An Ultrastructural Analysis of Endothelial Change Paralleling Platelet Aggregation in a Light/Dye Model of Microvascular Insult

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Those microvascular endothelial events that parallel the evolution of platelet aggregation were evaluated in a well-controlled animal model. Cat pial microvessels were observed through a cranial window while local platelet aggregation was produced by intravenous injection of sodium fluorescein and simultaneous exposure of the pial vessels to light from a filtered mercury lamp that excited the fluorescein. The vessels were fixed *in situ* when the *in vivo* observations of a preselected vessel indicated early, intermediate, or advanced aggregation in that vessel. The preselected vessel was then harvested for ultrastructural study together with adjacent vessels from the illuminated field. These vessels and appropriate controls were compared in semiserial thin sections. The onset of platelet aggregation in both venules and arterioles was accompanied by focal endothelial lucency, vacuole formation, luminal membrane rupture, and swelling of the nuclear envelope. These changes were not found in control material. With intermediate aggregation these changes were more common, while with advanced aggregation these abnormalities occurred together with focal endothelial denudation. Thus, in this model denudation occurred only with advanced aggregation and was not a prerequisite for aggregation. (Am J Pathol 1983, 110:148–160)

CURRENT CONCEPTS of platelet aggregation suggest that endothelial cell loss ("denudation") is followed by adhesion of platelets to exposed subendothelium, followed by the release reaction and recruitment of additional platelets to form platelet aggregates.[1-5] This concept appears to dominate the literature in spite of ultrastructural data indicating that exposure of the subendothelial basal lamina may result in adhesion of platelets without significant aggregation.[6] Whether the basal lamina induces only adhesion or adhesion followed by aggregation, traditional emphasis has been on the importance of basal lamina collagen as the initiator of the platelet response; but recent evidence shows that such collagen is distinct from other collagens, in that it does not induce either platelet adhesion or aggregation.[7,8] Because of the past emphasis on the role of endothelial denudation and basement membrane collagen in eliciting or not eliciting platelet aggregation, relatively little attention has been paid to the possibility that lesser degrees of endothelial cell injury may elicit adhesion and aggregation. Limited electron-microscopic studies[9-11] have suggested such a possibility, although this was not necessarily the conclusion of those publishing the study.[10]

The purpose of our own work, presented below, was to describe the sequence of ultrastructural changes occurring in the walls of cerebral vessels in a new model of microvascular injury[12,13] where the progress of platelet aggregation could be followed *in vivo* and the vessels fixed at known stages during the accumulation of aggregating platelets. In this model we produced aggregation in the microcirculation by exposing the vessels to filtered light from a mercury vapor source in the presence of sodium fluorescein. Our initial publication and subsequent studies emphasized the use of this model for investigation of drug effects on platelet aggregation *in vivo*[14-17] and

Supported by NIH Grants NS-12587, HL-18932, CA-26316, and HL-21851.

Accepted for publication September 14, 1982.

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0002-9440/83/0207-0148$01.45

also on the changes in vascular diameter that accompanied injury and aggregation.[18] The original ultrastructural data was scant but illustrated that denudation could occur in the model.[12] From this scant data we assumed that denudation was a prerequisite for adhesion and aggregation. We performed the original studies in the mouse utilizing the surface vasculature of the brain (pial vessels). Since then our efforts to perform more extensive ultrastructural investigations have been hampered because of the small caliber of the mouse pial vessels, which makes it difficult to isolate and process the precise segment in which the platelet aggregate has been produced and identified *in vivo*. Moreover, as intense platelet aggregation occurred within 60 seconds of exposure to the noxious stimulus, we were limited in our ability to harvest the pial microvessels during earlier stages of platelet aggregation. However, during our continued attempts to gain more ultrastructural data from the mouse we were struck by the rarity of endothelial alterations and the frequency with which aggregation occurred in the absence of denudation. During the same period a cranial window technique was developed in cats, which allowed for the precise morphologic analysis of directly visualized, larger pial microvessels in that species. The latter technique has permitted the electron-microscopic evaluation of pial vessels in situations where platelet aggregation does not occur[19] and, in addition, has generated numerous control studies of the cat pial microvessels fixed and processed under standardized conditions.[20] Therefore, we decided to apply the light plus dye injury to the cat pial vessels and found that the generation of platelet aggregates occurred much more slowly than in the mouse. Since this slower sequence of platelet aggregation permitted us to obtain vascular specimens during the early, middle, and late stages of aggregation, and since the cranial window technique allowed us to isolate and process vascular segments fixed during selected stages of platelet aggregation, we felt that a controlled and systematic analysis of aggregation in such a situation would help define the relationship between aggregation and endothelial damage. Our data are reported below. They indicate that adhesion and aggregation are initiated at a time when definite but minimal ultrastructural alterations of endothelium can be recognized, and that denudation is not a prerequisite for adhesion or aggregation in this model.

Materials and Methods

Six adult cats of both sexes were used in the present investigation. After initial anesthetization with pentobarbital (30 mg/kg intravenously), the femoral artery and vein were cannulated, tracheostomy was performed, and the animals were artificially ventilated with a positive pressure respirator. During the course of the investigation expiratory CO_2 concentrations were continuously monitored and maintained at 30 mmHg. The animals were placed in a stereotaxic frame, and a craniectomy 11 mm in diameter was performed over the left middle suprasylvian and ectosylvian gyri. Upon removal of the bone flap, the underlying intact dura was visualized and was carefully resected. With removal of the dura mater the underlying pial vasculature could be directly observed coursing within the subarachnoid space. Mock cerebrospinal fluid[21] was kept over this site with the aid of a dam of dental acrylic attached to the bone. A Leitz Ultropak microscope was used for observation of the pial vessels.

