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Colloids and Surfaces B: Biointerfaces 11 (1998) 67–77

COLLOIDS AND SURFACES B

Platelet interations with surface-adsorbed plasma proteins: exposure of CD62P induced by von Willebrand factor

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Received 28 October 1997; accepted 3 March 1998

Abstract

The adhesion of isolated platelets to surface-adsorbed plasma proteins and subsequent activation of the cells was studied by immunofluorescence. The spreading of the cells, formation of F-actin and exposure of CD62P, a marker for α-granule release, were detected by specific antibodies and FITC-labelled phalloidine. The plasma proteins used were albumin, complement factors C1q and C3, fibrinogen, thrombin and von Willebrand factor. Normal plasma was used as a positive control. Of the pure plasma proteins adsorbed, von Willebrand factor was the only protein which induced a marked exposure (>70%) of CD62P. Platelets adhering to fibrinogen and thrombin only showed a marked exposure (>70%) of CD62P when von Willebrand factor was added to the platelet suspension. This addition of von Willebrand factor inhibited the adhesion of platelets to surface-adsorbed C1q, C3 and albumin. The finding that pure von Willebrand factor was the only protein which induced a high exposure of CD62P was verified by the adhesion of platelets to surface-adsorbed factor-VIII deficient plasma. F-actin was seen around cell edges at all surfaces, although there was a difference in spreading. We conclude that CD62P exposure on the cell surface of adhering platelets is induced mainly by von Willebrand factor but could be influenced by other proteins, and that the spreading of platelets is not correlated to the exposure of CD62P. © 1998 Elsevier Science B.V.

Keywords: Platelet; CD62; V. Willebrand Factor; Biomaterials; Protein adsorption

1. Introduction

Blood encounters foreign materials in an increasing number of clinical situations, a contact which without exceptions leads to a non-self reaction with blood coagulation and inflammatory response. The inflammatory reaction as such, being a part of both healing and rejection, cannot be judged as harmful, but an understanding of the difference between non-self recognition leading to healing or rejection must be achieved. In order to map the time-sequence of events during blood–material contact, we have studied the initial blood reactions and found that platelet adhesion occurs within seconds of blood material contact, and that exposure of CD62P, a marker for α-granule release, can be detected within minutes [1,2].

A strong correlation has been found in several studies between the adsorption of fibrinogen and the surface adhesion of platelets [3–5]. The adhesion of platelets does not necessarily comprise activation of the cells. Platelet adhesion to surface-adsorbed fibrinogen has been reported to result in activation of tyrosine phosphorylase kinase and reorganisation of the cytoskeleton, but not in the secretion of serotonin [6], generally considered as a marker of platelet activation.

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PII S0927-7765(98)00026-5

Interactions between complements and a number of different receptors on platelets are known [7]. The specific interaction between C1q and platelets has also been reported previously [8,9]. The aim of the present study was to elucidate explicitly the reactions between isolated platelets and surface-adsorbed proteins, with special emphasis on the specificity of induced CD62P-exposure and the polymerisation of actin.

2. Materials and methods

2.1. Chemicals

Lyophilized platelet-poor plasma and lyophilized platelet-poor plasma with factor-VIII deficiency as well as fibrinogen, human thrombin, human albumin, human factor C1q, human factor C3 and fluorescein isothiacyanate (FITC) labelled phalloidine were purchased from Sigma Chemical Company (St. Louis, MO). Von Willebrand factor (vWF) was purchased from Calbiochem-Novabiochem Corporation (La Jolla, CA). Percoll was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). All chemicals used for buffer solutions were obtained from Merck (Darmstadt, Germany).

2.2. Preparation of platelets

Venous blood from healthy human donors who were not on medication was collected with a 0.6 mm needle into 0.6 ml of CPD (citrate, phosphate, dextrose) anticoagulant to a final volume of 5 ml. The platelets were separated by a one-step Percoll technique. A modified version of Braide and Bjursten's [10] method for leukocyte preparation was used [11]. In short, the blood was mixed 1:1 with 400 mOsm phosphate-buffered saline (PBS) containing 4 mM $MgCl_2$. The mixture was transferred to a Percoll gradient. The platelets were collected from the first layer after centrifugation ($750 \times g$, 5 min, 20°C), after which they were washed twice with a washing buffer. A modified version of Dulbeccos' PBS containing 4 mM $MgCl_2$, no $CaCl_2$ and 1 mg ml^{-1} albumin (pH 7.4) was used. After washing the cells were resuspended and pooled in unmodified Dulbeccos' PBS (D-PBS) (0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, pH 7.4) containing albumin (1 mg ml^{-1}). Only polystyrene cups and tubes were used during the isolation. The cell concentration was determined by counting in a Bürker chamber.

