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Involvement of Fibronectin, Von Willebrand Factor, and Fibrinogen in Platelet Interaction With Solid Substrata

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The proteins fibronectin (FN), Von Willebrand factor (VWF), and fibrinogen are believed to play a role in platelet function. They are distributed between the plasma and the platelet pool in the resting state and undergo redistribution upon platelet activation. We have studied their expression on the surface of the platelet and their mobilization following platelet binding to substrata. For the purpose of studying protein expression on the surface of intact platelets either adherent to a substratum or in suspension, the enzyme-linked immunosorbent assay (ELISA) was elaborated and modified. Using this technique as well as immunofluorescence, we found that antiserum raised against carefully washed human platelets recognized FN, VWF, and fibrinogen as well as platelet surfaces. However, specific antisera against these three proteins failed to bind to the surface of unactivated gel-filtered platelets. When gel-filtered platelets were exposed to plastic or fibrillar collagen, they adhered and spread. Such platelets did bind antibodies against FN, VWF, and fibrinogen, Moreover, when the adherent platelets were incubated with FN or with VWF in the absence of ristocetin, they bound these proteins in a concentration-dependent fashion. The patterns of the bound proteins were not similar, suggesting a different spatial distribution of binding sites. These findings indicate that platelet activation by adhesion to substrata mobilize both endogenous and exogenous pools of these proteins, thereby making them surface associated and probable participants in further binding properties of the activated platelet.

Key words: platelet, fibronectin, Von Willebrand factor, fibrinogen, cell adhesion, ELISA, extracellular matrix

While it is established that fibronectin (FN) [1,2], FVIII-VWF [3], and fibrinogen [4] are present in the blood platelet, it is generally maintained that the surface of the unactivated washed human platelet is free of all three proteins [5-8] though there are some reports to the contrary at least in the case of fibrinogen [9]. Thrombin activation of the washed platelet causes release of its stored FN [2,5] fibrinogen [7], and FVIII-VWF [10,11] to the medium and expression of fibronectin antigen on the platelet surface [5]. Binding of exogenous proteins to the surface of the thrombin activated platelet was shown to be Ca^{++}-dependent in the case

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of fibrinogen [12] and ristocetin-dependent in the case of human FVIII-VWF [13]. FN also binds to the membrane of thrombin-activated platelets [14].

The effect of platelet adhesion to collagen or to surfaces on the interaction of these three proteins with the platelet membrane has been less well studied. It has been shown, however, that FN [2], fibrinogen [15], and FVIII-VWF [10, 11] are released as a result of collagen stimulation. In this study we investigated the effect of surfaces and collagen adhesion of gel-filtered platelets on the expression of FN, FVIII-VWF, and fibrinogen on the cell surface, as well as on binding of these proteins added exogenously. For this purpose the sensitive technique of enzyme-linked immunosorbent assay (ELISA), developed for proteins [16], was modified to be used with whole cells, either in suspension or absorbed to a solid substratum.

MATERIALS AND METHODS

Platelet Preparation

Fresh human blood was obtained from healthy volunteers, anticoagulated with ACD 1:9 and processed within 1 h of withdrawal. The platelets were separated from other blood cells and proteins by differential centrifugation and gel filtration through Sepharose 2B (Pharmacia Fine Chemicals). The column was prewashed with modified Tyrode's buffer (NaCl 140 mM, KCl 2.7 mM, NaH_2PO_4 0.32 mM, $NaHCO_3$ 12 mM, glucose 0.1%, EGTA 1 mM, pH 7.2), and the platelets were eluted in the same buffer, Platelet concentration was determined by counting in the haemocytometer.

Antibodies

Antihuman plasma fibronectin antiserum (αpFN) was raised in rabbits as described [17]. Antiplatelet antiserum (αplt) was raised in rabbits by injecting 1 ml packed washed cells that had been prepared as follows: Platelet-rich plasma obtained by differential centrifugation was centrifuged at 1,100g for 15 min, and the platelets were resuspended in modified Tyrode's buffer. This procedure was repeated three times. The cells were injected in complete Freund's adjuvant. The rabbit was boosted after 6 weeks and again after 6 more weeks. Antihuman FVIII-VWF (αVIII) was obtained from Calbiochem-Behring Corp., California, and antihuman fibrinogen (αfib) and fluorescein-conjugated goat antirabbit IgG, $F(ab')_2$ fragment, from Cappel Labs, Pennsylvania. Antivimentin antiserum (α58K) was raised in rabbits as described earlier [18].

