The Pathogenesis of Bleomycin-Induced Pulmonary Fibrosis in Mice

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Administration of 0.5 mg bleomycin to mice twice weekly for 4 weeks induced pulmonary fibrosis. The initial site of injury was the intima of pulmonary arteries and veins where endothelial cells became edematous and were separated from the underlying basement membrane by large blebs. These lesions occurred after 2 weeks and were associated with infiltration of perivascular spaces by lymphocytes and plasma cells. Capillary endothelial blebbing and interstitial edema were observed after 4 weeks, when multifocal necrosis of type 1 alveolar epithelial cells was accompanied by fibrinous exudation into the alveoli. The process of repair was characterized by proliferation and metaplasia of type 2 epithelial cells, fibroblastic organization of alveolar fibrin and fibrosis of the interstitium within 8 to 12 weeks. The consistent induction of changes similar to those of diffuse pulmonary fibrosis or fibrosing alveolitis in man suggests that bleomycin-induced injury may provide a suitable model for the investigation of this ill-defined group of diseases (Am J Pathol 77:185–198, 1974).

BLEOMYCIN, a complex of water-soluble peptides extracted from *Streptomyces verticillatus*,[1] has been found to be an effective agent in the control of a number of human cancers.[2] It has been particularly useful in the treatment of skin tumors, and the absence of hematopoietic toxicity or immunosuppressive activity have been cited as advantages of this therapeutic agent.[3] With increasing use, however, it has become apparent that diffuse pulmonary fibrosis has been recognized as a severe and puzzling complication of bleomycin therapy.[4,5] The pathologic end stage of the toxic reaction, pulmonary fibrosis, has been described in both humans[5,6] and animals,[7] but no coherent description of the pathogenesis of this reaction to bleomycin has been reported. The present study describes the sequential changes observed by light and electron microscopy following the administration of toxic doses of bleomycin to mice.

Materials and Methods

Groups of 10 male albino mice (25 g) were injected twice a week for up to 8

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Table 1—Response of Mice (Groups of 10) to Various Doses of Bleomycin

	Dose of bleomycin (mg, twice per week intraperitoneally)					
	0.01	0.02	0.04	0.1	0.5	1.0
No. of injections	16	16	16	16	16	10
Time of death or sacrifice (wks)	8–20	8–20	8–20	8–20	8–20	5
Mortality	0/10	1/10	1/10	6/10	3/10	10/10
Pulmonary lesion	0/10	3/10	3/10	7/10	7/10	10/10
Perivascular edema and cell infiltrate	No	Yes (3)	Yes (3)	Yes (7)	Yes (7)	Yes (10)
Fibrosis	No	No	No	Yes (2)	Yes (2)	Yes (2)
Epithelial metaplasia	No	No	No	Yes (3)	Yes (2)	Yes (2)

weeks with one of the following doses of bleomycin: 0.01 mg, 0.02 mg, 0.04 mg, 0.1 mg, 1 mg (Table 1). A control group that received 1 ml water at the same time was also studied. Mice were sacrificed at the end of the injection period and at 4-week intervals to 20 weeks. Complete autopsies were performed on these animals and on any that died during the experiment. Following a tracheotomy, the lungs of each mouse were expanded with 4% glutaraldehyde, postfixed in formalin, dehydrated through alcohols and embedded, 1 lobe per block, in glycol methacrylate. Sections 0.75 mμ thick were cut and stained with toluidine blue, hematoxylin and eosin or silver methenamine. Standard histologic examination was made on heart, kidney, liver, intestine, muscle, spleen and pancreas.

From the mortality figures and the changes in pulmonary morphology observed in this experiment, it was decided that a dose of 0.5 mg/mouse administered twice weekly for 4 weeks would produce pulmonary lesions that could be studied serially. A group of 30 mice was injected by this regimen and sacrificed weekly during the injection period and at 2 week intervals up to 12 weeks postinjection. Three experimental and 1 control mouse were examined at each time. The lungs were prepared as above for light microscopy, and in addition small samples of each lung were taken after glutaraldehyde fixation, postfixed in osmic acid, dehydrated and embedded in Spurr for electron microscopy.