2.3. Prepartion of hydrophobic glass slides

Hydrophobic slides (methylated silica) were prepared by cleaning glass slides for microscopy (80% quartz) for 30 min in 70% ethanol containing 0.35 M HCl. After rinsing with water (3×10 min), the slides were dried with an air current. The clean slides, which were hydrophilic, were made hydrophobic by methylation of the surfaces in dichloromethane containing 0.1% 1,1,1,3,3,3-hexamethylsilazane for 7 min. The contact angle with water was $>85°$ at the slides.

2.4. Surface adsorption of plasma proteins

Lyophilized platelet-poor plasma and plasma with factor-VIII deficiency were rehydrated and allowed to stand for at least 10 min before use. According to the manufacturer's specification, citrate had been used as an anticoagulant at the time of preparation. Albumin, the complement factors C1q and C3 and the coagulation factors fibronigen and vWF were dissolved in D-PBS to a concentration of 100 μg ml^{-1}. Thrombin was dissolved to a concentration of 240 μg ml^{-1}. Dots ($V=$ 30 μl) of each protein solution were incubated on hydrophobic slides for about 30 min in a humid chamber at room temperature. The plasma dots were incubated for 15 min in a humid chamber at room temperature. The surfaces of the slides were then rinsed with D-PBS. The protein- and plasma-coated slides were used within 1 h. For recalcification of the rehydrated lyophilized plasmas, the plasma was mixed 4:1 with $CaCl_2$ solution to achieve a normal calcium-ion level. During the coating time the normal platelet-poor plasma coagulated, while no difference in the viscosity could be seen for plasma with factor-VIII deficiency.

2.5. Adhesion and activation of platelets

The platelet solution was diluted to a concentration of 2.5×10^7 platelets ml^{-1}, and 30 μl dots

were transferred to each protein or plasma spot on the slides. The slides were then incubated for 10 min in a humid chamber at 37°C. The samples were washed with D-PBS before they were fixed at -20°C ethanol for 10 min or more. After fixation the samples were rehydrated and washed with D-PBS. The effect of soluble vWF was investigated by adding vWF to a concentration of 10 μg ml^{-1} at the time of platelet adhesion.

2.6. Immunofluorescence

The number of adhering platelets and the coverage were determined with antibodies. For primary detection, rabbit-anti-pan platelet antibodies (DAKO Patts A/S, Glostrup, Denmark) and monoclonal mouse-anti-human antibodies against CD62P (Serotec, Oxford, UK) were used. The samples were incubated for 30 min with the antibodies on a cooling plate (0°C, HISTO-LAB, Sweden) and rinsed with D-PBS before a second incubation with FITC-conjugated swine-anti-rabbit antibodies of FITC-conjugated rabbit-anti-mouse antibodies (DAKO Patts A/S). All antibodies were diluted in D-PBS. After a second rinsing the samples were assembled with 1,4-diaza-bicyclo[2,2,2]octane mixed with glycerol (DABCO) to keep the fluorescence from fading. Viewing and photography was perfomed within 12 h.

2.7. Microfilament staining

FITC-labelled phalloidine was used to detect F-actin filaments in the platelets. The samples were fixed in 1% formalin for 15 min at room temperature and rinsed with D-PBS before treatment with 0.01% Triton 100 for 3 min. After a second rinsing, they were incubated for 40 min with FITC-labelled phalloidine at a concentration of 0.005 g l^{-1} at room temperature. After a final excessive rinsing, the samples were assembled with DABCO. Viewing and photography was performed within 12 h.

2.8. Anlaysis

All samples were photographed in a Zeiss 3RS fluorescence microscope. Two to three photos were taken on each protein spot using black and white Kodak T-max 400 pro film. The exposure time was kept constant at 1 min. The photographs for analysis were taken at a magnification of 10×20. Detailed pictures were taken at a magnification of 10×100. After development, the black and white negatives were scanned into Adobe Photoshop on a Power Macintosh 8100/80 desktop with Sprint Scan 35. Some data were transferred to NIH Image on the same computer. The percentage coverage and the number of platelets adhering to the surface were analysed in Adobe Photoshop or NIH Image. The area for scanning was kept constant at a size of about 10 000 μm^2. The spreading index (SPI) was calculated as the percentage coverage multiplied by 100 hundred divided by the number of cells. For compact platelets with native morphology the SPI was about 1.5, and for a surface covered with only fully spread platelets the SPI was about 12.2. The SPI was used to give an idea of the spreading differences at different surfaces. The expression of CD62P for the platelets was calculated as normalized values from the number of CD62P positive platelets divided by the total number of platelets adhering to the surface.

2.9. Statistical evaluation

All statistical evaluations were performed with Student's *t*-test, and the number of observations was nine if not stated otherwise. The values presented are given as mean $\pm$ standard error of the mean (SEM).

