Severe Microvascular Injury Induced by Lysosomal Releasates of Human Polymorphonuclear Leukocytes

Increase in Vasopermeability, Hemorrhage, and Microthrombosis Due to Degradation of Subendothelial and Perivascular Matrices

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The purpose of this study was to assess the nature of the lesions in the microcirculation of the dermis of rabbits induced with lysosomal releasates of human polymorphonuclear leukocytes (PMNs). No attempt was made in the studies presented in this publication to deal with the offending agent in the releasate. Four parameters of microvascular injury were quantitated: increase in vascular permeability with ^{125}I-labeled serum albumin, hemorrhage with ^{59}Fe-labeled erythrocytes, accumulation (aggregation) of platelets with ^{111}In-labeled platelets. In one experiment accumulation of ^{51}Cr-PMNs was investigated. The lysosomal releasate induced a rapid increase in vasopermeability, but both hemorrhage and exudate formation peaked 1 hour after intradermal injection. Platelet accumulation was also demonstrable in these lesions, and microthrombosis was a very prominent feature. The microvascular injury, including microthrombosis, could be elicited also in animals rendered leukopenic with nitrogen mustard. Simultaneous injection of prostaglandin E_2 with the releasate enhanced the microvascular injury. The morphologic changes in the microcirculation of the rabbit's dermis were assessed in lesions 5 minutes to 5 hours old. Several changes were encountered, primarily in the wall of venules and small veins and to a lesser degree in small arteries and capillaries. Ultrastructurally very early lesions (up to 15 minutes) had gaps or spaces in the endothelium, resembling those induced by mediators such as histamine or bradykinin. Older lesions were different, quite characteristic, and represent the hallmark of these lesions. Lysis and disappearance of vascular basement membrane, of perivascular collagen, and of the internal elastic lamina were a frequent finding, best demonstrable when microthrombi did not abut on vessel walls. Cellular components of vessels (endothelium, pericytes, smooth muscle) showed fragmentation, leading to complete disappearance of cellular elements. These lesions were usually walled off by platelet aggregates and fibrin. At times microthrombi occluded an entire vessel. These changes were interpreted as hemostasis. The mild accumulation of PMNs at the site of injury did not contribute significantly to the microvascular injury. The findings indicate that the unique changes in the microcirculation, not described before, may occur quite frequently, when the microvascular injury is elicited primarily by release of lysosomal constituents by phagocytic or nonphagocytic stimuli.

One can conclude that the hallmark of this type of injury is disappearance of basement membrane followed secondarily by disintegration of the vascular wall, followed in turn by hemorrhage and microthrombosis. The authors also conclude that this is the nature of microvascular injury associated with immune complex-induced and of infective inflammation, associated with phagocytosis and release by PMNs, the most common form of inflammation. (Am J Pathol 1985, 121:404–417)

MICROVASCULAR injury with increase in vasopermeability and hemorrhage is encountered when polymorphonuclear leukocytes (PMNs) undergo phagocytosis and release in the course of an acute inflammatory reaction. This occurs whether the phagocytosed material consists of killed microorganisms[1] or whether the material *per se* is inert, such as immune complexes.[2,3] When killed *Escherichia coli* are deposited in the dermis of decomplemented rabbits, there is less enhancement of vasopermeability than in normals, but PMN accumulation and hemorrhage are not significantly reduced, whereas in leukopenic animals PMN accumulation and vascular injury are abrogated.[4]

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Immune complex vasculitis[5] and pneumonitis[6] are entirely complement- and PMN-dependent; and in a classic immune-complex–induced inflammation, the Arthus reaction, both leukopenia and complement depletion prevent both exudation and hemorrhage.[3,7] In all these experimental models accumulation of PMNs is crucial and microvascular injury is PMN-mediated, although in the immune-complex–initiated process PMN accumulation is mediated entirely by host-derived factors, whereas in the *E coli*-induced lesion parasite-derived factors seem to play a role.

A number of PMN-derived substances could play a role in microvascular injury. The release of a variety of proteases, such as collagenase, elastase, cathepsin G, and other lysosomal proteases, from PMNs is a common feature of many inflammatory reactions. Although the mechanism of microvascular injury is not well understood, there is evidence that *in vitro* basement membrane can be degraded by PMN lysosomal proteases.[8] Release of proteases from inflammatory cells, predominantly PMNs, and tissue injury by these proteases, have been proposed by a number of investigators (see reviews[7,9,10]). In addition to proteases, oxygen-derived free radicals and metabolites have gained attention in PMN-dependent inflammatory reactions (see review[9]). These may cause cell injury and may interfere with the natural inhibitors of PMN-derived proteases.

Enhanced vascular permeability had been observed with PMN lysosomal releasates or lysates.[11–14] However, in these early studies quantitation of a complex inflammatory reaction was not possible. In fact, even enhanced vasopermeability was assessed only semiquantitatively by measuring the diameter of a blue spot induced by extravasation of Evans blue. Hemorrhage was observed but could not be quantitated.[14] We were interested in the effects of PMN lysosomal releasates on the microvasculature. In recent years it has become possible to quantitate in the whole animal a number of parameters of the inflammatory response, eg, enhanced vasopermeability,[15] accumulation of leukocytes[16] and platelets,[17] and of microhemorrhage.[18] With the currently available methods it is also possible to assess the kinetics of inflammatory lesions. We were particularly interested in enhanced vasopermeability and hemorrhage. The latter is particularly characteristic of PMN-mediated vascular injury.[7] In the course of the experiments a marked accumulation of platelets was also encountered. Another aim was to ascertain the nature of microvascular injury. Were these the classic gaps between adjcent endothelial cells which came to be generally known in the early 1960s as the "histamine" or "mediator" type? Or were the lesions more complex? In addition to swelling due to leakage, there was also grossly visible hemorrhage due to extravasation of erythrocytes. For assessment of these changes tissue was examined by light and transmission electron microscopy and the morphologic observations correlated with the functional quantitative assays.

Materials and Methods

Preparation of PMN Lysosomal Releasate

The PMNs were isolated from ACD blood obtained from the Canadian Red Cross, by the method of Henson,[19] as described before.[20] Briefly, in a typical experiment, we centrifuged approximately 1 liter of human blood in citric acid, trisodium citrate, dextrose solution at 500 *g* for 10 minutes to obtain platelet-rich plasma, which was discarded. The buffy layer, which contained most of the mononuclear cells and 25–30% of the PMNs, was also removed. The packed erythrocytes and the remaining 70–75% of the PMNs were sedimented at 1.0 *g* at room temperature for 30 minutes in 2.5% gelatin in pyrogen-free buffered saline (pH 7.4). The supernatant, containing over 95% PMNs, was centrifuged at 400 *g* for 10 minutes at room temperature. The pellet was resuspended in 0.84% ammonium chloride (pH 7.4) and left to stand for 5 minutes at room temperature so that the erythrocytes could lyse, then centrifuged at 200*g* for 10 minutes at 4 C. The pellet containing 95% PMNs was washed with sterile Tyrode solution, containing 0.1% gelatin, and resuspended in Tyrode-gelatin.

These cells were then induced to release their lysosomal contents (PMN-lysosomal releasate) by exposure to bovine serum albumin (BSA)–rabbit anti-BSA precipitates (final concentration of PMNs, 10^8; antibody protein in precipates, 1.5 mg/ml) in the presence of cytochalasin B (5.0 μg/ml) at 37 C for 30 minutes. After incubation, the PMNs and nonphagocytosed immune precipitates were removed by centrifugation(650*g* for 20 minutes).[20] The supernatant was injected intradermally. All protein determinations were performed by the method of Lowry et al.[21] The lysosomal releasates had a protein concentration of 3–3.5 mg/ml.

Induction of Lesions

All experiments were performed on randomly bred female New Zealand albino rabbits (2.5–3.0 kg). The

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hair on the back of the rabbits was removed with electric clippers, and PMN lysosomal releasate (0.2–0.5 ml) and appropriate controls were injected intradermally in duplicate or triplicate. The volume of the controls in each experiment was the same as that of the releasate. Two controls were used, sterile Tyrode–gelatin and heat-inactivated lysosomal releasate (boiled for 1 minute). In the quantitative assays the minimal increase in vasopermeability induced by the heat-inactivated releasate (versus Tyrode-gelatin) was subtracted from the values given by the untreated lysate, and the latter is presented as net values.

Measurement of Hemorrhage

Hemorrhage was measured with the aid of ^{59}Fe-labeled erythrocytes. These cells were prepared in donor rabbits, who were made moderately anemic by the bleeding of about 20 ml of blood and then the use of intravenous ^{59}Fe-labeled ferrous citrate, as described before.[18] However, in order to obtain erythrocytes with a very high specific activity, instead of administering 300 μCi of ^{59}Fe-citrate, we gave the donors 500 μCi twice at 3-day intervals. This protocol raised the specific activity of the ^{59}Fe-erythrocytes from 6×10^5 cpm/ml to 10×10^6 cpm/ml of packed erythrocytes. In most experiments 60×10^6 cpm were injected. In all previously reported studies from this laboratory, hemorrhage was measured as a cumulative parameter, since the ^{59}Fe-labeled erythrocytes were administered to the experimental rabbits at the beginning of the experiments. In the present studies the ^{59}Fe-erythrocytes were given as a pulse 15 minutes before the animals were killed.

