A Pathway to Pulmonary Fibrosis: An Ultrastuctural Study of Mouse and Rat Following Radiation to the Whole Body and Hemithorax

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DIFFUSE PULMONARY FIBROSIS is a recognized sequel to thoracic radiation.[1,2] The fibrogenic response is thought to be related to septal edema and cellular proliferation but, although the early and late lesions have been characterized,[3,4] the complete sequence from initial cell injury to the deposition of collagen has not been demonstrated. The possibility that the long-term effects of radiation may be the result of an immunologic reaction has been stimulated by a suggestion that there may be a latent interval between the primary injury and the development of fibrosis.[5]

There are several factors which make the study of this problem particularly difficult. In humans, the reaction is influenced by the underlying disease and by secondary infection;[6] in animals, there is a high mortality rate and the effects of radiation may be difficult to distinguish from those induced by bacterial pneumonia.[7]

The control of bacterial proliferation by the administration of chlortetracycline before and after radiation greatly enhances the survival rate of mice.[8] There is no evidence of pneumonia in the survivors, so that the pulmonary changes may be considered to be the result of pure radiation injury.

In the study to be presented, rats and mice protected by chlortetracycline were given high doses of radiation to the thorax and whole body respectively. The evolution of the pulmonary lesions was studied by electron microscopy.

Materials and Methods

Whole-Body Radiation. A group of 50 male Wistar albino mice (20 g), housed in individual sections of a plastic cage, received 650 rad whole-body X-radiation

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in 7 min. The animals were at a distance of 54 cm from an x-ray machine operating at 220 KV, 15 ma with 1 mm Cu + 1 mm Al filters. Chlortetracycline (2 g/l) was added to the drinking water 2 weeks before radiation and up to the time of sacrifice. A second set of 50 mice received 1100 rad whole-body radiation in 12 min under identical conditions. Animals were sacrificed on the following days after radiation: 1, 2, 3, 5, 7, 10, 14, 21, 28 and then at monthly intervals to 6 months.

Local Radiation. 25 male Charles River rats (150–200 g), under pentobarbital anesthesia, received 3000 rad to the right hemithorax while the rest of the body was protected by lead shielding. The dose was achieved in 32 min under the above conditions. The animals were given chlortetracycline and were sacrificed at the following days after radiation: 2, 5, 7, 14, 28 and at monthly intervals to 6 months.

Controls. One nonradiated animal, maintained under identical conditions, was sacrificed at each of the times indicated above. Further controls were provided by the left (shielded) lungs of the radiated animals.

Tissue Preparation. Radiated animals and nonradiated controls were sacrificed by intraperitoneal injection of sodium pentobarbital. A tracheotomy was performed, the thorax was opened and the lungs were re-expanded by fixative. From the 650 rad group only, blood samples were taken and platelet counts made. For each time interval at least 2 radiated animals were sacrificed. The lungs of 1 were fixed by buffered formalin and paraffin sections were made; the lungs of the other were fixed by buffered osmic acid, prestained by uranyl acetate, dehydrated and embedded in Vestopal W. Thick (0.5 μ) sections were stained by toluidine blue or silver methenamine, thin sections were stained by lead hydroxide for 15 min and examined in a Philips electron microscope EM200.

Electron microscope autoradiography was carried out on selected mice. They were injected with 40 μc tritiated thymidine (specific activity 1.9 c/mM) 2 hr before sacrifice. Unstained thin sections were coated with Ilford L4 emulsion by the loop technique,[9] exposed for 8 weeks, processed photographically, and stained with lead hydroxide for ½ hr.

Results

The administration of chlortetracycline before and after radiation resulted in a zero mortality rate following the 650 rad dose and 12% after the other two doses in the 6 month period. Control animals on chlortetracycline showed no pulmonary lesions.

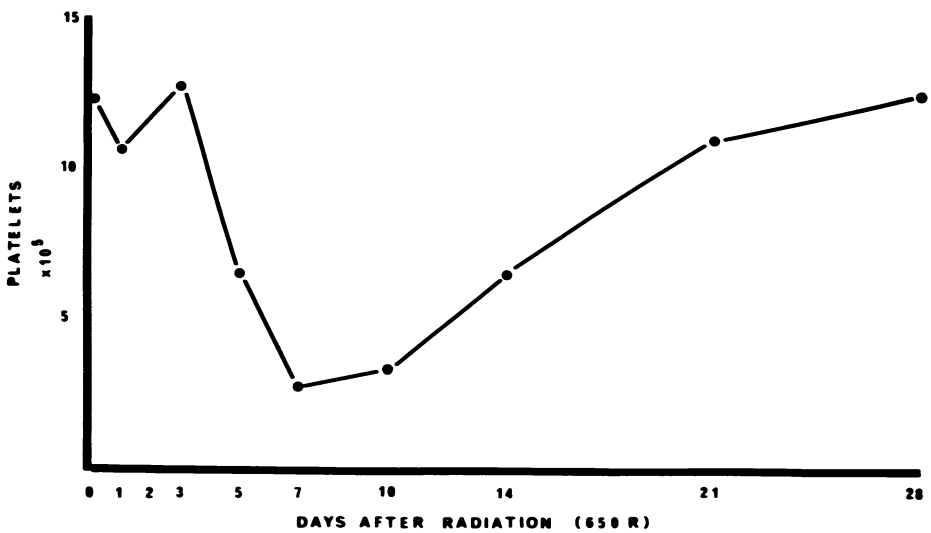
The primary site of radiation injury was the capillary. The lesions, which were focal and widespread throughout the lung, were qualitatively similar following whole-body and local radiation, although the timing of the sequential changes was dose dependent. The earliest evidence of cell injury was observed 2 days after 1100 rad and 5 days after 650 rad. In the rat, pathological changes were confined to the radiated lung.

