Thrombogenesis of the Rabbit Arterial Plaque

An Electron Microscopic Study

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Rabbit arteries, de-endothelialized with an intravascular balloon catheter and allowed to heal for 4 weeks, showed intimal changes that were similar to the preatherosclerotic fibromusculoelastic plaques of man. Reinjury of the healed vessels by balloon catheter produced marked quantitative and qualitative alterations of hemostasis, as compared to that in previously uninjured vessels. The most apparent modification of thrombogenesis 10 minutes after injury to the plaque was a large increase in the size of the thrombotic deposits. Features of this exaggerated response were the major participation of fibrin in thrombus formation and greater platelet accumulation. Some platelets and fibrin strands appeared to penetrate into and beneath the neointima. By 3 hours, these deposits had diminished in size, although the hemostatic mass remained larger in the doubly injured vessels (Am J Pathol 73:7–26, 1973).

WHEN THE ARTERIAL VESSELS of rabbits and monkeys are deprived of their endothelium, profound early and late intimal changes are known to occur.[1-4] Diffuse, highly reproducible, selective de-endothelialization can be readily achieved by rapid passage of a balloon catheter, inflated to modest pressures, through the vessel lumen.[1,4,5] Removal of the endothelium results in the exposure of intimal connective tissue to the circulating blood. The first observable event of thrombogenesis is adhesion of platelets to this connective tissue surface. Ten minutes after the balloon injury, the de-endothelialized intima is covered by a layer of platelets which have spread upon this surface and have lost their intracytoplasmic granules.[1,4,6] This mural platelet accretion is virtually free of fibrin and is usually not greater than two platelets in depth. Occasionally, accumulations of larger platelet aggregates are seen randomly distributed on the de-endothelialized intimal surface, forming nonocclusive mural platelet thrombi. In this study, thrombi are defined as adhering aggregates of platelets, with or without associated fibrin deposition.

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Soon after the platelet cover has formed, polymorphonuclear and mononuclear leukocytes are found on the luminal aspect of the adhering platelets. The leukocytes do not migrate from the lumen into the media, and no morphologic alteration is noted in the media, indicating that damage to the vessel has been confined to the intima.[1,4] By approximately 7 days, the vessel has been relined with cells similar to endothelial cells, and few remnants of the platelet deposit can be identified morphologically.[1,2,4] However, the intima, instead of reverting to its previous state, becomes thickened at least partly by the luminal migration of medial smooth muscle cells across the internal elastic lamina.[4]

In the present study, the intima was examined 4 weeks following endothelial removal, at which time the hyperplastic intima approximated the media in thickness. Morphologic features of this rabbit "plaque" are reminiscent of the preatherosclerotic fibromusculoelastic intimal change of man.[4,8–11] Removal of plaque endothelium by a second balloon catheter injury induced a thrombogenic response notably different from that of vessels injured only once. Major features of thrombus formation following the second injury which differed from the first injury included: a) a significant increase in the number of mural platelet thrombi and b) an accumulation of large amounts of fibrin. These observations suggest that a previously injured vessel provides a surface for an augmented thrombotic response.

Materials and Methods

Endothelial Removal

Fourteen New Zealand rabbits, weighing between 2.2 to 3.1 kg, were anesthetized with intravenous pentobarbital, 5 mg/kg, supplemented with ether as needed; the vascular endothelium was dislodged by passage of a balloon catheter.[1,4–6] The right femoral artery was exposed and cannulated with a 3F modified thin-walled Fogarty Embolectomy Catheter (balloon wall thickness .006 inches, Edwards Laboratory, Santa Ana, Calif). With the catheter in the artery, the balloon was inflated with air to a pressure of approximately 450 mmHg. The catheter was then promptly and rapidly pushed through the right femoral artery and the right iliac artery into the abdominal aorta to approximately the level of the diaphragm and was quickly withdrawn. The entire transit time for the inflated balloon was less than 10 seconds. The balloon was deflated once it had again entered the right femoral artery. The wound was sutured, and the lesion was allowed to heal for 4 weeks, at which time a similar balloon catheterization was performed from the left side. These procedures left the animals with: a) a 4-week healed lesion of the right iliac artery, b) a fresh lesion of the left iliac artery and c) a 4-week healed injury of the aorta with a superimposed fresh injury. At 10 minutes or 3 hours following the second procedure, the animals were anesthetized, and sacrificed by perfusion fixation through their abdominal aortas,[12] and their arteries were processed for examination by light and electron microscopy.

Control aortas and iliac arteries were obtained from sham-operated rabbits perfused with fixative in a similar manner.

Electron Microscopy

The abdominal aorta and both common iliac arteries were prepared for examination by perfusion fixation with 0.1 M cacodylate-buffered 2.5% gluteraldehyde–2% paraformaldehyde [4,13] for 20 minutes at 120 mmHg pressure and room temperature. This and all other procedures were carried out at pH 7.4. The arteries were carefully dissected free and were immersed in the same fixative at 4 C for 1 hour. The vessels were cut into 1-mm rings and returned to the fixative for another hour. They were placed in 7% sucrose–0.1 M cacodylate buffer overnight and washed repeatedly in 0.1 M cacodylate prior to postfixation. The tissues were postfixed in 2% osmium tetroxide buffered with cacolylate for 1 hour, followed by *en bloc* staining with 1% uranyl acetate for one-half hour. The rings were dehydrated through ethanol and were flat embedded in Epon 812.

Approximately 1-μ cross-sections of entire vessel rings were stained for light microscopy with basic fuchsin–methylene blue.[14] Morphometric measurements were made on these sections, and areas of interest were selected for thin sectioning and examination by electron microscopy. The thin sections were stained with 6% uranyl acetate followed by lead hydroxide and were examined in a Siemens 1-A Elmiskop electron microscope.

Morphometrics

Multiple .8-μ sections from each experiment were evaluated by phase contrast and bright field microscopy for mural platelet thrombotic accumulation.[15] Only thrombi with platelets spread upon and conforming with the vessel surface and with platelets in close association with each other were counted. Rare vessel cross-sections with any remaining endothelium were excluded. The accuracy of the evaluation was confirmed by electron microscopy of selected areas from each of the thick sections. The platelet thrombi on each vessel ring were evaluated by identifying three different types, depending upon size and number of platelets. The three types were as follows: a) aggregates of three to five platelets, not extending more than 4.5 μ from the surface; b) aggregates of six to ten platelets, not extending more than 10 μ from the surface and c) aggregates of greater than ten platelets, measuring over 10 μ from the surface. These criteria are summarized in Table 1.

