')_2$ fragments of goat anti-human fibronectin

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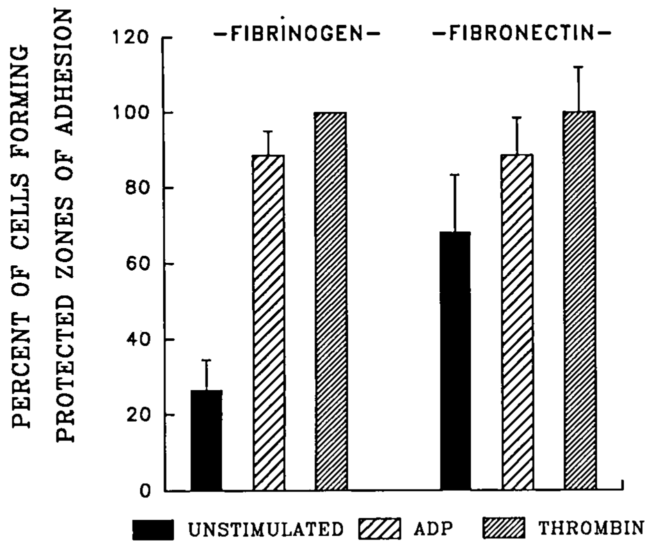

from beneath adherent Plts. Fixation of these preparations before or after incubation with anti-fibronectin F(ab')$_2$ did not significantly affect the appearance of the protected zones of adhesion formed by ADP- or thrombin-stimulated Plts (Fig. 3, *C* and *F*).

Although 85% of ADP-stimulated Plts and >99% of

Figure 2. Stimulation of Plts affects the percent of Plts that form protected zones of adhesion on protein-coated surfaces. Unstimulated, ADP- or thrombin-stimulated Plts were prepared and allowed to adhere to either Fg- or fibronectin-coated glass for 5 min as described under Materials and Methods. The Plts were fixed and the glass surfaces were incubated for 30 min with either fluorescein-conjugated F(ab')$_2$ fragments of goat anti-human Fg or fluorescein-conjugated F(ab')$_2$ fragments of goat anti-human fibronectin. The percent of Plts that formed protected zones of adhesion was determined as described under Materials and Methods by counting at least four fields from three separate wells. The data are the averages and SEM of three separate experiments.