3. Results

3.1. Adhesion and spreading on protein and plasma films

We studied C1q and C3 from the complement system and fibrinogen, thrombin and vWF from the coagulation cascade. Albumin-coated surfaes were used as non-reactive controls. The coverage of platelets at the different surfaces is given in Fig. 1. Platelets were also allowed to adhere to surfaces coated with normal plasma, recalcified normal plasma, plasma with factor-VIII deficiency

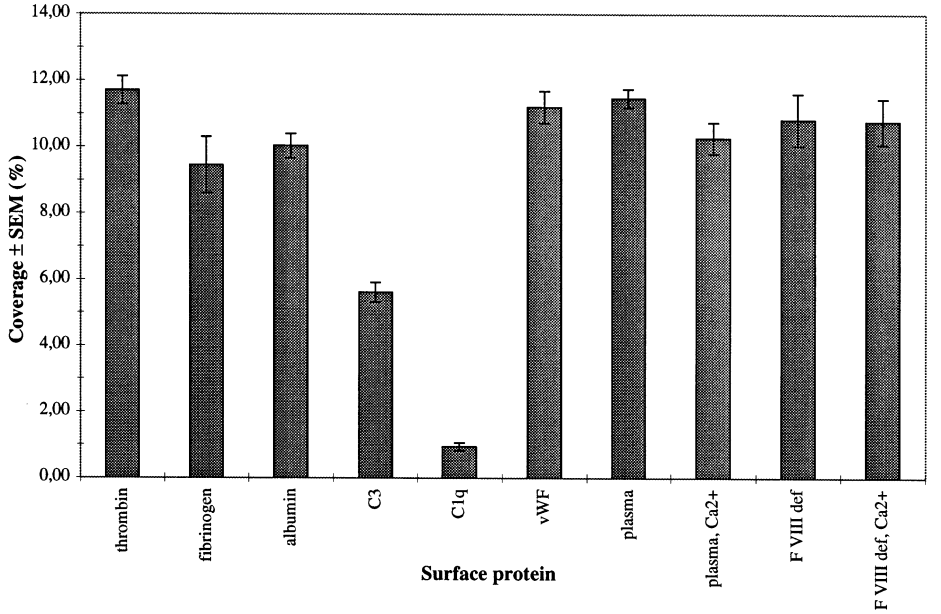


Fig. 1. The coverage of platelets adhering to a surface coated with plasma or plasma proteins after 10 min of incubation at 37°C. The platelet concentration used was 2.5×10^7 platelets ml^{-1}. F VIII def = plasma with factor VIII deficiency. A recalcification of the plasma surfaces was also done (Ca2+) (mean ± SEM).

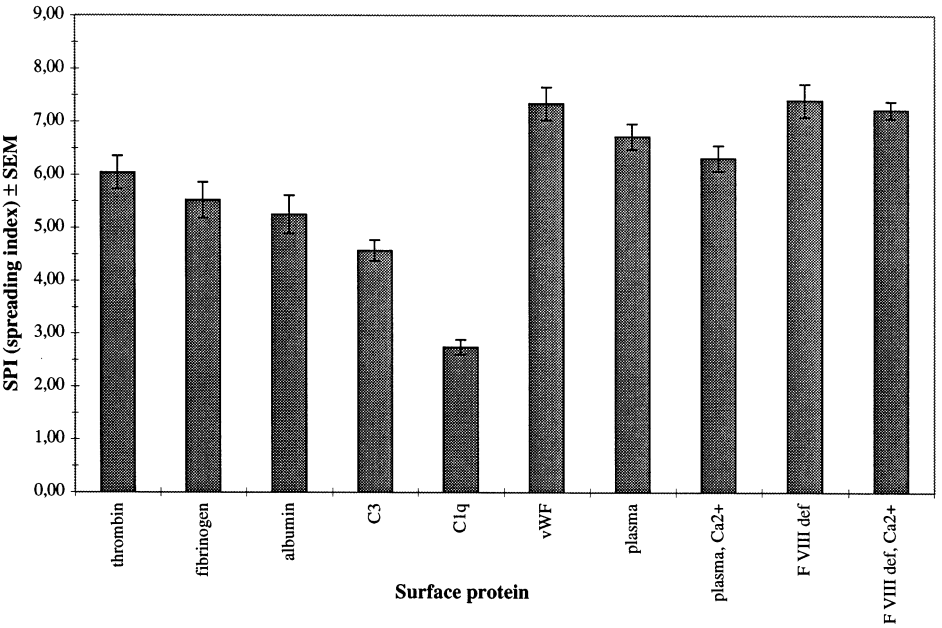


Fig. 2. The spreading index for platelets adhering to a surface coated with plasma or plasma proteins after 10 min of incubation at 37°C. The platelet concentration used was 2.5×10^7 platelets ml^{-1}. F VIII def = plasma with factor VIII deficiency. A recalcification of the plasma surfaces was also done (Ca2+). The spreading index for compact platelets is 1.5, and for fully spread platelets it is 12.2 (mean ± SEM).