A pulmonary alveolus of a control mouse is shown in Fig 1; the vessel is lined by endothelium, which is separated by basement membrane from the two types of alveolar epithelial cells. All of these structures were affected by radiation. The reaction originated in the capillary endothelium with the appearance of vacuoles in the cell cytoplasm (Fig 2). Progression of the intracellular vacuolation often distended

the cytoplasm into thin ribbons, so stretching the cell membrane that it met the opposite capillary wall, blocking the lumen (Fig 3). Subendothelial swelling, which was most severe after 1100 rad, also contributed to capillary blockage by displacing the entire endothelium into the capillary lumen.

After a few days, many of the vacuolated and distended cells ruptured and were stripped off the basement membrane. Simultaneously, there was focal swelling, necrosis and sloughing of the squamous Type I epithelial cells, leaving a completely denuded basement membrane as the only barrier between air and blood at these sites (Fig 4 and 5). These lesions were maximal 10 days after 1100 rad and 14 days after the other two doses. There was no visible change in the Type II alveolar epithelial cells at this or any other time during the investigation (Fig 3, 6, and 8); the ciliated and nonciliated cells of the respiratory bronchioles were also unaltered.

The adherence of platelet masses to basement membranes at the sites of endothelial destruction initiated the process of focal thrombotic occlusion in the pulmonary capillaries (Fig 6). The number of platelets in the blood dropped to a minimum 7 days after 650 rad (Text-fig 1) and had recovered to 650,000/cu mm, one-half the control value, by Day 14 when platelet thrombi were observed. Some capillaries showed the complete spectrum of injury with endothelial vacuolation and stripping, basement membrane swelling, necrosis of squamous epithelium and platelet aggregation.



TEXT-FIG 1. Blood platelets per cu mm following 650 rad (mouse, whole body)

The subsequent reactions involved the accumulation of amorphous ground substance in the basement membrane region and the deposition of collagen fibers within the capillary lumen, resulting in occlusion and nodular fibrosis (Fig 7). Focal subepithelial fibrosis was observed (Fig 8) and there was an increased number of mononuclear cells in the interstitium. The cytokinetic implications of the latter finding are discussed in a separate paper.[10]

Resolution of the capillary lesions occurred with recanalization and the establishment of small central channels lined by regenerated endothelium (Fig 9). Active proliferation of endothelial cells was indicated by an increased number of nuclei and their incorporation of tritiated thymidine (Fig 10). The basement membrane of the new endothelium was separated from remnants of the original endothelium and basement membrane by fibroblastic cells and collagen fibers (Fig 11). Duplication of basement membranes and capillary fibrosis were demonstrated in the light microscope at this stage. These structural changes were observed 28 days after 1100 rad and 2–3 months following the other two doses. There was no further progression up to 6 months after radiation. The vascular lesions were limited to the capillaries, no sclerosis or occlusion of larger vessels was observed.

Discussion

Radiation injury to the human lung is characterized by two main reactions; the first involves the formation of hyaline membranes in the alveolar ducts; the second, the development of septal edema with cellular proliferation and fibrosis.[4] The reaction in experimental animals is somewhat different in that the interstitial reaction predominates and hyaline membranes are rarely observed.[3,11] Interstitial fibrosis is thought to be related to vascular sclerosis[5] but there are conflicting reports regarding the nature of the changes in the blood vessels and the site of the initial ultrastructural injury. Phillips demonstrated, in rats, that the capillary endothelium was particularly sensitive to radiation injury whereas the epithelium was not affected.[5] Goldenberg and coworkers, using parabiotic rats, found little endothelial change but extensive epithelial damage including the Type II granular pneumonocytes[12] and a similar reaction has been reported in dogs.[11] Such variability of response is probably related to different experimental conditions, species variation, the reaction to intercurrent infection and the times at which the observations were made.

In the present series of experiments, the complication of bacterial infection was eliminated by the administration of antibiotic. The possi-

bility of viral infection cannot be excluded but the low mortality rate and the predominant vascular reaction with minimal interstitial involvement make this extremely unlikely. The lesions were therefore considered to represent "true" radiation injury.

The pulmonary reaction was found to be independent of whether radiation was administered locally or to the whole body and by making serial observations the complete spectrum of radiation injury was observed in the mouse and the rat. The early changes differed from those previously described. The capillary endothelium was severely damaged and the epithelium showed a differential response with focal swelling and necrosis of the squamous Type I cells while the Type II cells and bronchiolar epithelium, including the Clara cells, remained unaltered. Regeneration of the squamous alveolar lining cells sppeared to occur in situ; there was no proliferation of Type II cells such as occurs after exposure to oxygen and nitrogen dioxide.[13,14]

Reconstitution of the epithelial lining of the air sacs was accomplished with very little subepithelial scarring; fibrosis was related almost exclusively to the capillaries. Destruction of the capillary endothelium with stripping of the basement membrane coincided with the intravascular agglutination of platelets. This suggests that the adherence of platelets was related to their interaction with collagen of the basement membrane and not to primary coagulation of the blood.[15] Subsequent growth of thrombi by the progressive accumulation of fibrin and blood cells was followed by fibroblastic invasion and the deposition of collagen. This thrombotic response of the pulmonary capillaries is not specific to radiation injury since similar results have been reported following endothelial damage induced by anthrax toxemia.[16] The resolution of lumenal fibroblastic tissue was accompanied by active proliferation of endothelial cells as demonstrated by nuclear labelling with tritium. The process of recanalization, which was seldom complete, ensured partial recovery of the capillary bed.