Before any experimental interventions were initiated, a detailed sketch of all the vessels in the field was prepared, and a single arteriole and venule were selected for continued detailed monitoring. Then we switched from the tungsten lamp, under which the microscopic observations were being made, to a filtered mercury light as previously described.[12] The filters included a Leitz KG-1 and two Leitz dichroic reflecting filters, which virtually excluded all wavelengths below 350 or above 800 lambda. In addition, a Leitz BG-12 exciter filter was used with a broad peak transmission at 400 lambda. Thus, harmful ultraviolet wavelengths have been removed from the light emitted by the mercury lamp. This explains why no harmful effects were seen even after 40 minutes of exposure unless the fluorescein was present and excited by the light passed by the BG-12 filter. We have recently suggested that the endothelial injury may be initiated by free radicals produced when the dye is excited by the light.[22] It should be noted that this light was focused by the Ultropak on *only* one microscopic field within the craniectomy site. Within seconds of changing to the illumination from the mercury lamp we initiated the infusion of 2% sodium fluorescein at a rate of 1.2 ml/min via the femoral vein, and within approximately 1 minute the pial vessels in the illuminated field fluoresced brightly as previously described.[12,13] A maximum of 20 ml of fluorescein was infused. Within 10 minutes of the onset of the light plus dye insult, platelet aggregates were visualized first in the venule and later within the adjacent arteriole. Many other arterioles and venules adjacent to the preselected pair of vessels also contained aggregates. As previously described, these aggregates were brightly fluorescent and were not seen outside the illuminated field.[12,13] By continuous observation

of the preselected vessels, aggregation could be identified and followed at its onset, at its more intermediate stages, and at its advanced stages when the aggregates had totally blocked the vessel lumen. Moreover, since the venular platelet aggregation always preceded that occurring within the arterioles, it was frequently possible to obtain in the same animal a venule in one stage of aggregation while the adjacent arteriole manifested an earlier aggregatory stage. For example, with prolonged exposure to the light plus dye insult, the illuminated venule demonstrated frank occlusion, whereas the adjacent illuminated arteriole frequently demonstrated only the initial or intermediate stages of aggregation. Of additional interest was our ability occasionially to arrange the field so that only one vessel wall was illuminated while the other lay outside the field, thereby effectively inducing aggregation on one side of the vessel while sparing the other.

The absence of aggregates outside the illuminated fluorescing field was originally[12] cited by us as evidence that it was endothelial injury, rather than platelet damage, that precipitated aggregation. If platelets were injured, we would expect aggregates to be seen both inside and outside the illuminated fields, since they pass through the illuminated area in less than a second. In fact, this short exposure to the light would itself mitigate against platelet injury by the noxious stimulus. One might reason that a given platelet would return many times to the illuminated site prior to the onset of aggregation, but in mice aggregation occurs in 30 seconds, even though the chance of a platelet circulating back to the same field in this short period of time is vanishingly small. As a further check on the ability of the filtered light to injure fluoresceinated platelets, we placed a glass capillary tube containing fluoresceinated platelet-rich plasma (PRP) within the light beam for 5 minutes and then expelled the contents into a platelet counting chamber for counting under phase microscopy. No aggregates were seen, and the platelet count was the same as that in a sample that was not illuminated. Even when placed at the focal point of the filtered light beam, no aggregates were produced in the capillary tube of fluoresceinated PRP. Finally, a cuvette of fluoresceinated PRP was placed in an aggregometer, and its optical density was determined. The cuvette was then placed in the path of the filtered light for 5 minutes. This failed to alter the optical density of the PRP.

When the desired stage or stages of aggregation, i.e., initial, intermediate or advanced, were present within the respective venules and arterioles, the site and/or sites of aggregation were marked on the previously drawn sketch, the illumination was switched back to the tungsten lamp, and the infusion of the sodium fluorescein was discontinued. The craniectomy site was sealed with a glass ring and petroleum jelly, and the animals were prepared for transcardial perfusion. A rapid thoracotomy and pericardiotomy were performed, and the left ventricle was pierced with a cannula, through which normal saline was perfused. After 2 minutes of saline perfusion, the craniectomy site was reopened, and the site was flushed with topically applied fixative composed of 2% paraformaldehyde and 2.5% glutaraldehyde in a 0.1 M sodium phosphate buffer. Concomitant with this topical lavage of fixative, the saline perfusion was discontinued, and 1000 ml of the fixative was now perfused. The rationale for this bimodal topical and transcardial fixation was that the perfused fixative will fix those vessels that remain patent after experimental intervention, while the topically applied fixative assures adequate fixation of those pial vessels occluded with platelet aggregates. Upon termination of the fixation procedure, the brain, with its meningeal investments, was removed from the cranial vault and placed in the same fixative as used in the perfusate. After 2 hours the brains were transferred to 0.1 M sodium phosphate buffer, and the site under the craniectomy was identified. Employing a protocol described in detail elsewhere,[19] we stripped the pia mater and its related vasculature from this site and placed it in buffered osmic acid. After 2 hours of osmication, this tissue sample was placed under a dissecting microscope; and on the basis of the previously drawn sketch, those precise arteriolar and venular segments previously demonstrating *in vivo* platelet aggregation were identified and dissected free of the pia mater. These included both the preselected pairs of vessels and any of their branches as well as neighboring vessels in which aggregates had been incidentally noted *in vivo* and included on the sketch. Other vessels within the illuminated field were also harvested for possible study, even though their status had not been noted *in vivo*. As shown later, such vessels did prove useful. For the purpose of control analyses, arterioles and venules lacking aggregation were harvested from a nonilluminated site. Furthermore, pial arterioles and venules from the hemisphere contralateral to the craniectomy were taken as additional control samples. Finally, we could obtain even more critical controls in select cases by identifying a vessel wall lying outside the illuminated field and comparing it with the opposite wall *of the same vessel* lying within the illuminated field. The chosen microvascular segments were then dehydrated in chilled ethanols and propylene oxide and embedded

in Medcast resin (Ted Pella Inc., Tustin, CA). In 6 cats, the embedded vessels were transferred to the stage of an ultramicrotome, where the vessels were cut in a plane parallel to their long axis. Alternate thick sections, followed by multiple serial thin sections, were cut through the vessels' entire extent. In this fashion we obtained and examined 150–300 sections from each of the preselected pair of vessels in each of 6 cats, as well as 20–30 sections from all other harvested vessels. The serial thin sections were collected in serial order on membrane-coated slotted grids, which were then stained with uranyl acetate followed by lead citrate. All thin sections were viewed and photographed on a Hitachi HU-12 electron microscope. The arterioles ranged in diameter from 80 to 225 μ, whereas the venules ranged from 100 to 200 μ in diameter.

In 6 additional cats, segments of comparably injured vessels as well as vascular segments from noninjured sites were not processed for transmission electron microscopy but, rather, were bisected, critical-point-dried, coated with gold, and examined and photographed in a Hitachi S-500 scanning electron microscope. Such scanning electron microscopic analysis was employed to survey the adhering and aggregating platelets in order to see whether the platelets were fragmented or displayed any other features not associated with normal aggregatory phenomena. This information represented yet another check against the possibility that the light plus dye directly damaged the platelets.

