THE ROLE OF THE ENDOTHELIUM IN THE INITIAL PHASES OF THROMBOSIS

AN ELECTRON MICROSCOPIC STUDY

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After a long period during which little of real significance was added to the classic work of the last century on the nature of platelet agglutination and the mechanisms of blood coagulation beginning with Virchow,[1-5] studies have only recently begun to provide additional insight into the early phases of thrombosis. It has become apparent, for example, that adenosine diphosphate (ADP) produces platelet clumping both *in vitro* and *in vivo,* and that other agents capable of inducing similar aggregation—thrombin, for example—may operate through this common pathway.[6] Unlike ADP, however, thrombin is also capable of inducing platelet metamorphosis, and activation of this enzyme is essential for the formation of the fibrin which binds together the loose platelet mass forming at a site of injury into a more stable thrombus. Although all of these changes can be induced by appropriate injury to the vessel wall, it has not as yet been clearly established what the exact nature of this injury must be, or whether the endothelium plays an active or a passive role in initiating these changes or in preventing them under normal conditions. The following studies were therefore undertaken in an effort to resolve some of these questions.

METHODS

Male Sprague–Dawley rats weighing 250 to 500 gm each and fed *ad libitum* were anesthetized with intraperitoneal Nembutal® and the femoral vein was gently separated from the surrounding structures. In 10 rats, no further manipulation was done and fixative was introduced into the vein distal to the isolated segment 10 minutes after the procedure was completed. A smaller group of 5 animals in which the undisturbed vein wall was fixed prior to isolation served as normal controls for the mild injury produced by the manipulative procedures.

More severe mechanical trauma was achieved in 11 rats by pinching the anterior surface of the femoral vein with a fine-pointed ophthalmic forceps. Pinching was performed under dissecting microscope view with force sufficient to produce visible

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edema but not bleeding. Following fixation the effects of different degrees of trauma were studied by sectioning the vessel either longitudinally or transversely at varying distances from the point of pinching to include areas of both maximal and minimal injury. Five of these animals were sacrificed by the introduction of fixative distal to the isolated segment 10 minutes after pinching and 3 were sacrificed after a period of 2 hours in order to permit as many platelets as possible to come into contact with areas of injury to the wall. In the remaining 3 animals, 1,000 units of heparin were injected intravenously immediately before the vein was pinched and blood was again permitted to flow through the segment for 2 hours before the fixative was introduced.

Stasis thrombi were induced in the isolated vein segments in 10 rats using the method of Wessler and associates.[7] This was accomplished by injecting rat serum, 7.5 ml per kg, into a contralateral vein followed 30 seconds later by gentle clamping of both ends of the isolated segment. The development of thrombosis in the clamped segment was confirmed by direct observation with the dissecting microscope. After 10 minutes, the clamps were released and the thrombus was permitted to leave the segment. Fixation was initiated either 10 minutes (5 rats) or 2 hours (5 rats) after release and the isolated segment was then removed for study.

In a final group of 2 rats, adenosine 5′-diphosphate (Sigma Chemical Co., St. Louis, Mo.) in concentrations of 25 and 250 μg per ml in Ringer's solution respectively was continuously infused into the vein distal to the isolated segments over a period of 3 to 5 minutes.

Following each experiment, the animal was sacrificed by slow infusion into the segment of 3 per cent glutaraldehyde in 0.2M cacodylate buffer at pH 7.4 [8] from a point distal to the area under study. When breathing ceased, additional fixative was applied to the segment externally and was permitted to remain *in situ* for 1 to 2 hours. The segment was then removed, appropriately sectioned, and postfixed in 1 per cent osmium tetroxide in 0.2M phosphate buffer at pH 7.4 for 2 hours. Following dehydration in graded alcohols and propylene oxide, the tissues were embedded in Epon 812.[9] Thin sections for electron microscopic study were stained with lead[10] or with uranyl acetate and were examined with a Philips EM200 electron microscope. In some cases, grids were selected to permit study of uninterrupted fields that sometimes consisted of an entire cross section of the vein. Adjacent sections, 1 μ in thickness, were stained metachromatically with toluidine blue and azure II [11] for study by light microscopy.

Results

Control Studies

Examination of sections of the femoral vein which had been left undisturbed prior to fixation showed a well preserved endothelium (Fig. 1). Single or small groups of platelets were observed close to the endothelial cell membranes in rare instances but these were of normal appearance and no fibrin was evident.

Although every effort was made to free the femoral vein segment from the surrounding tissues as gently as possible, mild alterations of the endothelium were, nevertheless, often noted following this procedure. Within 10 minutes some of the cells showed vacuolization (Fig. 2) or, in extreme instances, a loss of portions of the cytoplasm (Fig. 3). The plasma membranes of these cells invariably remained intact, however, and no changes were demonstrable in the subendothelial tissues.