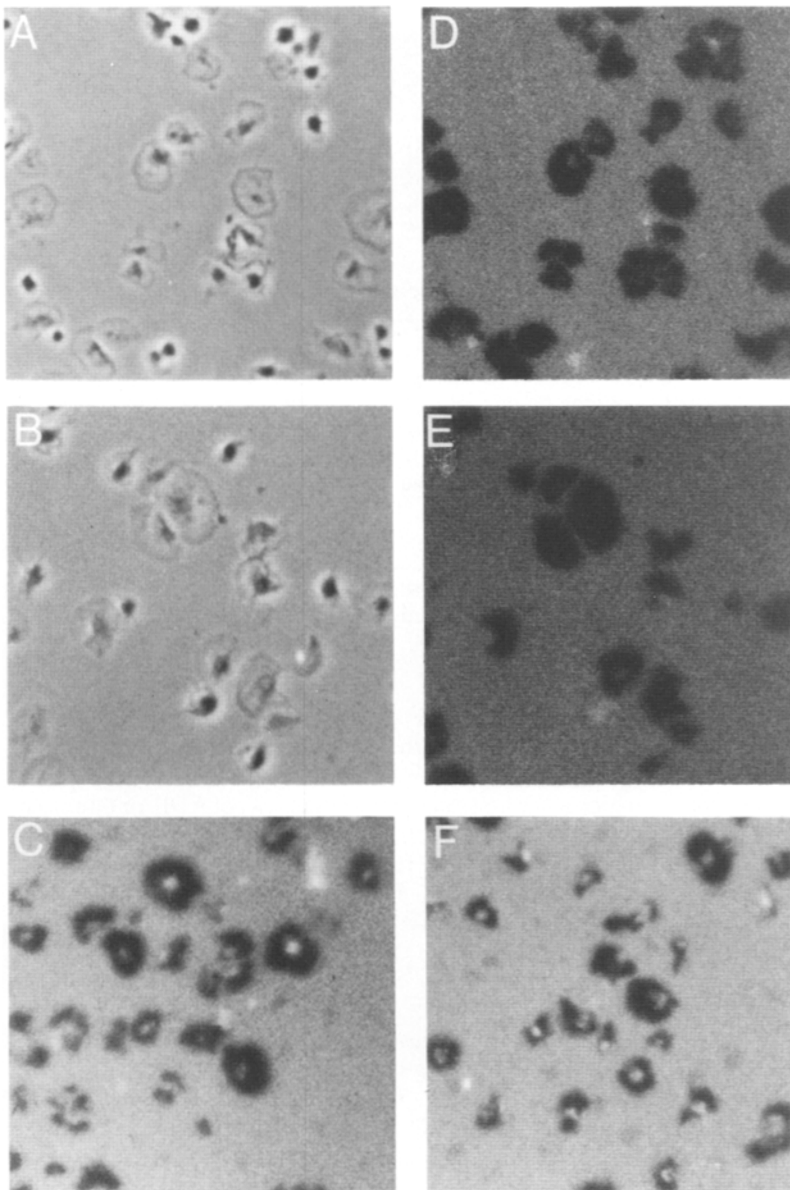

Figure 3. F(ab')$_2$ is excluded from zones of contact between stimulated Plts and fibronectin-coated surfaces: ADP- or thrombin-stimulated Plts were prepared and allowed to adhere to fibronectin-coated glass wells for 5 min as described under Materials and Methods. As indicated in the text, the fibronectin-coated surfaces (~3.5 μg/well) were visualized by incubating the wells with fluorescein-conjugated F(ab')$_2$ fragments of goat anti-human fibronectin and were observed under phase or fluorescence microscopy. *A* and *D* are phase and fluorescence micrographs, respectively, of ADP-stimulated Plts adherent to fibronectin-coated surfaces. *B* and *E* are phase and fluorescence micrographs of thrombin-stimulated Plts adherent to fibronectin-coated surfaces. *C* and *F* are fluorescence micrographs of ADP and thrombin-stimulated Plts that were allowed to adhere to fibronectin-coated surfaces, incubated for 30 min at 37°C with F(ab')$_2$ fragments of goat anti-human fibronectin, and then fixed with formaldehyde.

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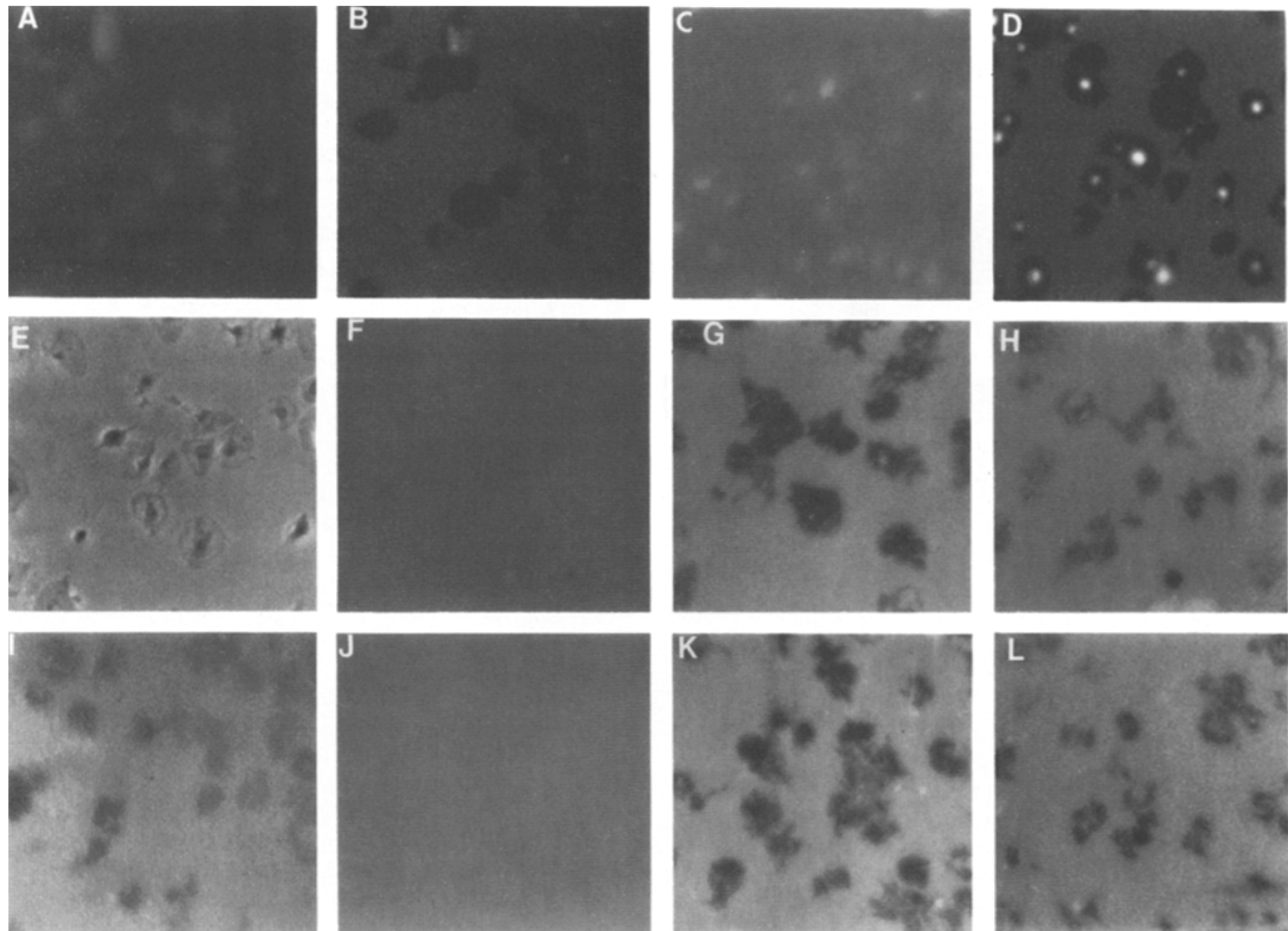