Results

Controls

When the pial vessels harvested from nonilluminated sites on the ipsilateral and contralateral hemispheres were studied at the ultrastructural level, no evidence of cellular damage was discerned. The tunica intima, media, and adventitia of both the arterioles and venules appeared unremarkable. In particular, vacuolization of the endothelial cells, rarefaction of the endothelial cell cytoplasm, and endothelial luminal membrane rupture were absent, as previously reported by us[20] and others.[23] Naturally degranulated platelets or platelet aggregates were not seen.

Experimental

Scanning Electron Microscopy

With scanning electron microscopy multiple intact platelets with prominent pseudopodia were observed adhering to the vascular luminal surface of all the injured vessels (Figure 1). Scattered red and white blood cells were identified in relation to these aggregated platelets. No fragmented cells, platelets or otherwise, were observed. Since the platelets naturally obscured the luminal endothelial surface from detailed analysis, further scanning electron microscopic studies were not pursued, nor was transmission electron microscopic study of these specimens carried out. Rather, the bulk of the present investigation focused on the transmission electron microscopic study of the pial vasculature from 6 additional cats.

Transmission Electron Microscopy

For the purpose of clarity the following passages will be divided to reflect the three stages of platelet aggregation. Specifically, we will first consider the onset of platelet aggregation in both venules and arterioles. Second, we will consider the intermediate stages of platelet aggregation in these vessels; and finally we will consider the advanced stages of platelet aggregation in which vessels were occluded by the aggregates.

Onset of Platelet Aggregation

In one animal the light plus dye insult was stopped as soon as any aggregates were recognized. These aggregates were located in a venule. At the ultrastructural level, numerous platelets in association with scattered red blood cells and occasional white blood cells could be observed in close proximity to the venular endothelial wall. The red cells demonstrated normal ultrastructural features. This was always the case throughout our study, as exemplified by the red cells in Figures 11 and 12, taken from more advanced stages of platelet aggregation and endothelial damage. The platelets displayed a variety of forms consistent with the onset of normal platelet aggregation (Figure 2). Discoid platelets containing granules, spheroid degranulated platelets, and platelets that had formed large pseudopodia were recognized. All of these forms were seen both in close proximity to and adherent to the venular endothelium (Figures 2–5). Occasional cellular profiles were tentatively identified as platelets but may be white cells (Figures 2 and 3, lower right). These are in addition to the widely scattered white cells that could definitely be identified (eg, Figure 11). Other amorphous membrane-bound profiles were not identified (eg, Figures 2 and 3). Despite the conspicous presence of platelet aggregation, initial ultrastructural analysis suggested that the associated endothelial cells were unaltered. However, after extensive analysis of serial sections, it

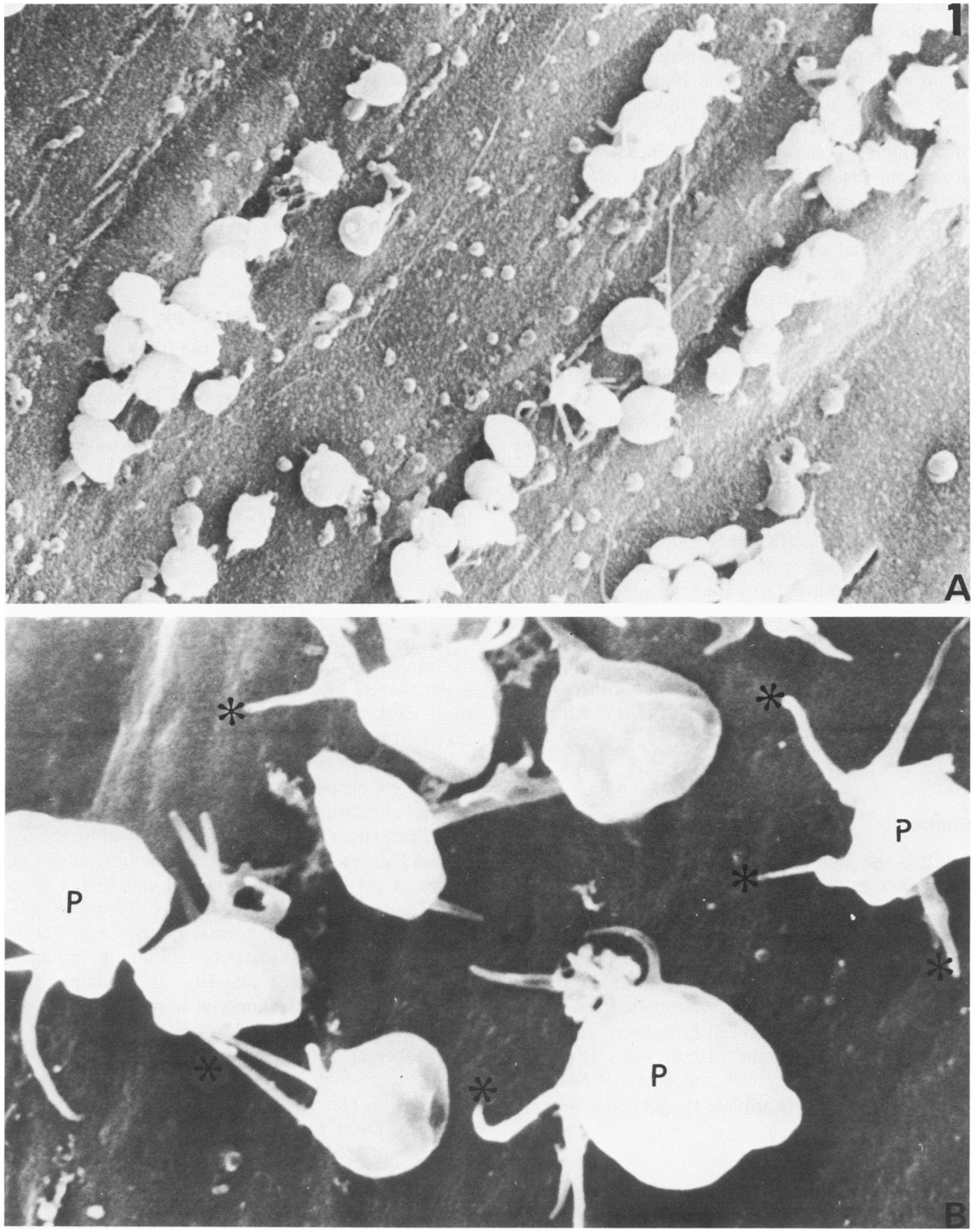


Figure 1A – In this scanning electron micrograph numerous intact platelets are visualized adhering to the endothelial surface. (×4000) **B** – With scanning electron microscopy the aggregating platelets (*P*) are observed to demonstrate normal morphologic features. Note the numerous pseudopodia (*). (×12,000)