The accumulation of platelet thrombi was expressed as the percentage of de-endothelialized surface covered. For every vessel ring, the circumference and the surface covered for each platelet type were measured in microns. The percentage of surface covered by each platelet thrombus type was determined by dividing the length covered by the vessel's circumference. The total percentage of denuded surface covered by platelet thrombi was the sum of these percentages. Measurements of the rings were initially attempted in a double-blind fashion, but this was soon discontinued because the different types of vessels could easily be identified by their characteristic morphology. Thirty-six vessel rings were counted, and the averages for these determinations are seen in Text-figure 1.

Results

Uninjured Vessels

The morphology of the abdominal aortas and iliac arteries was similar to that previously described.[6,12] The intima is the innermost layer of

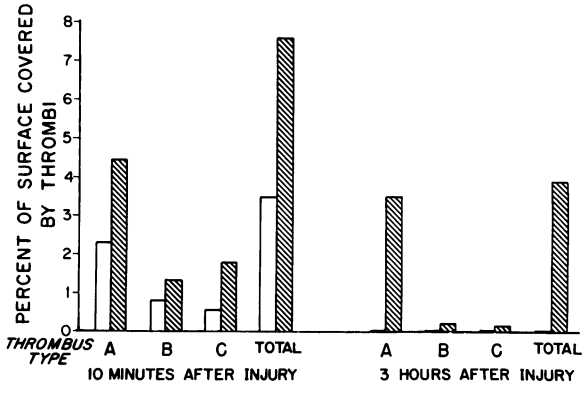
Table 1—Characteristics of the Three Thrombus Types

Thrombus type	No. of platelets	Thrombus height (μ)
A	3–5	2.5–4.5
B	6–10	4.6–10
C	>10	>10

the vessel, being composed of those elements between the lumen and the internal elastic lamina. In the uninjured rabbit arteries, this zone was composed of the endothelial cells and the connective tissue residing between the endothelial cells and the internal elastic lamina (Figure 1). The connective tissue components of this zone consisted of: a) vascular basement membrane, b) microfibrils of elastin and c) elastin. The vascular basement membrane was discontinuous, being present in some regions and completely absent from others.[6,12] The microfibrils were found to be present over the entire surface of the internal elastic lamina. The elastin of the intima was continuous with the internal elastic lamina and was considered the dividing line between the intima and the internal elastic lamina. Only rare collagen fibrils were seen in the connective tissue of the normal intima. The endothelial cells formed junctions with one another and were characterized by the presence of rod-shaped tubular bodies.[16]

Plaque

Four weeks after de-endothelialization with a balloon catheter, the healed intima of the right iliac arteries was markedly thickened, varying from ten to fifteen cells in depth, and extended from the vessel lumen to the internal elastic lamina. The luminal surface of the vessel was lined by a continuous layer of endothelial cells. These cells



TEXT-FIG 1—Percent of denuded surface covered by thrombi. (Single injury, *open bars;* double injury, *hatched bars*).

contained the usual number of pinocytotic vesicles and organelles, including rod-shaped tubular bodies;[16] they formed junctional complexes with each other.[4] Usually, large amounts of rough endoplasmic reticulum were scattered throughout the endothelial cells, and occasional myelin figures appeared near the abluminal surface. Such myelin figures were not present in control vessels.

The neointimal connective tissue in the healed lesion consisted of a granular basement-membrane-like material,[17] collagen fibrils and elastin fibers with associated microfibrils. These elements were distributed in a characteristic manner, with collagen being furthest from the lumen and near the internal elastic lamina and basement membrane closest to the lumen; elastin was distributed evenly throughout the neointima. In this arrangement, the connective tissue components just beneath the endothelium were individual elastin fibers intermixed with basement membrane (Figure 2). Collagen fibrils were not present in the intimal connective tissue layer close to the endothelium.

The predominant cells of the newly formed neointima were smooth muscle cells.[4,18] These cells were typical in appearance except for increased amounts of rough endoplasmic reticulum in those cells closest to the lumen. The smooth muscle cells contained myofilaments and were surrounded by an incomplete basement membrane. The arrangement of these neointimal smooth muscle cells was without pattern in relation to the vessel axis; they contained no lipid vacuoles.

The original internal elastic lamina was easily identified and was intact. There was no morphologic evidence of cell death, nor was debris noted on either side of the internal elastic lamina. The smooth muscle cells of the media retained their typical orientation to the vessel.

Ten Minutes Following Primary Endothelial Injury

The changes in the left iliac artery were those which resulted from a single injury. Ten minutes after balloon injury, the endothelium was absent. In its place was an almost continuous layer of adhering platelets (Figure 3) in close proximity to the exposed intimal connective tissue. The space between the platelet membrane and connective tissue varied from 200 to 400 Å. The adhering platelets contained few granules; their disc shape was lost, and they appeared to be irregularly flattened, spread upon and conforming to the intimal connective tissue surface, which was itself contiguous with the internal elastic lamina. Mural platelet thrombi, as defined previously, were randomly scattered over the surface, and were easily distinguished from the monolayer of platelets that covered most of the de-endothelialized intima (Figure

4). The platelet thrombi consisted of tightly packed aggregates which were close to the exposed intimal connective tissue. Only aggregates which included three or more platelets were designated as platelet thrombi. Platelets furthest from the vessel wall retained more intracytoplasmic granules and were more disc-shaped than the platelets nearer the vascular surface.

Fibrin was not seen at the de-endothelialized intimal surface. Fibrin strands intermixed with platelets could be identified in the mid-portion of B- and C-type thrombi; fibrin was rarely seen in the A type.

There was no morphologic evidence of medial injury—no cell debris or leukocyte migration was seen.

Ten Minutes Following Plaque Endothelial Injury

Ten minutes after balloon injury to vessels with healed intimas, the endothelium was no longer present. Instead, there was exposed intimal connective tissue irregularly covered by thrombotic accumulations consisting of platelets intermixed with fibrin strands (Figures 5 and 6). The platelets of the basal adhering layer, those platelets closest to the vessel, conformed to the highly irregular exposed intimal connective tissue of elastin fragments and vascular basement membrane, and they extended pseudopods into this connective tissue layer (Figure 7). Where de-endothelialization exposed the basement membrane of smooth muscle, platelets accumulated; in contrast, in the occasional regions where naked smooth muscle cells without basement membrane were directly exposed to the lumen, platelets were absent. Morphologically, the adhering and aggregating platelets shared the same morphologic characteristics as those of the once-injured vessels.