Monospecificity of Antibodies

This was tested by conventional ELISA [16] where 1 ml of 5 μg/ml solutions of plasma FN (pFN) prepared as described [19], 97% clottable human fibrinogen (a kind gift from Dr. D. Waugh, MIT) or a crude preparation of FVIII-VWF ("Profilate"-antihemophilic factor, Alpha Therapeutic Corp., Calif), were incubated in polystyrene tubes overnight at 4°C, the tubes washed, and the antibody of interest introduced for 3–6 h at room temperature. A second antibody conjugated to alkaline phosphatase was then introduced for 3–6 h; p-nitro-phenyl-phosphate was used as a substrate, and the color intensity developed was read at 400 nm. Ouchterlony and immunoelectrophoresis were also used to verify the results. It was found that αpFN

gives a single line in Ouchterlony against pure pFN, plasma, and crude FVIII-VWF ("Profilate"). In ELISA it recognized pure pFN and Profilate but not pure fibrinogen. Antifibrinogen gave a single band in immunoelectrophoresis against plasma but not against serum. It recognized pure fibrinogen, but not pFN in ELISA. Profilate also interacted strongly with αfib in ELISA.

Anti-FVIII-VWF gave a single line against Profilate in Ouchterlony. In ELISA it recognized Profilate but not pure pFN nor pure fibrinogen. This confirmed the purity of the pFN and fibrinogen preparations and the monospecificity of their respective antisera. Anti-FVIII-VWF antiserum was also monospecific. The commercial preparation of FVIII-VWF, however, was contaminated with fibrinogen and with fibronectin. Gel electrophoresis of this preparation showed bands at the molecular weights corresponding to these proteins.

Enzyme-Linked Immunosorbent Assay (ELISA)

Detection of specific antigens on the surface of the gel filtered platelets was done using a modification of the ELISA method. Platelet suspension, 0.3 ml, at 10^7 cells/ml was incubated at the bottom of polystyrene tubes (Falcon) for 45 min at room temperature. The unbound platelets were removed, and the tube with the bound platelets was washed three times with modified Tyrode's buffer. The bound platelets were lightly fixed with 3.7% formaldehyde for 10 min, the formaldehyde quenched with 0.1 M tris buffer, and the tubes washed with phosphate buffer. When appropriate, at this stage the tubes were incubated with exogenous protein in phosphate buffer containing 1% ovalbumin (Sigma) and 0.05% Tween 20 (Schwartz Mann) overnight at 4°C. The tubes were then washed three times and incubated 3–6 h at room temperature with the first (rabbit) antibody, in 1% ovalbumin, 100 μg/ml goat IgG and 0.05% Tween 20 when appropriate. After washing the first antibody away, a goat antirabbit IgG, conjugated with alkaline phosphatase by the method of Engvall and Perlmann [16], was incubated in the tubes for 3–6 h at room temperature, and unbound antibody was removed by washing. Finally, p-nitrophenyl-phosphate (Sigma) was introduced for 1 h, the color development was stopped with 0.1 M NaOH (final concentration), and the intensity was read at 400 nm. In all experiments, tubes without platelets were run in parallel as controls. A high titer antiserum against vimentin (58K protein of fibroblasts) or nonimmune rabbit serum (NRS) was used as control for nonspecific antibody binding. When ELISA was performed on unactivated gel-filtered platelets, 1 ml suspension of 10^7 cells/ml was fixed for 10 min by addition of 100 μl 37% formaldehyde to the suspension shortly after gel filtration. The formaldehyde was quenched with 0.1 M Tris buffer, and the fixed platelets were washed three times in phosphate buffer containing 1% ovalbumin, 100 μg/ml goat IgG, and 0.05% Tween 20 when appropriate. All subsequent steps were similar to those used with adherent platelets, the only difference being that the platelets were in suspension in Eppendorf tubes and centrifuged for washing.

We found that in the system gel-filtered platelets–dilute antisera, the presence of 0.05% Tween 20 gave comparable results to those obtained in the presence of carrier protein and excess IgG. (Both were significantly lower than those obtained with buffer alone.) However, when plasma proteins were present in significant amounts, carrier protein was not as effective in reducing nonspecific adhesion to

substrata as was 0.05% Tween 20. Treatment of spread and fixed platelets with buffer, 0.05% Tween 20 and 1% Triton X-100 were compared for the exposure of binding sites for antiactin antibody. It was found that while permeabilization with 1% Triton X-100 increased antiactin binding significantly, platelets treated with 0.05% Tween 20 bound only background levels of antibody as did platelets not treated with any detergent. 0.05% Tween 20 was therefore used in all experiments where ELISA was used for detection of binding of exogenous antigens to platelet surfaces.