Results

The first phase of the investigation was concerned with the determination of a sublethal dose of bleomycin which would induce pulmonary lesions in a reasonable number of animals. The dose level required to produce significant pulmonary fibrosis in mice was found to be considerably higher per kilogram body weight than has been reported for dogs.[7] The administration of 1 mg of bleomycin to mice twice per week was uniformly fatal within 5 weeks (Table 1). In the dose range 0.1 to 0.5 mg, about half of the animals died, and pulmonary lesions, including fibrosis, were observed in the majority. The precise cause of death could not be determined, since the only consistent extrapulmonary change observed at autopsy was lymphoid follicular depletion of the spleen; animals sacrificed at specific intervals during the experiment did not

show this change, and organs other than lung were histologically normal.

The evolution of the pulmonary lesions was studied in mice given 0.5 mg bleomycin twice weekly for 4 weeks. The earliest changes, observed 2 weeks after the start of the injections, involved the endothelium of pulmonary arteries and veins. Subendothelial blebs bulging into the vascular lumen produced severe attenuation of the endothelial cytoplasm (Figure 1); in addition some of the endothelial cells showed severe intracytoplasmic edema (Figure 2). These changes were observed by electron microscopy, but by the fourth week, endothelial lesions were occasionally visible by light microscopy. At this time perivascular edema with lymphocytic and plasma cell infiltrates was also seen (Figure 3). The latter feature, usually limited to the peribronchial and perivascular regions, was a constant accompaniment of the developing lesions in the lung. Although lesions of the larger pulmonary vessels were observed as early as the second week, capillary involvement was not found before the fourth week of bleomycin administration, when endothelial and subendothelial blebbing similar to that described in larger vessels was seen (Figure 4). Diffuse interstitial edema was also observed, and the air sacs contained numerous vacuolated macrophages (Figure 4).

Changes in the alveolar epithelium were first observed after 4 weeks, at which time there was focal necrosis of type 1 cells but the type 2 epithelium appeared normal (Figure 5). Necrosis of type 1 cells was associated frequently with intraalveolar aggregates of fibrin, and occasionally the origin of the fibrin could be traced to a localized defect in the epithelium covering a blood vessel (Figure 6). These changes were observed with the electron microscope; hyaline membranes were not seen with the light microscope. From the fourth week onward, mitotic activity and focal proliferation of type 2 alveolar epithelial cells were observed (Figures 7 and 8), accompanied by excessive accumulation of lamellar debris in the alveoli (Figure 8). After 6 weeks, aggregates of epithelial cells with microvilli but no lamellar bodies were observed (Figure 9); occasionally, alveoli were lined by flattened ciliated cells, a change that was most prominent around small bronchioles (Figure 10).

Intraalveolar and septal fibrosis, first observed at 4 weeks, became progressively more severe and extensive. Initially, invasion of intraalveolar fibrin by fibroblasts resulted in the formation of collagenous nodules (Figures 11 and 12). From the sixth week on, intraalveolar fibrosis was readily demonstrable by light microscopy (Figure 13), and

interstitial fibrosis was observed by electronmicroscopy within the alveolar septa and in the walls of blood vessels. From 8 to 12 weeks these changes progressed to involve extensive areas of the lung (Figure 14).

Discussion

Pulmonary fibrosis has been reported as a significant complication in patients receiving bleomycin for the treatment of various forms of cancer.[4-6] The particular susceptibility of the lung to injury by this agent may be related to the selective retention of the drug by pulmonary tissues.[3] The end stage of the reaction, diffuse pulmonary fibrosis, has been described in man [4-6] and dogs; [7] in the present investigation, the pathogenesis of this condition has been described in a sequential study in the mouse.