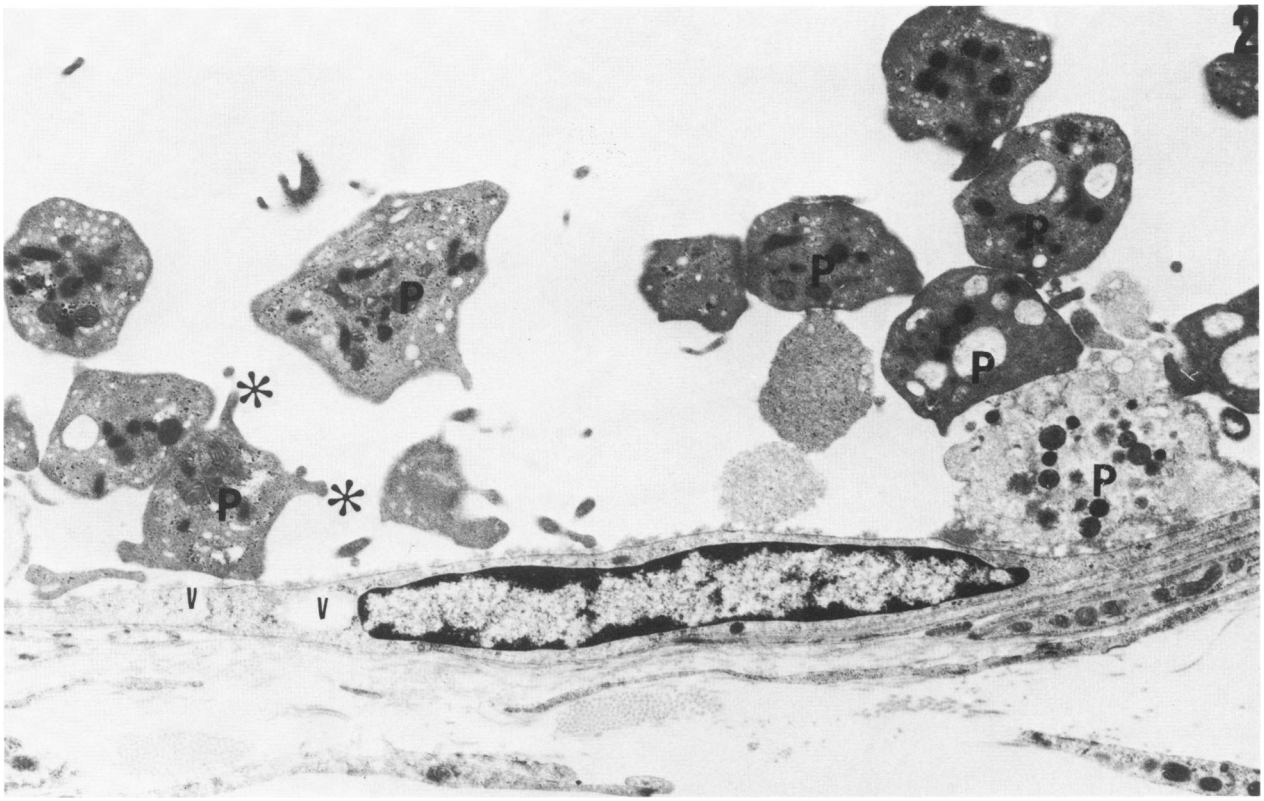


Figure 2—This electron micrograph demonstrates a portion of a venular wall harvested at the onset of platelet aggregation. Note that platelets (*P*) demonstrating pseudopodia (*) or other forms consistent with aggregation overlie an endothelial cell, which appears unremarkable except for vacuolization (*V*). (× 10,000)

became apparent that certain subtle endothelial abnormalities were spatially associated with the observed aggregates. These abnormalities included increased endothelial lucency, swelling of the nuclear envelope, rupture of the luminal membrane, and vacuolization of the endothelium (Figures 2–5). Frequently, the observed vacuoles displayed no limiting membrane. If only a single section had shown such changes, we might have been inclined to dismiss them as artifacts; however, in our material such changes were readily followed in serial sections (Figures 2 and 3). Despite exhaustive examination of the venular endothelial wall, which entailed the examination of all the luminal surface associated with the aggregation, no evidence of endothelial denudation could be found. The associated perivascular basal lamina, fibroblasts, smooth muscle cells, and leptomeningeal cells demonstrated no evidence of change (Figures 2–5).

In 2 cats the experiment was terminated at the onset of aggregation in preselected arterioles. This occurred 5 or more minutes after aggregation was noted in the venules. Although the arteriolar aggregation occurred after longer exposure to the noxious stimulus, the associated arteriolar endothelial abnormalities were reminiscent of those seen in the venule harvested in the early stages of platelet aggregation. Again, no evidence of endothelial denudation was recognized; and the perivascular basal lamina, as well as the cellular components of the media and adventitia, did not demonstrate any abnormalities. In both of these cats the study of the initial phase of aggregation within the pial arterioles provided an internal control, because these vessels demonstrated platelet aggregation on one wall, while the opposite wall, which was kept outside the illuminated field, was spared (Figure 6). The wall demonstrating aggregates revealed the presence of subtle endothelial change (Figures 6A, 7, and 8) whereas the opposite uninvolved wall failed to demonstrate evidence of endothelial alteration (Figures 6B and 9).