Although the number of platelet groups noted close to the endothelial

surface was clearly increased, their sizes were not appreciably larger than those seen in undisturbed preparations. They consisted of loosely organized arborizations displaying 1 to 6 unaltered platelets in any one section. Serial sections performed on a number of blocks from one animal, however, showed more extensive groups in which the more remote members were well removed from the endothelial surface. No good correlation was noted between the occurrence of these groups and the observed endothelial changes. The distance between the platelets or between platelet and endothelial cell membrane was never less than 200 Å although wider spaces were frequently seen. In close contacts, the spaces were often obscured by a structureless electron-dense material thought to be serum protein (Fig. 4). Fibrin could not be identified near these platelet masses.

When blood was permitted to flow through the isolated segment for 2 hours prior to fixation, edema was observable in the subendothelial tissues but the appearance of the endothelium and the number, size and character of the platelet groups remained essentially unchanged. The anterior surface of the vein, which was subjected to less manipulation during the course of isolation, was most consistently free of the endothelial changes described and was accordingly the site selected for the subsequent studies.

Effect of Mechanical Trauma: Pinch Preparations

Sections obtained from portions of the isolated vein well removed from the pinched area showed essentially the same features as the controls. As the area of injury was approached, however, edema of the subendothelial tissues was apparent even after 10 minutes and evidence of endothelial cell damage became more prominent and consistent. The platelets in such areas, however, retained their normal shape, density and organelles, and no fibrin was seen.

In areas of more severe trauma, the endothelium was disrupted or absent permitting access of the constituents of the blood into the subendothelial tissues. Here fibrin formation was prominent and the platelets in contact with the subendothelial tissues or in close proximity to fibrin were often extensively altered, showing marked irregularity in shape and loss of alpha granules and mitochondria (Fig. 5). Such mingled masses of fibrin and changed platelets could sometimes be followed for considerable distances into the lumen in 2-hour specimens. In these same preparations, on the other hand, platelets remote from visible fibrin or the subendothelial connective tissues retained their normal appearance (Fig. 5). Conversely, traces of fibrin, and, in some cases, clumps of unchanged platelets over areas of slight injury or apparently normal

endothelium could be shown to be in contiguity with foci of changed platelets and fibrin over zones of endothelial disruption when the field of examination was expanded or adjacent sections examined. This could be readily shown by photo-montage techniques in which large segments of the vein wall could be reconstructed in continuity. Occasional areas of denuded vascular wall tended to be free of both platelets and fibrin although both were present in areas immediately adjacent. After 2 hours neutrophils were prominent on such denuded surfaces and were also present in the subendothelial tissues adjacent to these areas.

Effect of Mechanical Trauma in the Presence of Heparin

The injection of a massive dose of heparin into the animal immediately prior to pinching the vein failed to alter the appearance of the wall or that of the platelets accumulating at the surfaces of the intact endothelial cells. In areas of endothelial disruption, however, those platelets in contact with the subendothelial tissues continued to exhibit varying degrees of alteration, degranulation and aggregation (Fig. 6) whereas those in the lumen remained normal in appearance (Fig. 7). No fibrin was seen in any of these sections.

Effect of Induction of a Serum Thrombus

Following induction of a thrombus in the isolated segment by means of serum, and its subsequent release into the circulation, the appearance of the vessel resembled in all respects that of the isolated control segments. Even after 2 hours, the endothelial surface was intact, no edema was seen, and the small groups of platelets noted were unchanged. No residual fibrin was apparent.

Effect of ADP Infusion

Infusion of ADP both in low (25 mg per ml) and high (250 mg per ml) concentrations resulted in the aggregation of large numbers of loosely aggregated platelets near the intact endothelium. No fibrin was seen. These large masses were readily dislodged and it was usually necessary to reduce the rate of flow in the segment by clamping the vessel proximally in order to permit their consistent preservation in contact with the vein wall. Conventional fixation and embedding procedures resulted in the loss of most of these fragile masses in paraffin sections.

Discussion

The results of these experiments indicate that platelets accumulating at a site of injury remain essentially unchanged if the plasma membranes of adjacent endothelial cells are intact and in contact with each other.

Under these circumstances, there is no evidence of platelet alteration or degranulation nor is there visible evidence of associated fibrin formation. This remains true even though the endothelial cells show manifestations of severe injury and considerable edema of the vein wall is present. On the other hand, in every instance in which the platelets show evidence of alteration or loss of granules and in which fibrin is seen at a site of mechanical injury, it is also possible to demonstrate breaks in the endothelial continuity at or near these points. Such breaks include either total loss of cells or loss of contact between adjacent cells resulting in exposure of the underlying subendothelial connective tissues to the constituents of the blood. In the case of platelets which enter the subendothelial tissues, these changes are particularly obvious and can sometimes be observed to extend in continuity for varying distances into the lumen of the vessel.