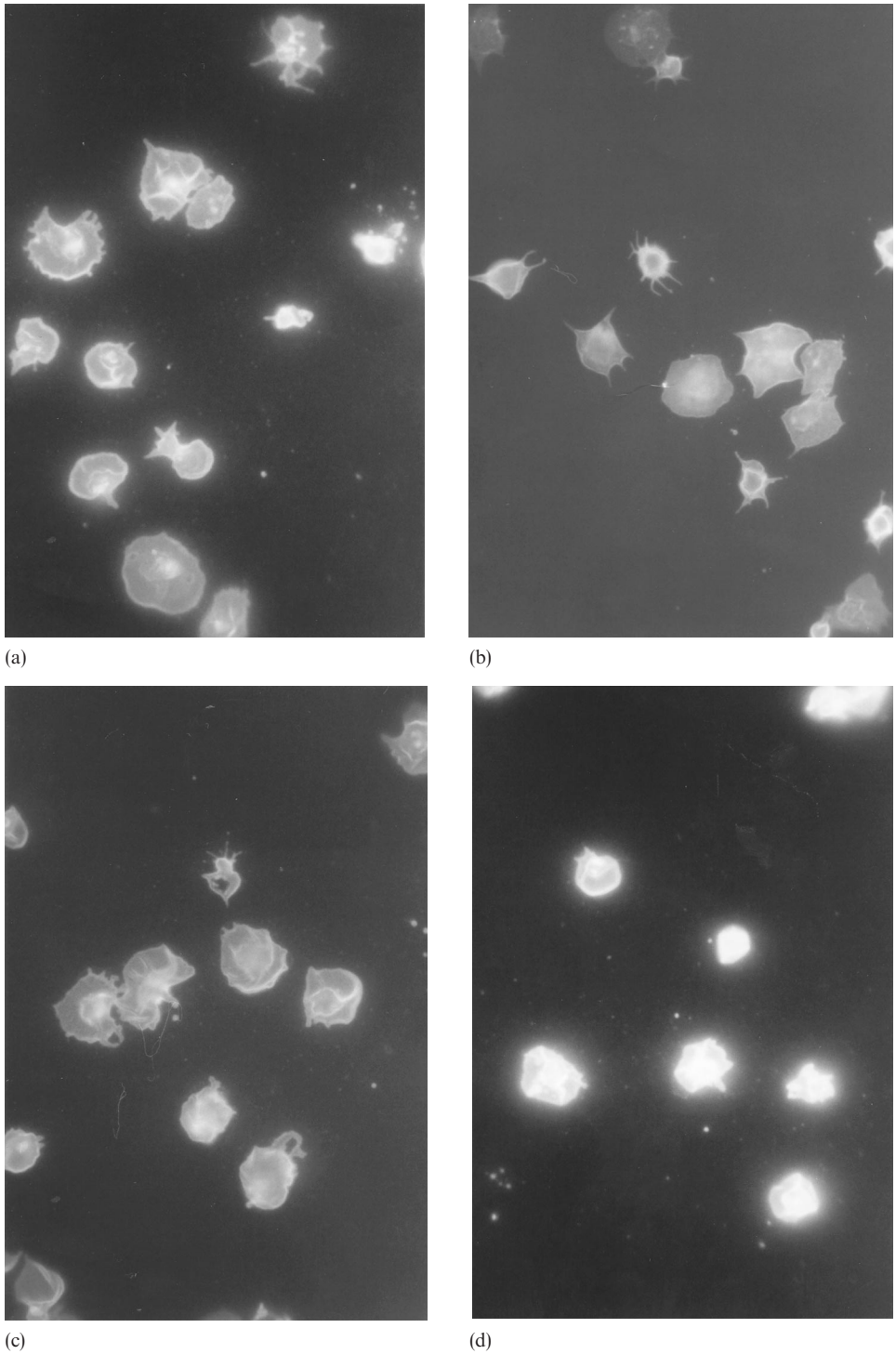
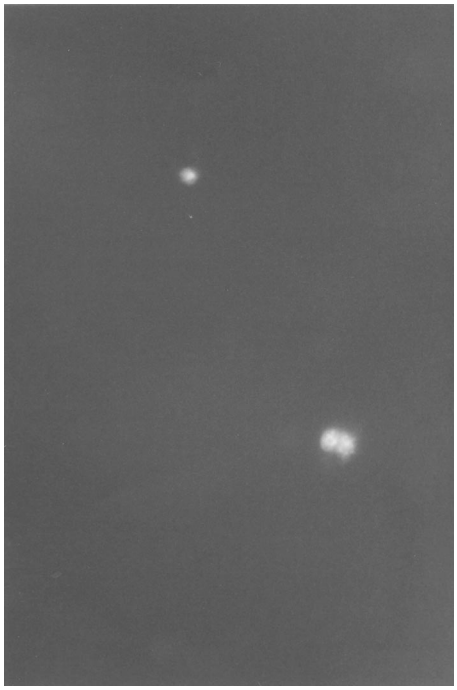


Fig. 3. (a) Platelets at a thrombin-coated surface. (b) Platelets at a fibrinogen-coated surface. (c) Platelets at an albumin-coated surface. (d) Platelets at a C3-coated surface. (e) Platelets at a C1q-coated surface. All after 10 min of incubation at 37°C. The platelet concentration used was 2.5×10^7 platelets ml^{-1}. Original magnification $\times 2000$. Rabbit-anti-human platelet antibodies were used.



