Mechanisms of Platelet Adhesion to the Basal Lamina

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The human glomerular basal lamina (HGBL) is composed of collagenous and noncollagenous glycoproteins. We assessed the role played by each constituent in platelet-basal-lamina interactions by selective cleavage and removal of each component by clostridial collagenase or by pepsin. When noncollagenous proteins are removed from HGBL, human platelets exhibit little reactivity toward the residual collagen framework of the isolated basal lamina. With the noncollagen matrix of basal lamina, after removal of the bulk of the collagen, platelet adhesion and spreading proceed normally in the presence of divalent cations, similar to what occurs on intact basal lamina. No platelet degranulation or aggregation is observed. The results indicate that the basal lamina collagen, even in its native packing arrangement, lacks affinity for platelet adhesion and is incapable of triggering platelet release reactions. Platelet adhesion and spreading on the basal lamina appears to depend primarily on the presence of the noncollagen components and to require divalent cations. The data suggest the presence on platelets of receptors for basal lamina distinct from those for interstitial collagens. These receptors activate a unique modulation of platelet behavior, ie, adhesion and spreading without degranulation. A difference in biologic function of the basal lamina and interstitial collagens is apparent in these experiments. (Am J Pathol 92:99–110, 1978)

ADHESION AND SPREADING OF PLATELETS on the endothelial basal lamina are important modes of platelet function in maintaining vascular integrity following endothelial loss. Although the basal lamina contains a collagen-type protein as one of its major constituents, the outcome of platelet interaction with basal lamina seems to be distinct from that of skin, tendon, and vascular collagens in that it is nonthrombogenic; there is no platelet degranulation, adenine nucleotide release, or aggregation.[1] The mechanisms of platelet adhesion to the physical substrate and the consequent modulation of platelet behavior are of fundamental importance to our understanding of thromboembolic and other vascular diseases in humans.[2-5] We report here further observations that platelets fail to adhere to the basal lamina after partial cleavage of the noncollagen component of basal lamina by pepsin, indicating that the determinants of platelet-basal-lamina adhesion and spreading reside in the noncollagen component of the basal lamina. Divalent cations are required. In this respect, the platelet-basal-lamina interaction is similar to the endothe-

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lium-basal-lamina interaction and distinct from the platelet-collagen-fiber interaction.

Materials and Methods

Highly purified human glomerular basal lamina (HBGL) and insoluble human tendon collagen (HTC) were prepared according to methods previously described.[1]

Preparation of the Collagen Matrix of Basal Lamina

The insoluble HGBL collagen was obtained by repeated pepsin cleavage of the non-collagen component of the basal lamina. HGBL (110 mg) was suspended in 5 ml of 0.1N acetic acid, to which 10 mg of pepsin (porcine stomach, crystallized twice, Worthington Biochemical Corp., Freehold, N.J., lot number PM 33K865) in 1 ml of 0.1N acetic acid was added. The enzymatic cleavage was carried out at 4 C for 24 hours with gentle stirring. At the end of the period, the reaction mixture was centrifuged and the HGBL pellet was resuspended and digested with fresh pepsin solution twice more; the total period of pepsin cleavage was 72 hours. This was followed by rinsing three times with cold 0.5 M NaCl buffered with 0.02 M phosphate, pH 7.4, dialysis against distilled water at 4 C, and lyophilization.

Preparation of the Noncollagen Matrix of Basal Lamina

The insoluble noncollagen matrix of HGBL was obtained by clostridal collagenase cleavage of HBGL. The collagenase solution was prepared by dissolving 5 mg of highly purified collagenase (CLSPA IDA, Worthington Biochemical Corp.) containing 2000 units of collagenase activity in 20 ml of 0.1 M tris-acetate buffer, pH 7.4, with 0.005 M calcium acetate. To inactivate clostridiopeptidase B, 0.022 ml of 30% hydrogen peroxide was added.[6] Purified HGBL (600 mg) was suspended in 600 ml of 0.1 M tris-acetate buffer, pH 7.4, in 0.005 M calcium acetate. To the suspension, 10 ml of the collagenase solution was added together with three drops of toluene to prevent microbial growth. The cleavage was carried out at 37 C in a shaking water bath. At 24 and 48 hours, an additional 5 ml of the enzyme solution was added. The incubation was terminated at 72 hours and the reaction mixture was centrifuged at 40,000*g* for 30 minutes. The insoluble residue was washed three times with distilled water and was lyophilized.