To study the kinetics of the erythrocyte extravasation in response to injected lysosomal releasate, we followed the protocol given in Figure 1.

Measurement of Vascular Permeability

Enhanced vasopermeability was measured by means of intravenous injection of 20 μCi/kg of ^{125}I-labeled human serum albumin,[15] which was also administered with the radiolabeled erythrocytes (Figure 1).

Measurement of Platelet Accumulation

Accumulation (aggregation) of platelets was quantitated by administration of ^{111}In-labeled isolated platelets[17] 1 hour before the animals were killed, as shown in the diagram illustrating the protocol. The platelets were isolated from 40 ml of blood.

Measurement of Leukocyte Emigration

In a pilot experiment accumulation of ^{51}Cr-labeled leukocytes was examined, with the use of the procedure described by Issekutz and Movat.[16] The radiolabeled cells were injected 1 hour before the animal was killed.

Conversion of CPM into Volume of Extravasated Blood and Plasma

A few minutes before the experimental animals were killed, small aliquots of blood were drawn. To estimate the quantity of extravasated blood, we counted ^{59}Fe in whole blood; and to quantitate the volume of extravasated plasma, we measured the ^{125}I in the separated plasma. After the rabbits were killed the lesions were punched out and counted as described earlier.[1,4] From the counts per minute per lesion and the counts per minute per microliter of blood or plasma, the volume of extravasated blood or plasma was calculated.

Preparation of Tissue for Light and Electron Microscopy

For the preparation of semithin (1–2 μ) and ultrathin sections dermal tissue obtained at the end of the experiments was used, as described in the experimental protocol.

Blocks of skin measuring $10 \times 5 \times 2$ mm were placed into fixative consisting of 0.01 M phosphate-buffered (pH 7.4) glutaraldehyde (final concentration, 2%) and formaldehyde (final concentration, 10%), as described before.[4] Smaller fragments (1.5–2.0 mm in diameter) were fixed in 2.0–3.0% glutaraldehyde. One-half of the small blocks were fixed at 4 C, and the other half at

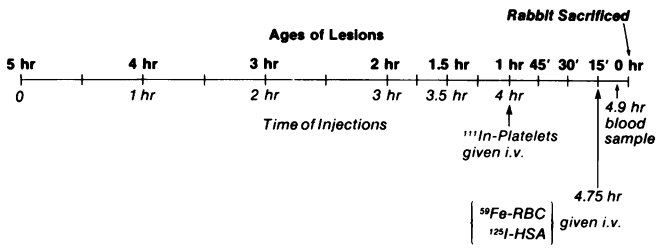


Figure 1—Experimental protocol. PMN lysosomal releasate and appropriate controls were injected intradermally in duplicate or triplicate in the back of rabbits at the times indicated in the upper half (age of lesions). In the lower half the time of intravenous injections is illustrated, consisting of ^{111}In-platelets given 60 minutes and ^{59}Fe-erythrocytes and ^{125}I-human serum albumin given 15 minutes before sacrifice. Immediately before sacrifice a sample of blood was taken.

20 C, for 2 hours, followed by overnight fixation at 4 C. To enhance the penetration of the fixatives, the vials containing the tissue and fixatives were placed on a turntable and rotated in an oblique position. The small fragments were rinsed and kept in buffer for 1 hour and postfixed for 2 hours in 1.0% osmium tetroxide in phosphate buffer. The large blocks were kept in buffer at 4 C (two changes of 30 minutes each), dehydrated, and embedded in hydroxyethyl methacrylate; semithin sections were cut on a du Pont-Sorvall JB-4 microtome. The dehydrated small blocks were infiltrated and embedded in an epxoy resin consisting of 20 parts Araldite 502, 25 parts Epon 812, and 60 parts dodecylsuccinic anhydride. The resin was polymerized by 45 C for 1 day and at 60 C for 4–7 days.

For light microscopy the semithin sections prepared from the large blocks were stained with buffered (pH 7.0) azure-eosin. The semithin sections of the small blocks were stained with alkalinized Azure II. Some sections were first stained with azure-eosin, examined, and photographed. The coverslips were then allowed to float off in xylene, and the sections were destained in acid-alcohol, restained in alkalinized Azure II, reexamined and when desirable rephotographed. For electron microscopy the sections were stained with uranyl and lead salts or with the latter only. They were examined in a Philips EM-400 transmission electron microscope. In 2 rabbits with dermal lesions of varying ages, Gleptosil (gleptoferron; Fisons Corp., Ltd., Toronto, Ontario, Canada) was administered intravenously as an electron-dense tracer 15 minutes before death. According to the manufacturer (Fisons, Loughborough, Leics, England) gleptoferron is a macromolecular complex of β-ferric oxyhydroxide and dextran glucoheptonic acid with an approximate molecular weight of 2 million (average size of particles, 50 × 20 nm). It is used clinically in anemic piglets. On the basis of the therapeutic dose used in veterinary medicine, 0.2–0.5 ml/kg was administered intravenously to rabbits without any visible toxic effects.

Results

Increase in Vascular Permeability: Exudation

Intradermal injection of PMN-lysosomal releasate induced a rapid increase in vascular permeability and accumulation of plasma and ^{125}I-albumin at the injected site within minutes (Figure 2). The vessels remained leaky for several hours, as shown in Figure 2, but the rate of plasma and ^{125}I-albumin escape was highest during the first hour of the experiment. We injected a large amount of lysate (0.5 ml) in this experiment to obtain an intense response. When increasing concentrations of lysosomal lysates were injected, a proportionate increase in the exudation was observed, reflecting a direct relationship between lysosomal contents and the extent of exudation.

Extravasation of Erythrocytes: Hemorrhage

Unlike the increase in vascular permeability, only a small amount of hemorrhage was noted in the earliest lesions, but like exudation, hemorrhage peaked at 1–1.5 hours after injection of the lysosomal releasate, and it also persisted for several hours (Figure 2). As in the permeability studies, increasing concentrations of lysosomal lysate induced a proportionate extravasation of erythrocytes.

Platelet Accumulation

Platelet accumulation in the affected vessels was first observed morphologically. Therefore, a quantitative study with labeled platelets was carried out in 1- and

Figure 2—Kinetics of enhanced vascular permeability and hemorrhage induced in the dermis of rabbits given injections in duplicate of 1.5 mg (0.5 ml) of PMN lysosomal releasate. The quantitation of the two parameters measured is described in Materials and Methods. In this and all other experiments the radiolabeled serum albumin and erythrocytes were in the animals' circulation for 15 minutes before sacrifice. The increase in vasopermeability induced by heat-inactivated releasate was minimal and was subtracted from the vasopermeability induced by unheated releasate. The data on hemorrhage also represent net values.

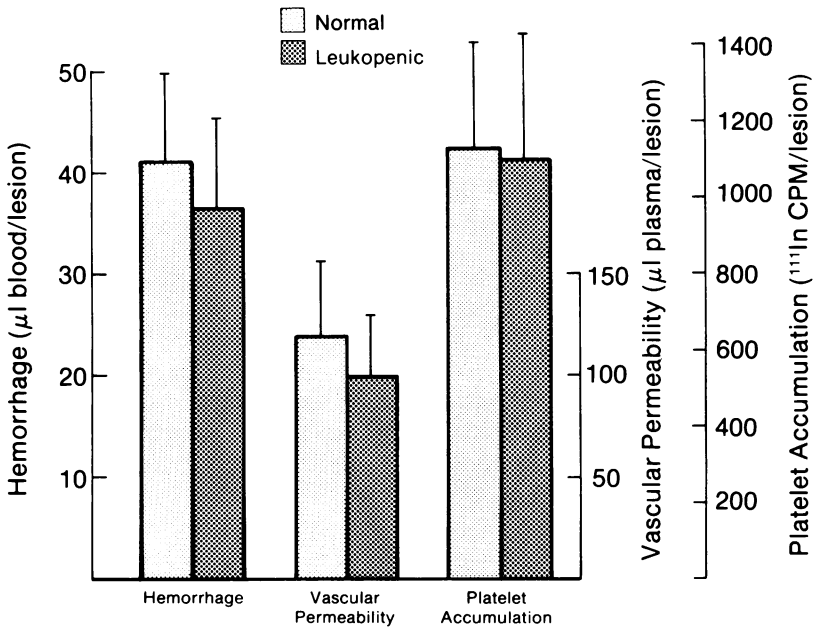


Figure 3—Microvascular injury in normal and leukopenic rabbits. This illustration shows that there was a slight, but not significant, decrease in rabbits rendered leukopenic, when tested for hemorrhage, increase in vascular permeability, and accumulation of platelets, when injected in triplicate 1 hour before sacrifice with 1.5 mg of lysosomal releasate (0.5 ml).