Fibrin appeared to participate to a greater extent in thrombus formation in twice-injured vessels (Table 2). Fibrin strands were intermixed with the platelet accretions and were often interposed between the exposed intimal connective tissue and adhering platelets. In addition, fibrin strands with typical electron microscopic characteristics were seen beneath the exposed intimal connective tissue, penetrating as much as 1 μ into the connective tissue (Figure 8). These findings were not seen in once-injured vessels, where the only fibrin strands seen were those associated with the mid-portion of some platelet thrombi. At places, the area immediately abluminal to the exposed intimal connective tissue appeared to be edematous. Moreover, there was evidence of smooth muscle cell damage, with mitochondrial swelling, as well as loss of pinocytotic vesicles and cytoplasmic lucency. However, there was no leukocyte migration from the lumen at 10 minutes, and the general arrangement of the neointimal elements did not ap-

Table 2—Comparison of the Thrombogenic Response of the Subendothelium

	Thrombi at 10 minutes	Thrombi at 3 hours	Fibrin	Platelet adhesion
Once injured	Present	Absent	Absent at subendothelial surface	Spread and conform to IEL
Healed reinjured (plaque)	Very plentiful (three times as plentiful as once-injured vessel)	Present	Present at the subendothelial surface	Penetrate into the subendothelium

pear to be disturbed; the cells beneath the edematous layer remained intact.

Three Hours Following Primary Endothelial Injury

Three hours after endothelial removal by the balloon catheter, platelets covered the denuded surface. These platelets were spread on the intimal connective tissue, and they retained few intracytoplasmic granules. Polymorphonuclear leukocytes (PMNs) were seen at the luminal surface of the internal elastica, as previously described.[1,7] The platelet layer was morphologically similar to that in the 10-minute injury, except that platelet thrombi were not present (Table 2). These vessels showed no morphologic evidence of medial injury, either by cell necrosis or leukocyte migration, and the internal elastic lamina remained intact (Figure 9).

Three Hours Following Plaque Endothelial Injury

Three hours after the second balloon injury, platelets covered most of the de-endothelialized surface. Small areas of the exposed intimal connective tissue were covered only by fibrin strands. The number of thrombi had diminished, but those remaining were similar morphologically to those seen in the 10-minute plaque injury (Figure 10). Some smooth muscle cell disruption was seen in the region immediately beneath the luminal layer of connective tissue.

PMN's appeared with less frequency in the 3-hour plaque-injured vessels as compared to the 3-hour once-injured preparations. There were occasional PMNs seen within the thrombotic mass, and some were near the luminal surface of the neointima.

Quantitation of Thrombus Deposits

In all lesions except the 3-hour double injury, there was almost complete covering of the denuded surface by hemostatic material. These

3-hour plaque lesions showed occasional small areas either entirely free of platelets and fibrin, or covered only by fibrin strands (Table 2). The constituents of the intimal connective tissue in these regions were morphologically the same as those found elsewhere.

By either bright field or phase microscopy, whole platelets could be identified, and the width and height of the thrombotic accumulation could be measured. As shown in Text-figure 1, after 10 minutes there was increased platelet deposition on the double-injured vessels and an increase in every designated platelet thrombus type. In the 3-hour lesions, no platelet thrombi were present on the once-injured vessels. At this interval after injury, platelet thrombi were seen in the double-injured arteries, but they were diminished as compared to those of the 10-minute injury. It is to be noted that comparison was made between the twice-injured aortas and the once-injured iliac vessels. The luminal diameters of the aortas were about twice those of the iliacs in the uninjured state but approximated the size of the uninjured iliacs after injury and healing. They remained somewhat larger than the uninjured iliacs, but this minor difference in luminal circumference could not account for the marked increase in the surface covered by the platelet thrombi.

Discussion

Endothelial cells provide the vasculature with a nonthrombogenic surface.[19] When the endothelium is removed, cellular elements of the blood react with the underlying connective tissue and form a thrombus. The immediate and late morphologic events of thrombus formation and resolution have been detailed for intima not previously traumatized.[1–5,7] After 4 weeks of healing, a fibromusculoelastic plaque that resembles a human arteriosclerotic plaque covers the lumen of the rabbit vessel. Similarities include the morphology of the cellular and extracellular elements involved in the intimal change,[6,8,18,20] as well as similarities in glysocaminoglycans and collagen content.[21]

The present observations indicate that plaque de-endothelialization induces a notable enhancement of the thrombotic response. In both previously injured and uninjured vessels, there is a virtually uninterrupted cover of platelet material on the exposed intima. However, the exposed surface of the doubly-injured vessel is covered by a much larger and more persistent hemostatic mass. Ten minutes after injury, the exposed surface of the once-injured vessel is the site of platelet thrombi, but by 3 hours, this surface, although still covered by a monolayer of platelets, is free of significant aggregates. The dy-

namics of thrombus formation differ in the doubly-injured vessel. With the second injury, platelet thrombi are more numerous and are still present 3 hours after injury. Although the doubly injured vessel continues to show platelet thrombi at three hours, occasional zones of the de-endothelialized intima show fibrin strands without platelets. The lack of platelet cover in these regions is most readily explained by embolization. Whether damage to the healed intima results in greater embolization than from the once-injured vessel remains to be determined.

The alteration in thrombogenesis of the plaque may be related to an interplay of the following: a) alteration of flow, b) enhanced porosity of the altered intimal connective tissue, c) blood contact with a highly reactive connective tissue component and d) availability of clot-promoting tissue substances.

Interruption in the usual flow pattern appears to enhance the deposition of blood hemostatic material.[22,23] Fibrin in the once-injured vessel is morphologically evident only within platelet aggregates, and this is not seen 3 hours after de-endothelialization. This contrasts with the extent of the fibrin formation within the platelet thrombi of the injured neointima. In this lesion, generous amounts of fibrin are seen in both the 10-minute and 3-hour stage of thrombus evolution. Arterial flow under usual conditions is considered to be streamlined; irregularities in the vessel lumen can alter streamlining and may even create areas of low flow known as "captured vortices."[23-26] Disruption of the endothelial cover in the doubly injured vessels exposes a highly irregular surface which may be sufficiently deformed to generate such flow disturbances. Static regions, which preclude the scouring effect of high flow, might thereby promote the accumulation of hemostatic materials, including activated clotting factors.[26]

In the healed lesion, adhering platelets have no access to the relatively "smooth" and continuous internal elastic lamina; instead they cover intimal connective tissue consisting of individual units of small elastic fibers and basement membrane. The adhering platelet layer penetrates this surface by extending "pseudopods" deep into the intimal connective tissue, suggesting that it is spongy. The deposition of fibrin within the plaque intimal connective tissue is further evidence of its highly porous nature. Fibrin strands are intermixed with the adhering platelet layer and also appear to be buried within the exposed intimal connective tissue, as noted. This porous quality may act to sequester platelets as well as circulating procoagulants, thus promoting the formation of fibrin.