Figure 4. The entry of different sized molecules into the zones of contact between Plts and Fg- or fibronectin-coated surfaces. ADP- or thrombin-stimulated Plts were prepared and allowed to adhere to either Fg- or fibronectin-coated glass for 5 min as described under Materials and Methods. As indicated in the text, proteins adherent to the glass surfaces were visualized by incubating them with different fluorochrome-conjugated probes. (*A* and *B*) ADP-stimulated Plts on Fg-coated surfaces were incubated with fluorescein-conjugated Fab fragments of goat anti-human Fg and $F(ab')_2$ fragments of mAb 4A5, respectively. (*C* and *D*) thrombin-stimulated Plts adherent to Fg-coated surfaces were incubated with fluorescein-conjugated Fab fragments of goat anti-human Fg and $F(ab')_2$ fragments of 4A5, respectively. *E* and *F* show phase and fluorescence micrographs, respectively, of thrombin-stimulated Plts adhering to Fg-coated surfaces and incubated with rhodamine-conjugated PEG of 10 kD. *G–J* show ADP-stimulated Plts adhering to fibronectin-coated surfaces and incubated with rhodamine-conjugated PEG of 10, 8, 5, and 3.5 kD, respectively. *K* and *L* show thrombin-stimulated Plts adherent to surfaces coated with a mixture of Fg and fibronectin and incubated with rhodamine-conjugated PEG of 10 and 8 kD, respectively.

thrombin-stimulated Plts formed protected zones of adhesion on fibronectin-coated surfaces (Fig. 2), ∼70% of unstimulated Plts formed protected zones of adhesion on fibronectin-coated surfaces. Thus, prior activation of Plts is not essential for the formation of protected zones of adhesion on fibronectin-coated surfaces. This was in marked contrast to the results obtained with Fg-coated surfaces on which only 26% of adherent unstimulated Plts formed protected zones of adhesion (Fig. 2).

Permeability of Macromolecules into Protected Zones of Adhesion Formed by Platelets

As shown in Figs. 1 and 3, molecules of 100 kD (i.e., fluorescein-conjugated $F(ab')_2$ fragments of goat anti-human antibodies), were excluded from the zones of contact formed between stimulated Plts and either Fg- or fibronectin-coated surfaces. To examine the permeability properties of these protected zones of adhesion in greater detail we determine whether molecules of <100 kD could enter into these contact regions. We first examined the capacity of ADP- or thrombin-stimulated Plts to exclude Fab fragments (50 kD) of anti-Fg antibody (4A5) from regions between adherent Plts and the underlying substrate. Fab fragments of 4A5 readily penetrated into the contact zones between ADP (Fig. 4 *A*), or thrombin (Fig. 4 *C*), treated Plts and Fg-coated surfaces. Parallel experiments confirmed that $F(ab')_2$- fragments of goat anti-human Fg or of 4A5 were excluded from the protected zones of adhesion formed by thrombin- or ADP-stimulated Plts on Fg-coated surfaces (see Fig. 1, *B* and *D* and data not shown). Thus, molecules of 50 kD permeated into protected zones of adhesion formed by Plts on Fg-coated surfaces while molecules of 100 kD did not permeate into these zones. The observation that Fab fragments of 4A5

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stained the Fg located beneath the Plts confirm the data reported above (with SDS-treated Plts) that the Fg in these regions remains immunoreactive.

To investigate the permeability properties of protected zones of adhesion formed between Plts and fibronectin-coated surfaces, we conjugated rhodamine to PEG polymers of molecular weights varying between 3.5 kD and 10 kD. The rhodamine conjugated PEG probes chromatographed on SDS PAGE at ∼2 to 2 1/2 times the nominal molecular weight of the PEGs from which they were synthesized. For example, the rhodamine conjugate of 10 kD PEG migrated in SDS PAGE like a globular protein of ∼20–25 kD (not shown). Rhodamine-conjugated PEG probes are especially useful for these experiments because they bind to both Fg- and fibronectin-coated surfaces but do not bind to uncoated glass or to Plt membranes (not shown).