2-hour-old lesions. At both times accumulation of ^{111}In was demonstrable (Figure 3).

Accumulation of Leukocytes

In a pilot experiment PMN lysosomal releasate was injected (0.5 ml) in duplicate into the dermis of a rabbit 1, 2, and 4 hours before sacrifice and ^{51}Cr-labeled leukocytes were administered intravenously. In addition to the controls mentioned in Materials and Methods, this rabbit was given 0.2 ml of 10^{-5} M formylmethionyl-leucyl-phenylalanine and zymosan-activated rabbit plasma (ZAP), as a source of C5a des Arg.[22] Compared with the chemotaxin-induced accumulation of leukocytes at the site of injection, the accumulation induced by the lysosomal releasate was very small. This was confirmed morphologically in lesions ranging between 5 minutes and 5 hours. The results are shown in Table 1. Because of the variation in the peak response to chemotaxins (see Colditz and Movat[22] the accumulation of ^{51}Cr-PMNs varied with each agent injected.

Experiments With Leukopenic Rabbits

The vascular injury induced by the PMN lysosomal releasate lasted for 3–4 hours (Figure 2). Although PMN accumulation in the lesions was not intense, possible contribution to microvascular injury by the accumulated cells[4,7,23,24] was given consideration. Three rabbits were rendered leukopenic with one intravenous injec-

Table 1—Accumulation of PMNs Induced by Chemotaxins and PMN Lysosomal Releasate

Test substance injected	Time of ^{51}Cr-leukocytes in circulation*	Accumulation of ^{51}Cr-PMNS (cpm/lesion; mean ± SEM)†
FMLP (10^{-5} M) in 0.2 ml	1–2 hours after injection	2180 ± 165
ZAP (90%) in 0.2 ml	2–3 hours after injection	3628 ± 358
PMN lysosomal releasate (1.5 mg protein) in 0.5 ml	1–2 hours after injection	420 ± 18
PMN-lysosomal releasate (1.5 mg protein) in 0.5 ml	2–3 hours after injection	403 ± 22
Heat inactivated PMN lysosomal releasate (1.5 mg protein) in 0.5 ml	2–3 hours after injection	342 ± 17
1% gelatin in Tyrode in 0.5 ml	2–3 hours after injection	292 ± 25

* ^{51}Cr-leukocytes circulated for 1 hour before sacrifice.

† The accumulating leukocytes were identified as over 95% PMNs in histologic sections.

Intradermal injections were timed to achieve exposure of the lesions to the circulating radiolabeled cells at various times for 1 hour, on the basis of results in Colditz and Movat.[22] FMLP, formylmethionyl-leucyl-phenylalanine; ZAP, zymosan-activated plasma.

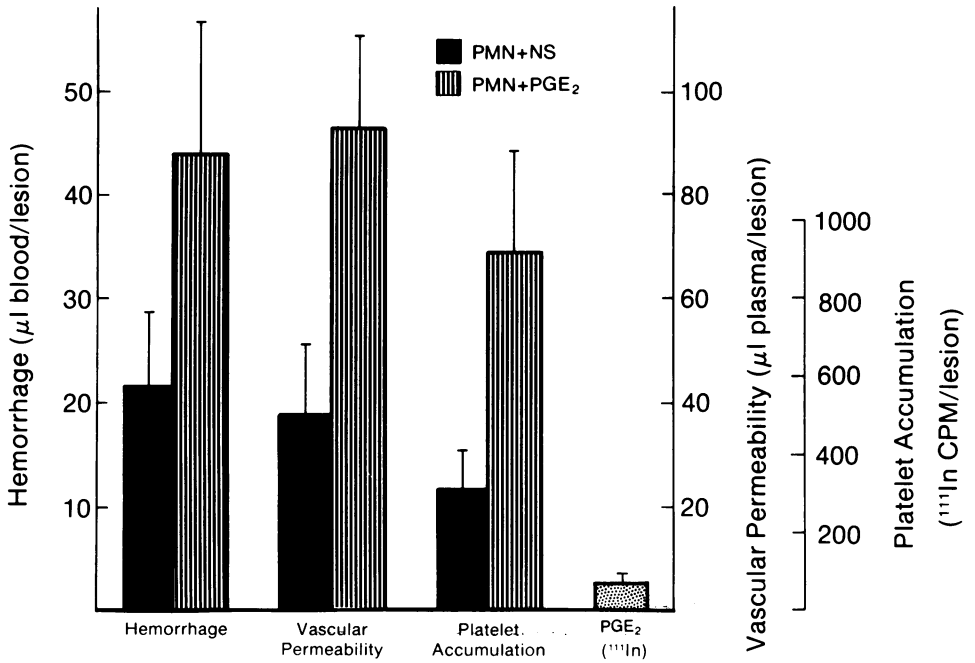


Figure 4—Effect of a PMN lysosomal releasate on dermal vessels in the presence or absence of simultaneously injected PGE_2 (1.0 μg of PGE_2). The PGE_2 was injected intradermally in triplicate with 0.6 mg (0.2 ml) of lysosomal releasate in one-half of the back of rabbits. Into the second half of the back 2% ethyl alcohol (in which the PGE was dissolved) was injected with the releasate. The three parameters illustrated were quantitated. The rabbits were killed 1 hour after the intradermal injections. The PGE_2 caused a significant increase in the three parameters tested ($P < 0.05$). In this experiment the PGE_2 injected by itself caused no increase in vasopermeability and hemorrhage, but it induced a minimal accumulation of platelets. *PMN + NS*, releasate containing 2% ethanol in normal saline.

tion of nitrogen mustard (Mustargen, Merck Sharpe and Dohme, Montreal, Quebec, Canada, 1.75 mg/kg) and the animals were used 72 hours later.[25] At this time the total white blood cell count was less than 10^9/l (<1000/μl) of blood, and differential counts showed almost no granulocytes; whereas the platelet counts were reduced by less than 30%. The results are shown in Figure 3. The vascular injury induced by the lysosomal releasate was slightly, but not significantly, diminished in the leukopenic rabbits (see also Morphologic Observations).

Effect of PGE_2

In previous experiments it was observed that increase in vascular permeability,[26] accumulation of leukocytes, and hemorrhage[27] can be enhanced by prostaglandins of the E class. Triplicate lesions were induced with a mixture of 1.0 μg PGE_2 (Upjohn, Toronto, Ontario, Canada) and PMN lysosomal releasate in 3 rabbits on one half of the back and releasate and 2% alcohol (the final concentration in the PGE_2) on the other half of the back. The sites injected with the PGE_2 containing releasate demonstrated a significant increase in all three parameters measured, compared with the controls (Figure 4). The minimal accumulation of platelets induced by PGE_2 was seen also, but to an even lesser extent, at sites at which 2% ethanol in normal saline was injected.

Morphologic Observations

The most impressive alteration affecting the entire dermis, when examined grossly and microscopically at

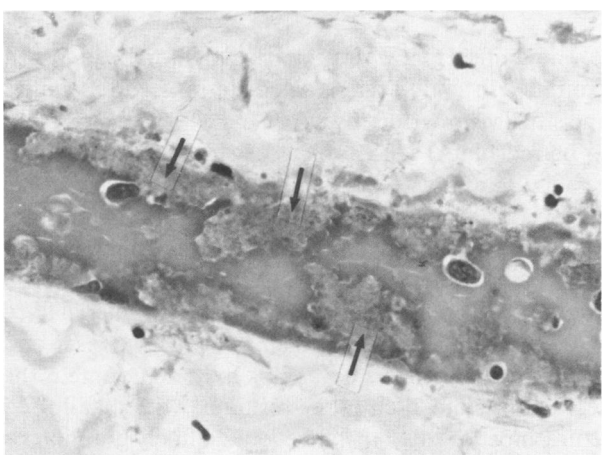


Figure 5—Part of a small vein in a 2-hour-old lesion of a leukopenic rabbit. The lumen is filled with very densely packed red blood cells. The *arrows* point to granular masses (platelet aggregates) which abut on the wall and protrude into the lumen. (Methacrylate-embedded tissue, stained with Azure II, ×560)