Immunofluorescence

Glass coverslips were incubated with soluble rat tail collagen (a generous gift from Dr. Linsenmayer, Massachusetts General Hospital) in 0.5 M acetic acid overnight at 4°C under NH_3 atmosphere. This treatment lead to fibrillogenesis, and, by phase microscopy, collagen fibers could be clearly seen adherent to the glass even after extensive washing. The coverslips were then washed thoroughly in modified Tyrode's buffer and 20 μl gel-filtered platelet suspension was layered over them and incubated 45 min at room temperature in a humid atmosphere. After thorough washing, the bound platelets were fixed for 10 min with 3.7% formaldehyde, washed and incubated with the first antibody (raised in rabbit) for 30 min at 37°C. The second antibody, also incubated for 30 min at 37°C with the washed coverslip, was fluorescein conjugated $F(ab)_2$ fragment of goat antirabbit IgG. Visualization and photography of the fluorescent samples were done on a Zeiss photomicroscope III.

RESULTS

Fibronectin (FN), Fibrinogen, and FVIII-VWF Antigens in the Blood Platelet

Antiplatelet antiserum, obtained with carefully washed platelets and studied by conventional ELISA, recognized pure pFN and fibrinogen and a crude preparation of FVIII-VWF (Fig. 1). These results imply the presence of FN, fibrinogen, and FVIII-VWF (with reservations concerning FVIII-VWF due to contamination of this preparation as discussed in Methods) within the blood platelet, confirming earlier reports [1–4]. This finding does not, however, distinguish between surface-bound and internally localized antigens, and this procedure cannot detect redistribution of the antigen due to cell activation.

To address the question of expression of these antigens on the platelet surface before and after spreading on a substratum the ELISA technique was modified to be used with whole cells.

Development of ELISA for Detection of Antigens on Cell Surfaces

The study of protein redistribution on the surface of the platelet can be hampered by the sensitivity and excitability of this cell. Almost any manipulation of the platelet suspension gives rise to some extent of shape change, release of granular material, and probable expression of new antigens on the surface. Fixation of the platelets shortly after filtration seemed a necessary step prior to any further manipulation when studying expression of antigens on the surface of the unactivated cell. As will be discussed below, fixation of the platelet soon after spreading was also necessary in experiments where low concentrations of nonionic detergent

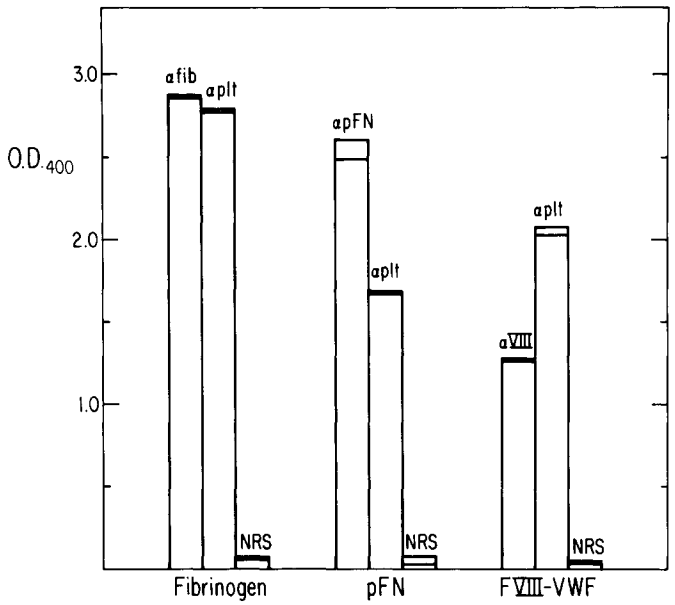

Fig. 1. Recognition of fibrinogen, plasma fibronectin (pFN) and factor VIII-Von Willebrand factor (VIII-VWF) by antiplatelet antiserum. Antigen solution at a concentration of 5 μg/ml was incubated in plastic tubes at 4°C overnight. The unbound antigen was washed away, and the bound was reacted with the respective antibody using the ELISA method (αfib-antifibrinogen; αplt—antiplatelet; NRS—nonimmune rabbit serum; αpFN—antiplasma fibronectin; αVIII—anti-factor VIII-Von Willebrand factor). All antisera were used at a dilution of 1:200. The variation between duplicate samples is indicated by the double horizontal lines.

were used. Therefore the effect of formaldehyde fixation on antigen-antibody interaction on the platelet surface was studied with platelets prepared in three different ways: 1) Gel filtered platelets were fixed in suspension soon after filtration (fixed unactivated); 2) gel-filtered platelets were allowed to spread on polystyrene, the spread platelets washed and then fixed (spread and fixed); 3) the spread platelets were washed but not fixed (spread). Comparison of the interaction of antiplatelet antiserum with these three platelet preparations showed that all three preparations bound antiplatelet antibodies (Fig. 2). Both preparations of spread platelets bound somewhat more antibody than did those fixed prior to activation and the spread-fixed samples bound slightly more than did the spread-unfixed. Platelets fixed after spreading bound significant amounts of other antibodies as well, even though some decrease was observed in comparison with the binding to spread but not fixed platelets (Fig. 2). We therefore conclude that the precedure used for fixation did not seriously interfere with antigen-antibody interactions and could be used for detection of antigens on the surface of platelets.