It has been proposed by a number of investigators [12-14] that the presence of denuded collagen at the endothelial surface may account for the occurrence of thrombosis following a variety of pathologic processes. Our own observations provide direct evidence that, in the case of mechanical trauma at least, this is probably so, and that one function of the endothelium may be that of a passive barrier separating the procoagulants of the blood from tissue activators capable of inducing fibrin formation and platelet alteration. It is known that contact with tissue thromboplastin results in prothrombin activation via the extrinsic pathway and that collagen is capable of activating factor XII (Hageman factor) via the intrinsic pathway.[15] In addition, considerable evidence now indicates that platelets adhere rapidly to collagen [12,16] and that collagen is capable of inducing rapid platelet transformation.[17] Exposure of blood to the subendothelial tissues and particularly to collagen, therefore, can readily account for all of the observed phenomena leading to the development of a stable thrombus at a site of injury.

The accumulations of unaltered platelets over an injured but unbroken endothelium closely resemble the aggregations induced by ADP both *in vitro* and *in vivo* and could be readily duplicated in these experiments by the infusion of ADP into the vein segment. The augmenting effect of ADP in inducing visible platelet accumulation following minimal mechanical injury to cerebral vessels has also been clearly demonstrated by Honour and Mitchell [18] who were able to block the effect with specific inhibitors. Evidence is now accumulating that the release of ADP may be the final common pathway for the induction of platelet clumping by a variety of agents including thrombin, adrenalin and serotonin,[6,19] and the demonstration by Hovig and others [20,21] that ADP, together with other soluble components, is liberated from platelets in the presence of

collagen suggests that collagen may also produce its clumping effect by way of ADP released on contact. The capacity of such specific inhibitors as adenosine and adenosine monophosphate to inhibit platelet aggregation by collagen fibers [22] provides further evidence for this mechanism. The release of ADP, however, cannot easily account for the occurrence of platelet alteration or degranulation since there is no evidence in these experiments or in the work of others [6,23] that ADP alone can induce such changes.

Thrombin, on the other hand, also produces rapid platelet changes similar to those observed in the case of platelets adhering to collagen fibers [22,24,25] and it is entirely possible that traces of thrombin may account for them. This possibility is supported by reports indicating that platelets tend to be a more sensitive indicator of thrombin than is visible fibrin formation [26] and by the fact that fibrin monomers cannot be identified by electron microscopy. Even the fact that large doses of heparin, presumably far in excess of the amount required to block the release of thrombin, failed in these experiments to prevent the occurrence of extensive platelet alteration on contact with the connective tissues does not completely exclude the possibility that traces of thrombin may be present in spite of the heparin. On the other hand the fact that platelet alteration can be induced in certain species by agents other than thrombin including heparinoid, antigen–antibody complexes and endotoxin [27,28] suggests that collagen may also be capable of inducing these changes in some fashion other than by a thrombin-dependent mechanism. It also raises the possibility that the coagulation mechanism can at times be accelerated by the entry of platelets into the disrupted vascular wall followed by clumping and alteration on contact with the collagen.

Once thrombin has been generated, it seems evident that many of the changes seen subsequently can be attributed to this agent. The extension of fibrin and altered platelets into the vascular lumen is entirely consistent with a focus of enzymatic activity centering on the area of endothelial disruption. Furthermore, the lack of change in those platelets noted at the periphery of such areas (Fig. 5) suggests that the continuing blood flow, by neutralizing and removing the newly formed thrombin and continuously replacing it with inactive precursors, limits the size of the thrombus while at the same time helping to sweep away those more loosely aggregated unchanged platelets presumably clumped by the ADP mechanism. Such aggregations, lacking the fibrin meshwork and foci of platelet fusion required to provide stability, tend to be easily dislodged. This has been repeatedly demonstrated by direct observation on the living circulation beginning with Wharton Jones.[2] It has also been pointed out that such "white bodies" or platelet emboli, once

they have been torn free from their sites of formation by the flowing blood, do not grow [18,29] and may, in fact, undergo disaggregation to return intact to the circulation.[30] Similar unstable intraluminal platelet clumps also occur in the heparinized animal in which fibrin is not seen and in which alteration is limited only to those platelets contiguous with exposed collagen in areas of injury. They are also seen with considerable frequency over areas of milder injury (Fig. 4) or apparently intact endothelium in the non-heparinized animal. The fact that their number and size bear no clear relation to the degree of underlying endothelial damage is probably attributable, in part at least, to their high turnover rate.