(e)

Fig. 3. (*continued*)

and recalcified plasma with factor-VIII deficiency. Very few platelets adhered to the C1q-coated surface ($p<0.001$). The coverage on the C3-coated surface was also significantly lower ($p<0.001$) than on the other surfaces, whic had very equal coverages.

The degree of spreading differed between surfaces, as indicated in Fig. 2. On the C1q-coated surface the adhering platelets were compact, while on the other surfaces the cells spread to different degrees (Fig. 3). At the fibrinogen- and C3-coated surfaces the platelets showed a high degree of short dendritic extrusions (Fig. 3B and D).The spreading of platelets at the plasma- and the vWF-coated surfaces were very similar, and only slightly more than at the thrombin-coated surface.

3.2. Exposure of CD62P

The exposure of CD62P on platelets at protein- and plasma-coated surfaces is shown in Fig. 4. Platelets at the albumin-coated surfaces exposed very little CD62P on the platelet surfaces, and could be said to be unactivated. The data for C1q should be regarded as an indication, because the number of adhered platelets was low.

The CD62P exposure for platelets adhering to normal plasma was much higher than for platelets adhering to factor-VIII deficient plasma, both with and without calcium present at the time of plasma coating (Fig. 4). A higher degree of CD62P exposure was seen when calcium was present, both for normal and factor-VIII deficient plasma. With calcium present, the degree of exposure was only about 40% for the factor-VIII deficient plasma. The exposure of CD62P on platelets at normal recalcified plasma reached 93%. At the surface coated with vWF and CD62P, an exposure of around 80% was seen. Such high values were not seen for the other pure plasma proteins used in this study.

3.3. Effects of soluble vWF

When soluble vWF was added to the platelet suspension, only a few platelets adhered to surfaces coated with albumin, C3 and C1q (Fig. 5). The reduction was significant ($p<0.001$) even for C1q, where the coverage was low even without the addition of vWF. At the surfaces coated with fibrinogen, thrombin and factor-VIII deficient plasma, the exposure of CD62P increased and reached a level of more than 85% (Table 1).

3.4. Microfilament staining

After actin filament staining, the outlines of the cells adhering to the different surfaces could be seen clearly (Fig. 6). In most platelets it was also

Table 1
Number of platelets and CD62P positive platelets and the activity after adhesion to different adsorbed protein when vWF ($10\ \mu g\ ml^{-1}$) was present in the platelet solution. Incubation: 10 min at 37°C (mean ± SEM)

Adsorbed protein	Platelets	CD62P+	Activity (%)
F VIII def. plasma	99.2 ± 5.7	95.2 ± 4.3	95
Thrombin	161.9 ± 4.5	133.7 ± 9.0	83
Fibrinogen	96.5 ± 5.6	95.3 ± 11.4	99