Amino Acid Analysis

The amino acid analysis was carried out with a Beckman 120 C amino acid analyzer.[7,8] 3-Hydroxyproline was estimated according to the method of Piez and associates.[9]

Interaction of Human Platelets and Basal Lamina Preparations

Human-platelet-rich plasma (PRP) was obtained by centrifuging blood from healthy volunteers; this blood contained an anticoagulant consisting of one of the following: a) 1 part 3.8% sodium citrate and 9 parts blood, b) 1.5% EDTA and 9 parts blood, or c) 20 units of heparin per milliter of blood.[1]

Interaction of the test materials with platelets was carried out in a Payton aggregometer (Payton Associates, Inc., Buffalo, N.Y.) at 37 C, stirring at 900 rpm. One-half millilter of PRP was placed in a cuvette. After establishing a stable baseline record for 1 minute, 0.5 ml of Tyrode solution containing 2 mg of the test materials was rapidly added. The interaction was continuously monitored by light transmission at 609 nm. After 10 minutes, 1 ml of Karnovsky's fixative [10] at room temperature was added to terminate the interaction. The remaining procedures for electron microscopy were performed as previously described.[1]

Results

Human Glomerular Basal Lamina

Platelets in heparin- or citrate-PRP avidly adhered to and spread on HGBL. The spreading cytoplasm was devoid of secretory granules and seemed to be filled with microfilaments. When numerous platelets adhered to a fragment of basal lamina, overcrowding often prevented platelets from spreading; the basal lamina was paved with a single layer of densely populated platelets (Figure 1). Secretory granules, both α-granules and very dense granules, remained intact; aggregometry failed to register evidence of platelet aggregation. These observations were consistent with previous findings.[1] Platelets in EDTA-PRP failed completely to adhere to basal lamina (Figure 2).

Insoluble Collagen Matrix of Human Glomerular Basal Lamina

After 72 hours of pepsin digestion, the remaining protein matrix showed an enrichment of hydroxyproline, a marker amino acid for collagenous proteins, from 87.6 to 137.9 residues/1000 amino acid residues. Hydroxylysine and glycine were also increased, indicating substantial enrichment of collagenous protein in the insoluble matrix (Table 1). The estimated collagenous protein content was approximately 75%.

Electron microscopy of the interaction between the collagen matrix of HGBL and platelets in heparin-, citrate-, or EDTA-PRP revealed no evidence of platelet adhesion to the basal lamina or degranulation (Figure 3). Aggregometry also failed to show platelet aggregation.

Insoluble Noncollagen Matrix of Human Glomerular Basal Lamina

The insoluble HGBL matrix after 72 hours of clostridial collagenase cleavage was largely noncollagenous protein, as reflected by reduction of hydroxyproline and hydroxylysine and enrichment of half-cystine, glutamic acid, and aspartic acid (Table 1). The estimated collagen content was only 5.2%, compared with 52% in the native HGBL.[11]

Human platelets in heparin- and citrate-PRP adhered to and spread on the noncollagen HGBL matrix, again without degranulation or aggregation (Figure 4). Absence of divalent cations as in EDTA-PRP prevented platelets from adhering to the noncollagen matrix. The results were identical to those for native HGBL.