The presence in the blood vessel wall [31] and more specifically in endothelial cells,[32] of an active enzyme capable of splitting ATP, ADP and other nucleotides raises the possibility that the endothelium may play a role in this process by catalyzing the local release or degradation of the active agent. This concept finds further support in the observation of Jorgensen and Borchgrevink [33] that the adhesion of platelets to endothelial cells is impaired in von Willebrand's disease, a condition in which the response of platelets to minute amounts of ADP is abnormal.[34] On the other hand, in those veins subjected to injury in which comparatively large segments are examined in continuity, or in which serial sections are performed, it is evident that arborization of platelet masses, so often described in the earlier literature, could readily account for some of these phenomena. Small groups of 3 or 4 unchanged platelets seen in the lumen can sometimes be shown to be in direct contact with masses lying in close relation to the endothelium in adjacent sections indicating that the point of initial interaction can be readily overlooked; in some instances these areas of contact show evidence of endothelial disruption and focal platelet alteration. The possibility that disruption of the plasma membrane and an exposure of the underlying collagen, however minute, may be a necessary prerequisite to the release of ADP is also raised by the occasional lack of platelet masses over severely injured endothelial cells (Fig. 3). In such cases, however, the possibility of artifactual displacement of pre-existing loosely clumped platelet masses by the manipulative procedures of fixation and embedding cannot be entirely discounted.

Serum-induced stasis thrombi, unlike those due to vascular injury, are coagulation thrombi triggered by activated factors introduced with serum at a distant point. As such they contain only those few platelets that happen to be trapped within the static segment.[7] Such platelets as are present, however, show evidence of both clumping and extensive alteration presumably induced by thrombin. If flow is permitted to resume, the platelets accumulating on the surface of the thrombus also show ex-

tensive alteration and intermingling with fresh fibrin probably attributable, in part at least, to residual thrombin within it.[35] The endothelium in the static segment, on the other hand, shows no evidence of alteration confirming previous impressions obtained by light microscopy that the local endothelium plays no discernible role in the formation of thrombi by this mechanism.

Summary

In the rat varying degrees of mechanical injury were applied to the femoral vein; these ranged from gentle dissection to pinching with a fine-pointed forceps. Fibrin formation together with alteration and degranulation of the platelets accumulating over the altered endothelial surfaces occurred only when the endothelial barrier was interrupted and the underlying connective tissues were exposed. Even endothelial injury sufficiently severe to produce extreme vacuolization of the cell failed to produce these changes if the plasma membrane remained intact. Transformation of platelets in direct contact with collagen, on the other hand, occurred even in the presence of doses of heparin sufficiently large to prevent fibrin formation.

These studies strongly suggest that the platelet transformation and fibrin formation necessary for the production of a stable thrombus at a site of injury require interruption of the endothelial barrier permitting contact between the procoagulants of the blood and the subendothelial connective tissues, especially the collagen. Contact with tissue thromboplastin and with collagen results in prothrombin activation via both the extrinsic and intrinsic pathways as well as in direct platelet transformation and degranulation.

Platelet clumping also occurs, presumably through the mediation of ADP, over injured but apparently unbroken endothelium, but the absence of platelet change and fibrin formation results in an unstable mass which is readily dislodged and disaggregated. Thrombi induced by serum in areas of stasis, on the other hand, depend for their formation entirely on prothrombin activation via the intrinsic system by the activated factors present in the serum and the local endothelium appears to play no role in their genesis.