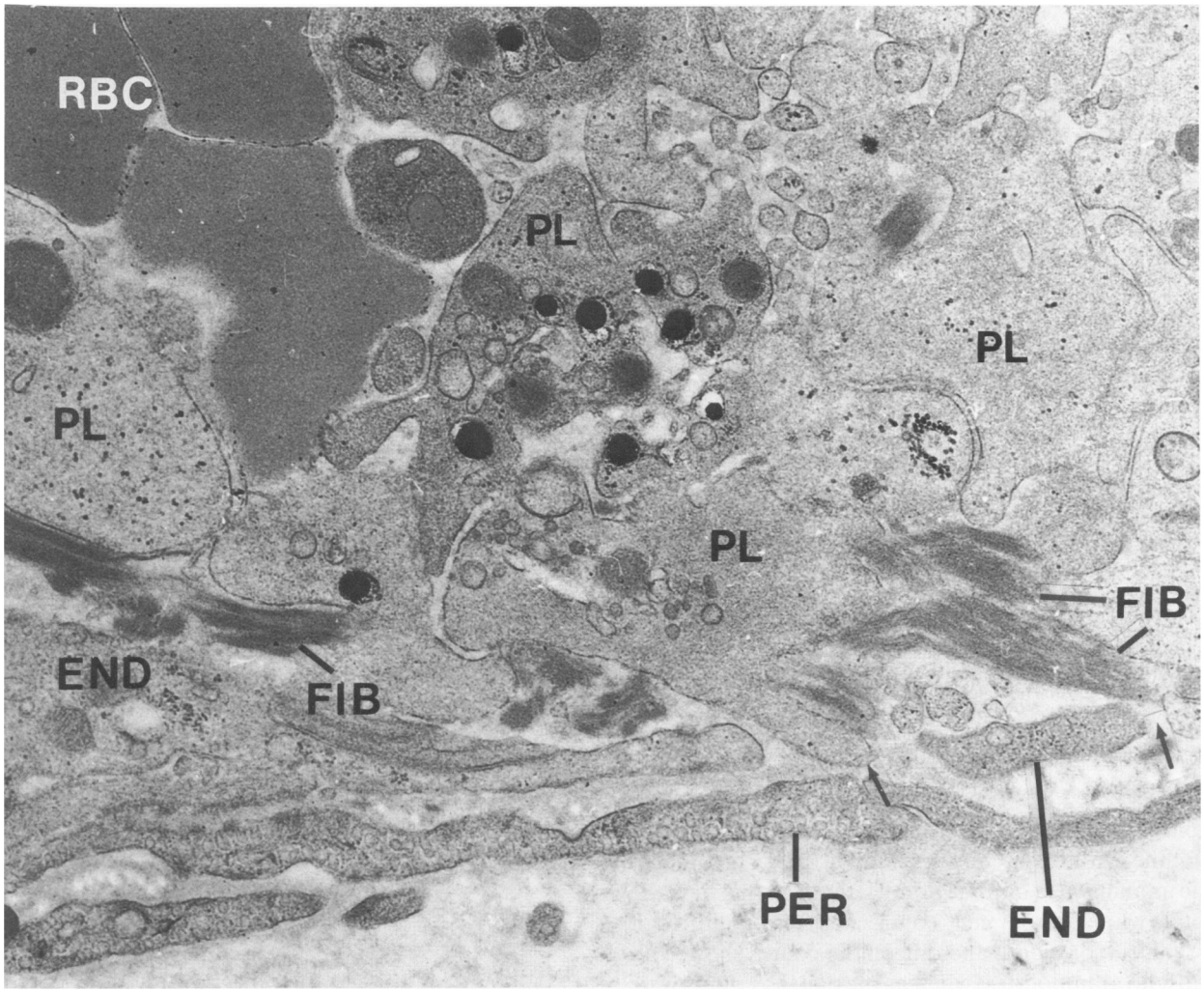
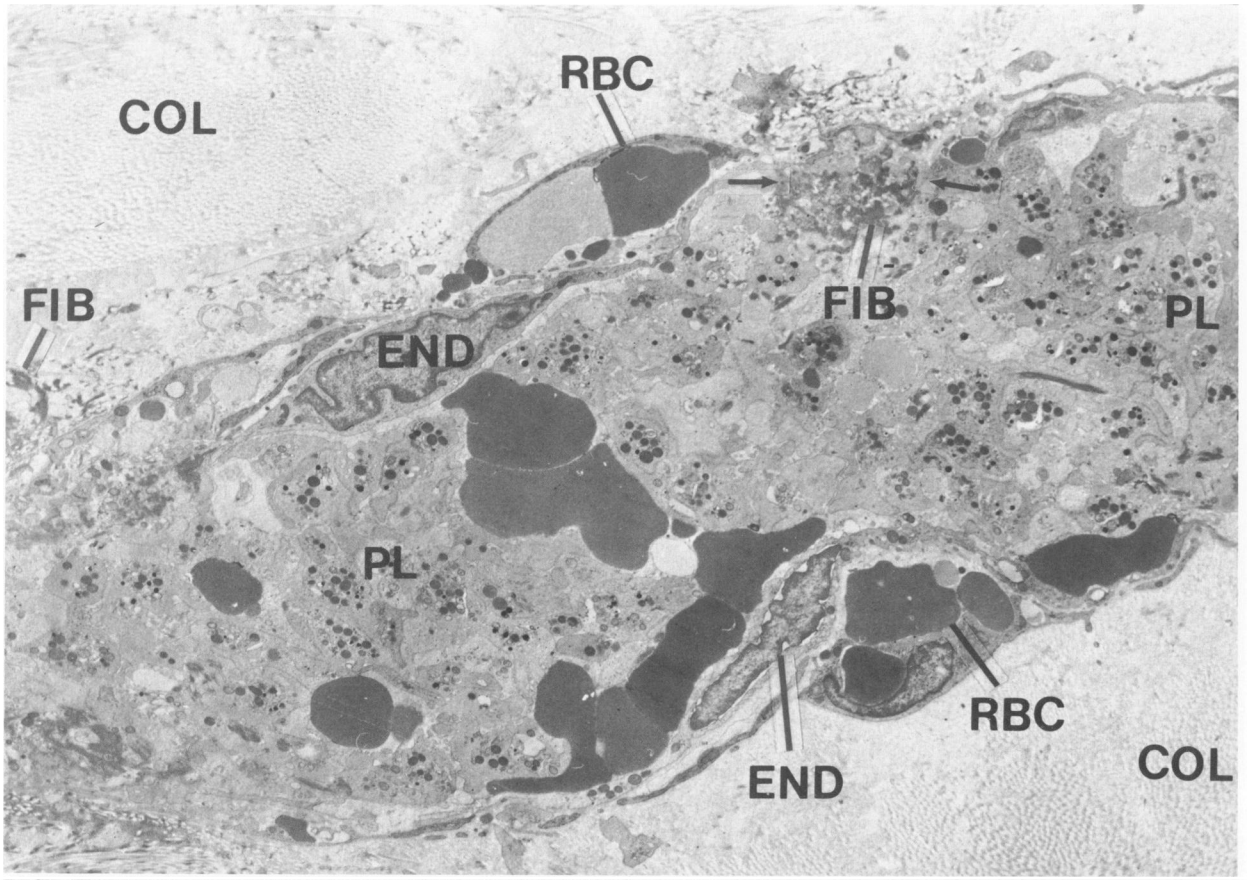


Figure 6—Electron micrograph of part of a medium-sized venule in a 5-minute-old lesion. Part of the wall is identified as endothelium (*END*) and as a periendothelial cell or pericyte (*PER*). Platelets (*PL*) and fibrin strands (*FIB*) abut on the endothelium. Some of the platelets are degranulated. At one of the *arrows* a cytoplasmic process of a platelet fills the gap, and at the second *arrow* fibrin abuts on another gap in the endothelium. (×19,250)

low magnification, was the marked hemorrhage. When examined in azure-eosin-stained sections, some venules and small veins were densely packed with what appeared to be red blood cells, typical of stasis. Small arterial vessels, as well as some venules and small veins, were relatively free of erythrocytes. In addition, some venules and small veins contained a homogeneous pink mass, lighter than the red blood cells and found mostly abutting on the endothelium. At times this material filled the vessels. It resembled plasma, but was denser and hyalin-like. When the coverslips were demounted, the sections destained and then restained with alkalinized azure, most of this substance looked finely granular or punctate and protruded like polyps into the lumen (Figure 5). By electron microscopy these masses corresponded primarily to platelets. Examining the platelet aggregates where they abutted on the endothelium revealed, in lesions not older than 15 minutes, discontinuities of the endothelium resembling the gaps encountered when histamine or bradykinin are injected intradermally but obscured by platelets and fibrin abutting on the endothelium (Figure 6).