Are There FN, FVIII-VWF, and Fibrinogen on the Platelet Membrane?

Platelets fixed shortly after gel filtration showed no detectable amounts of FN, FVIII-VWF, or fibrinogen, above nonspecific background, on their surfaces (Fig. 2, panel A). These result confirmed the absence of the antigens in question

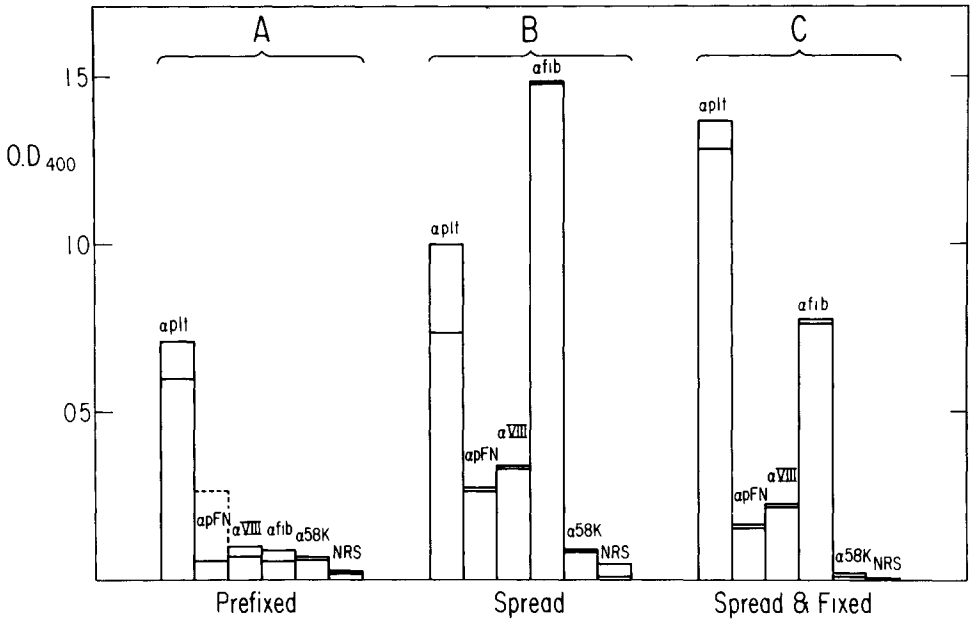

Fig. 2. Expression of pFN, FVIII-VWF, and fibrinogen on the surface of substratum activated platelets. Gel-filtered platelets were treated in three different ways: A) fixed unactivated in suspension (prefixed); B) allowed to spread on a plastic surface (spread); C) allowed to spread on a plastic surface and then fixed (spread and fixed). Antisera specific to gel-filtered platelets (αplt), plasma fibronectin (αpFN), factor VIII-Von Willebrand factor (αVIII), fibrinogen (αfib), and vimentin (α58K) as well as serum from nonimmune rabbit (NRS), each at a 1:200 dilution, were incubated with the different platelet preparations. Binding of the antibodies was detected by the modified ELISA method. The variation between duplicate samples is indicated by the double horizontal lines. No anti-FN, FVIII-VWF, or fibrinogen bound to the surface of the unactivated platelet at levels higher than those of the unrelated antiserum α58K. (In three different ELISA experiments the level of pFN detected was always insignificantly different from that of α58K. The one case in which it was different, depicted here in dotted lines, is an exception rather than the rule.) Antiplatelet, on the other hand, bound at significantly higher level than that of α58K. The binding of α58K and of NRS to the spread as well as to the spread and fixed platelet did not increase, whereas αpFN, αVIII, and αfib binding increased significantly upon spreading. The binding of αplt increased significantly in one case but not in the other.

from the surface of the unactivated platelet, and indicated that unactivated platelets do not adsorb these proteins from the medium in which they are suspended.

Expression of Surface Antigens Upon Platelet Binding and Spreading

As shown by Ginsberg et al [5] thrombin activation of platelets in suspension exposes FN on their surface. Release of internal FVIII-VWF and of fibrinogen to the medium was also demonstrated with the same activator [7, 10, 11]. To test whether the platelets' binding to and spreading on a substratum with no other exogenous activator present would have a similar effect, platelets were allowed to adhere and spread either on polystyrene, where antigen expression was studied by ELISA, or on fibrillar collagen type I adsorbed on glass coverslips where antigen ex-

pression was studied by immunofluorescence. It was found that platelets spread on both types of substrata expressed all three antigens on their surface. As can be seen in Figure 3, there was a clearly detectable specific immunofluorescent signal of all three antigens on the platelet surface. In ELISA (Fig. 2), a significant increase in signal, as compared with fixed unactivated platelets, was observed with antibodies to the antigens in question but not with a high titer antibody against an irrelevant antigen (a 58,000-dalton fibroblast protein that was not detected in the platelet). These experiments demonstrate exposure of previously sequestered (presumably intracellular) antigens upon platelet adhesion and spreading. Treatment of the spread and fixed platelets with 0.05% Tween 20 or with 1% Triton X-100 did not alter the amount of antifibronectin bound as compared with its binding to platelets treated with buffer only (data not shown), indicating that the platelet pool of fibronectin becomes completely exposed for antibody binding upon platelet adhesion.