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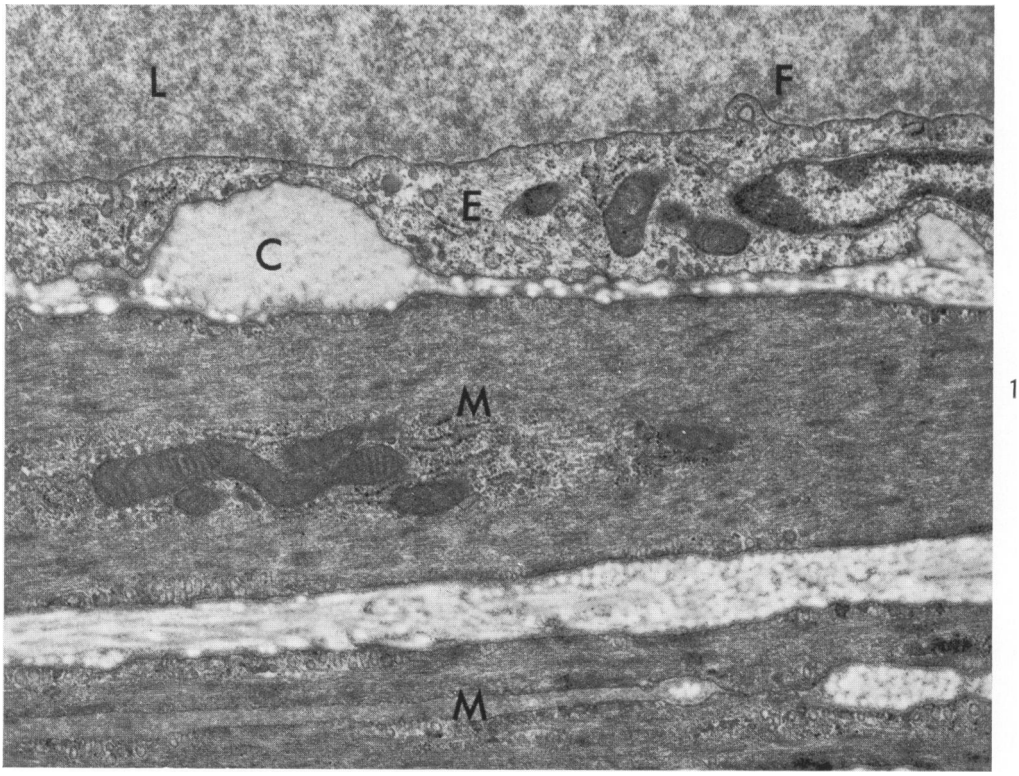
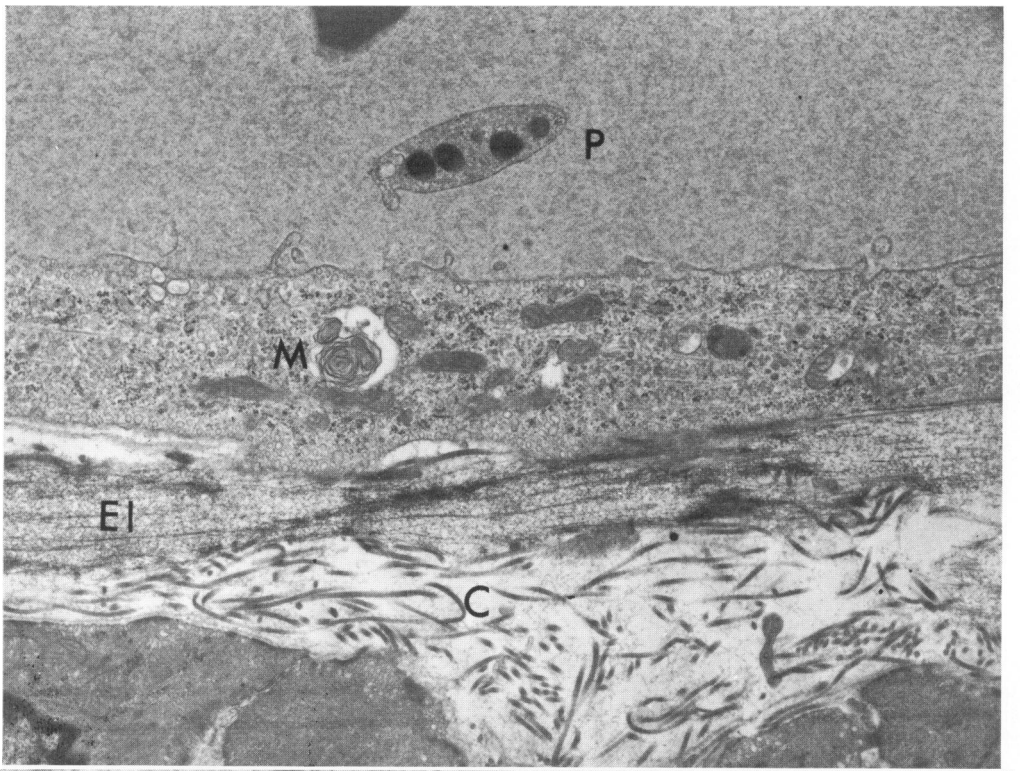


FIG. 1. Vein wall with normal endothelium (E) and smooth muscle cells (M). Collagen (C) appears as negatively stained areas between the cells. A normal endothelial flap (F) extends into the lumen of the vessel. The amorphous material in the lumen (L) represents precipitated plasma protein. Lead stain. × 7,000.

FIG. 2. A myelin figure (M) is seen within an intact endothelial cell showing minimal traumatic injury due to gentle vein dissection. Edema of the subendothelial layers has resulted in separation of positively stained collagen fibers (C) and fibrils thought to be elastic tissue (E1). An unchanged platelet (P) is seen in the lumen. Stained with lead and uranyl acetate. × 9,000.

FIG. 3. A severely injured endothelial cell (E) is seen between two dense endothelial cells (C). The cell is disorganized but has an apparently intact plasma membrane (PM). Fibrin and platelets are absent. Lumen (L). × 20,000.