All of the rhodamine-conjugated PEGs readily penetrated underneath thrombin- or ADP-stimulated Plts adherent to Fg-coated surfaces (Fig. 4, *E* and *F*, and data not shown). In contrast, rhodamine-conjugated PEG of 10 and 8 kD were excluded from the protected zones of adhesion formed by either ADP- or thrombin-stimulated Plts on fibronectin-coated surfaces (Fig. 4, *G–I*, and data not shown). Rhodamine-conjugated PEG of 3.5 kD readily permeated into the contact zones between Plts and fibronectin-coated surfaces (Fig. 4 *J*). Subtle differences were noted in the permeation of 10-, 8-, and 5-kD probes into the protected zones of adhesion formed by these Plts. The protected zones of adhesion defined by the 8- or 5-kD PEG polymers were smaller and their borders were less crisply demarcated than those observed when a 10-kD rhodamine-conjugated PEG probe was used (compare *G–I* in Fig. 4). We believe this reflects partial penetration of 8 and 5 kD rhodamine-conjugated PEG into the contact regions between the Plts and the underlying substrate. These results show that Plts generate a much tighter or closer association with the substrate when they adhere to fibronectin-coated surfaces than when they adhere to Fg-coated surfaces; they imply that the composition of the matrix determines the tightness of the seal formed between Plts and protein-coated surfaces.

Fixation did not affect the permeability properties of protected zones of adhesion formed on fibronectin-coated surfaces. Thrombin-stimulated Plts adherent to fibronectin-coated surfaces were first incubated with rhodamine-conjugated PEG of 10 kD and then fixed with formaldehyde. These Plts excluded this probe from their zones of contact with protein-coated surfaces as efficiently as Plts fixed before incubation with this probe (not shown).

Protected Zones of Adhesion Formed by Platelets on Surfaces Coated with Both Fibrinogen and Fibronectin

A solution of equal amounts (by weight) of Fg and fibronectin was used to coat glass surfaces. The presence of each protein on the surface was detected by staining the surface with the corresponding antibody. ADP- or thrombin-stimulated Plts formed protected zones of adhesion on Fg/fibronectin-coated surfaces as measured by their capacity to exclude fluorescein-conjugated $F(ab')_2$ fragments of goat anti-human Fg antibodies from zones of contact between the Plts and the substrate. The permeability of these protected zones of adhesion was identical to that observed when stimulated Plts were plated on surfaces coated with fibronectin alone; i.e., the Plts excluded rhodamine-conjugated PEG polymers of 10 and 8 kD (Fig. 4, *K* and *L*), but were permeable to rhodamine-conjugated PEG polymers of 3.5 kD (not shown). Surfaces were also coated with Fg/fibronectin solutions in which the Fg concentration was maintained at 125 μg/ml, but the fibronectin concentration was reduced by fivefold to 25 μg/ml. Even under these conditions, the permeability of protected zones of adhesion formed by thrombin-stimulated Plts was similar to that observed on surfaces coated with a solution of 250 μg/ml of fibronectin alone (not shown). These results indicate that fibronectin exerts a dominant effect when Plts adhere to surfaces coated with both Fg and fibronectin; they suggest that the permeability characteristics of protected zones of adhesion formed by Plts on substrates containing two different ligands will reflect those of the ligand that promotes formation of the tightest zones of contact.

We examined the relative contribution of $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_1$ integrins to the formation of protected zones of adhesion between Plts and surfaces coated with both Fg and fibronectin. Plts first were stimulated with thrombin for 5 min, treated with PPACK (to inactivate the thrombin), incubated in suspension for 1 h in the absence (Fig. 5, *A* and *B*), or presence (Fig. 5, *C* and *D*), of 5 μg/ml of anti-$\alpha_{IIb}\beta_3$ antibody (7E3), and then allowed to adhere to surfaces coated with both Fg and fibronectin. mAb 7E3 reduced the number of adherent Plt by at least 80%. Both anti-Fg $F(ab')_2$ and 10 kD PEG permeated into the region between the Fg/fibronectin-coated substrate and the 20% of Plts that did adhere (Fig. 5, *A–D*). mAb 7E3 inhibited thrombin-stimulated Plt adhesion and the formation of protected zones of adhesion on surfaces coated with Fg alone or with fibronectin alone (Fig. 1, and data not shown). These results indicate that this antibody prevents the formation of protected zones of adhesion between stimulated Plts and either Fg- or fibronectin-coated surfaces.