Tissue factor antigen has recently been reported to be associated with cellular elements of the vasculature.[27] Lack of fibrin formation at or beneath the intimal connective tissue in the once-injured vessel may be explained by a structural barrier formed by the adjacent internal elastic lamina. The neointima of the healed vessel has no comparable nearby structure, and its absence might permit more ready access to tissue factor or other factors promoting coagulation contained within the vessel, especially if blood products are trapped by the porous connective tissue.

Another possible factor contributing to the enhanced thrombogenicity may be a neointimal connective tissue component, not found in the virgin vessel, which may enhance the accumulation of platelets and the formation of fibrin. Basement membrane is more prominent in the 4-week lesion, but rabbit basement membrane preparations show little affinity for platelet deposition.[28] Collagen fibrils, among the connective tissue materials studied, are uniquely reactive with platelets,[6,12] and can initiate blood coagulation by activation of factor XII;[29] however, these fibrils are easily recognized by electron microscopy and are not associated with the exposed intimal connective tissue of the fibrous plaque. Perhaps there is a connective tissue material that was undetected by electron microscopy with the physiologic characteristics of collagen fibers. A glycoprotein might act as a nidus for platelet thrombus formation and be unobserved with the usual electron microscopic technics. The role of this group of materials in hemostasis is unclear at this time.[22,30,31]

The results of this study indicate that thrombus formation is augmented by an intima which has been injured and allowed to heal. The healed intima appears similar to the arteriosclerotic lesion of man, which may be a prodroma of the atherosclerotic plaque.[9,32,33] This indicates that the connective tissue changes which accompany arteriosclerosis and atherosclerosis may predispose the affected vessel to the development of significant thrombosis or occlusion.

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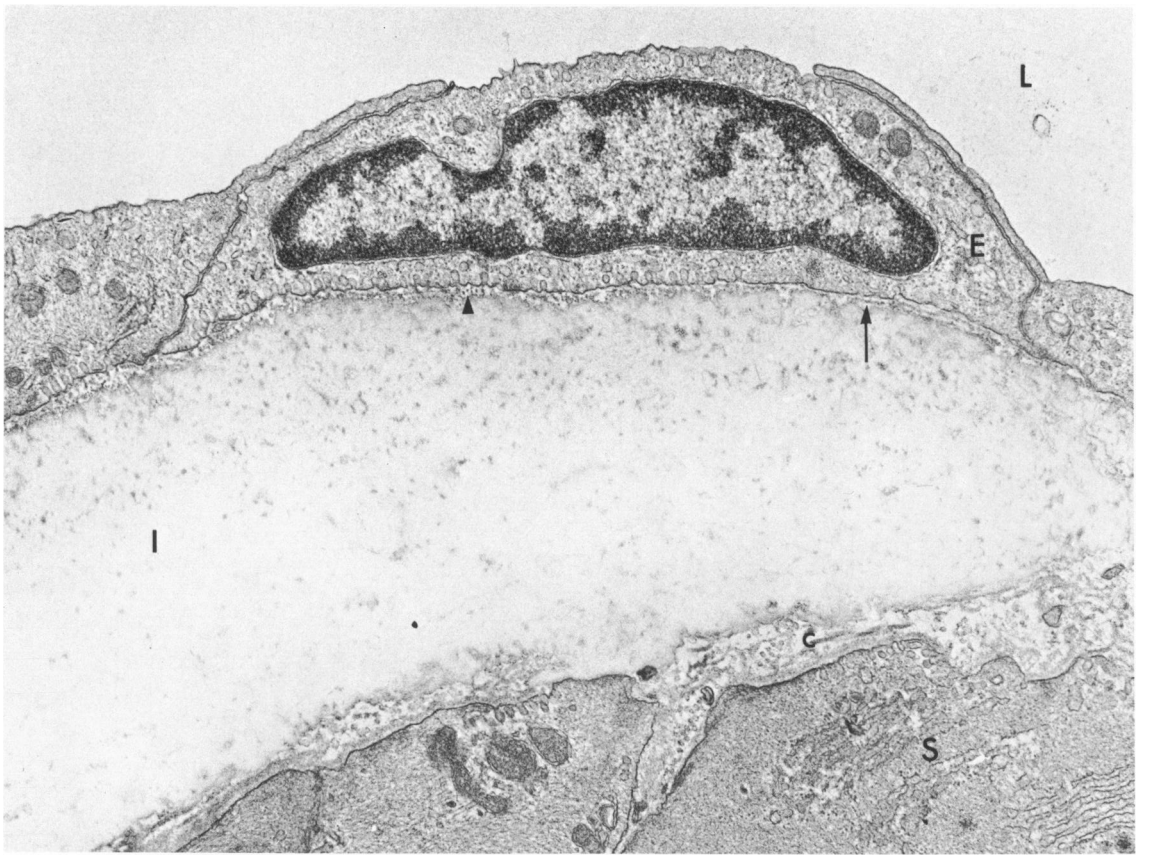

Fig 1—Electron micrograph of an uninjured rabbit iliac artery. The lumen (*L*) is at the top of this and all other illustrations. The endothelium (*E*) rests on intimal connective tissue consisting of microfibrils (*small arrowheads*), basement membrane (*arrow*) and elastin which is contiguous with the internal elastic lamina (*I*). Beneath the internal elastica are collagen fibers (*c*) and smooth muscle cells (*S*) (× 18,000).