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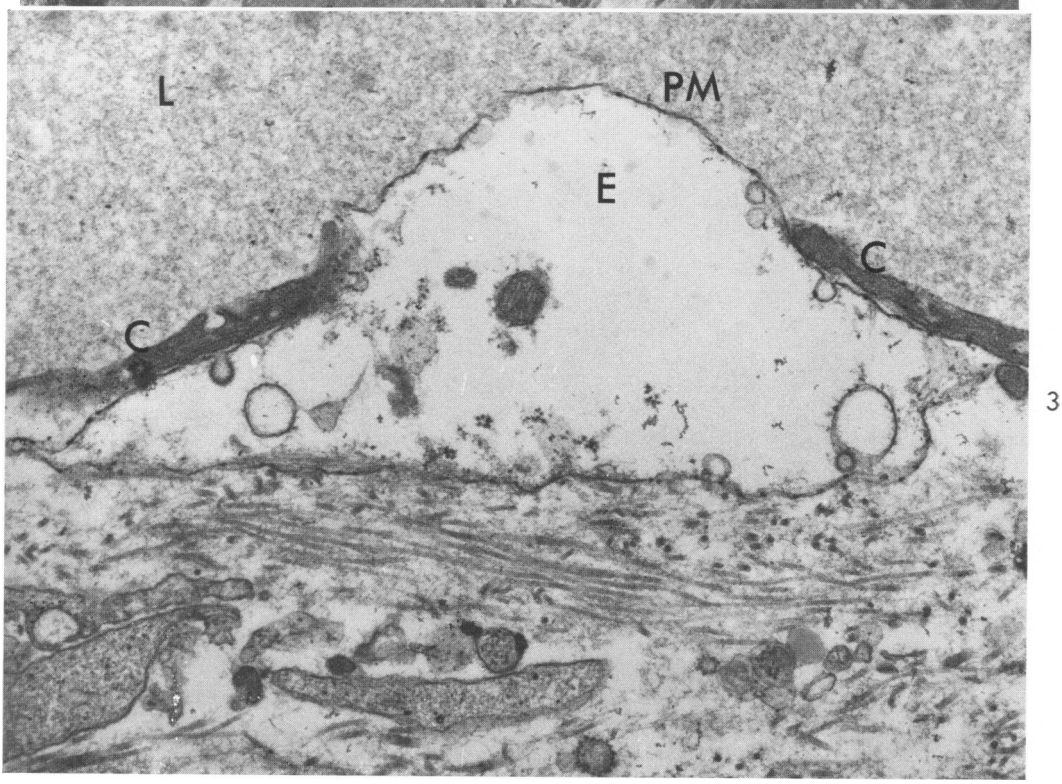

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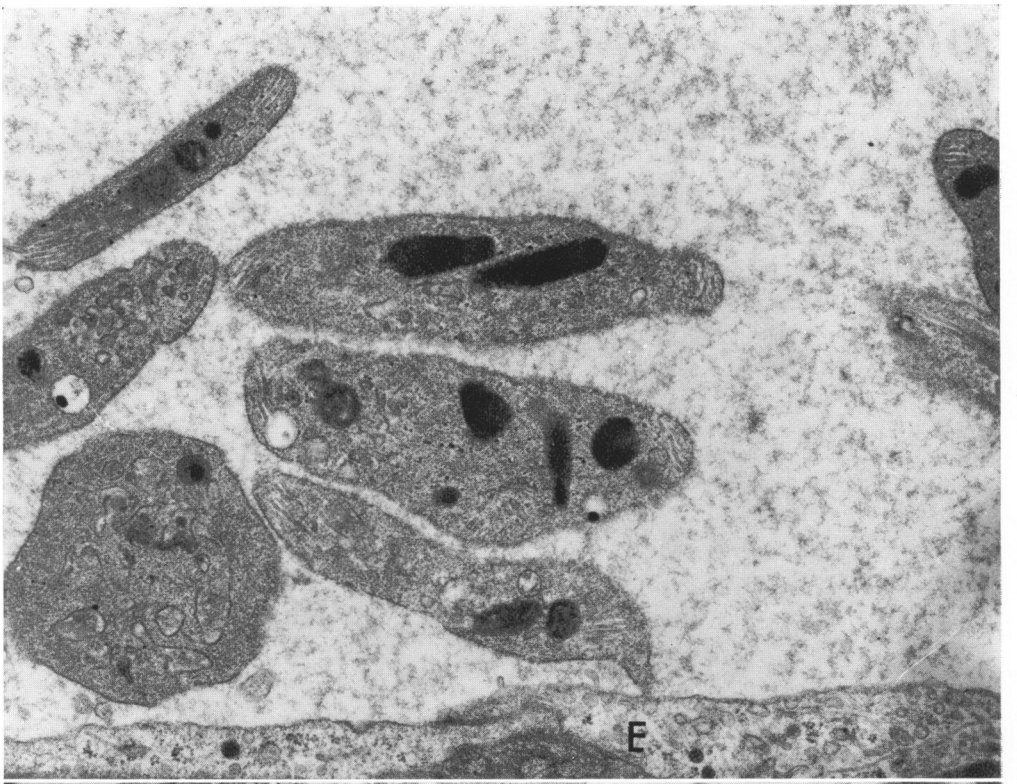
FIG. 4. A loose aggregation of unchanged platelets is seen near intact endothelial cells. The platelets are as near as 200 Å, but no closer contact is noted. The juncture is often obscured by a condensation, presumably of plasma protein as in this figure. × 14,000.