In two cats we also performed ultrastructural studies of many vessels that were within the illuminated field but that had not been selected for *in vivo* monitoring. We looked particularly for changes in the walls of vessels that did not show platelet aggrega-

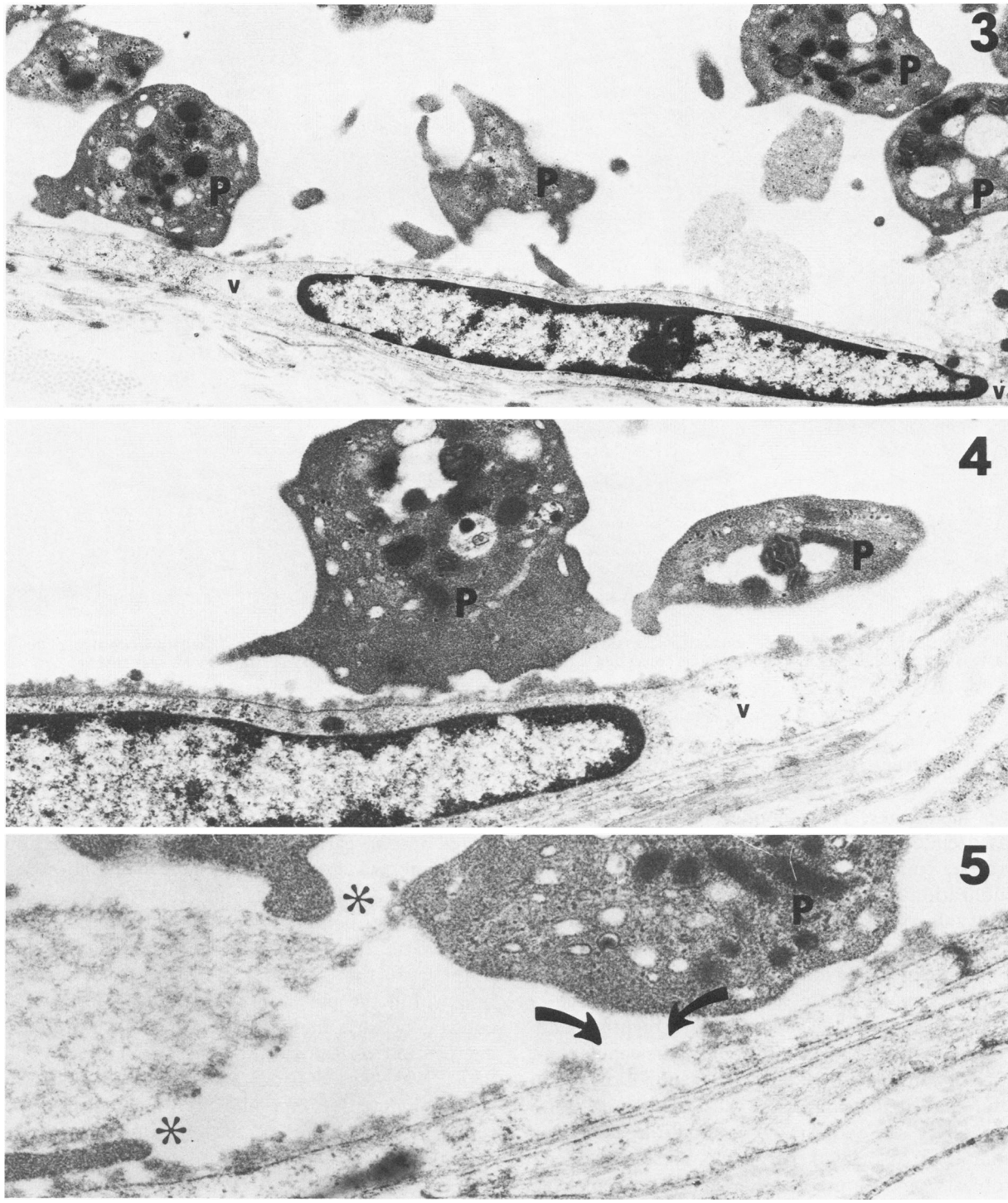


Figure 3—This electron micrograph demonstrates a serial section taken several segments away from that section illustrated in Figure 2. Again, notice the pseudopod-forming platelets (*P*) as well as the prominent endothelial vacuolization (*V*). (×12,000) **Figure 4**—This venular segment harvested at the onset of platelet aggregation also displays limited change reflected in a conspicuous zone of vacuolization (*V*). (×20,000) **Figure 5**—This venule was harvested at the onset of platelet aggregation. One of the platelets (*P*) seen in the field overlies an area of endothelial cell lucency and luminal membrane rupture (between *curved arrows*). Additionally, notice the platelet pseudopodia (*). (×25,000)

tion in their lumens. We identified two vessels in which endothelial lesions identical to those already described were seen. It should be stressed once again that we never saw such changes in vessels from the nonilluminated sites.

Intermediate Stages of Platelet Aggregation

We next considered those endothelial alterations seen during a period intermediate between the very onset of aggregation and the advanced stages of platelet aggregation. Such an intermediate stage was recognized within several minutes of the onset of aggregation and was manifested by multiple foci of aggregation, which effectively altered and redirected the blood flow through the vascular lumen. Intermediate stages of aggregation were first recognized in venules and then, after a lapse of several minutes, within the adjacent arterioles. These intermediate stages of aggregation were assessed in 2 cats. At the ultrastructural level the vessels demonstrated tightly packed platelet aggregates, in which the majority of platelets were degranulated and/or forming pseudopodia. Red and white blood cells appeared unaltered. The associated venular endothelial cells demonstrated abnormalities comparable to those seen at the initial phase of aggregation; however, now these changes were far more frequent. Again, endothelial lucency, perinuclear envelope swelling, endothelial cytoplasmic vacuolization, and fragmentation of the luminal endothelial cell membrane were observed, and, in addition, distinct endothelial swelling was now recognized. No evidence of endothelial denudation was seen; however, sites of luminal membrane fragmentation appeared so extensive as to suggest the onset of the denudation process. It was of interest that in contrast to the situation in the venular endothelium the arteriolar endothelial cells displayed proportionally fewer endothelial abnormalities (Figure 10).

Advanced Stages of Aggregation

This stage of frank vascular occlusion appeared several minutes after the onset of the intermediate aggregatory phase. Venules were occluded first with the occlusion of the arterioles following several more minutes of continued insult. This advanced stage of aggregation was seen in the venules of 2 cats in which the light plus dye insult was continued until arteriolar aggregation was initiated. It was also seen in some of the venular branches in 2 cats in which the insult was prolonged long enough to assure at least an intermediate stage of aggregation in both the arterioles and venules. In 1 additional cat we prolonged the