The results of previous investigations have suggested that the initial vascular injury following radiation was succeeded by a latent interval before the development of cellular proliferation and fibrosis.[5] Phillips demonstrated that the endothelial damage observed in the first month after radiation was followed by a latent interval of about 1 month before the appearance of further endothelial change and a septal infiltrate of plasma cells and mast cells.[5] Capillary occlusion with recanalization was observed 6 months after radiation. The apparent delay in the tissue response introduced the idea that pulmonary fibrosis following radiation may be the direct result of immunologic injury to the

lung capillaries. Support for this proposal is provided by the demonstration of large amounts of globulins in rabbit lung 3 months after local radiation.[17] It is known, however, that immune responses are depressed following radiation; for example, the induction of antibodies in mice is delayed for 26 days following 1100 rad.[18,19] In the present investigation the lesions produced by 1100 rad progressed from endothelial injury to fibrosis entirely within the refractory period. There was no quiescent or latent interval. It is unlikely, therefore, that an immunologic process was involved. The sequential changes that were observed indicate that the pathogenesis of radiation injury to the lung is directly related to multifocal capillary thrombotic occlusion and the intraluminal deposition of collagen.

Summary

The ultrastructural changes which follow radiation of the whole body and thoracic cage were studied in mice and rats. The administration of chlortetracycline significantly lowered the mortality rates and by the elimination of bacterial pneumonia, allowed the observation of pure radiation damage.

The earliest injury following 650 rad or 1100 rad to the whole body in mice involved vacuolation and subsequent destruction of capillary endothelium. Necrosis and stripping of the squamous (Type I) epithelium was an immediate sequel; Type II alveolar cells were not affected. The sequential intracapillary response involved platelet aggregation, focal occlusion, fibrosis, and recanalization. The changes were qualitatively similar to those observed in rats given 3000 rad to one lung. In both models the vascular changes and deposition of collagen occurred as a continuum. There was no latent interval between the initial cell injury and the mesenchymal reaction; fibrosis was established before the expected time of immunologic recovery. It is concluded that radiation-induced pulmonary fibrosis is the direct result of the primary vascular injury rather than a delayed immunologic response.

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[*Illustrations follow*]

Legends for Figures

Fig 1. *(upper)* Lung of a control mouse. Capillary *(CAP)* is lined by endothelium *(EN)* with its basement membrane *(BM)*; alveoli *(ALV)* are lined by two types of epithelium *(EP)*, Type 1 squamous, and Type 2 with lamellar bodies. × 9000.

Fig 2. *(lower)* Capillary wall, 7 days after 650 rad (mouse, whole body). Vacuolation of endothelium *(EN)* had ballooned cytoplasm into capillary lumen *(L)*. Subendothelial swelling has separated endothelial cell from basement membrane *(BM)*. Epithelium *(EP)* × 80,000.