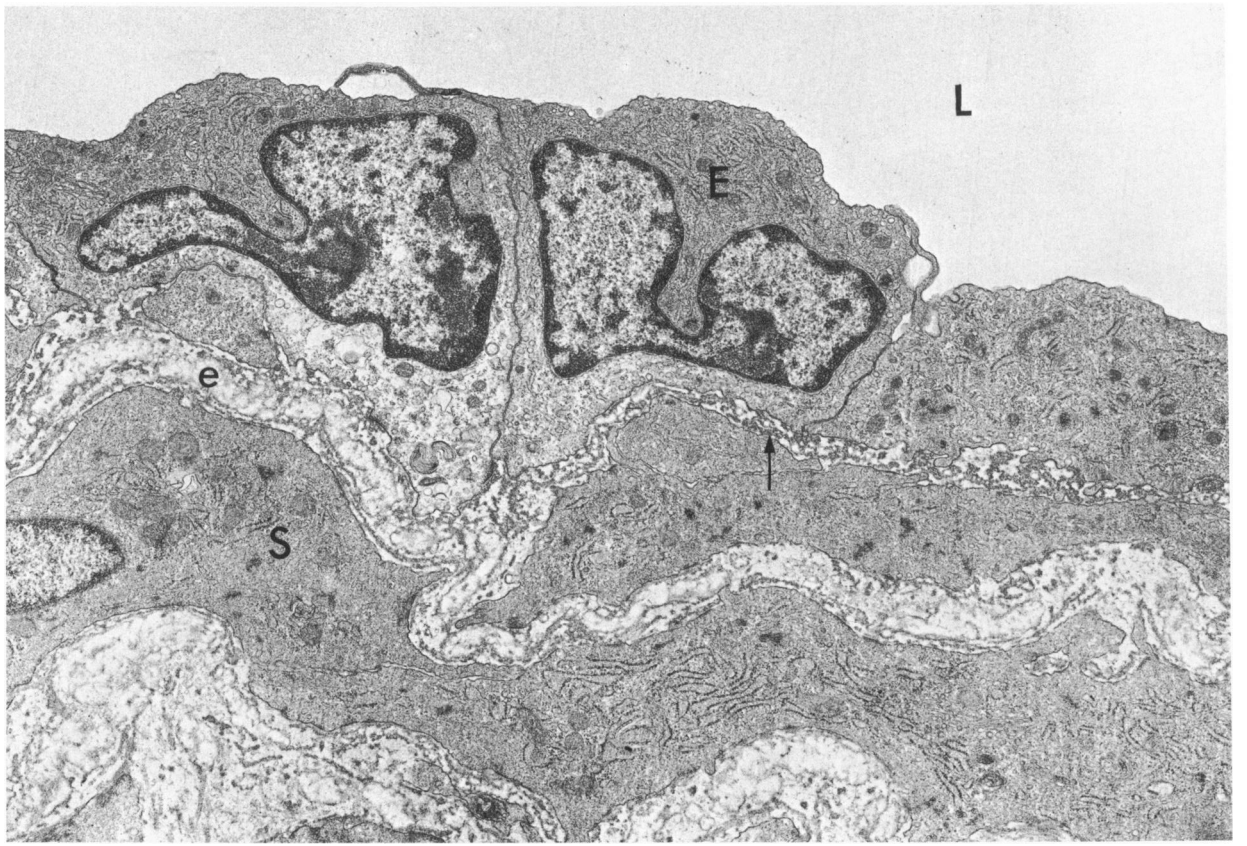

Fig 2—Electron micrograph of a rabbit plaque 4 weeks after endothelial desquamation. The endothelial cells (*E*) covering the plaque contain large quantities of rough endoplasmic reticulum, few pinocytotic vesicles and extensive areas of cytoplasmic lucency. The intimal connective tissue beneath the endothelium consists of fragments of elastin fibers (*e*) and basement-membrane-like material (*arrow*). Smooth muscle cells (*S*) with large complements of rough endoplasmic reticulum, free of lipid, are positioned beneath the proximal connective tissue layer and are themselves surrounded by an incomplete basement membrane and other extracellular elements of the neointima. This picture should be compared to Figure 1 for the morphologic alteration in the intima following endothelial injury and healing (× 10,000).

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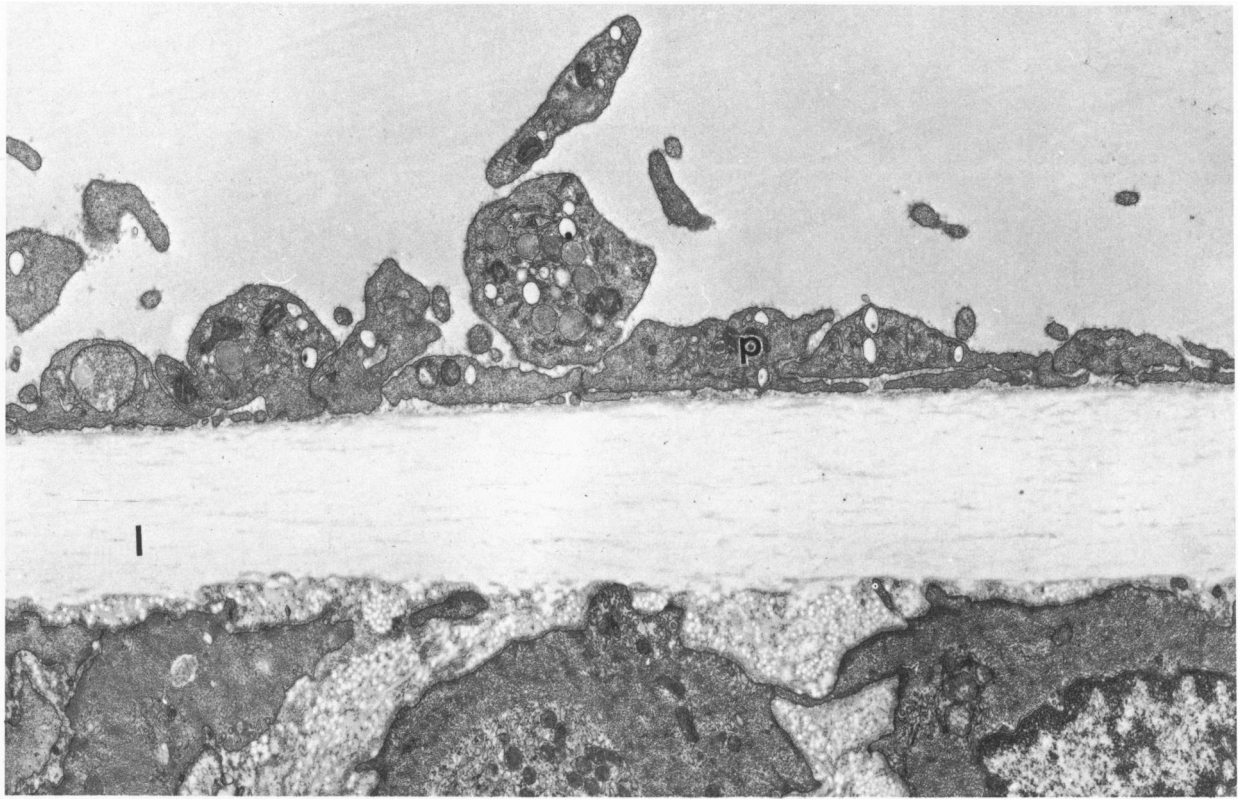

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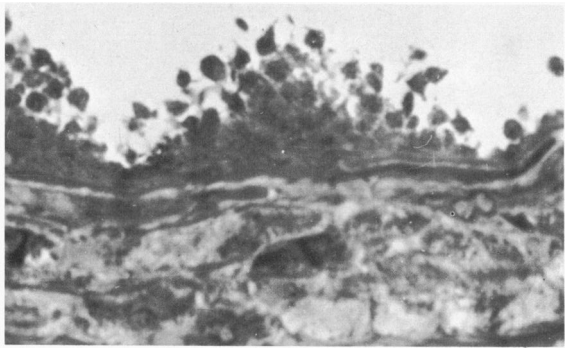

Fig 3—Platelet layer closely adhering to the exposed connective tissue of a vessel 10 minutes following balloon removal of the endothelium. The platelets (*p*) spread onto and conform with the surface of the internal elastic lamina, IEL, (*I*). The IEL and the smooth muscle cells lying beneath it are intact (× 12,000). **Fig 4**—Light micrograph of a 0.8-μ Epon section showing thrombus formation on a once-injured vessel (× 1500).