Human Tendon Collagen Fibers

On electron microscopy, human tendon collagen fibers caused platelet degranulation and aggregation in citrate-PRP. The aggregated platelets,

Table 1—Amino Acid Composition of Native and Peptidase-Cleaved Human Glomerular Basal Lamina

Amino acid	Native	Pepsin-treated	Collagenase-treated
Asp	68.4	63.7	89.6
Thr	37.2	27.5	51.4
Ser	48.9	46.7	61.4
Glu	96.7	81.5	107.8
Gly	233.5	284.8	106.9
Ala	56.8	39.0	79.4
1/2-Cys	17.1	0.2	31.8
Val	36.2	25.3	58.5
Met	14.4	11.1	17.4
Ileu	29.5	30.8	67.0
Leu	64.7	59.2	79.3
Tyr	14.7	14.0	25.5
Phe	29.3	26.8	37.5
Pro	70.0	60.8	61.8
3-Hypro	17.8	29.8	0.6
4-Hypro	69.8	108.1	6.9
Hylys	18.4	36.5	3.0
Lys	16.1	12.0	41.3
His	14.6	9.8	18.7
Arg	45.8	32.6	54.1

Residues per 1000 amino acid residues

with embedded collagen fibrils between them, contained no secretory granules, neither α-granules nor very dense granules. Occasional mitochondria, canaliculi, and disorganized microtubules were discernable (Figure 5). The platelet aggregation was further verified by aggregometry. The results obtained by using heparin–PRP were the same as those using citrate–PRP.

When EDTA–PRP was employed, platelet adhesion to collagen fibers and degranulation took place as in heparin– and citrate–PRP. However, platelet aggregation was not observed (Figure 6). Treatment of the human tendon collagen fiber with pepsin had no effect on its interaction with platelets. The results are summarized in Table 2.

Discussion

Interaction of platelets with structural elements of vascular wall exhibits a high degree of specificity. Platelets adhere avidly to basal lamina, microfibrils, and collagen fibers [1,5,12-14] but not to the amorphous component of elastic fibers.[15] Following adhesion, platelets spread on the basal lamina forming a platelet pavement resembling that of endothelium; this occurs without thrombus formation.[1,5] Interaction of platelets with collagen fibers triggers the hemostatic sequence causing platelet degranulation and aggregation with ultimate formation of thrombus.[16,17] Since two

Table 2—Platelet Interaction With Human Glomerular Basal Lamina and Collagens

	Heparin- or citrate-PRP		EDTA-PRP	
	Adhesion	Degranulation	Adhesion	Degranulation
Basal lamina	+	−	−	−
Basal lamina noncollagen matrix	+	−	−	−
Basal lamina collagen matrix	−	−	−	−
Tendon collagen fiber	+	+*	+	+

* With aggregation

modes of platelet function must have such different physiologic and pathologic implications,[1] the elucidation of underlying mechanisms, especially the macromolecular components involved in the platelet-basal-lamina interaction, should shed light on determinants which may activate various modes of platelet behavior on physical substrate.

Low Platelet Reactivity With the Collagen Matrix of the Basal Lamina

Basal laminas in general consist of collagen and noncollagen types of proteins.[18,19] The two constituent sets of molecules are present in approximately equal proportion in human glomerular basal lamina.[11] In our previous report[1] we presented evidence that the purified soluble HGBL collagen, in contrast to human skin or tendon collagen, failed to induce platelet release or aggregation. This has been amply confirmed by others.[20,21] The HGBL collagen preparation disclosed a preponderance of monomeric collagen molecules on sodium dodecyl sulfate–polyacrylamide gel electrophoresis.[11] Since the native packing arrangement (quaternary structure) is required for interstitial collagens to induce platelet degranulation and subsequent ADP-mediated aggregation,[22-24] the failure of the HGBL collagen preparation to induce platelet degranulation may be due to the absence of native quaternary structure in the test condition. The native packing arrangement of the monomeric basal lamina collagen is still unknown. We have been unsuccessful, as have others,[25] in attempts to reconstitute collagen fibrils with characteristic periodicity from purified soluble HGBL collagen preparations either by thermal gelation [23,25] or by dialysis against phosphate-buffered neutral 0.17 M NaCl solution at room temperature.[26,27] However, our evidence indicates that the insoluble basal lamina collagen matrix in its native state of molecular organization can be obtained by cleavage of the noncollagen component by proteolytic enzymes that do not cleave collagen. Table 1 shows that pepsin cleavage of HGBL at 4 C preferentially removed the noncollagen component, resulting in substantial enrichment of collagen in the insoluble residue. Plate-