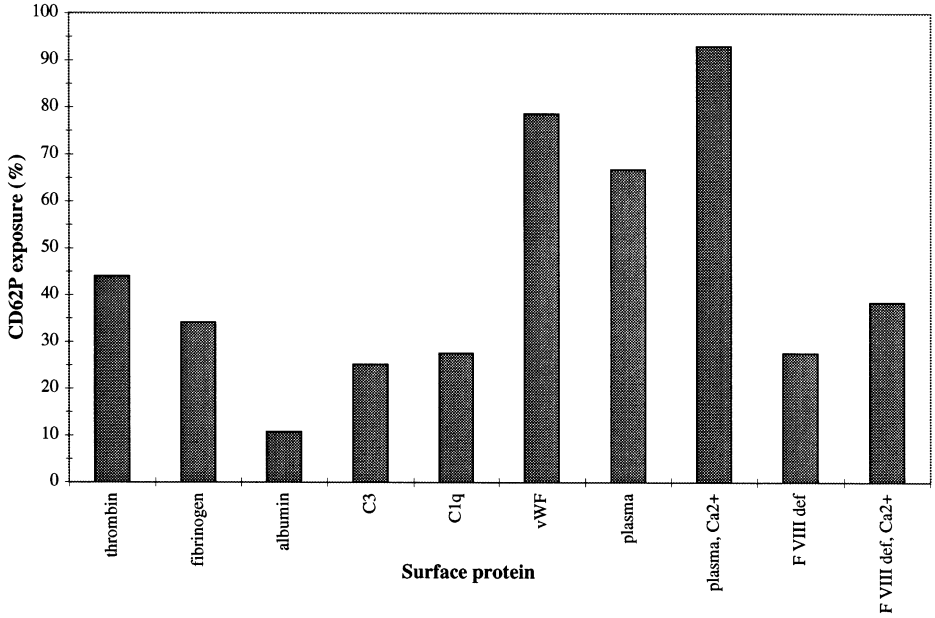


Fig. 4. Percentage of platelets exposing CD62P after adhesion to surfaces coated with plasma or plasma proteins after 10 min of incubation at 37°C. The platelet concentration used was 2.5×10^7 platelets ml^{-1}. F VIII def = plasma with factor VIII deficiency. A recalcification of the plasma surfaces was also done (Ca2+).

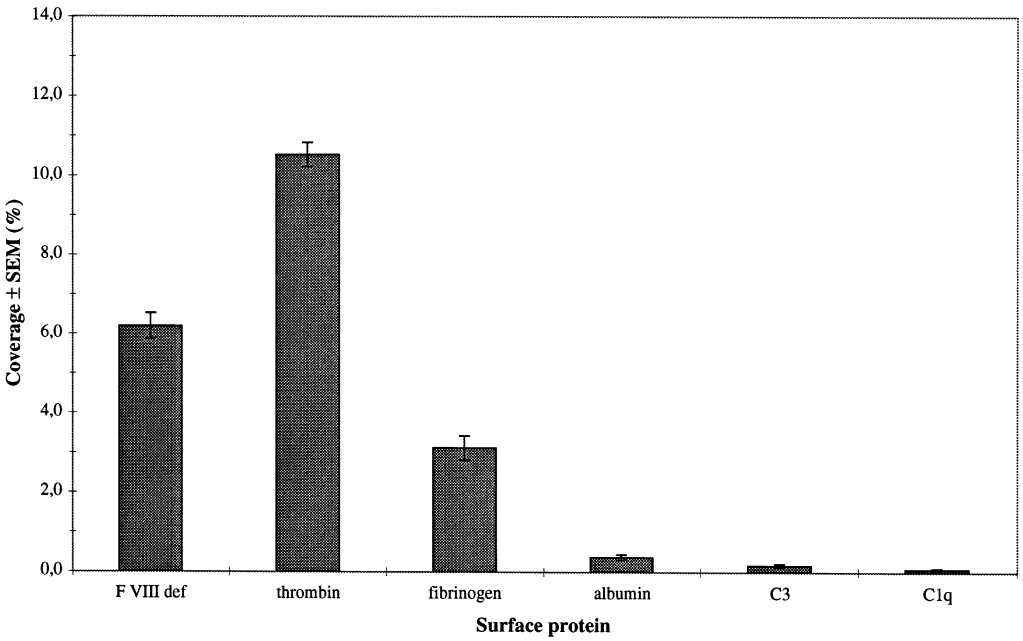


Fig. 5. The coverage of platelets adhering to a surface coated with proteins or plasma with factor VIII deficiency (F VIII def) after 10 min of incubation at 37°C. The platelet concentration used was 2.5×10^7 platelets ml^{-1} and the concentration of vWF in the platelet solution was 10 μg ml^{-1} (mean ± SEM).