Rendering the rabbits leukopenic did not prevent development of the vascular injury with exudation of plasma, hemorrhage, stasis, and formation of platelet aggregates (Figure 5). The red blood cells were often

Figures 7 and 8—Electron micrographs of part of a small vein in a 1-hour-old lesion of a leukopenic rabbit. **Figure 8** is a higher magnification of the disrupted wall (*arrows*) illustrated in **Figure 7** and filled with fibrin (*FIB*), interwoven with extensions of platelets. The vessel is completely occluded by platelets (*PL*). Several red blood cells are in the lumen, and those marked (*RBC*) are in the vessel wall. Cross-sectioned collagen fibrils (*COL*) are seen outside the vessel. (**Figure 7**, ×4125; **Figure 8**, ×19,250)



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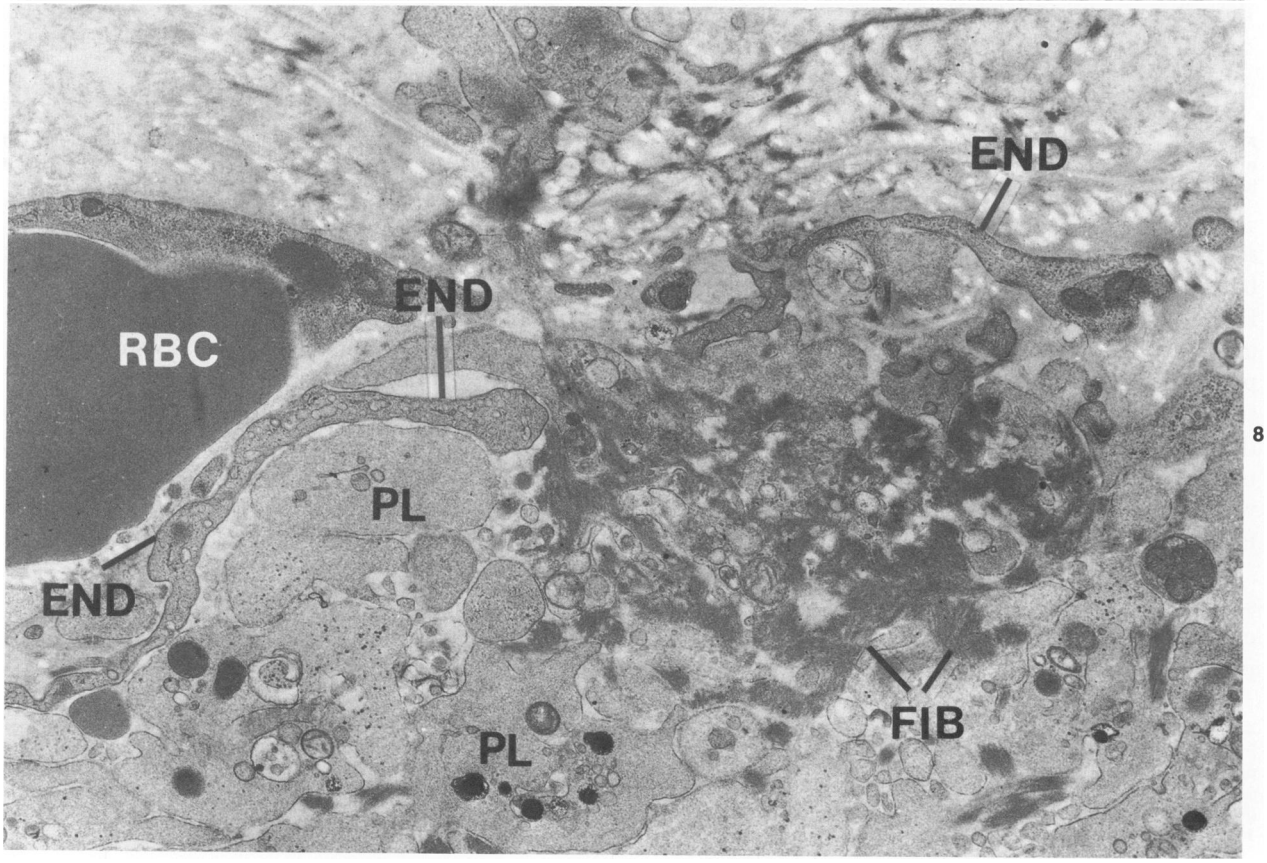

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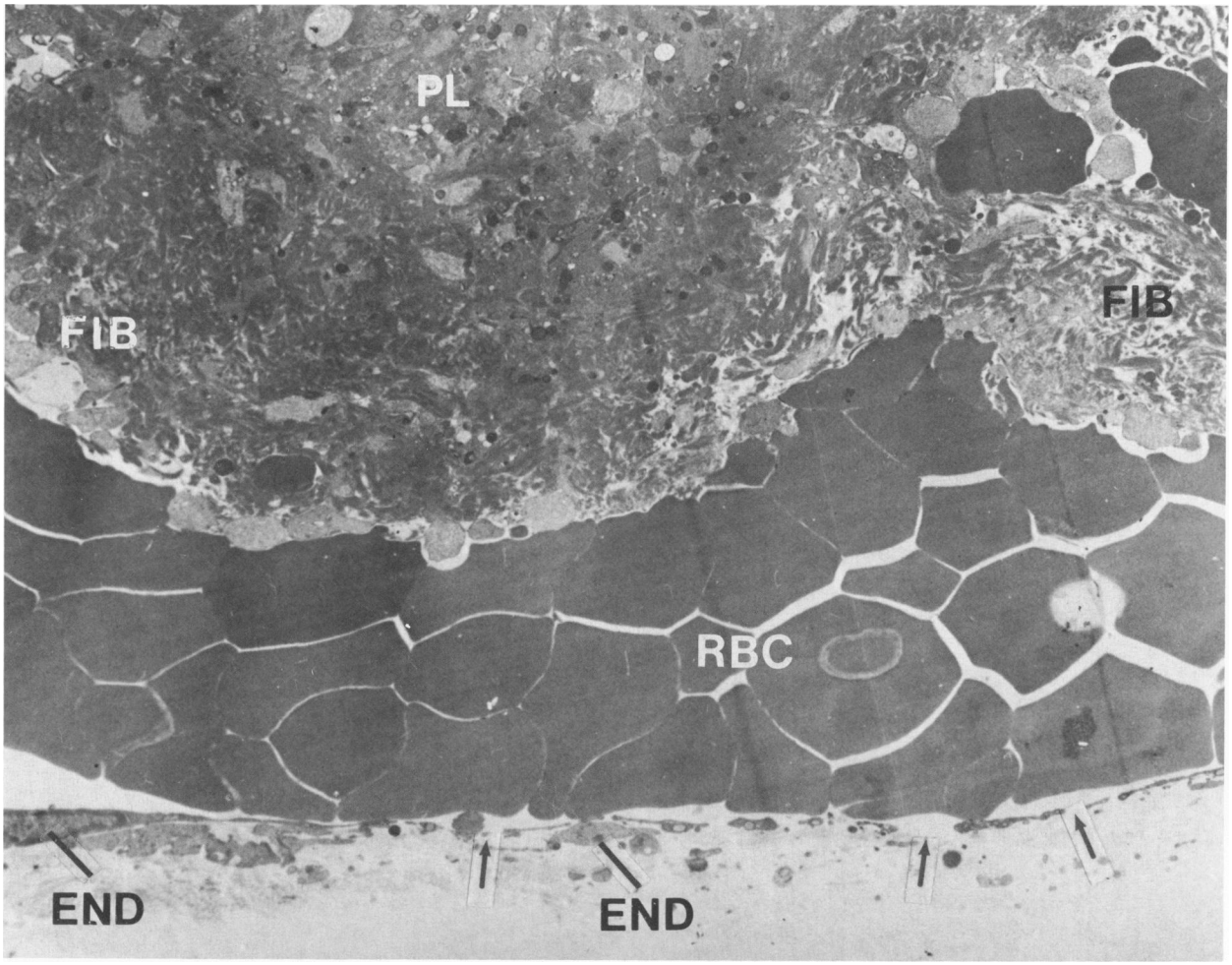


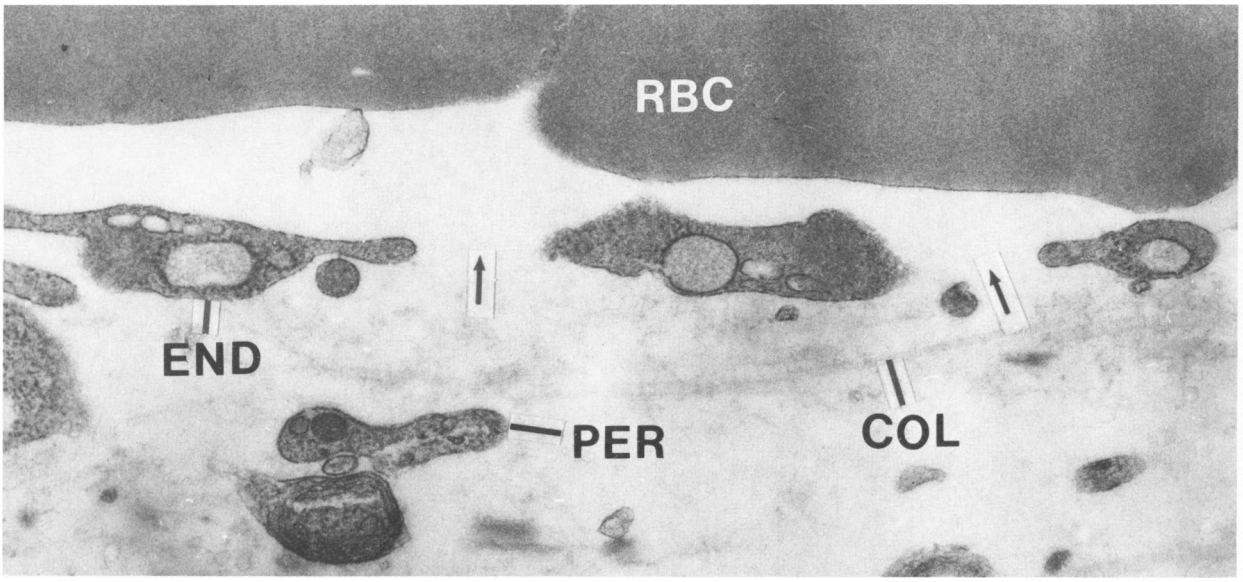
Figure 9—Part of a small vein from a 1-hour-old lesion which contains a thrombus, consisting of platelets (*PL*) in the center and fibrin (*FIB*) at the periphery. Between the thrombus and remnants of endothelium (*END*) there are red blood cells (*RBC*). No basement membrane can be recognized, and there is continuity (*arrows*) between the lumen and perivascular tissue, which is devoid of collagen. (×4950)

so densely packed that individual corpuscles could barely be recognized by light microscopy (Figure 5). Electron microscopy, however, showed much more detail (Figure 6). The vessel illustrated in Figure 5 was completely occluded by platelets, a few red blood cells, and some fibrin (Figure 7). Erythrocytes were entrapped in the lumen, but also in the wall of the small vein, just outside the endothelium. At higher magnification there was disruption of the wall, and this was walled off by fibrin and fragments of platelets (Figure 8).