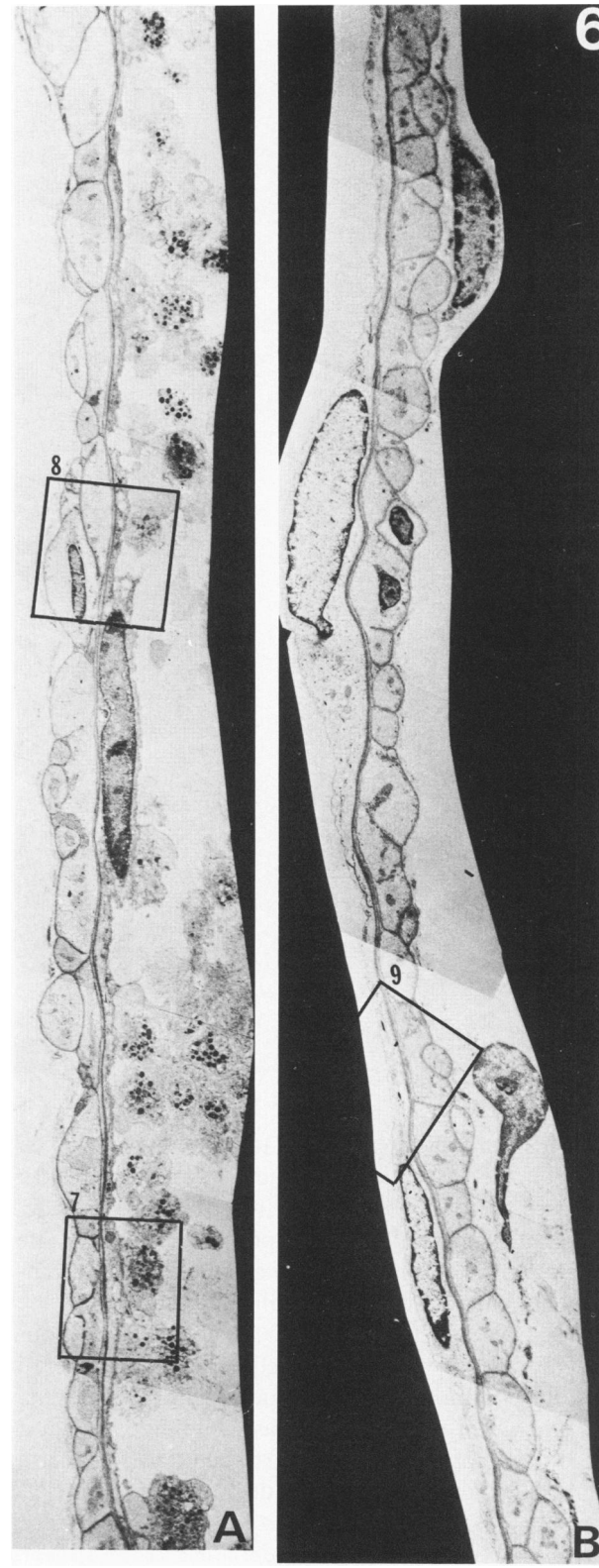
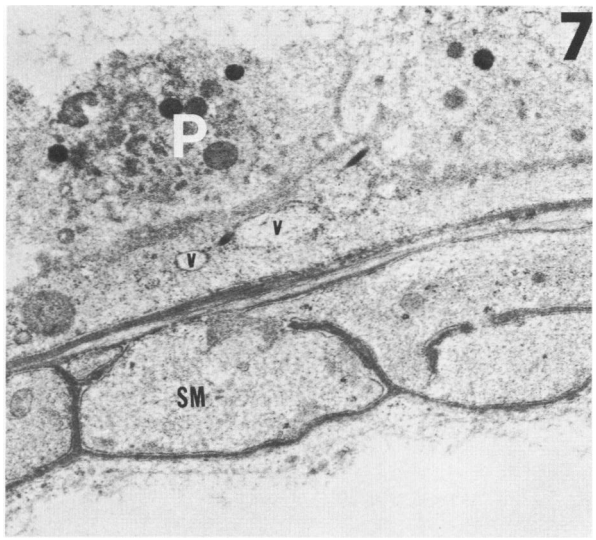
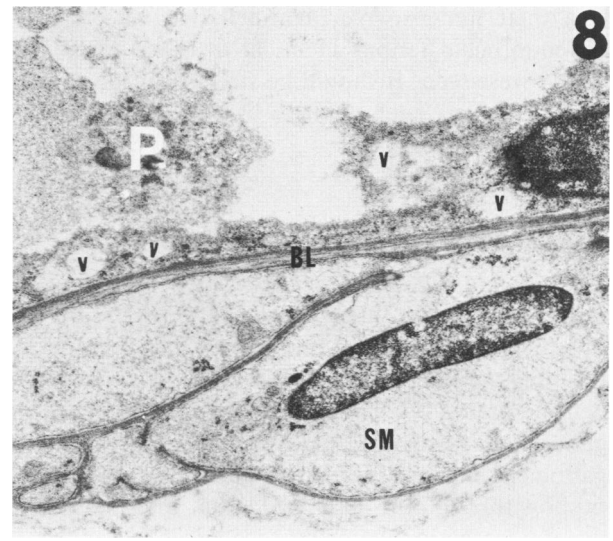


Figure 6 — In this electron-microscopic montage both walls of an arteriole 90 μ in diameter are mounted side by side. One wall was in the illuminated field, while the other was not. Notice that while the illuminated side (*A*) demonstrates an initial stage of aggregation, the opposite wall (*B*) demonstrates neither aggregation nor platelet adhesion. (×2500)