Binding of Exogenous Plasma Proteins to Spread Platelets

The question of binding of proteins added exogenously to the bound and spread platelet was also approached with the same methods used for studying exposure of endogenous proteins. Immunofluorescence visualization of antigens on the surface of platelets adherent to collagen fibers indicated that binding did take place, since a clear increase in fluorescence intensity could be seen. This increase, however, was above a background of a preexisting signal due to the expression of endogenous antigens on the spread platelet. It was therefore not possible to draw quantitative conclusions from these observations. However, the pattern of the fluorescence in the case of fibronectin was distinctly different from that of FVIII-VWF (Fig. 4), a fact that may indicate a difference in surface distribution. When binding was studied by ELISA, which is semiquantitative, the increase in signal above endogenous antigen expression could be more easily visualized, and the concentration dependence of binding could be studied. As can be seen in Figure 5 for the cases of pFN and FVIII-VWF (the case of fibrinogen could not be resolved owing to high background problems), these two antigens bound to the surface of the spread platelet when added exogenously. This binding was well above the amount of antigen absorbed nonspecifically to the tube; it was also above the level of expression of the endogenous antigen and was concentration-dependent.

DISCUSSION

Two aspects of platelet activation have received attention in recent years, namely whether adhesion proteins such as FN, FVIII-VWF, or fibrinogen participate in the platelet interaction with matrices, and whether the platelet pool of these proteins plays a role in such interaction.

Fibronectin

In vitro aggregation of normal platelets by collagen occurs without addition of exogenous source for FN. Yet a recent case study of the genetic disorder Ehlers-Danlos syndrome [2] shows that normal pFN restores to the patient's plasma its ability to support platelet aggregation by collagen, thereby implying a necessity for FN in the normal platelet–collagen interaction.