In contrast, an antibody against $\alpha_5\beta_1$ (CP13) (5 μg/ml), reduced thrombin-stimulated Plt adhesion to surfaces coated with Fg and fibronectin or with fibronectin alone by ∼30% (not shown). Greater inhibition of adhesion by this anti-fibronectin receptor antibody was not observed because the Plts can still attach to fibronectin via the $\alpha_{IIb}\beta_3$ integrin (Philips et al., 1991). However, the permeability of the protected zones of adhesion formed between thrombin-stimulated Plts and surfaces coated with Fg and fibronectin or with fibronectin alone was markedly altered in the presence of CP13 (Fig. 5, *E* and *F*, and data not shown). Rhodamine-conjugated 10 kD PEG penetrated the contact regions between CP13-treated Plts and the underlying surfaces coated with Fg and fibronectin or with fibronectin alone (Fig. 5 *F*), whereas $F(ab')_2$ anti-Fg was excluded (Fig. 5 *E*). These results show that formation of a protected zone of adhesion that excludes low molecular weight probes (rhodamine-conjugated PEG) requires the participation of both $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_1$ receptors.

Formation of Protected Zones of Adhesion Between Platelets and Surfaces Coated with Fibrin

Thrombin- and ADP-stimulated Plts formed protected zones of adhesion on fibrin-coated surfaces (Fig. 6, *A* and *C*

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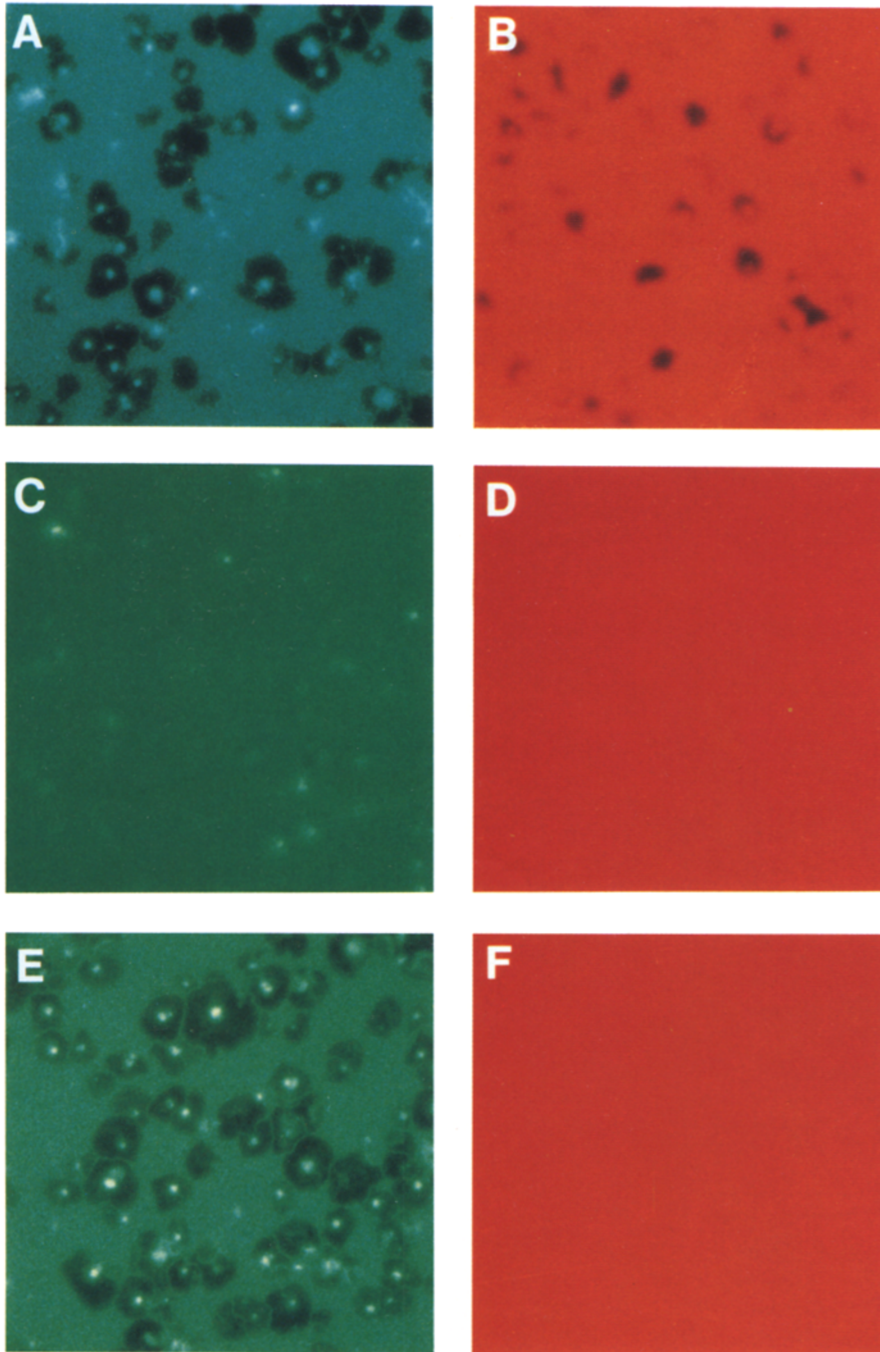