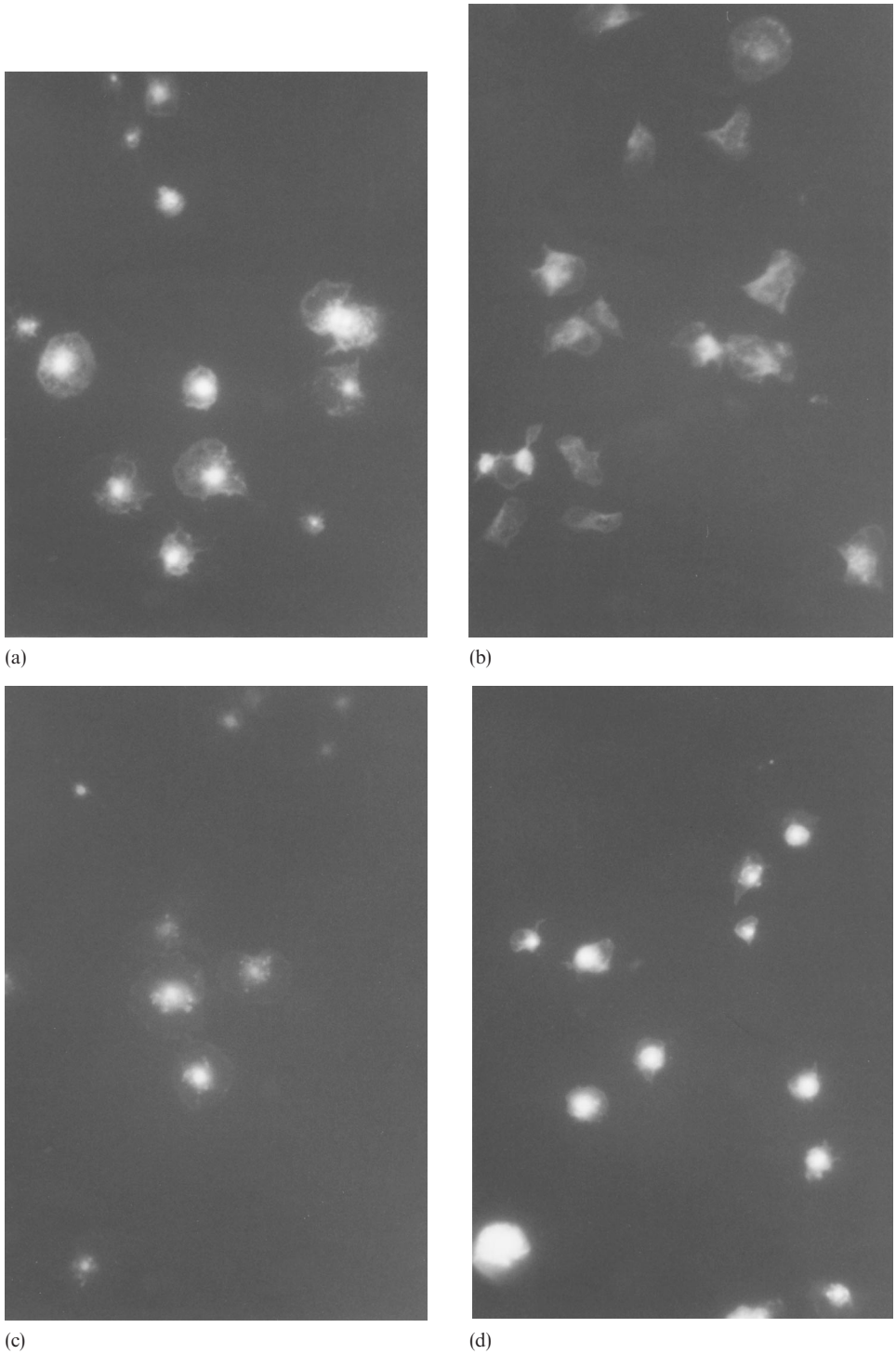
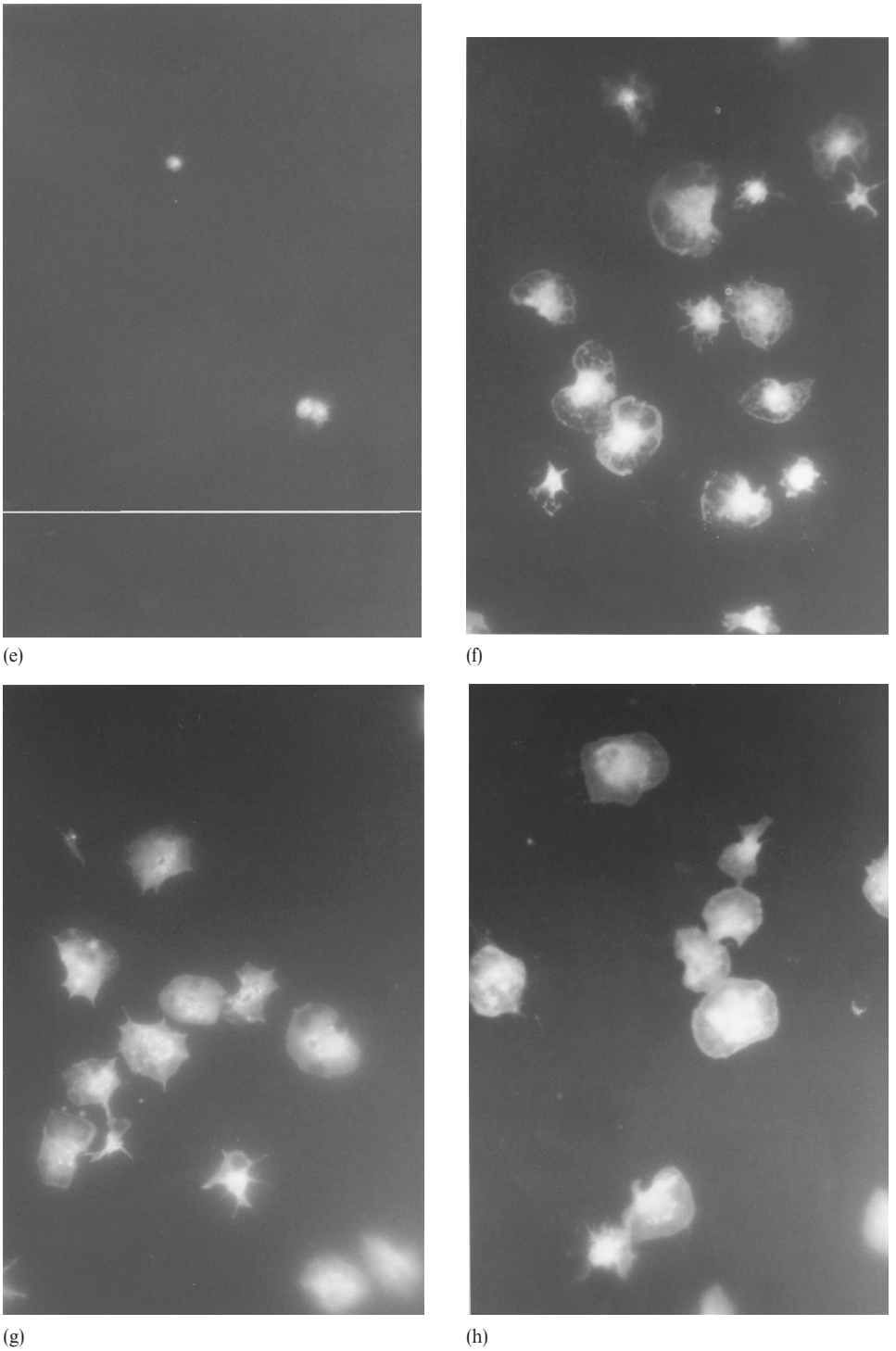