FIG. 5. A focus of endothelial disruption appears between the arrows. A mesh of fibrin and degranulated platelets (F) extends from the subendothelial tissues into the lumen. Occasional platelets beneath the endothelium (P_1) or more remotely placed in the lumen (P_2), tend to remain unchanged. × 8,500.



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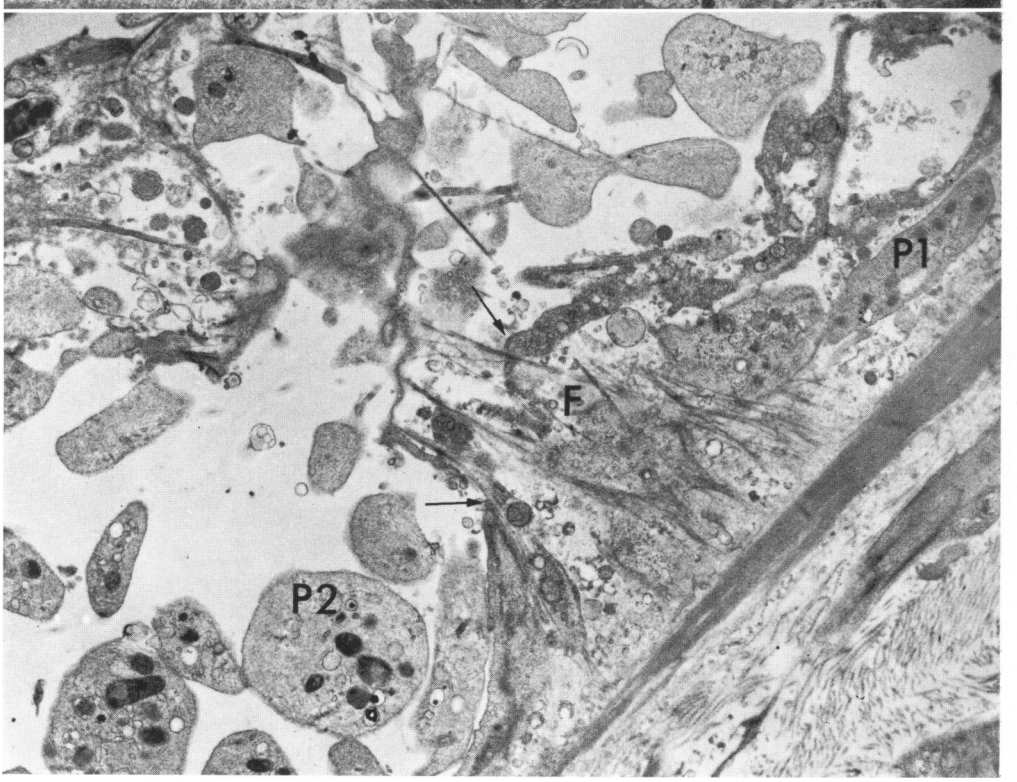