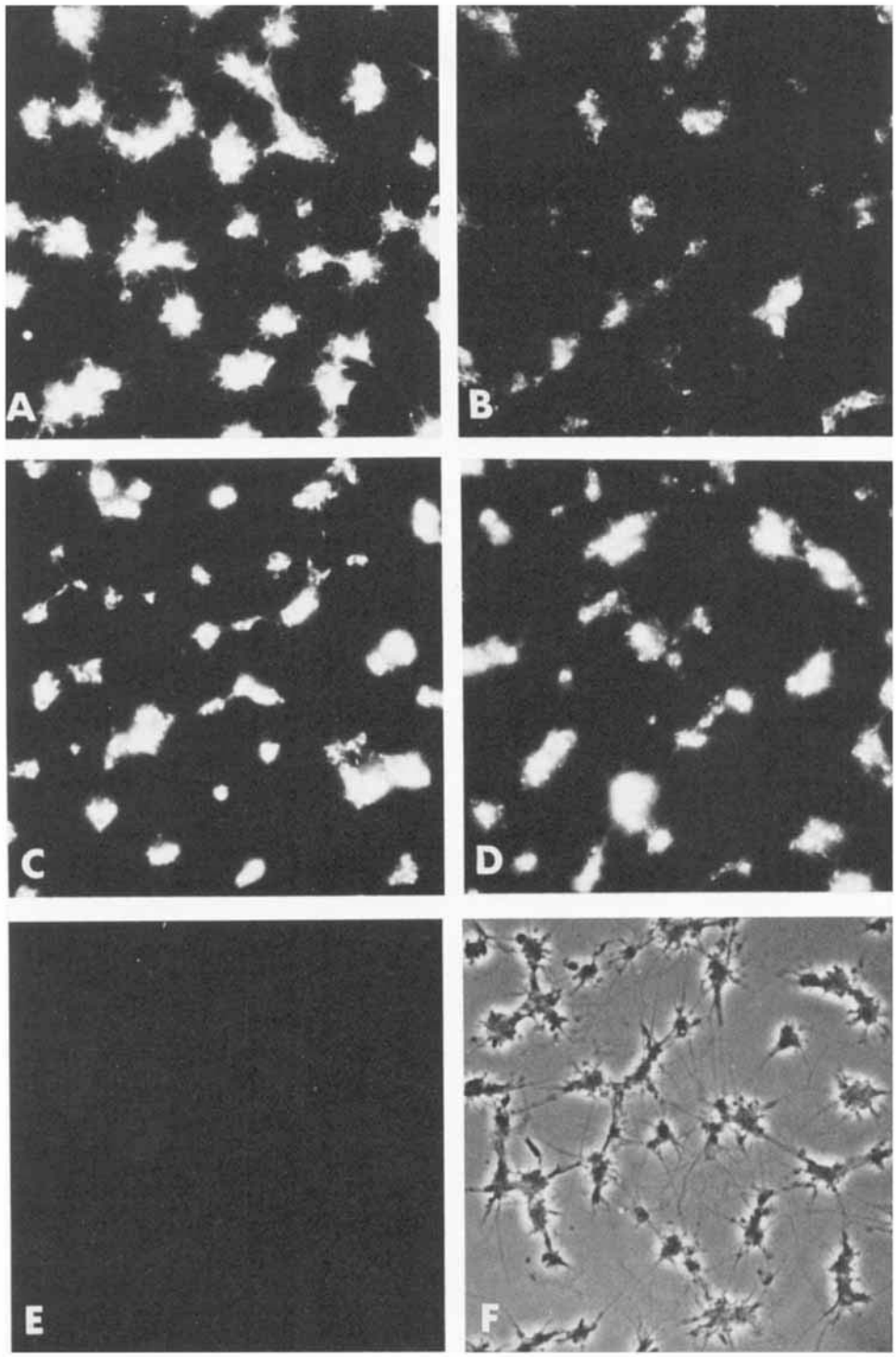

Fig. 3. Expression of pFN, FVIII-VWF, and fibrinogen on the surface of the substratum activated platelet. Gel-filtered platelets were incubated with collagen fibers on glass coverslips for 45 min at room temperature. The unbound platelets were washed away and the coverslips incubated with anti-platelets (A), anti-pFN (B), anti-FVIII-VWF (C), antifibrinogen (D), and nonimmune rabbit serum (E), each at a 1:20 dilution. Antibody binding was detected with fluorescein-conjugated antirabbit $F(ab')_2$. The pattern of platelet adhesion to the collagen fibers is shown in a phase contrast photograph in panel F.