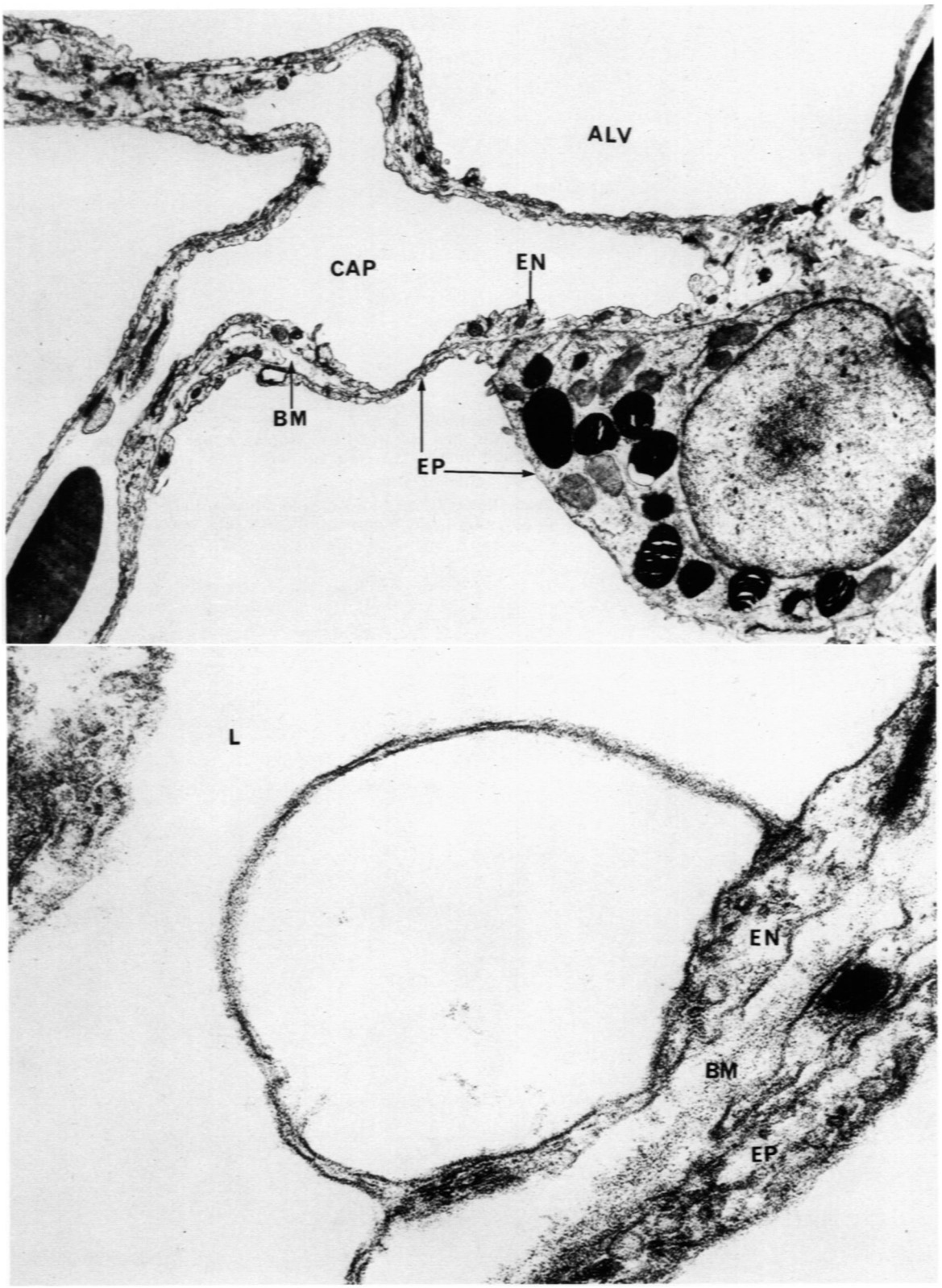

ALV
CAP
EN
BM
EP
L
EN
BM
EP

Fig 3. *(upper)* Capillary and adjacent epithelial cells 7 days after 650 rad (mouse, whole body). Endothelial vacuolation *(EV)* has so stretched cytoplasm that it touches opposite side of capillary and obstructs lumen *(L)*. The epithelial cells are normal. × 17,000.

Fig 4. *(lower)* Capillary wall, 10 days after 650 rad (mouse, whole body). Endothelium has stripped off basement membrane *(BM)*. Squamous epithelium *(EP)* shows focal swelling. × 51,000.