lets in heparin, citrate, or EDTA anticoagulant failed to adhere to the insoluble HGBL collagen matrix. The insoluble HTC similarly treated with pepsin remains as effective as untreated HTC in inducing platelet adhesions and degranulation; therefore, we conclude that the HGBL collagen, even in its native packing arrangement, lacks sufficient affinity for platelet adhesion and is incapable of triggering the platelet release reaction. A related and consistent observation has been made by Suresh, Stemerman, and Spaet,[28] who reported low platelet reactivity toward rabbit heart valve basal lamina following trypsinization of endothelium. The effect of trypsin should be similar to that of pepsin in cleaving noncollagen constituents from basal lamina.

Incubation of human tendon collagen fibers with heparin- or citrate-PRP resulted in the platelet adhesion, degranulation, and aggregation, as has been described.[29,30] Platelet adhesion to human tendon collagen fibers and subsequent degranulation proceeded normally in our system without divalent cations in EDTA-PRP, while the ADP-induced platelet aggregation requires divalent cations; these results are consistent with the findings of others. [12,30-33]

The amino acid composition of HGBL collagen (Table 1) is consistent with its being a different gene product than other collagens, although they share coiled-coil triple helical conformation characteristics of all collagen-type proteins.[34] The high specificity of the platelet-collagen-fiber interaction, requiring certain specific reactive sites in addition to the triple helical conformation of the molecule, is well established.[35,36] Our studies further emphasize the specificity of platelet-collagen-fiber interaction as it relates to the diverse biologic functions of chemically heterogeneous collagens from various tissues.

Interaction of Platelets With the Noncollagen Component of the Basal Lamina

Since peptic cleavage of noncollagen component from the HGBL completely abolishes the platelet-basal-lamina interaction and platelets adhere and spread on the noncollagen matrix as on the native HGBL, we conclude that the determinants of platelet-basal-lamina interaction reside in the noncollagen component of HGBL. The divalent cations, presumably calcium ions, are required for the interaction.

The adhesion of endothelium to basal lamina involves noncovalent molecular interactions. EDTA can readily dissociate endothelium from the basal lamina by chelating calcium ion.[37-39] The endothelium can also be dissociated from basal lamina by trypsinization, indicating that the proteins involved in endothelium-basal-lamina interaction are noncollagenous. Thus, there appears to be a similarity between platelet-

basal-lamina and endothelium-basal-lamina interactions [40] since both involve noncollagen proteins and divalent cations.

The platelet-basal-lamina interaction is a solid-phase reaction. Therefore, platelet receptor sites for adhesion and activation of the sequence of events leading to modulation of platelet behavior must be on the membrane surface. The presence of membrane surface glycoproteins, presumably membrane receptors, has been shown by lactoperoxidase-catalyzed radioiodine labeling of these molecules [41] and electron microscopic demonstration of a surface glycoprotein coat, 15 to 20 nm in thickness.[42] Based on our studies, one would expect the receptor sites for basal lamina to be distinct from those for collagen fibers due to differences in compositional and conformational characteristics of interacting ligands (noncollagen vs collagen), to the requirement for divalent cations, and to the subsequently expressed platelet behavior (spreading vs spreading and degranulation).

In its interaction with formed elements of blood, the basal lamina also exhibits a high degree of specificity. Only platelets interact avidly with basal lamina, although leukocytes are known to become very "sticky" and to adhere to endothelium in inflammatory reactions.[37] Selective interaction between cells and between cell and basal lamina is one of the fundamental phenomena in cell biology. Specificity of cell-basal-lamina interaction has also been observed in regeneration of skeletal muscle. The regenerating skeletal muscle and endothelial cells following ischemic necrosis reconstitute their respective basal lamina tubes with great accuracy.[43,44] Determinants of the specific interaction between muscle cell, endothelium, and their respective basal laminas in terms of macromolecular components of the basal lamina have not been identified. Our studies suggest that the noncollagen component may play an important, perhaps the cardinal, role in specific cell-basal-lamina interaction, while the collagen component probably serves as a mechanical backbone of the basal lamina.