Figure 5. Effects of anti-$\alpha_{IIb}\beta_3$ and anti-$\alpha_5\beta_1$ on the formation of protected zones of adhesion between Plts and surfaces coated with Fg and fibronectin. Plts were incubated in suspension for 5 min in the presence of thrombin, and further incubated in Tyrode's buffer containing PPACK in the presence or absence of 5 μg/ml of the specified antibody for 1 h. The Plts were then allowed to adhere for 5 min at 37°C to surfaces coated with both Fg and fibronectin as described under Materials and Methods. *A* and *B* show thrombin-stimulated Plts preincubated in suspension for 1 h at 37°C with no additions and allowed to adhere to surfaces coated with Fg and fibronectin. The surfaces with the attached Plts then were stained with fluorescein-conjugated F(ab')$_2$ fragments of goat anti-human Fg and rhodamine-conjugated PEG of 10 kD, respectively; *C* and *D* show thrombin-stimulated Plts preincubated with mAb 7E3 (5 μg/ml) in suspension and then allowed to adhere to surfaces coated with Fg and fibronectin. The surfaces with the attached Plts were then stained with fluorescein-conjugated F(ab')$_2$ fragments of goat anti-human Fg (*C*) or with rhodamine-conjugated PEG of 10 kD (*D*); *E* and *F* show thrombin-stimulated Plts preincubated with mAb anti-$\alpha_5\beta_1$ (10 μg/ml) and allowed to adhere to surfaces coated with Fg and fibronectin. The surfaces with the attached Plts were stained with fluorescein-conjugated F(ab')$_2$ fragments of goat anti-human Fg (*E*) or with rhodamine-conjugated PEG of 10 kD. (*F*).

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evidenced by exclusion of fluorescein-conjugated F(ab')$_2$ anti-human Fg from the zones of contact between the Plts and these surfaces. (The fluorescein-conjugated F(ab')$_2$ anti-human Fg used in these experiments stained fibrin-coated surfaces.) In contrast, rhodamine-conjugated PEG of 10 kD and Fab fragments of mAb 4A5 penetrated into these adhesive zones (Fig. 6, *B* and *D*, and data not shown), as observed when thrombin-stimulated Plts adhered to Fg-coated surfaces (Fig. 4, *E* and *F*). Thus, protected zones of adhesion formed between Plts and fibrin-coated surfaces and between Plts and Fg-coated surfaces exhibited similar permeability properties.

Discussion

ADP- and thrombin-stimulated Plts form "seals" with Fg-, fibrin-, and fibronectin-coated surfaces. These seals delineate protected zones of adhesion between Plts and protein-coated surfaces and prevent molecules in the surrounding medium from entering the cell-substrate interface. Stimulation of Plts with thrombin or with low concentrations of ADP significantly enhances their capacity to form protected zones of adhesion on Fg-coated surfaces. Because low dose ADP does not stimulate granule secretion (Kroll and Schafer, 1989; Loike, Silverstein and Silverstein, unpublished data),

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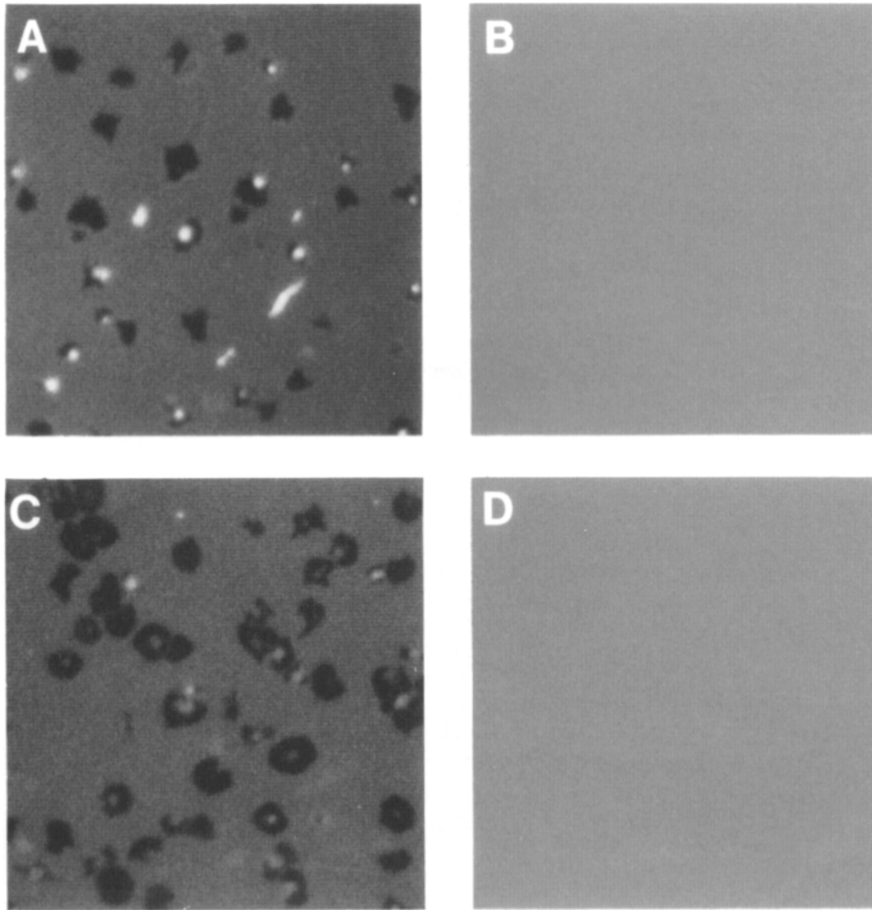