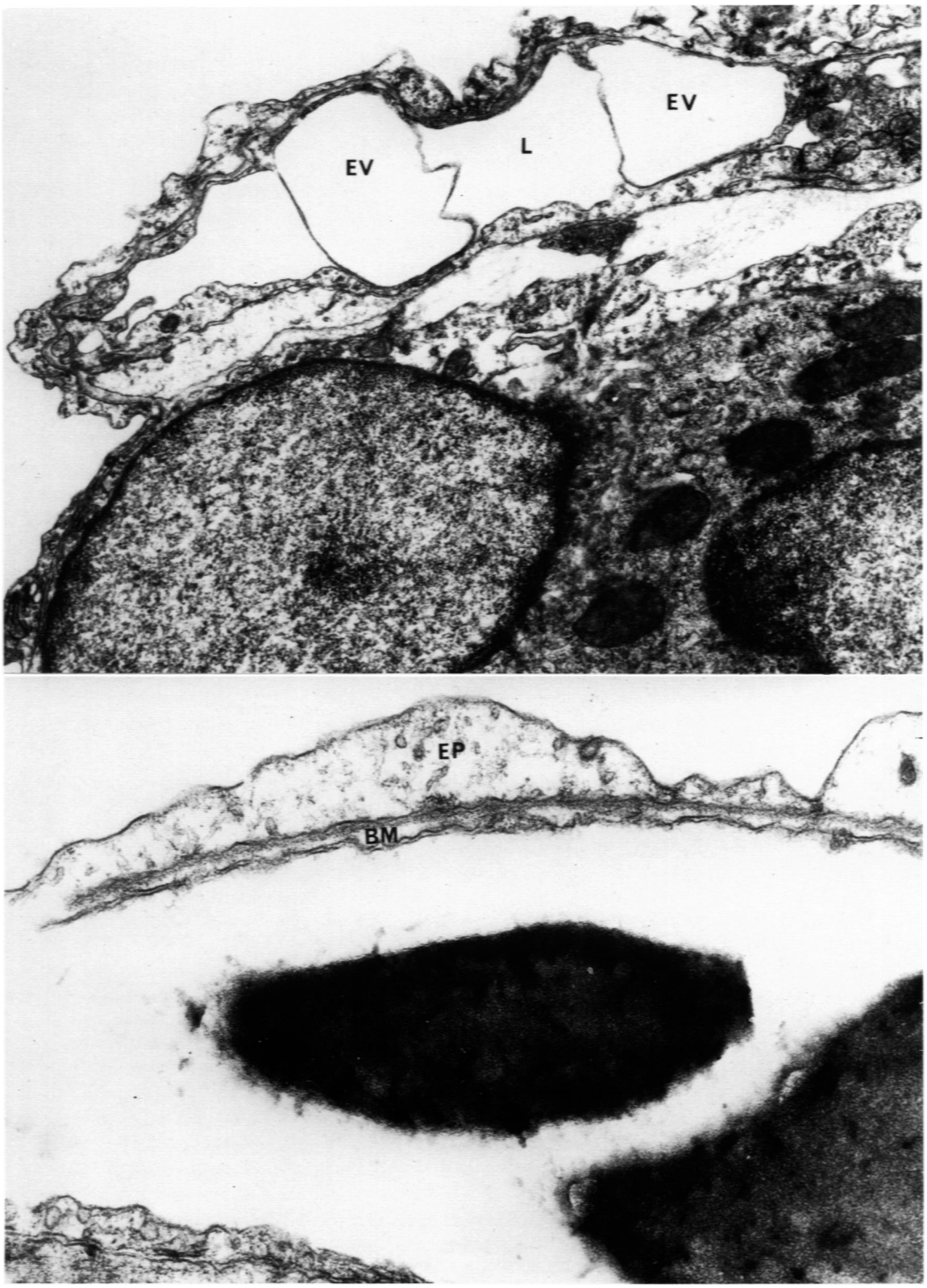
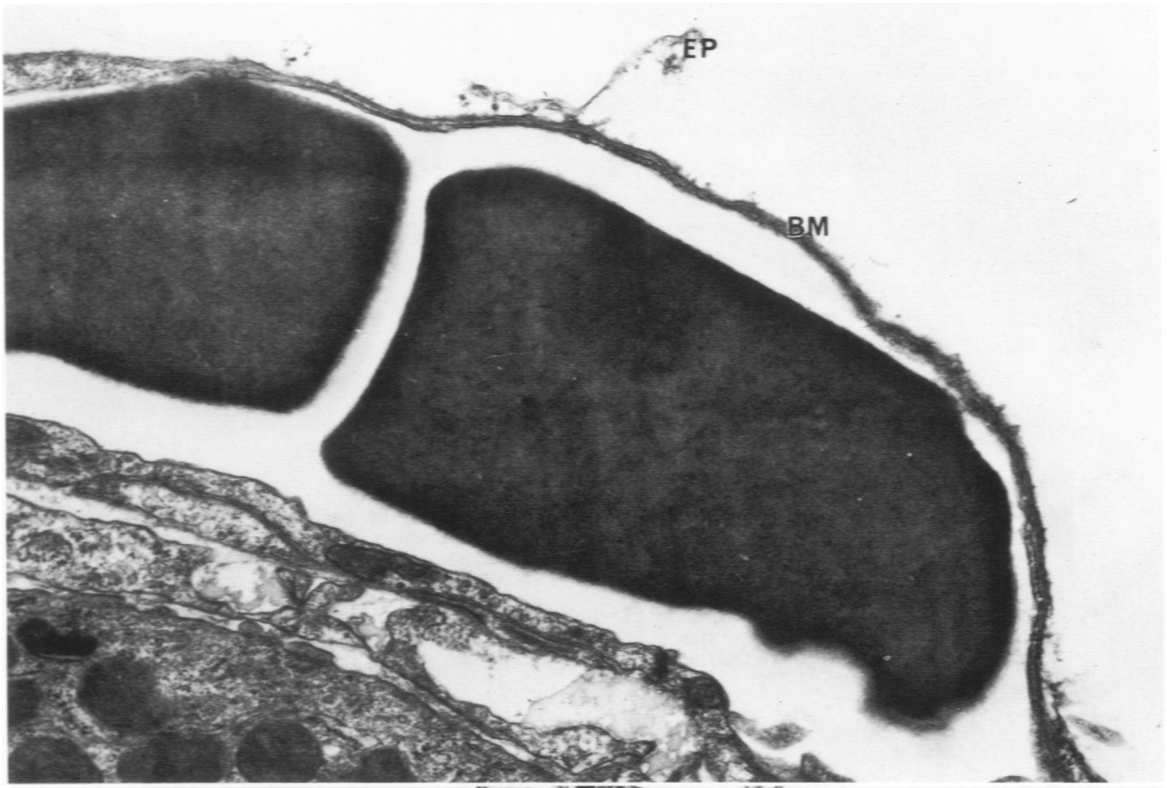

EV
L
EV
EP
BM

Fig 5. *(upper)* Capillary, 14 days after 650 rad (mouse, whole body). Endothelium and epithelium are stripped leaving only a few remnants of epithelium *(EP)* beside naked basement membrane *(BM)*. × 10,000.

Fig 6. *(lower)* Capillary and epithelial cells, 14 days after 650 rad (mouse, whole body). Platelet thrombus *(PT)* is attached to denuded basement membrane. Type 1 epithelium *(EP)* is swollen and shows loss of cytoplasmic structure whereas Type 2 epithelial cell appears normal. × 14,000.