Implication of Two Distinct Modes of Platelet Function

The platelet release reaction, which can be induced both by thrombin and by collagen fibers, has been shown to release factors promoting proliferation of smooth muscle cells [45,46] and may play a role in diffuse intimal thickening of elastic arteries following deliberate removal of endothelium.[47] However, in muscular arteries, such as coronary arteries, where a more complete endothelial basal lamina exists, and under physiologic turnover of endothelium or loss of endothelium due to chemical or hemodynamic injury, the predominant mode of platelet interaction with the vascular wall could be platelet-basal-lamina interaction, which does not

lead to platelet-release reaction. Therefore, extrapolation of the effect of platelet-collagen–fiber interaction to that of platelet-basal–lamina interaction may not be appropriate. The effect of platelet-basal–lamina interaction on vascular intima, which may be more relevant in understanding vascular reaction to injury, awaits further investigation.

A number of platelet inhibitors have been shown to reduce the incidence of thromboembolism in vascular diseases.[48,49] The pharmacologic effects of these agents are often evaluated *in vitro* on the basis of platelet interaction with collagen fibers. Their effects on platelet-basal–lamina interaction have not been explored. In view of the fact that the platelet pavement on the basal lamina may be a temporary substitute for the function of an endothelial lining and does not seem to lead to thrombosis, preservation of platelet-basal–lamina interaction by selective inhibition of platelet-collagen–fiber interaction may prove beneficial to patients undergoing long-term antiplatelet therapy. Based on our studies, the task seems feasible since the underlying mechanisms involved in the interactions are so distinct.

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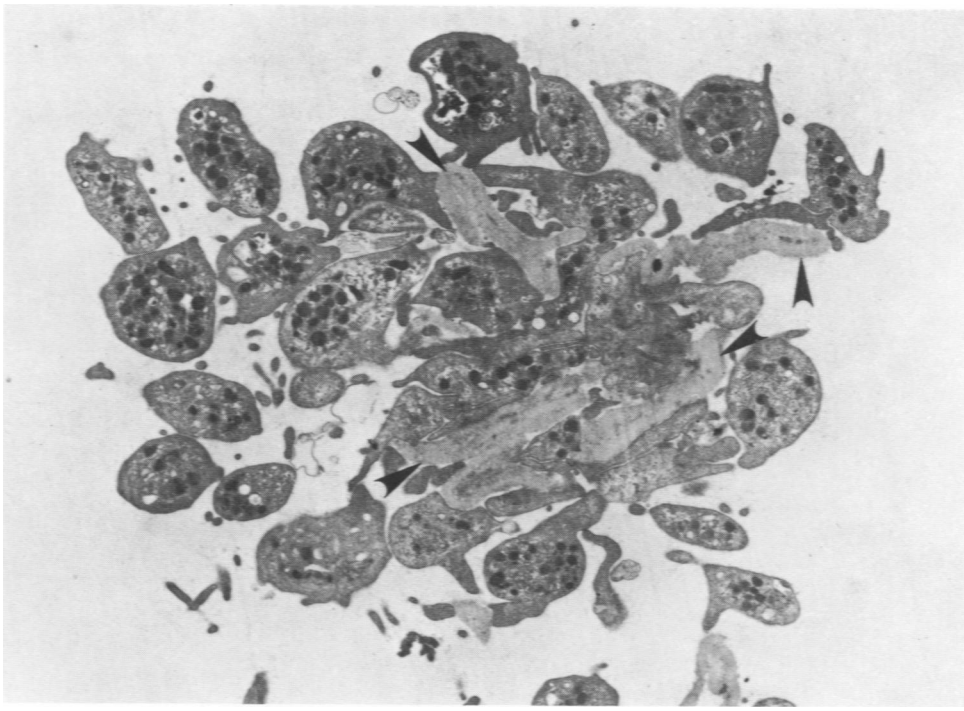