More severe loss of vessel wall constituents was observed in both normal and leukopenic rabbits. The changes in the vessel wall were more visible when the platelet–fibrin thrombi did not abut against the vessel (Figure 9). In such lesions the endothelium was fragmented, leaving spaces between the cytoplasmic fragments (Figure 10). No basement membrane was demonstrable, and the collagen which normally surrounds the vessels had almost completely disappeared (Figures 9 and 10). The large thrombi (Figure 9) were made up in part of swollen, degranulated, and partially fragmented or lysed platelets and of fibrin (Figures 11 and 12). These changes at the "blood–tissue barrier" (see Movat and Fernando[44]) constitute the hallmark of the changes observed.

The edema due to exudation was not as readily demonstrable histologically as the hemorrhage and the thrombosis. However, when colloidal iron (gleptofer-

Figures 10–12—Higher magnification of vessel illustrated in **Figure 9**. **Figure 10** shows details of the walls of the vein. The endothelium (*END*) and pericytes (*PER*) are fragmented and "float" between the lumen and perivascular tissue. No basement membrane and only a few collagen fibrils (*COL*) are seen. Discontinuities in the endothelium are marked by *arrows*. **Figure 11** represents part of the thrombus, consisting of degranulated platelets (*PL*) intermingled with fibrin (*FIB*). **Figure 12** illustrates fibrin at higher magnification with typical periodicity (~200 nm). (**Figure 10**, ×35,000; **Figure 11**, ×23,000; **Figure 12**, ×60,000)



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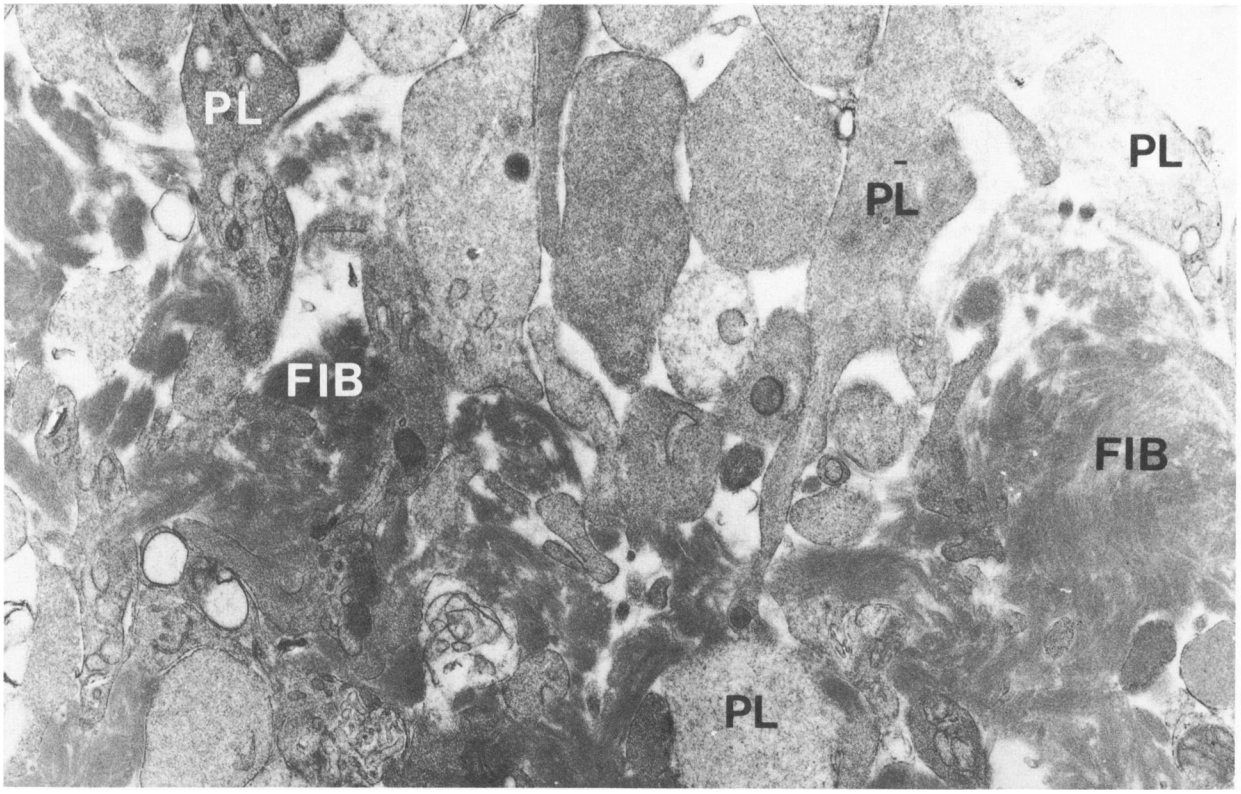

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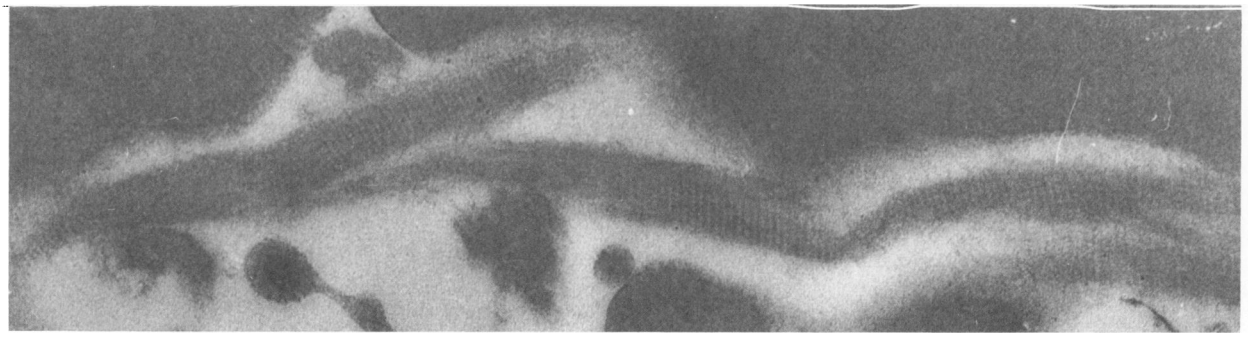