Figure 6. The entry of different sized molecules into the zones of contact between thrombin stimulated Plts and fibrin-coated surfaces. Fibrin-coated surfaces were prepared by adding 20 μl of a solution of Fg (250 μg/ml) to glass surfaces pretreated with 2 μl of a solution of thrombin (1,000 U/ml). Thrombin- (*A* and *B*) or ADP- (*C* and *D*) stimulated Plts were prepared and allowed to adhere to these protein coated surfaces for 5 min as described under Materials and Methods. As indicated in the text, the proteins on the surfaces were visualized by incubating them with different fluorochrome conjugated probes. (*A*) Preparations containing thrombin-stimulated Plts stained with fluorescein-conjugated goat $F(ab')_2$ anti-human Fg. (*B*) Preparations containing thrombin-stimulated Plts stained with rhodamine-conjugated PEG of 10 kD. (*C*) Preparations containing ADP-stimulated Plts stained with fluorescein-conjugated goat $F(ab')_2$ anti-human Fg. (*D*) Preparations containing ADP-stimulated Plts stained with rhodamine-conjugated Fab fragment of 4A5.

it seems likely that the formation of protected zones of adhesion does not require the release of secretory products from Plt granules.

The permeability properties of these protected zones of adhesion depend upon the nature of the protein (ligand) coating the surface (Table I). Stimulated Plts that spread on either Fg- or fibrin-coated surfaces exclude molecules of 100 kD (such as $F(ab')_2$) from the area between adherent Plts and the substrate, whereas molecules of 50 kD (such as Fab) readily permeate into these contact zones. In contrast, Plts adherent to fibronectin-coated surfaces or to surfaces coated with a mixture of Fg and fibronectin exclude molecules greater than 8–10 kD. Thus, protected zones of adhesion formed between Plts and fibronectin-coated surfaces are less permeable than those formed between Plts and Fg- or fibrin-coated surfaces.

The interaction of Plts with matrices other than Fg also is

*Table I. Summary of Permeabilities of Protected Zones of Adhesion (PZA)**

Matrix	Antibody	Size molecule excluded from PZA	Percent of cells‡ forming PZA that excludes >100 kD	>10 kD
Fg	none	100 kD	>95	<5
	anti-$\alpha_{IIb}\beta_3$	no PZA	0	0
	anti-$\alpha_5\beta_1$	100 kD	>95	<5
Fibronectin	none	8–10 kD	>95	70
	anti-$\alpha_{IIb}\beta_3$	no PZA	0	0
	anti-$\alpha_5\beta_1$	100 kD	82	<5
Fg/fibronectin	none	8–10 kD	>95	75
	anti-$\alpha_{IIb}\beta_3$	no PZA	0	0
	anti-$\alpha_5\beta_1$	100 kD	77	<5

* Thrombin-stimulated Plts were preincubated in suspension in the presence or absence of 5 μg/ml of either mAb 7E3 (anti-$\alpha_{IIb}\beta_3$) or CP13 (anti-$\alpha_5\beta_1$) for 60 min at room temperature. The Plts were then allowed to adhere to the surfaces coated with the indicated proteins as described under Materials and Methods and in Fig. 5. Protected zones of adhesion were determined as described under Materials and Methods. Protein-coated wells containing adherent Plts were stained with either fluorescein-conjugated $F(ab')_2$ fragments of goat anti-human Fg, fluorescein-conjugated $F(ab')_2$ fragments of goat anti-human fibronectin, rhodamine-conjugated 10 kD PEG or rhodamine-conjugated 8 kD PEG as described under Materials and Methods. PZA = protected zones of adhesion.

‡ The percent of Plts forming PZA was determined by counting the number of Plts that excluded each probe. Values represent the average (n = 5) percent of Plts that formed PZA that excluded either 100 kD ($F(ab')_2$ or 10 kD (rhodamine-conjugated PEG) probes.

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affected by the presence of fibronectin in the matrix. For example, Hynes et al. (1978) reported that in the presence of fibronectin, unstimulated Plts adhered, spread and clumped together on collagen-coated surfaces, whereas in the absence of fibronectin unstimulated Plts adhered poorly and did not spread or aggregate on these surfaces.