Fig. 6. Actin filaments in platelets adhering to different surfaces after 10 min of incubation at 37°C. The platelet concentration used was 2.5×10^7 platelets ml^{-1}. (a) Thrombin, (b) fibrinogen, (c) albumin, (d) C3, (e) C1q, (f) vWF, (g) normal plasma, (h) plasma with factor VIII deficiency. Original magnification $\times 2000$.



(e) (f)

(g) (h)

Fig. 6. (*continued*)

possible to detect a network of filaments. Pseudopodia and dendrites also contained actin filaments. The central part of most of the platelets were very filament dense. On the C1q-coated surfaces, the platelets had a native morphology and no filament network or pseudopodia could be seen. On the fibrinogen- and C3-coated surfaces, the majority of the platelets exhibited a spread morphology and a filamentous network was visible. Further, the platelets also showed the formation of short dendrites. These differences in the distribution of actin filaments on different surfaces were correlated to the differences seen in spreading, as seen in Figs. 2 and 3.

4. Discussion

Magnesium competes with calcium for binding to certain integrins, especially GPIIb/IIIa, and modulates their function [12,13]. According to Gawas et al. [14], a concentration of 4 mM Mg^{2+} will be sufficient to keep platelets from spreading, activation and aggregation. This inhibition is reversible, as shown by the massive CD62P expression after adhesion to normal plasma seen in this study. The reversibility makes magnesium ions an ideal inhibitor of platelet activation during separation and handling of the cells [11].

All proteins used in this study except vWF had very little effect on the exposure of CD62P. However, CD62P exposure was achieved when platelets adhered to surface-adsorbed proteins with the use of whole plasma or vWF. With factor-VIII deficient plasma, a low level of CD62P exposure was found. Goto et al. [15] have shown previously that the component of factor VIII responsible for CD62P exposure at platelets is vWF. This is in agreement with our results that surface-adsorbed vWF is sufficient for a massive α-granule release.

GPIIb/IIIa is the receptor responsible for the irreversible binding of platelets to immobilized fibrinogen [16]. As an effect of this binding, an extra pool of GPIIb/IIIa, located inside the platelets, is translocated to the surface [17]. The translocation also induces an activation of GPIIb/IIIa, which is necessary for binding to soluble vWF, which mediates aggregation [15]. This could explain the high CD62P exposure seen at platelets adhering to fibrinogen after contact with soluble vWF. This effect is also seen at the thrombin surfaces.

On the C1q-, C3- and albumin-coated surfaces, the aggregation effect could be stronger than binding, explaining the obseved decrease in coverage. The possibility of activated platelets leaving the surface should not be omitted.

The adhesion of platelets to surface adsorbed vWF has been reported to proceed via binding to GPIb, which provides a change in ligand recognition specificity for GPIIb/IIIa [16,18]. As indicated by the experiments with recalcified plasmas, the calcium-ion level is of importance for activation of platelets, but the difference between factor-VIII deficient and normal plasma remained. This is in accordance with Wu et al. [19], who found that divalent cations have no effect on the binding of platelets to vWF. However, adhesion to and activation by other proteins may be influenced by the Ca^{2+} concentration. From this study it was also possible to conclude that spreading of the platelets and α-granule release (CD62P exposure) are independent events.

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