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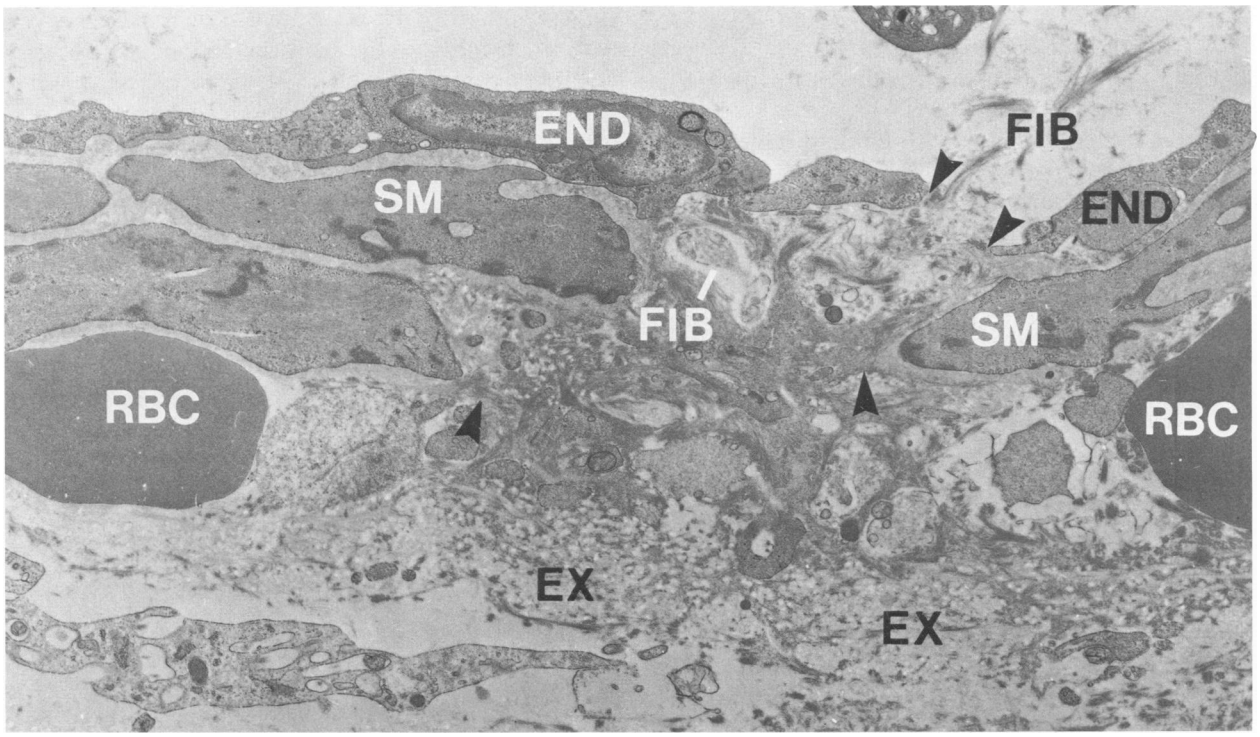

Figure 13—Disruption of the wall of a small arterial vessel. The disrupted portion is outlined by four *arrows*. Within this area endothelial (*END*) and smooth muscle cells (*SM*), internal elastica, and basement membrane are missing, and the channel created contains cellular fragments (probably platelets) and fibrin (*FIB*), which is seen also in the lumen. Protein-rich exudate (*EX*) is seen perivascularly. (×12,000)

ron) was administered intravenously, it was present in the lumens of all vessels. Only an occasional arterial vessel and some of the capillaries had iron particles beyond the endothelial lining, but a considerable amount had accumulated in venules and small veins outside the basement membrane. In older lesions it was present in the fibrin-rich exudate which had accumulated perivascularly and paravascularly.

As indicated, little electron-dense tracer had passed the endothelium of arterioles and small arteries, but there were occasional arterial vessels with disrupted walls containing fragmented platelets, fibrin, and tracer. The disruption had affected the endothelium, the internal elastica, the smooth muscle cells of the media, and the basement membrane surrounding the endothelium and smooth muscle cells (Figure 13).

Discussion

Lysis of basement membrane and cellular changes of the vessel wall, presumably secondary to those in the subendothelium and perivascular matrices, constitute the prime target of PMN lysosomal releasates.

Phagocytic stimuli (such as immune-complex–coated latex particles) have been reported to induce in PMNs *in vitro* primarily an intracellular (into digestive vacuoles) release of azurophil and an extracellular release of specific granule contents.[28] Ohlsson and Olsson[29] reported that upon incubation with immune complexes or yeast cells, in the presence of 10% fresh serum, human PMNs release 25–30% of both their contents of elastase (azurophil granules) and collagenase (specific granules) in addition to other lysosomal enzymes. When PMNs are subjected to frustrated phagocytosis of uningestable material, all the release is extracellular.[19,30] When PMNs, in suspension, are stimulated by phagocytosable particles, but phagocytosis is prevented by cytochalasin B, all the release occurs into the surrounding medium, and, furthermore, the release is enhanced.[20,31,32] Under such conditions exocytosis is demonstrable.[20] A phagocytosis-independent process is the recently described secretion of gelatinase from small, morphologically still unidentified storage organelles, distinct from azurophil and specific granules.[33] What happens *in vivo* is uncertain, but ultrastructural studies indicate that PMNs can become completely degranulated.[2]

In the studies presented in this publication, PMNs were incubated with immune precipitates in the presence of cytochalasin B, to achieve maximum enzyme

release and at the same time specific release or secretion. This material is referred to as "releasate." Such releasate, as well as a granule lysate of rabbit PMNs, has been shown to enhance vascular permeability.[11-14] A granule lysate of human PMNs induced hemorrhage when injected intradermally, and lysis of basement membrane was demonstrated both *in vitro* and by electron microscopy.[14] This is attributable primarily to elastase,[34] which is the most extensively studied PMN protease.[35] A PMN granule lysate and partially purified elastase degrade *in vivo* elastin of the aorta,[36] and *in vitro* highly purified elastase degrades elastin of lung[37] and cartilage proteoglycans.[38] The degradation of the endothelial basement membrane is probably the key factor leading to the increase in vascular permeability and hemorrhage. Such degradation by intact PMNs has been demonstrated recently *in vitro*.[8] In these studies it was shown that PMNs were able to use oxygen metabolites to potentiate their released elastase to solubilize endothelial basement membrane in the presence of α-1 proteinase inhibitor, the principal inhibitor of elastase.

When a large number of microorganisms or immune complexes accumulate in the tissues, numerous PMNs are chemoattracted and through phagocytosis are stimulated to release their lysosomal contents. This seemingly cannot be dealt with by the appropriate inhibitors; and, at least temporarily, the homeostatic balance is lost, which results in microvascular injury.[7,9,10] Only the earliest changes induced with a PMN lysosomal releasate resembled those referred to as the histamine type or mediator type.[39] This corresponds to the "immediate transient" response in injury induced by heat, the most common form of microvascular changes studied in the 1960s. This type of alteration is encountered also with other mediators which are derived from the cells and fluids of the host (see reviews[40,41]). It is confined to interendothelial gaps of venules and small veins, without visible endothelial necrosis, alterations in basement membrane, connective tissue matrixes, and other cellular components of vessels (pericytes and smooth muscle cells).

Whereas histamine and other mediators elicit a *functional response* from a cell, without damage, many inflammatory reactions are the result of *direct injury*. Direct microvascular injury is elicited by mechanical, chemical, and physical stimuli. It is characterized by a slow repair.[39] Based primarily on the type of vasopermeability induced, a "delayed-prolonged" and an "immediate sustained" type of leakage have been described.[42,43] In the latter, such as after heat injury, one encounters endothelial necrosis and sloughing (see review[40]).

With the PMN lysosomal releasate one encounters very severe injury of the microcirculation. Its hallmarks are marked enhancement of vasopermeability, hemorrhage and microthrombosis, and, ultrastructurally, lysis of the basement membrane and of perivascular connective tissue. It seems reasonable, therefore, to propose that we have outlined a *special kind of direct injury* of the microcirculation. The enhanced vasopermeability developed more rapidly than hemorrhage, but both peaked in 1-hour-old lesions (gap formation followed by disruption of the vascular wall). The kinetics of these changes in the microcirculation could be ascertained by admnistering the ^{59}Fe-labeled erythrocytes as a 15-minute pulse, like the ^{125}I-albumin. Microthrombosis (platelet accumulation) developed simultaneously.

Vascular injury by a PMN lysosomal lysate has been observed before. Janoff and Zeligs[14] reported a loss of basement membrane when venules were examined by electron microscopy and, *in vitro*, the lysate degraded isolated basement membrane. The complex vascular changes reported in the present study were not observed. Earlier reports describe "plugging" of interendothelial gaps by individual platelets[44] or "covering" of the gaps by small platelet aggregates.[39] The transient platelet accumulation observed by Issekutz et al[25] was believed to be PMN-mediated, since it did not develop when the inflammatory stimuli (C5a des Arg, *E coli, E coli* culture supernatant, the oligopeptide f-met-leu-phe, or endotoxin) were deposited intradermally into animals rendered leukopenic. Under these conditions, in the absence of PMNs, microvascular injury probably did not occur, and hence "plugging" or "covering" by platelets was not required. In the experiments reported in this publication the changes in the microcirculation, including thrombosis, were induced by the injection of a lysosomal releasate and hence could be elicited also in leukopenic rabbits. The few accumulating PMNs did not contribute appreciably to the development of these severe lesions.

One additional observation was made, that PGE_2 enhanced the parameters of acute inflammation that were quantitated. PGEs enhance the vascular permeability-inducing effect of histamine and bradykinin, because, as measured with radiolabeled microspheres, they enhance the blood flow of the microcirculation at the site of injection.[26] This finding was confirmed by using ^{133}Xe-clearance for quantitating blood flow.[45] It was further demonstrated that the enhanced blood flow induced by PGE_2 is associated with enhanced accumulation of leukocytes when zymosan-activated plasma (C5a des Arg) is deposited into the dermis as a chemotactic stimulus and that this is followed by enhanced hemorrhage.[27] Seemingly, because of vasodilatation, opening

up of more vessels, and enhanced blood flow induced by the injected PGE_2, all changes measurable in the microcirculation are enhanced.