EP
BM

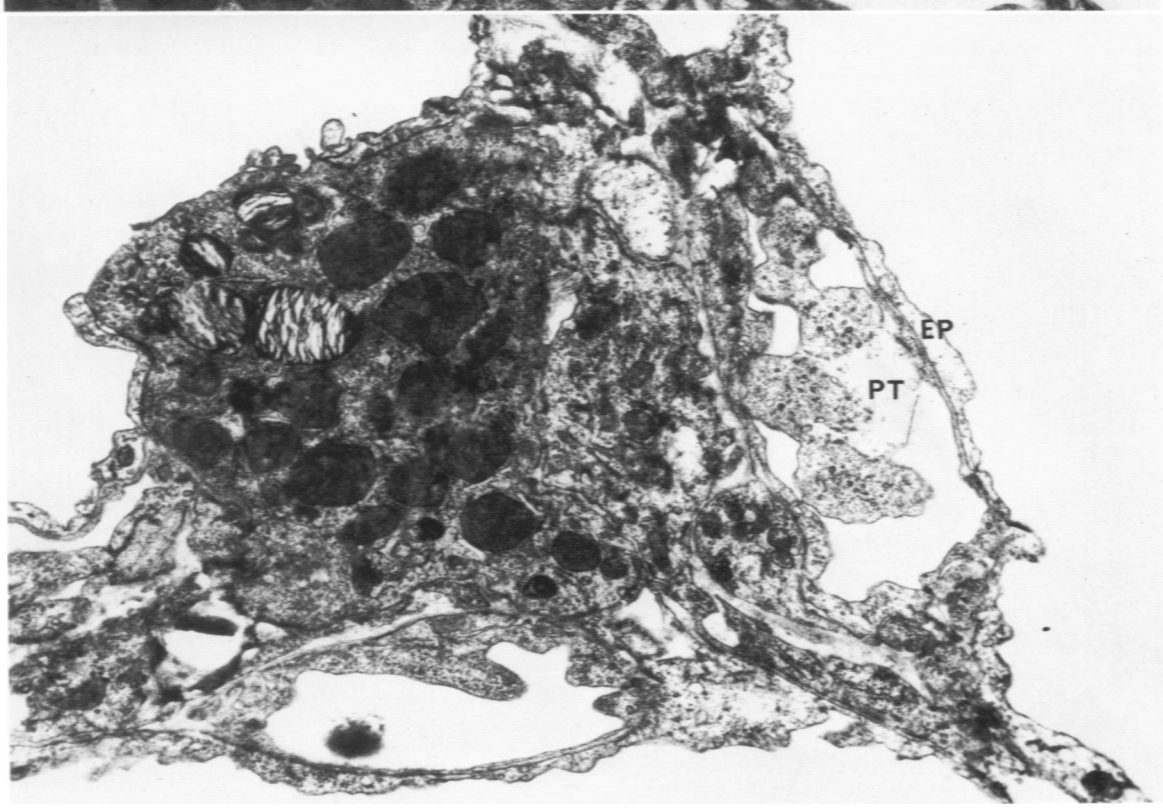

EP
PT

Fig 7. *(upper)* Capillary, 28 days after 650 rad (mouse, whole body). On the right, capillary lumen is patent although there is loss of endothelium and focal swelling of squamous epithelium. On the left, basement membrane is widened by deposition of ground substance *(GS)* and lumen is occluded by collagen *(C)*. × 17,000.

Fig 8. *(lower)* Subepithelial fibrosis, 21 days after 1100 rad (mouse, whole body). Fibroblast *(F)*, surrounded by many collagen fibers *(C)*, is seen beneath a normal Type 2 epithelial cell. × 14,000.