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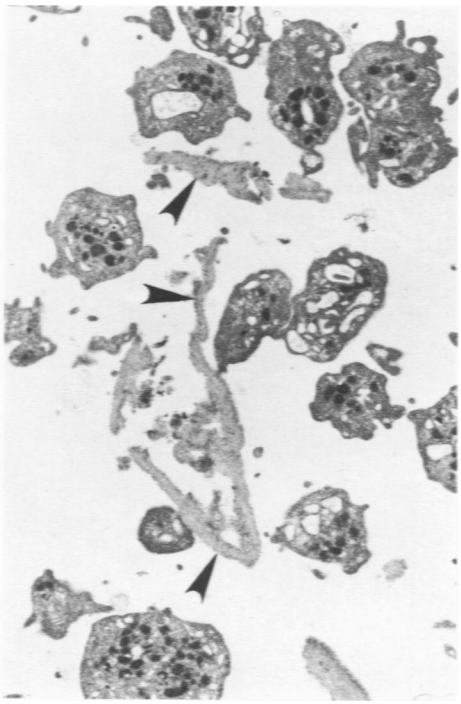

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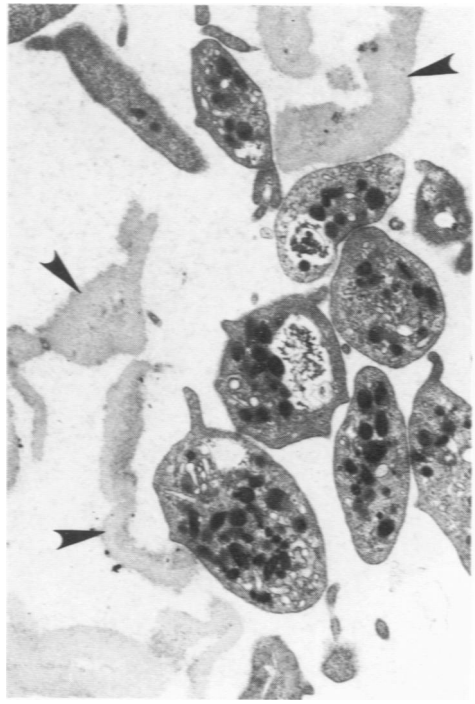

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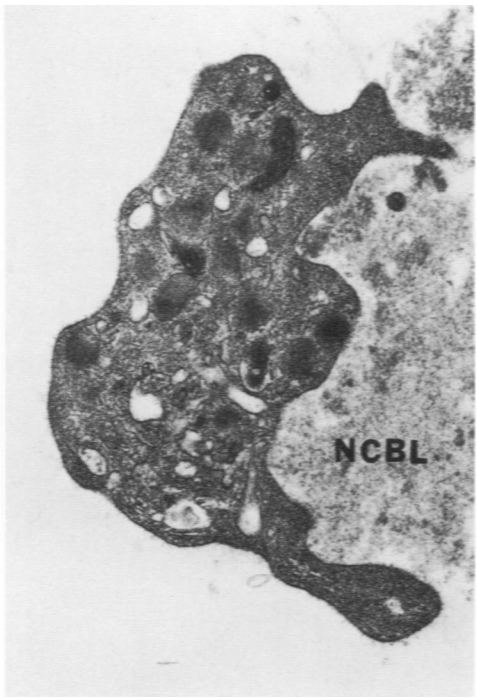
Figure 1—Platelet-basal-lamina interaction. Platelets adhere to and spread on basal lamina (*arrowheads*) without degranulation or aggregation. (Heparin-PRP, ×6000) **Figure 2**—Platelets fail to adhere to basal lamina (*arrowheads*) in the absence of divalent cations. (EDTA-PRP, ×5300) **Figure 3**—Platelets exhibit low reactivity toward collagen matrix of basal lamina (*arrowheads*). Platelets make physical contact without active adhesion or spreading as in Figure 1. Platelet granules are intact. (Heparin-PRP, ×8800)