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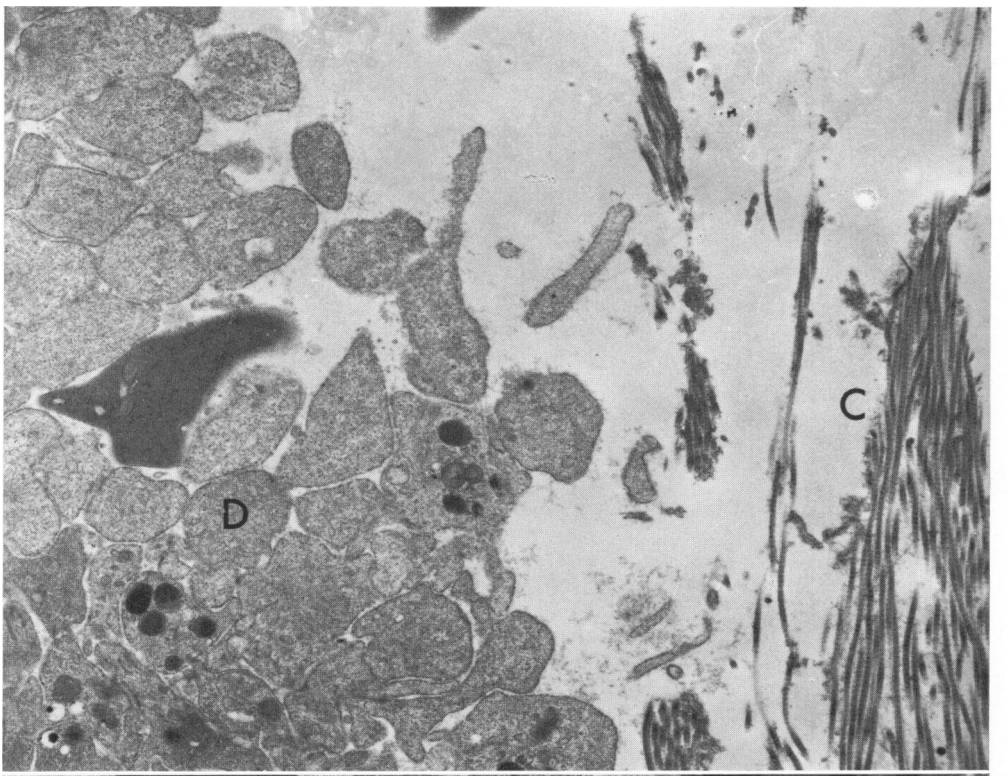
FIG. 6. Aggregates of degranulated platelets (D) appear within the wall of a disrupted vessel in an animal previously given a large dose of heparin. Fibrin is absent. Collagen (C). × 7,750.

FIG. 7. A segment of traumatized vessel wall denuded of endothelium from an animal previously given a large dose of heparin. Most of the platelets in contact with subendothelial tissues (D) are degranulated, whereas those seen in the lumen (P) are unchanged. Leukocyte (L). Red cell (R). × 7,900.



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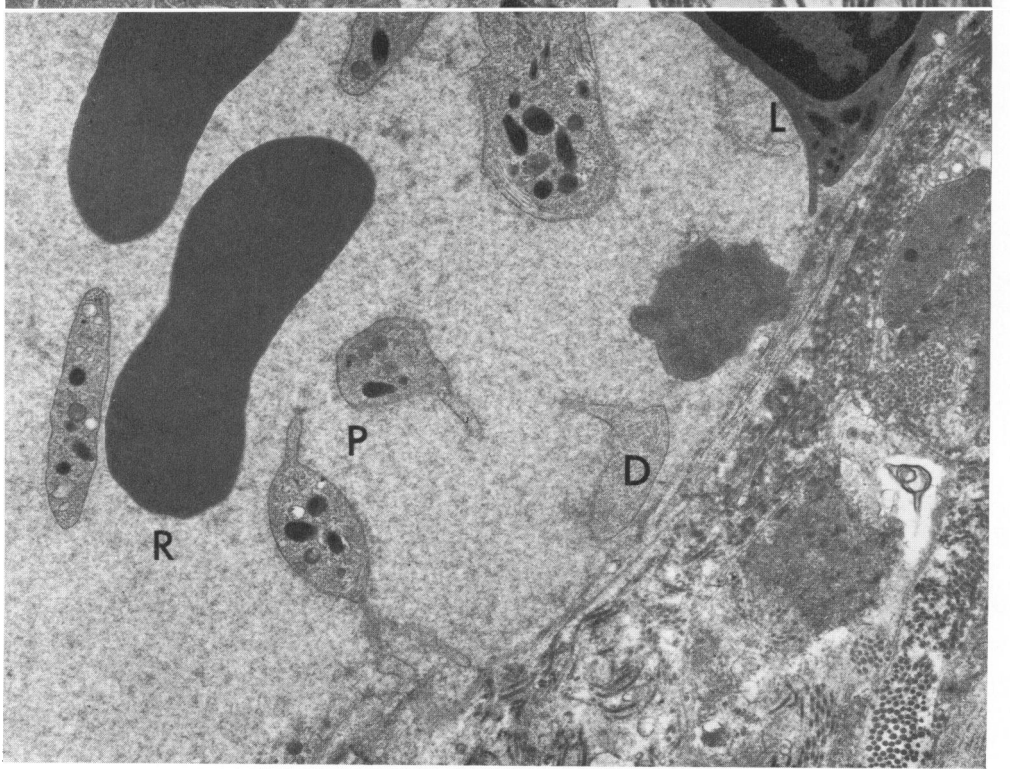

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