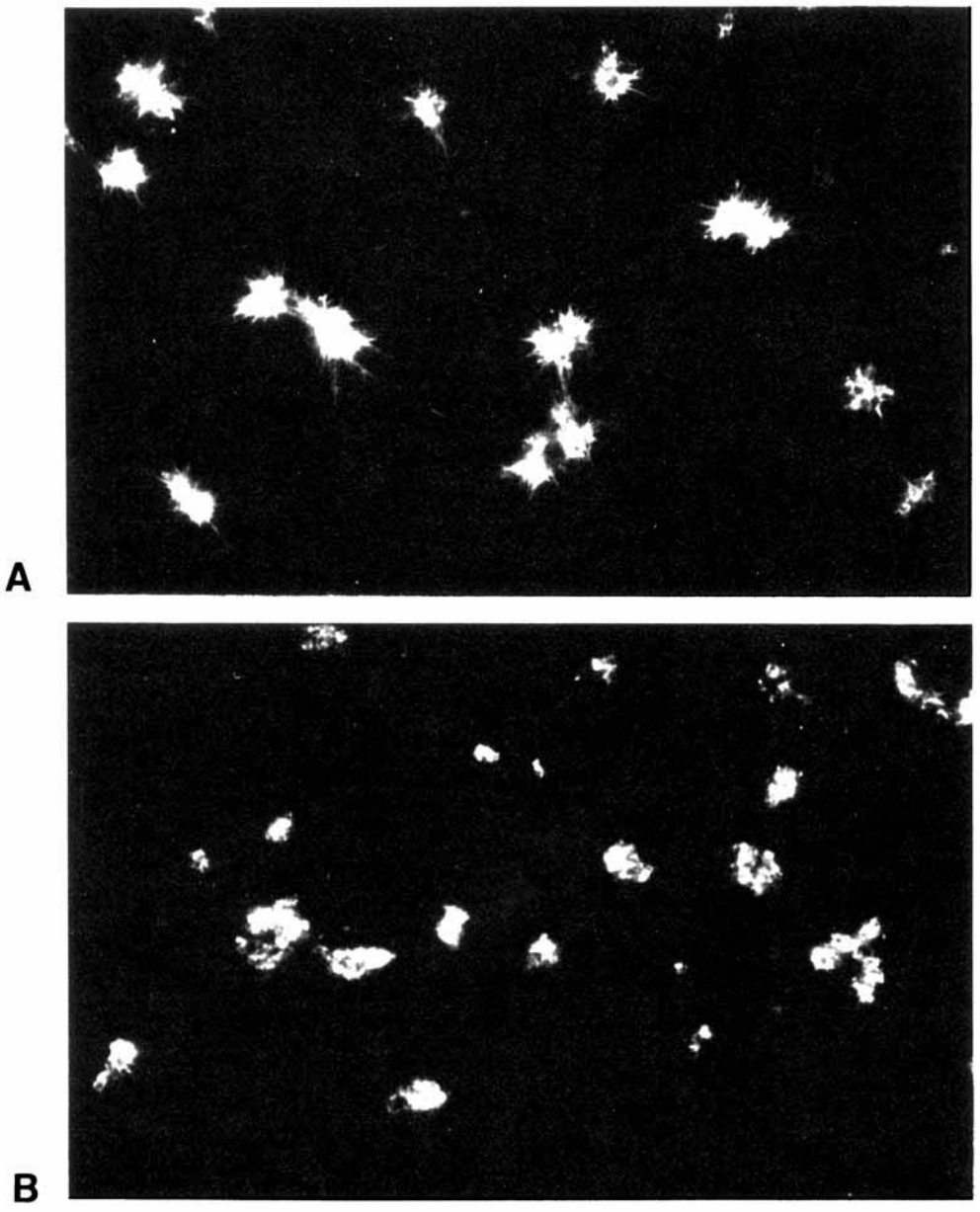

Fig. 4. Binding of exogenous pFN and FVIII-VWF by substratum-activated platelets. Gel-filtered platelets were incubated with human plasma and allowed to adhere to collagen fibers on glass coverslips. The nonadherent platelets were washed away and the coverslips incubated with antisera raised against pFN and FVIII-VWF. The bound antibodies were detected by fluorescein-conjugated antirabbit $F(ab')_2$. A) Platelets stained with anti-FVIII-VWF. B) Platelets stained with anti-pFN.

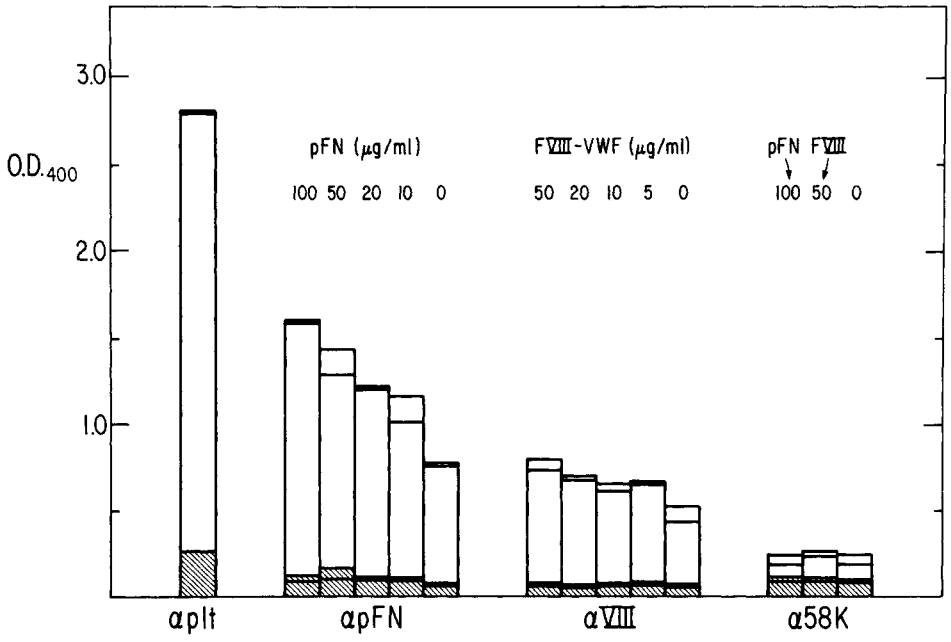

Fig. 5. Binding of exogenous FN and FVIII-VWF by substratum-activated platelets. Gel-filtered platelets were bound to a plastic surface as in Figure 2. Serially diluted purified pFN and partially purified FVIII-VWF (Profilate) were incubated with the spread platelets overnight at 4°C. The binding of the exogenously added proteins as well as the expression of endogenous proteins where none was added exogenously was detected by modifed ELISA. The persistence of the platelets on the surface in the presence of added antigens and antisera was confirmed by antiplatelets antibody. Relatively low nonspecific IgG binding was monitored with anti-58K. Specificity of binding of antigens to the platelet rather than to the plastic was checked by running in parallel a control assay without platelets. The level of detection of antigens on the plastic is shown in hatched bars. The variation between duplicate samples is indicated by the double horizontal lines.