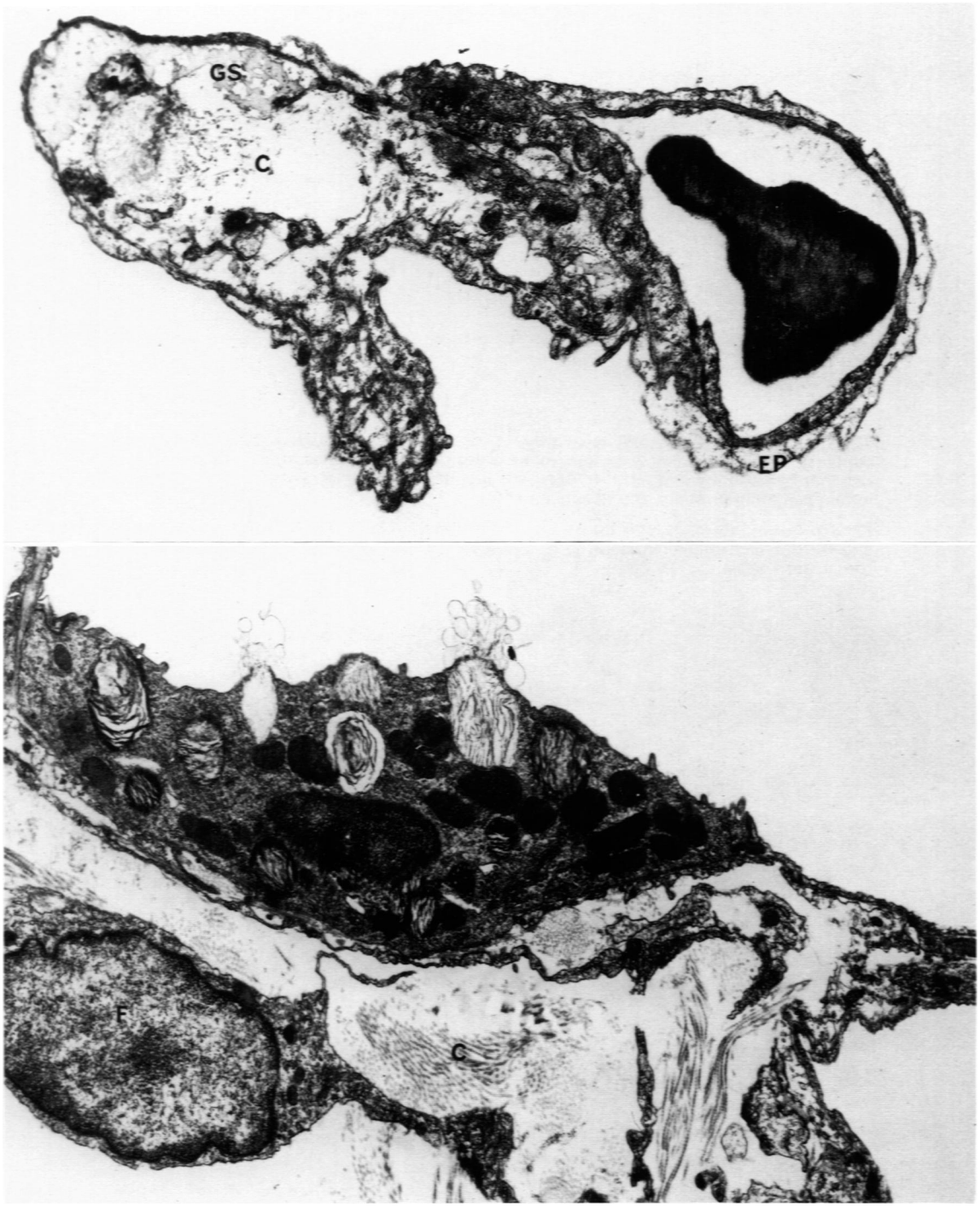
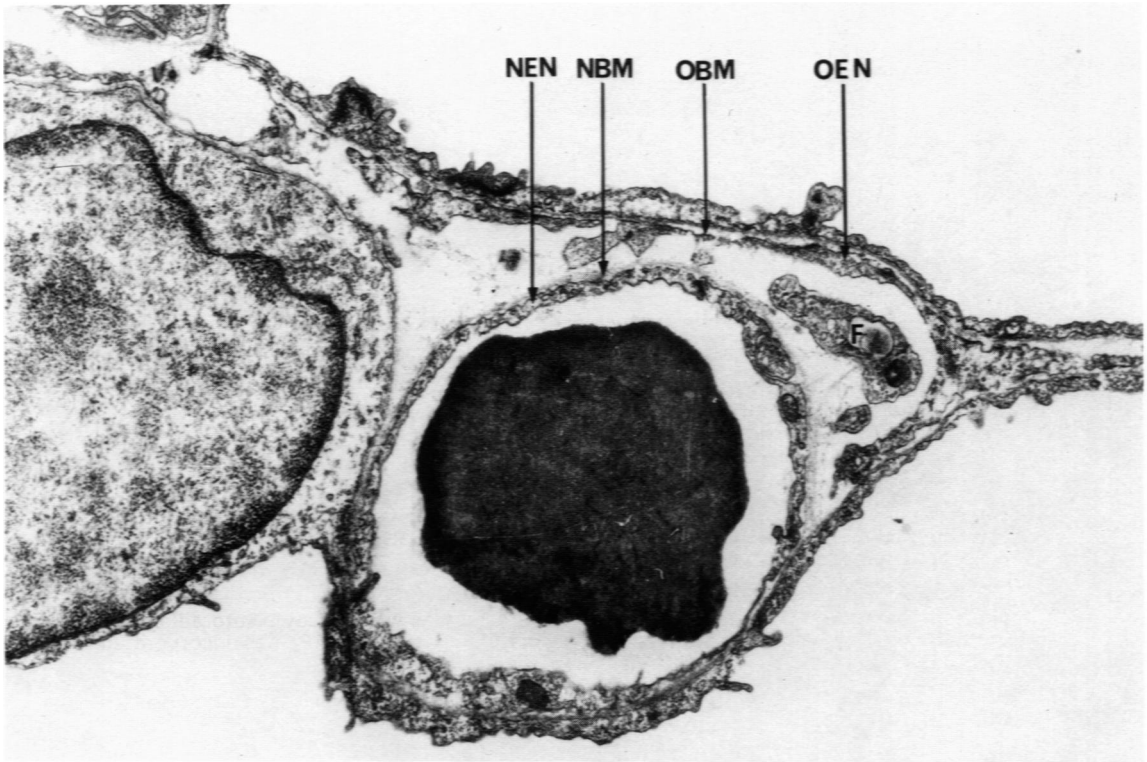
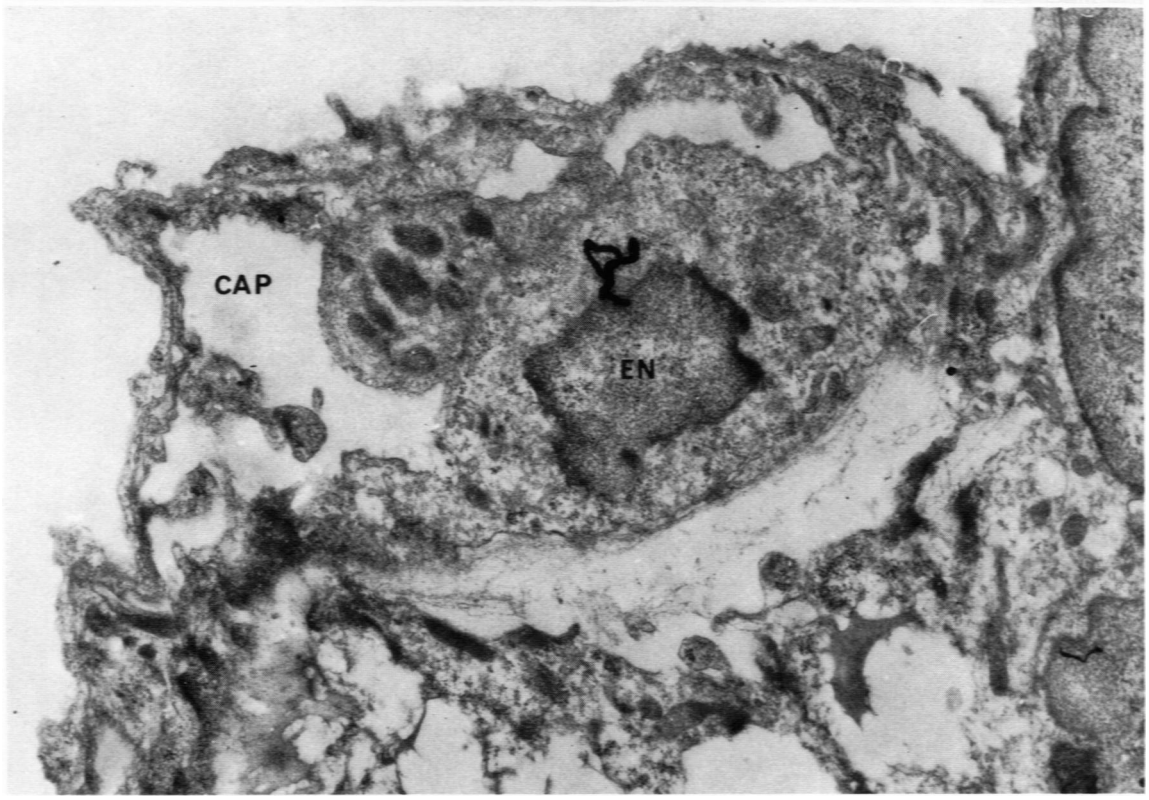