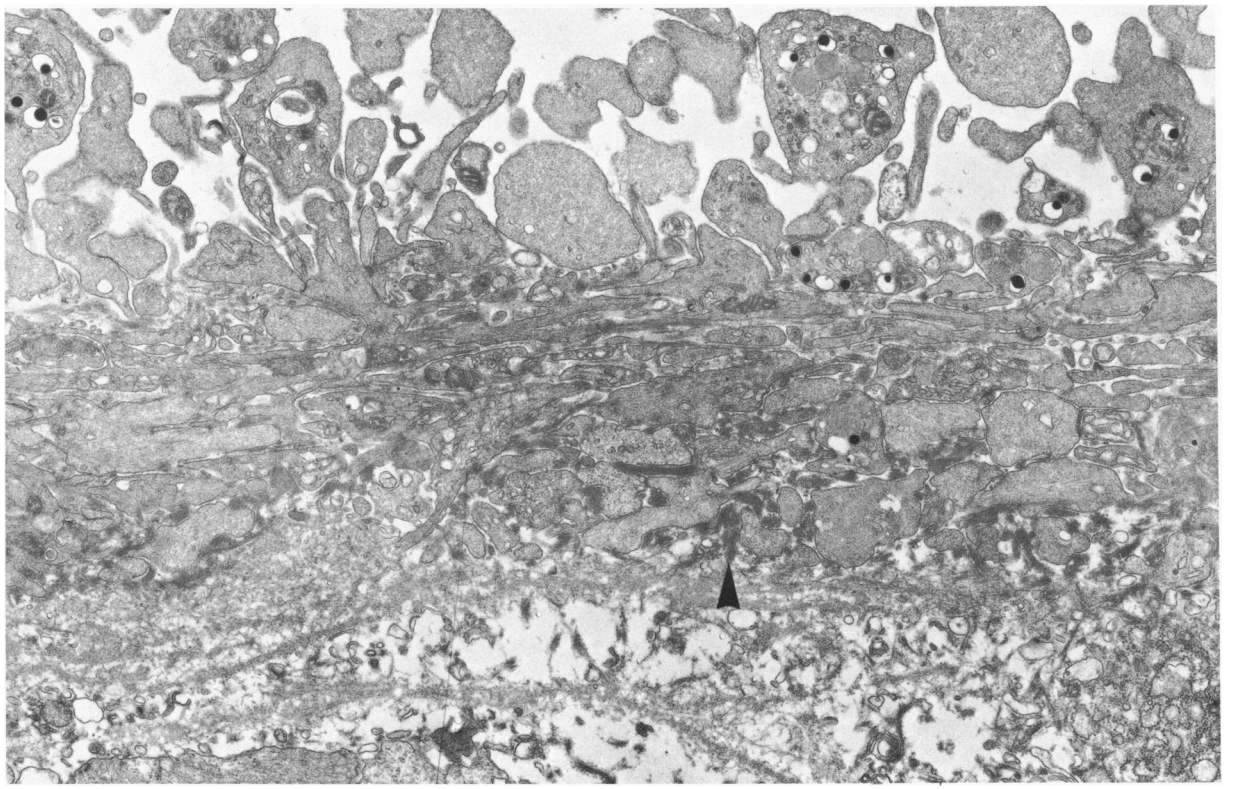

Fig 5—Electron micrograph of plaque 10 minutes following endothelial injury. There is a dense platelet layer covering the exposed connective tissue. In contrast to Figure 1, fibrin strands (*arrowhead*) are found mixed with this platelet mass and are found contacting the exposed connective tissue (× 12,000).

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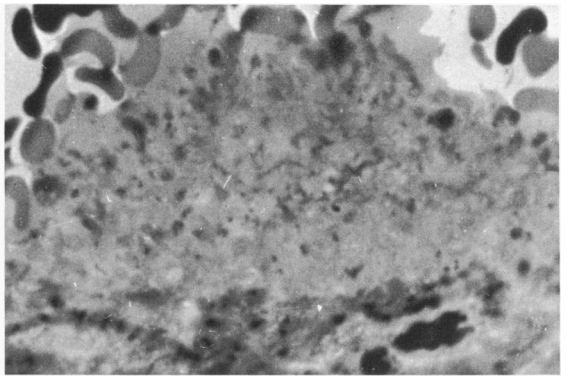

Fig 6—Light micrograph of a region similar to Figure 3, the large thrombus is resting on the plaque connective tissue and can be compared to Figure 2 (× 1500). **Fig 7**—Electron micrograph showing platelets adhering to the plaque connective tissue and extending a pseudopod into and beneath it (× 30,000).