Material antigenically similar to plasma and matrix FN has been detected inside the blood platelet after detergent permeabilization or extraction [1] or by subcellular fractionation [2]. No surface expression of fibronectin could be detected on the membrane of the unactivated platelet using lactoperoxidase catalyzed iodination [21] or immunofluorescence [22]. Lactoperoxidase catalyzed iodination of intact platelets, followed by lysis and radioimmune precipitation with anti-pFN antibody also failed to detect FN on the surface of the platelet (J. Lahav and R.O. Hynes, unpublished results). Thus our findings that antiplatelet antibody recognizes pFN and that platelets fixed in the resting state show no surface expression of FN corroborate the notion that platelet FN is contained within the platelet prior to activation. Binding and spreading of the gel-filtered washed platelets on collagen fibers and on artificial surfaces like polystyrene were found to be coupled to expression of FN antigen such that it could now be detected by immunofluorescence as well as by ELISA. The fluorescent pattern on collagen shows clearly that the FN is platelet-associated. Collagen fibers further away from the adherent platelet are much less fluorescent, and no background fluorescence was detectable on the glass, indicating that FN expression is a local event, limited to the platelet surface. In addition, we find that

when the spread platelets are exposed to an exogenous source of FN, they bind it to their surface. No binding of FN from an exogenous source could be detected on the resting platelet, either by radiolabeling studies [1] or by ELISA, as reported here. The recruitment of FN by the spread platelet brings the surface FN to a higher level than that reached by expression of endogenous FN (Figs. 4, 5) [22]. This binding is concentration dependent within the concentration range of plasma FN, happens both on collagen and on polystryrene bound platelets, and is limited to the area covered by the platelet. Since no kinetic studies were done, it is not known whether the expression of endogenous FN follows the same kinetics as the binding of exogenous FN. Thus it is not possible to postulate different roles for these two pools at this point. However, both the membrane expression and the concentration-dependent binding of pFN upon surface activation of the platelet suggest a possible participation of this protein in blood clotting steps associated with platelet membrane reactions. The fact that FN has binding sites for both collagen and fibrinogen lends further support to this idea.

Factor VIII-Von Willebrand Factor

The current understanding of the role of FVIII-VWF in platelet function is at a similar stage to that of FN. Here too a genetic bleeding disorder indicates involvement in the interaction of the platelet with components of the subendothelium in vivo [23], and in both cases no obvious role for the protein is detectable in control experiments with purified material in vitro. Platelet FVIII-VWF comprises 10–15% of the total circulating VIIIR:Ag [3, 24] and is totally contained within the platelet in its resting state [6]. Twenty to fifty percent of the platelet FVIII-VWF is released upon activation either by collagen or by thrombin [10,11].

The material released by collagen is electrophoretically similar to plasma FVIIIR:Ag [10]. Studies with radioactively labeled FVIII-VWF show that no VIII-VWF binds to platelets in suspension unless the antibiotic ristocetin is introduced [13, 25]. How, then, can it participate in normal platelet function? We find that when platelets adhere and spread on a surface, endogenous FVIII-VWF, previously undetectable on the platelet membrane, becomes expressed, and that under these conditions exogenous FVIII-VWF can also bind to the platelet in the absence of ristocetin in a concentration-dependent fashion. These findings indicate that there may be situations in which FVIII-VWF will function as a cell surface protein, and that activation by adhesion to substrata may be one such situation. Recent reports of FVIII-VWF interaction with collagen [26, 27] and with basement membrane [28] in the absence of ristocetin may provide the counterpart for such hypothesized role for FVIII-VWF in platelet function.

Fibrinogen

Binding of fibrinogen to the activated platelet has been studied rather extensively in recent years [29–31]. It has been shown that thrombin [32], epinephrin [33], and proteolytic enzymes [34] expose fibrinogen binding sites on the membrane of the activated platelet. At least the first activator causes release of endogenous fibrinogen from the platelet into the medium [7]. It will also cause aggregation of washed platelets without added fibrinogen [35]. This aggregation is interpreted to be due to the presence of endogenous fibrinogen on the membrane since it has been

shown that antifibrinogen will inhibit thrombin-induced aggregation of washed platelets [9]. However, no fibrinogen can be detected on the surface of carefully washed platelets [7, 8], and in the absence of Ca^{++} in the medium neither fibrinogen binding nor platgelet aggregation can be observed [12, 33, 34] upon platelet activation. Based on such observations, Marguerri et al [12] suggested a multistep model for the role of fibrinogen in the aggregation of platelets. We find that whereas indeed the resting platelet expresses no fibrinogen antigen on its membrane, platelets activated by surface binding express fibrinogen antigen on their surface, even in the absence of Ca^{++} and exogenous fibrinogen in the medium. This fibrinogen expression upon surface activation may indicate an added aspect of fibrinogen participation in platelet function, an aspect expressed perhaps in steps preceding platelet-platelet interaction that seem to be Ca^{++}-dependent.

In conclusion, we find that the platelet proteins FN, FVIII-VWF, and fibrinogen are redistributed upon platelet binding to substrata so as to be surface-associated. These proteins are also mobilized from exogenous pools when available. Both types of surface expression lend support to the hypothesis that adhesion proteins function in platelet activated by adhesion to the substratum.

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