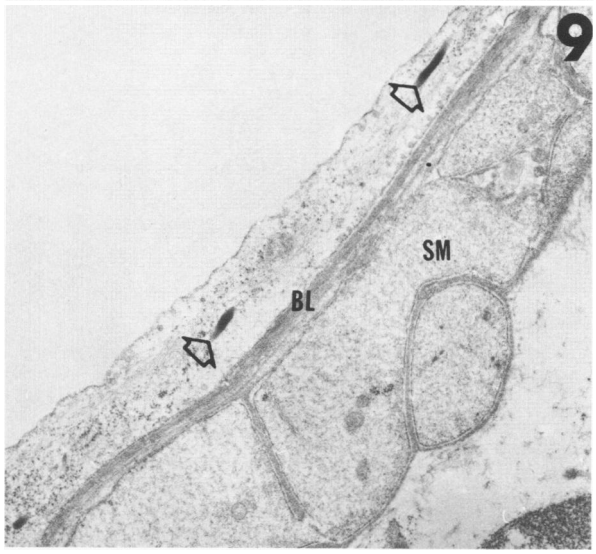
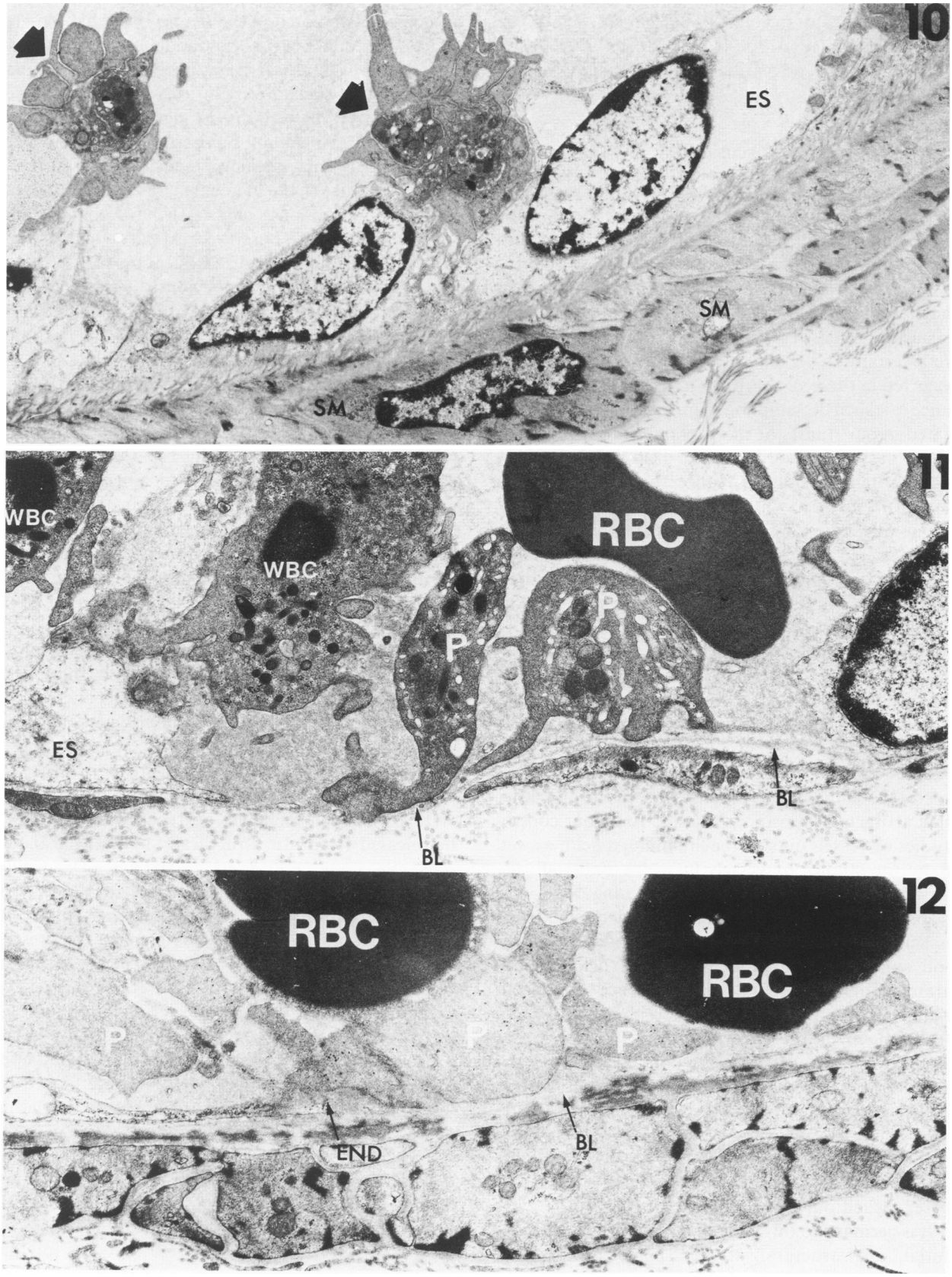


Figure 7—This electron micrograph is an enlargement of area 7, blocked out in Figure 6A. Notice the vacuolization (*V*) within the arteriolar endothelium associated with platelets (*P*) in varying stages of degranulation. Despite this endothelial change, the underlying smooth muscle cells (*SM*) appear unaltered. (× 12,500) **Figure 8**—This micrograph is an enlargement of area 8, blocked out in Figure 6A. Notice that the endothelial segment here, underlying a degranulated platelet (*P*), displays extensive vacuolization (*V*). Again note that although the endothelium displays extensive change, the underlying basal lamina (*BL*) and smooth muscle (*SM*) are unaltered. (× 12,500) **Figure 9**—This enlargement of area 9, blocked out in 6B, demonstrates the normal endothelial detail retained by the vascular wall not associated with aggregation. Weibel-Palade bodies are marked with *open block arrows*. (× 10,000)

stimulus for 5 minutes beyond the time at which even the arteriole was totally obstructed by aggregates. In all cases of total obstruction, the platelets were degranulated or forming pseudopodia (Figure 11). The venular endothelial surface revealed conspicuous endothelial change reminiscent of that described in the earlier phase of aggregation and now also demonstrated the presence of endothelial denudation. Serial sections commonly revealed such loci of endothelial absence with the underlying basal lamina exposed to the blood front (Figure 11). At such zones of basal laminar exposure, adhering degranulated platelets and/or platelets forming pseudopodia were commonly recognized. The underlying basal lamina and associated perivascular cellular elements appeared unaltered.

In contrast to the situation seen in the venules, our initial observations suggested that even when aggregation was advanced, endothelial denudation did not occur in arterioles. Only after careful examination of over 300 serial sections harvested from an approximately 1.22-mm longitudinal arteriolar segment did we definitively identify two loci showing denudation (Figure 12). At these sites the underlying

Figure 10—This arteriole was harvested at an intermediate stage of aggregation. Notice that focal platelet aggregates (*block arrows*) adhere to endothelium demonstrating vacuolization and endothelial swelling (*ES*). Note the unaltered basal lamina and smooth muscle cells (*SM*). (× 7500) **Figure 11**—Taken at the stage when platelets have occluded the vascular lumen, this venule demonstrates endothelial denudation and swelling (*ES*). Pseudopod-forming platelets (*P*) here adhere to a segment of exposed basal lamina (*BL*), while an unaltered red blood cell (*RBC*) and two white blood cells (*WBC*) lie trapped nearby. (× 15,200) **Figure 12**—Endothelial denudation can also be observed in this arteriole, harvested at the stage of complete occlusion by platelets. Notice that the vascular endothelial lining (*end*) is incomplete and that degranulated platelets (*P*) adhere to the exposed basal lamina (*BL*). *RBC*, red blood cells. (× 16,000)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basal lamina was associated with platelets that were degranulated and forming pseudopodia. The basal lamina was intact. All perivascular cellular elements within the media, adventitia, and leptomeninges were unaltered.