GS
C
EP
F
C

Fig 9. *(upper)* Capillary, 28 days after 1100 rad (mouse, whole body). Recanalized capillary has acquired new basement membrane *(NBM)* and endothelial lining *(NEN)*. Original basement membrane *(OBM)* and endothelium *(OEN)* are separated from new lumen by collagen fibers and fibroblast *(F)*. × 17,000.

Fig 10. *(lower)* Capillary, 28 days after 1100 rad (mouse, whole body). Autoradiograph shows that endothelial nucleus *(EN)* of recanalized capillary *(CAP)* has incorporated the tritium label. × 14,000.


NEN
NBM
OBM
OEN
F


CAP
EN

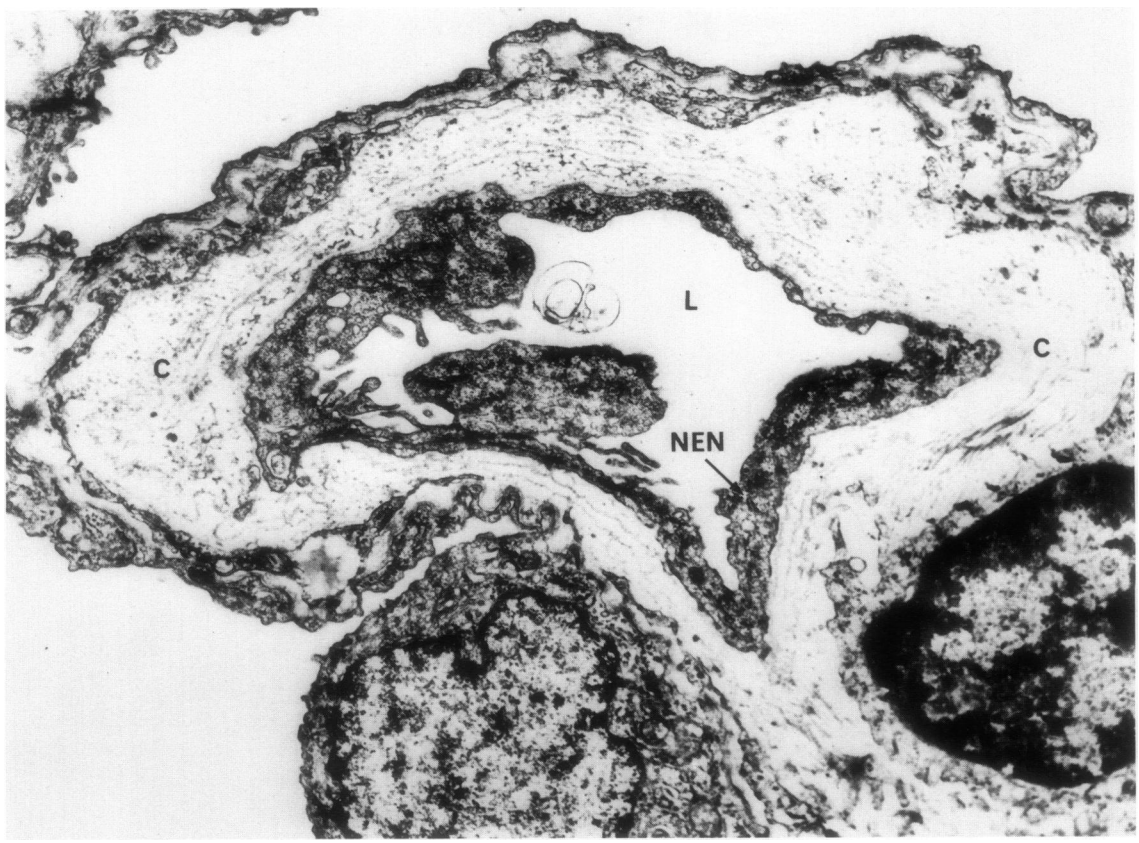


Fig 11. Capillary, 3 months after 3000 rad (rat, right hemithorax). Recanalized lumen *(L)* is lined by new endothelium *(NEN)* and surrounded by a thick band of collagen *(C)*. × 12,500.