We have explored the roles of $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_1$ integrins in the formation of protected zones of adhesion by Plts. mAb 7E3, which is directed against the ligand-binding domain of $\alpha_{IIb}\beta_3$, prevents Plts from forming protected zones of adhesion on Fg and fibronectin-coated surfaces, indicating that $\alpha_{IIb}\beta_3$ mediates this interaction. However, the engagement of $\alpha_5\beta_1$ integrins in addition to $\alpha_{IIb}\beta_3$ was required for stimulated Plts to form very tight (i.e., excludes 8 kD PEG) protected zones of adhesion on surfaces containing fibronectin (Table I). Additional work is required to determine whether formation of these very tight zones of adhesion requires only the physical interactions of $\alpha_5\beta_1$ integrins with fibronectin on the substrate or whether the engagement of $\alpha_5\beta_1$ integrin by fibronectin further activates the Plt cytoskeleton or modulates the adhesive properties of $\alpha_{IIb}\beta_3$. That the interaction of fibronectin receptors with substrate bound fibronectin can activate other integrins on the same cell has been reported by Wright et al. (1985) who showed that ligation of fibronectin receptors activates CD11b/CD18 on human monocyte-derived macrophages. Such activation is required for CD11b/CD18 to promote phagocytosis of C3bi coated particles.

Plts are not the only cells that form protected zones of adhesion. We and others (Campbell et al., 1982; Wright and Silverstein, 1984; Baron et al., 1985; Sandhaus, 1987; Weitz et al., 1987; Wright et al., 1988; Heiple et al., 1990; and Rice and Weiss, 1990) have shown that phagocytic leukocytes and osteoclasts form seals with substrates coated with a variety of proteins including Fg, fibrin, and fibronectin. Formation of these seals is mediated by a number of plasma membrane receptors including $\beta2$ integrins (Wright and Silverstein, 1984; Wright et al., 1988; and Loike et al., manuscript in preparation), $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_1$ integrins (current manuscript), and Fc receptors (Heiple et al., 1990; Wright and Silverstein, 1984).

Protected zones of adhesion formed by Plts may have significant physiological consequences in at least two situations: (*a*) Protected zones of adhesion could provide a confined space into which Plt secretory products (e.g., $\alpha2$ antiplasmin, or plasminogen activator inhibitor) are released and retained when Plts are stimulated to adhere to extracellular matrices. The extent to which these proteins diffuse out of these zones of adhesion will depend upon the permeability properties of the zones of adhesion. As we have shown, the permeability of protected zones of adhesion is determined in part by the nature of the protein on the surfaces to which cells adhere. (*b*) Protected zones of adhesion may restrict access of plasma proteins and therapeutic agents to at least three regions in a thrombus. First is the region between Plts and the subendothelial matrix, which contains collagen, laminin, von Willebrand factor and fibronectin (Sonnenberg et al., 1988). Our present results suggest that protected zones of adhesion formed by Plts in the regions of a thrombus that are rich in fibronectin, such as the subendothelial matrix, will be vastly less permeable to substances in plasma than the regions in which Plts interact with one another or with Fg/fibrin gels. Consequently, thrombolytic agents and plasma proteases (e.g., plasminogen/plasmin) of molecular weights between 50 and 100 kD may be excluded from the zones of adhesion formed between Plts and the subendothelial matrix. Second is the region between adjacent Plts in the Plt-rich aggregate termed the "white thrombus." Plts in this region bind to one another via Fg bridges. Third is the region between Plts and the Fg/fibrin meshwork that forms the outer region of a thrombus. If fibronectin (440 kD) does not penetrate into these latter two regions then we expect that the protected zones of adhesion formed would be permeable to molecules <50 kD but impermeable to molecules >100 kD. Because protected zones of adhesion may regulate the entry of proteases into zones of contact of Plts with one another, or with basement membranes, the permeability properties of these zones of adhesion deserve consideration in the design of thrombolytic agents.

This investigation was supported by grants from the National Institutes of Health DK39110 (first award to J. D. Loike), AI20516 (S. C. Silverstein), HL42540 and HL46403 (R. L. Silverstein), HL18828-Cornell National Institutes of Health Specialized Center of Research (SCOR) in thrombosis (R. L. Silverstein), Cystic Fibrosis Foundation Research Development Program at Columbia University, Columbia University National Institutes of Health Specialized Center of Research (SCOR) in atherosclerosis (S. C. Silverstein), a generous gift from from Samuel W. Rover, the Medical Research Council of Canada, and the Heart and Stroke Foundation of Ontario. J. I. Weitz is a Career Investigator of the Heart and Stroke Foundation of Ontario.

Received for publication 19 June 1992 and in revised form 23 February 1993.

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