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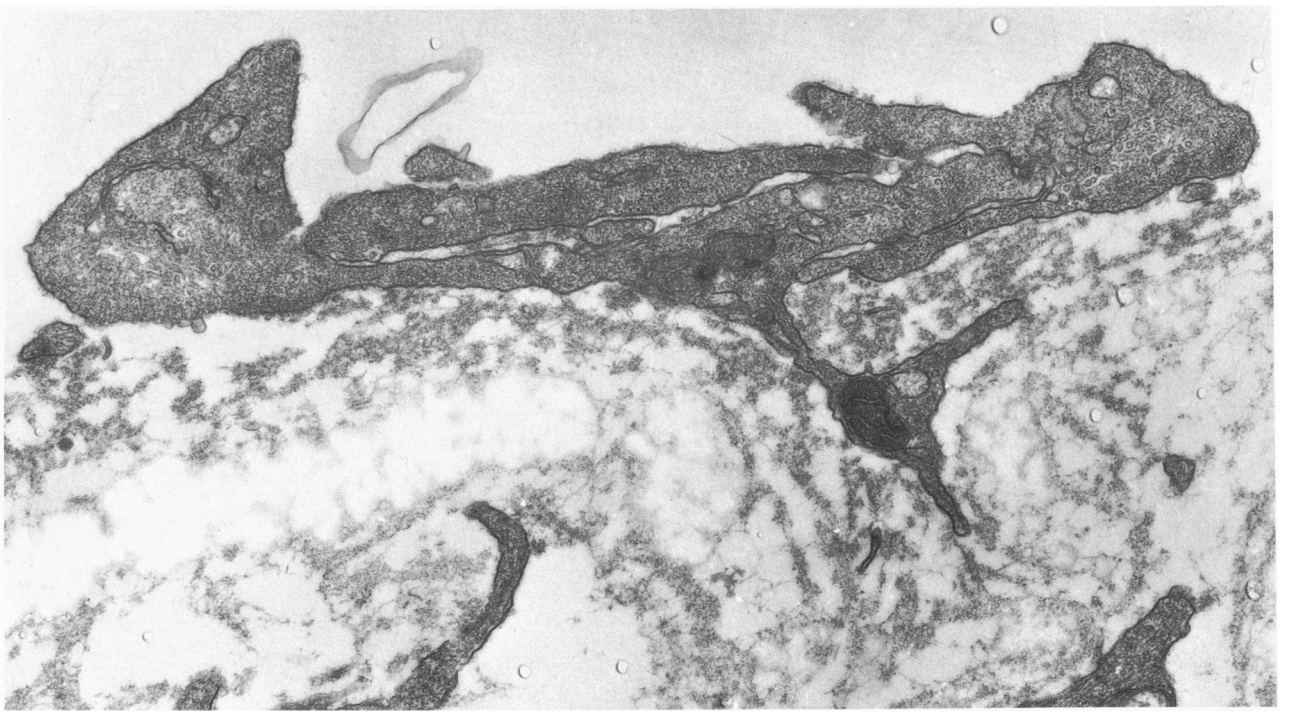

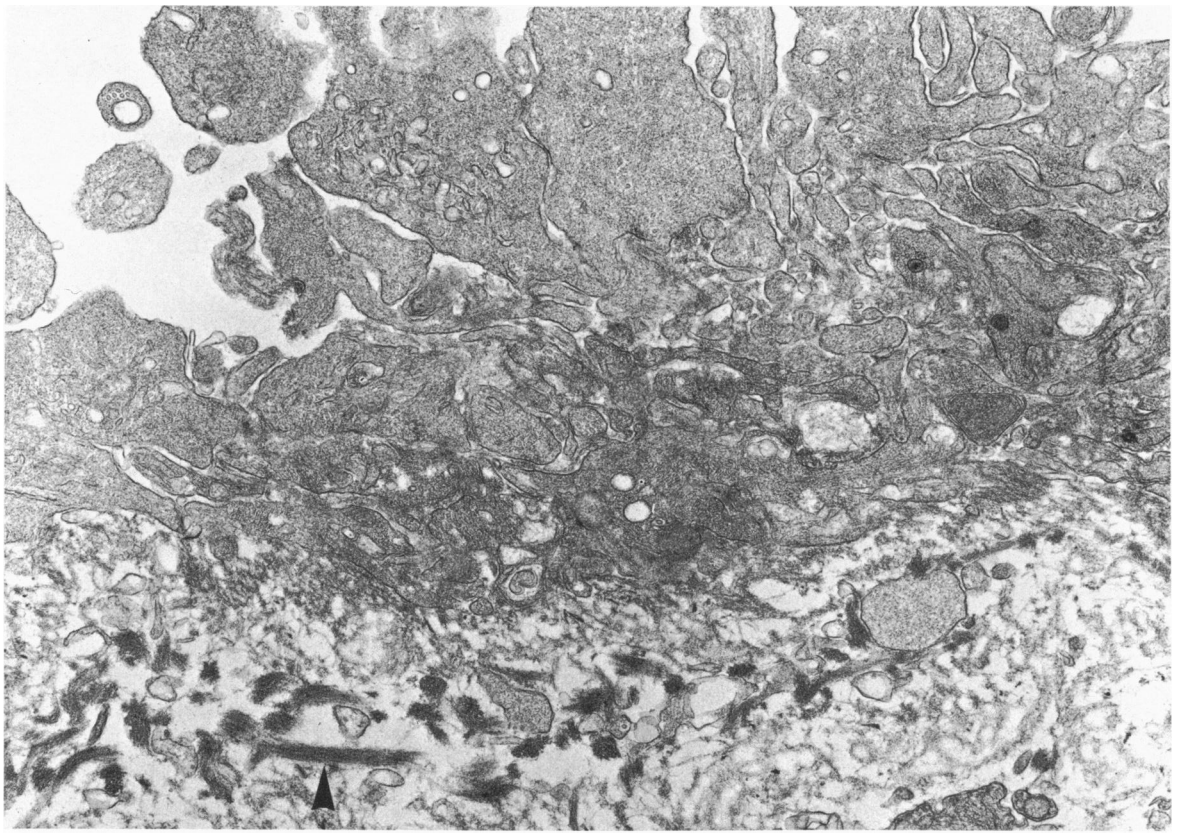

Fig 8—Electron micrograph of plaque 10 minutes after de-endothelialization. A large thrombus covers the exposed connective tissue and within and beneath it are seen strands of fibrin (*arrowhead*) with typical electron microscopic appearance (× 20,000).