References

1. Kopaniak MM, Issekutz AC, Movat HZ: Kinetics of acute inflammation induced by *E. coli* in rabbits: quantitation of blood flow, enhanced vascular permeability, hemorrhage, and leukocyte accumulation. Am J Pathol 1980, 98:485–498
2. Uriuhara T, Movat HZ: The role of PMN-leukocyte lysosomal enzymes in tissue injury, inflammation and hypersensitivity: I. The vascular changes and the role of PMN-leukocytes in the reversed passive Arthus reaction. Exp Mol Pathol 1966, 5:539–558
3. Crawford JP, Movat HZ, Minta JO, Opas M: Acute inflammation induced by immune complexes in the microcirculation. Exp Mol Pathol 1985, 42:115–193
4. Kopaniak MM, Movat HZ: Kinetics of acute inflammation induced by *E. coli* in rabbits: II. The effect of hyperimmunization, complement depletion and depletion of leukocytes. Am J Pathol 1983, 110:13–29
5. Ward PA, Cochrane CG: Bound complement and immunologic injury of blood vessels. J Exp Med 1965, 121:215–234
6. Johnson KJ, Ward PA: Acute immunologic pulmonary alveolitis. J Clin Invest 1974, 54:349–357
7. Cochrane CG, Janoff A: The Arthus reaction: A model of neutrophil and complement mediated injury, The Inflammatory Process. 2nd edition. Vol 3. Edited by BW Zweifach, L Grant, RT McCluskey. New York and London, Academic Press, 1974, pp 86–162
8. Weiss SJ, Regiani S: Neutrophils degrade subendothelial matrices in the presence of alpha-1-proteinase inhibitor: Cooperative use of lysosomal proteinases and oxygen metabolites. J Clin Invest 1984, 73:1297–1303
9. Janoff A, Carp H: Proteases, antiproteases, and oxidants: pathways of tissue injury during inflammation, Current Topics in Inflammation and Infection. Edited by G Majno, RS Cotran, N Kaufman. Baltimore and London, Williams & Wilkins, 1982, pp 62–82
10. Wasi S, Movat HZ: Phlogistic substances in neutrophil leukocytes: Their possible role *in vivo* and their *in vitro* properties. Curr Top Pathol 1979, 68:213–238
11. Movat HZ, Uriuhara T, Macmorine DRL, Burke JS: A permeability factor released from leukocytes after phagocytosis of immune complexes and its possible role in the Arthus reaction. Life Sci 1964, 3:1025–1032
12. Golub ES, Spitznagel JK: The role of lysosomes in hypersensitivity reactions: Tissue damage by polymorphonuclear neutrophil lysosomes. J Immunol 1965, 95:1060–1066
13. Seegers W, Janoff A: Mediators of inflammation in leukocyte lysosomes: VI. Partial purification and characterization of a mast cell-rupturing component. J Exp Med 1966, 124:833–849
14. Janoff A, Zeligs JG: Vascular injury and lysis of basement membrane by neutral proteases of human leukocytes. Science 1968, 161:702–703
15. Udaka K, Takeuchi Y, Movat HZ: Simple method for quantitation of enhanced vascular permeability. Proc Soc Exp Biol Med 1970, 133:1384–1387
16. Issekutz AC, Movat HZ: The *in vivo* quantitation and kinetics of rabbit neutrophil leukocyte accumulation in the skin in response to chemotactic agents and *Escherichia coli.* Lab Invest 1980, 42:310–317
17. Jeynes BJ, Issekutz AC, Issekutz TB, Movat HZ: Quantitation of platelets in the microcirculation: Measurement of indium-111 in microthrombi induced in rabbits by inflammatory lesions and related phenomena. Proc Soc Exp Biol Med 1980, 165:445–452
18. Kopaniak MM, Issekutz AC, Burrowes CE, Movat HZ: The quantitation of hemorrhage in the microcirculation of inflammatory lesions and related phenomena. Proc Soc Exp Biol Med 1980, 163:126–131
19. Henson PM: The immunologic release of constituents from neutrophil leukocytes: I. The role of complement on non-phagocytosable surfaces or phagocytosable particles. J Immunol 1971, 107:1535–1546
20. Movat HZ, Steinberg SG, Habal FM, Ranadive NS: Demonstration of a kinin-generating enzyme in lysosomes of human polymorphonuclear leukocytes. Lab Invest 1973, 29:669–684
21. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenon reagent. J Biol Chem 1951, 265–275
22. Colditz IG, Movat HZ: Kinetics of neutrophil accumulation in acute inflammatory lesions induced by chemotaxins and chemotaxinigens. J Immunol 1984, 133:2169–2173
23. Issekutz AC, Movat KW, Movat HZ: Enhanced vascular permeability and haemorrhage-inducing activity of rabbit $C5a_{des\ Arg}$: Probable role of polymorphonuclear leucocyte lysosomes. Clin Exp Immunol 1980, 41:512–520
24. Movat HZ, Rettl C, Burrowes CE, Johnston MG: The *in vivo* effect of leukotriene B_4 on polymorphonuclear leukocytes and the microcirculation. Comparison with activated complement (C5a des Arg) and enhancement by prostaglandin E_2. Am J Pathol 1984, 115:233–244
25. Issekutz AC, Ripley M, Jackson JR: The role of neutrophils in the deposition of platelets during acute inflammation. Lab Invest 1983, 49:716–724
26. Johnston MG, Hay JB, Movat HZ: The modulation of enhanced vascular permeability by prostaglandins through alterations in blood flow (hyperemia). Agents Actions 1976, 6:705–711
27. Issekutz AC, Movat HZ: The effect of vasodilator prostaglandins on polymorphonuclear leukocyte infiltration and vascular injury. Am J Pathol 1982, 107:300–309
28. Leffell MS, Spitznagel JK: Intracellular and extracellular degranulation of human polymorphonuclear azurophil and specific granules induced by immune complexes. Infect Immun 1974, 10:1241–1249
29. Ohlsson K, Olsson I: The extracellular release of granulocyte collagenase and elastase during phagocytosis and inflammatory processes. Scand J Haematol 1977, 19:145–152
30. Hawkins D: Biopolymer membrane: A model system for the study of the neutrophilic leukocyte response to immune complexes. J Immunol 1971, 107:344–352
31. Davies DT, Fox RI, Polysonis M, Allison AC, Haswell AD: The inhibition of phagocytosis and facilitation of exocytosis in rabbits polymorphonuclear leukocytes by cytochalasin B. Lab Invest 1973, 28:16–25
32. Zurier RB, Hoffstein S, Weissmann G: Cytochalasin B: effect on lysosomal enzyme release from human leukocytes. Proc Natl Acad Sci USA 1973, 70:844–848
33. Dewald B, Bretz U, Baggiolini M: Release of gelatinase from a novel secretory compartment of human neutrophils. J Clin Invest 1982, 70:518–525
34. Janoff A, Scherer J: Mediators of inflammation in leukocyte lysosomes: IX. Elastolytic activity in granules of human polymorphonuclear leukocytes. J Exp Med 1968, 128:1137–1155
35. Ohlsson K, Olsson I: The neutral proteases of human granulocytes. Isolation and partial characterization of granulocyte elastase. Eur J Biochem 1974, 42:519–527

36. Janoff A: Mediators of tissue damage in leukocyte lysosomes: X. Further studies on human granulocyte elastase. Lab Invest 1970, 22:228–236
37. Janoff A, Sloan B, Weinbaum G, Damiano V, Sandhaus RA, Elias J, Kimbel P: Experimental emphysema induced with purified elastase: Tissue localization of the instilled protease. Am Rev Resp Dis 1977, 115:461–478
38. Keiser H, Greenwald RA, Feinstein G, Janoff A: Degradation of cartilage proteoglycan by human leukocyte granule proteases—a model of joint injury: II. Degradation of isolated bovine nasal cartilage proteoglycan. J Clin Invest 1976, 57:625–632
39. Majno G: Mechanisms of abnormal vascular permeability in acute inflammation, Injury, Inflammation and Immunity. Edited by L Thomas, JW Uhr, L Grant. Baltimore, Williams & Wilkins, 1964, pp 58–93
40. Robbins SL, Cotran RS, Kumar V: Inflammation and repair, Pathologic Basis of Disease. Philadelphia, W. B. Saunders, 1984, pp 40–84
41. Movat HZ: The Inflammatory Reaction. Amsterdam, Elsevier Science Publishers, 1985
42. Cotran RS, Remensnyder JP: The structural basis of increased vascular permeability after graded thermal injury: Light and electron microscopic studies. Ann NY Acad Sci 1968, 150:495–509
43. Cotran RS: Delayed and prolonged vascular leakage in inflammation: III. Immediate and delayed vascular reactions in skeletal muscle. Exp Mol Pathol 1967, 6:143–155
44. Movat HZ, Fernando NVP: Allergic inflammation: I. The earliest fine structural changes at the blood–tissue barrier during antigen–antibody interaction. Am J Pathol 1963, 42:41–59
45. Williams TJ, Peck MJ: Role of prostaglandin-mediated vasodilatation in inflammation. Nature 1977, 270:530–532

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