Discussion

In this investigation we observed cat pial vessels injured by exposure to light from a mercury vapor source in the presence of intravascular sodium fluorescein. Platelet aggregation was initiated by this insult, and vessels were studied ultrastructurally in the presence of early, intermediate, and advanced aggregation. Since vessels were monitored *in vivo*, we were certain of the extent to which aggregation had occurred in these vessels prior to fixation. The importance of such a sampling approach can be appreciated in our studies of the very onset of aggregation where perfusion fixation could dislodge aggregates, making it impossible to realize that they had been present *in vivo*.

Our ultrastructural studies of the injured vessels demonstrated that aggregation progressed in parallel with the development of local endothelial alteration and that the initiation of aggregation could occur without the presence of endothelial denudation. Denudation ultimately did occur, but only after aggregation was well advanced. Since the lesser degrees of endothelial change were both spatially and temporally related to the platelet aggregates, it is reasonable to suggest that these events were causally linked. That the observed endothelial abnormalities constituted true endothelial responses to the insult rather than artifacts of fixation is attested to by our control studies of vessels outside the illuminated field, as well as by the studies of vessels in which one wall displayed aggregates, while the opposing wall was spared. These control walls consistently retained normal endothelial detail. In the initial phase of aggregation some vessels in the illuminated field demonstrated endothelial change in the absence of adjacent platelets. Since these changes were identical to those observed in the presence of platelets and were not seen in vessels outside the illuminated zone, we did not interpret these alterations as artifactual. Rather, this finding implies that the endothelial change precedes the development of aggregation, as one might expect. Of course, we cannot rule out the possibility that in such vessels platelets had been present but were washed out by the fixative.

It is germane to this issue that a reevaluation of our initial ultrastructural studies performed in the mouse[12] also supports our present concept. More specifically, we noted in the mouse both that platelet aggregation could occur without denudation and that denudation was an extremely rare event, when pial vessels of that species were injured by light plus dye. In fact, numerous sections through affected pial vessels in 7 mice, including semiserial sections in 3, revealed only a single denuded focus. Thus, it appears that in response to the mercury-light–sodium-fluorescein insult, the mouse and cat behave comparably, suggesting that the observed responses to the noxious stimulus are generalized phenomena.

Although others have previously advocated that aggregation can be induced without denudation,[11] their evidence seems less convincing. On the other hand, Hovig et al,[10] using a laser to initiate platelet aggregation, definitely showed aggregation in the presence of endothelial damage without denudation. However, extensive red cell damage was also observed, leading them to suggest that either the damaged endothelium could elicit adhesion and aggregation or that ADP released from the damaged red cells caused the platelets to become "more adhesive." Others[24] showed that the aggregatory effect of the laser model could be mimicked by intravascular injection of red blood cell stroma and/or ADP infusion, presumably without any endothelial damage. This, of course, cannot rule out the possibility that endothelial damage, when present, is important. The current investigation argues for the concept that primary endothelial damage without denudation can precipitate platelet aggregation. In contrast to the laser study,[16] we had no ultrastructural evidence of RBC damage. Furthermore, if it were assumed that ADP or any other platelet aggregatory factor could be released from ultrastructurally normal red blood cells, one could also assume that the ADP would diffuse randomly and result in aggregates that were randomly localized rather than consistently linked to sites of endothelial damage, as seen in the present study. Thus, the results of the present investigation support the suggestion of Hovig et al, that endothelial damage can initiate platelet adhesion and aggregation in the absence of denudation. Other models of inducing platelet aggregation should be reevaluated so that one can assess the role played by lesser degrees of endothelial damage in initiating aggregation.

The mechanism through which the damaged endothelial cells induced adhesion and/or aggregation cannot be deduced in the present investigation, nor do our findings explain how the endothelium is injured. However, it is of interest that the luminal

membrane damage, the nuclear envelope swelling, and the vacuolization all appear reminiscent of those changes seen with lipid peroxidation, perhaps mediated through free radical reactions.[25] In a recent parallel study of pial vessels in the cat we demonstrated that the mercury-light-sodium-fluorescein-induced platelet aggregation could be inhibited by pretreatment of the mice with either glycerol or dimethyl sulfoxide, both of which effectively scavenge the hydroxyl free radical.[22] Moreover, in this model of microvascular injury, both in the mouse[18] and in the cat (unpublished), the arterioles dilate. Chemically generated radicals damage cat pial vessels, and this damage is associated with dilation of the arterioles.[26,27] Hydroxyl scavengers prevent the dilation induced by the light plus dye insult in the mouse[22] and by radical generated damage in the cat.[26] All the data suggest that the light plus dye combination generates radicals that injure the pial vessels. It is possible that the free radicals stimulate the platelets directly and might initiate aggregation even without endothelial damage; but in view of the presence of this damage, it seems unwise to suggest that it is not the cause of the platelet adhesion and aggregation. Moreover, scanning electron microscopy of the adhering platelets failed to demonstrate any fragmentation that would suggest damage by the light plus dye. Subtle changes, of course, are not ruled out.

White cells were seen scattered among the aggregating platelets and normal-appearing red cells. Occasional agranular profiles could represent damaged white cells (e.g, unlabeled profiles in Figures 2,3, and 12), although they resemble profiles identified as empty platelet pseudopods by others.[28,29] Leukocytes are reported to adhere to damaged endothelium[30] and to alter it.[31] We cannot rule out the possibility that leukocytes, either injured by light and dye or adhering to endothelium damaged by light and dye, could in turn release materials that cause platelet adhesion and aggregation. However, leukocytes were a minor element adjacent to the lesions.

Finally, the observed differences between structural changes in the venules and the arterioles also are of interest. As noted, the venules demonstrated more rapid structural damage and ultimately displayed conspicuous endothelial denudation, whereas the arterioles always lagged temporally behind in the onset of aggregation and demonstrated less endothelial change and sparse foci of endothelial denudation even at the advanced stages of aggregation. The reasons for these differences are unclear. They may be partly accounted for by the thicker wall of the arterioles. In any case, the lesser degree of arteriolar endothelial damage may explain at least in part why aggregation occurred first in the venules, both in the cat and in the mouse,[12] and why the platelet aggregation is more susceptible to antiaggregative drugs in the arterioles, as compared with the venules.[12]

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