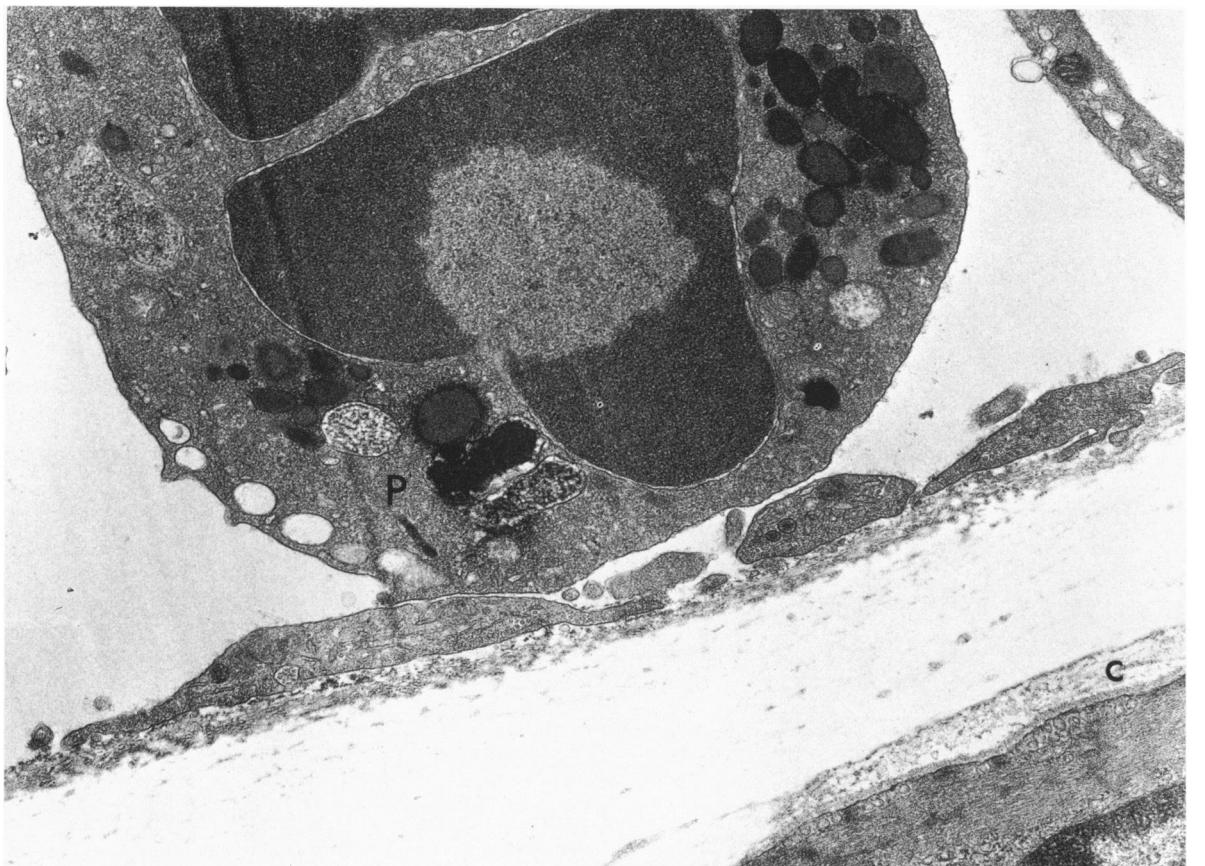

Fig 9—Electron micrograph of a once-injured vessel 3 hours after de-endothelialization. A monolayer of platelets, with gaps between them, covers the exposed IEL. No fibrin strands are seen. A polymorphonuclear leukocyte (*P*) is seen on the lumenal side of the adhering platelets. Beneath the IEL are collagen fibers (*c*) and a smooth muscle cell which appears morphologically intact (× 20,000)

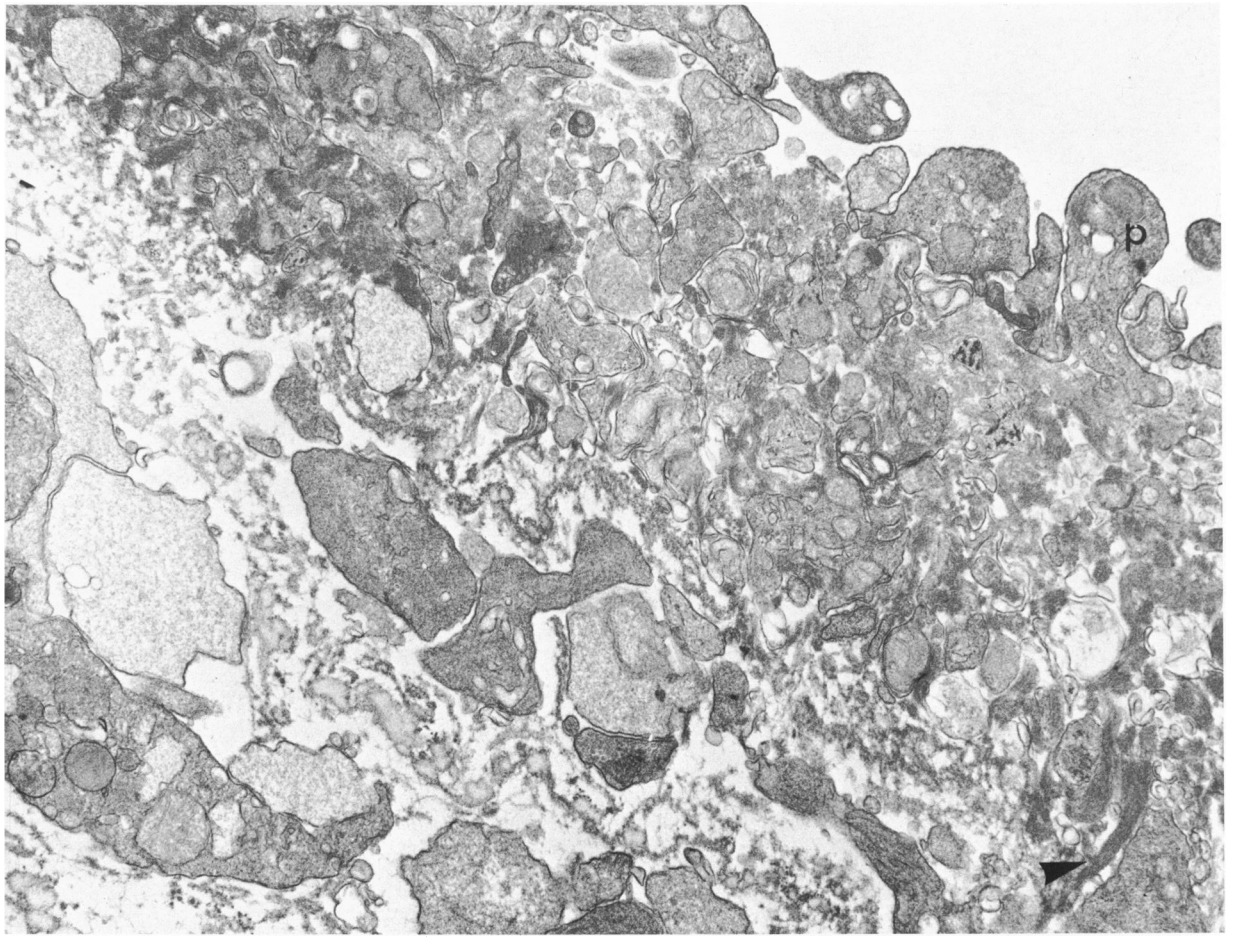

Fig 10—Electron micrograph of a plaque injury 3 hours after endothelial removal. The surface is covered by a mixed layer of platelets (*p*) and fibrin strands (*arrowhead*) (× 20,000).