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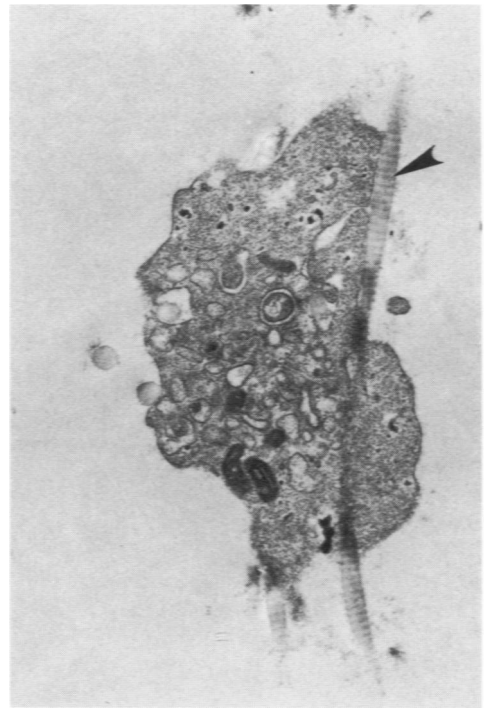

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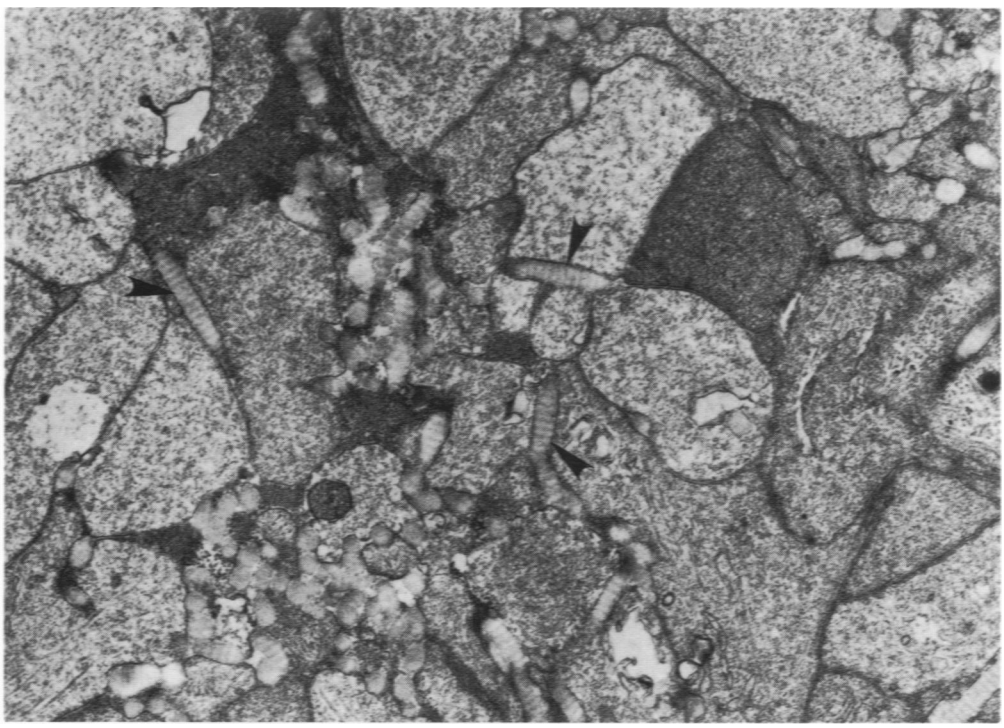

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Figure 4—Adhesion and spreading of a platelet on noncollagen matrix of basal lamina (*NCBL*). There is no platelet degranulation or aggregation. (Heparin-PRP, ×23,300) **Figure 5**—Platelet-collagen-fiber interaction. Platelet degranulation and aggregation are apparent. *Arrowheads* indicate collagen fibrils. (Citrate-PRP, ×11,600) **Figure 6**—Platelet-collagen-fiber interaction in the absence of divalent cations. Platelet adhesion, spreading, and degranulation proceed normally, although aggregation is not observed. *Arrowhead* indicates collagen fibril. (EDTA-PRP, ×17,000)