The initial sites of pulmonary injury were the endothelial cells lining veins and arteries; lesions in the capillary endothelium occurred somewhat later. The diffuse injury to vascular endothelium is probably related to the route of access of the drug to the lung. In this respect the anatomic distribution of lesions differs from that observed in oxygen poisoning where the cells on the route of maximum gaseous transfer, the capillary endothelium, are preferentially injured.[8,9]

The primary injury to endothelial cells, accompanied by diffuse interstitial edema, is probably a direct toxic effect of the drug. The continuing and progressive pulmonary lesions may well have an immunologic basis, since lymphocytic and plasmacytic infiltrates are consistently prominent. An immunologic mechanism for bleomycin toxicity has not been investigated, but the mixed peptide composition of the drug suggests that it might act as a foreign antigen. The collections of lymphocytes and plasma cells suggest a humoral mechanism, but electron microscopy revealed no evidence of dense deposits in basement membranes as might be expected if vascular injury were related to the deposition of antigen–antibody aggregates. The absence of renal lesions also militates against a humoral mechanism.

The multifocal proliferation of type 2 alveolar epithelial cells which follows necrosis of type 1 epithelium is similar to pulmonary injury by such diverse agents as oxygen,[9,10] nitrogen dioxide [11] and Freund's adjuvant.[12] It is concluded that this reaction is a common pathway of epithelial repair in the lung. We have previously shown that, in oxygen poisoning, repair of injured epithelium occurs by proliferation of type 2 cells with their subsequent transformation to the squamous type of epithelium.[10] This reparative process following an acute exposure to oxygen resulted in complete resolution of the lesions within 2 weeks.[10,13]

In the present experiment, in which bleomycin was administered over 4 to 8 weeks, the epithelial hyperplasia persisted and was accompanied in the subsequent weeks by metaplasia of alveolar epithelium to ciliated forms.

The fibrous reaction that was observed as part of the continuing response of the lung occurred in the interstitium and in the alveoli. Alveolar fibrosis was directly related to fibroblastic organization of fibrinous exudate that escaped through damaged type 1 epithelium. The interstitial fibrosis, which occurred at peribronchial, perivascular and alveolar septa, was invariably accompanied by edema and was frequently associated with lymphocytes and plasma cells. The end stage of the process, diffuse pulmonary fibrosis, is similar to the changes which have been described in bleomycin toxicity in humans.[4–6] This fibrotic reaction with reactive hyperplasia and metaplasia of alveolar epithelium is not specific to bleomycin toxicity because similar responses have been observed clinically in the various forms of diffuse pulmonary fibrosis or fibrosing alveolitis.[14,15] The consistency and rapidity with which pulmonary fibrosis is induced by bleomycin administration to animals suggests that this experimental method provides a suitable model for the investigation of pulmonary fibrosis in man.

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Legends for Figures

Fig 1—Pulmonary artery after 2 weeks bleomycin administration. Large blebs separate attenuated endothelial cytoplasm from internal elastic lamina (× 4000).

Fig 2—Pulmonary vein after 2 weeks bleomycin administration. Severe intracytoplasmic edema of endothelial cell is seen (× 6200).

Fig 3—Pulmonary vein after 4 weeks bleomycin injection. Endothelium is severely distorted by cytoplasmic and extracellular edema. Edematous perivascular connective tissue contains plasma cells and a few lymphocytes (H&E, × 1000).

Fig 4—Alveolar capillaries after 4 weeks bleomycin administration. Blebbing of capillary *(C)* endothelial cells with accumulation of interstitial edema *(ED)* has occurred. Several vacuolated macrophages *(M)* are seen in the alveoli *(A)* (× 6200).

Fig. 5—Alveolar epithelium after 4 weeks bleomycin injection. Cytoplasmic disintegration of type 1 cell is seen *(EP1)*, but the adjacent type 2 cell *(EP2)* is intact (× 9000).

Fig 6—Small blood vessel after 4 weeks bleomycin injection. Fibrinous exudate *(F)* appears to be escaping into alveolus through a defect in overlying type 1 epithelium *(EP1)* (× 9500).

Fig 7—Alveolar epithelium after 4 weeks bleomycin administration. A type 2 cell in mitosis is shown (× 9000).

Fig 8—Alveoli after 4 weeks bleomycin injection. Focal proliferation of type 2 cells with accumulation of lamellar debris in an air sac has occurred. (× 4000).

Fig 9—Alveolar epithelium 2 weeks after a 4-week administration of bleomycin. Alveolar cells have proliferated to form a small tubular structure; cells are cuboidal and contain microvilli but no lamellar bodies (× 6200).

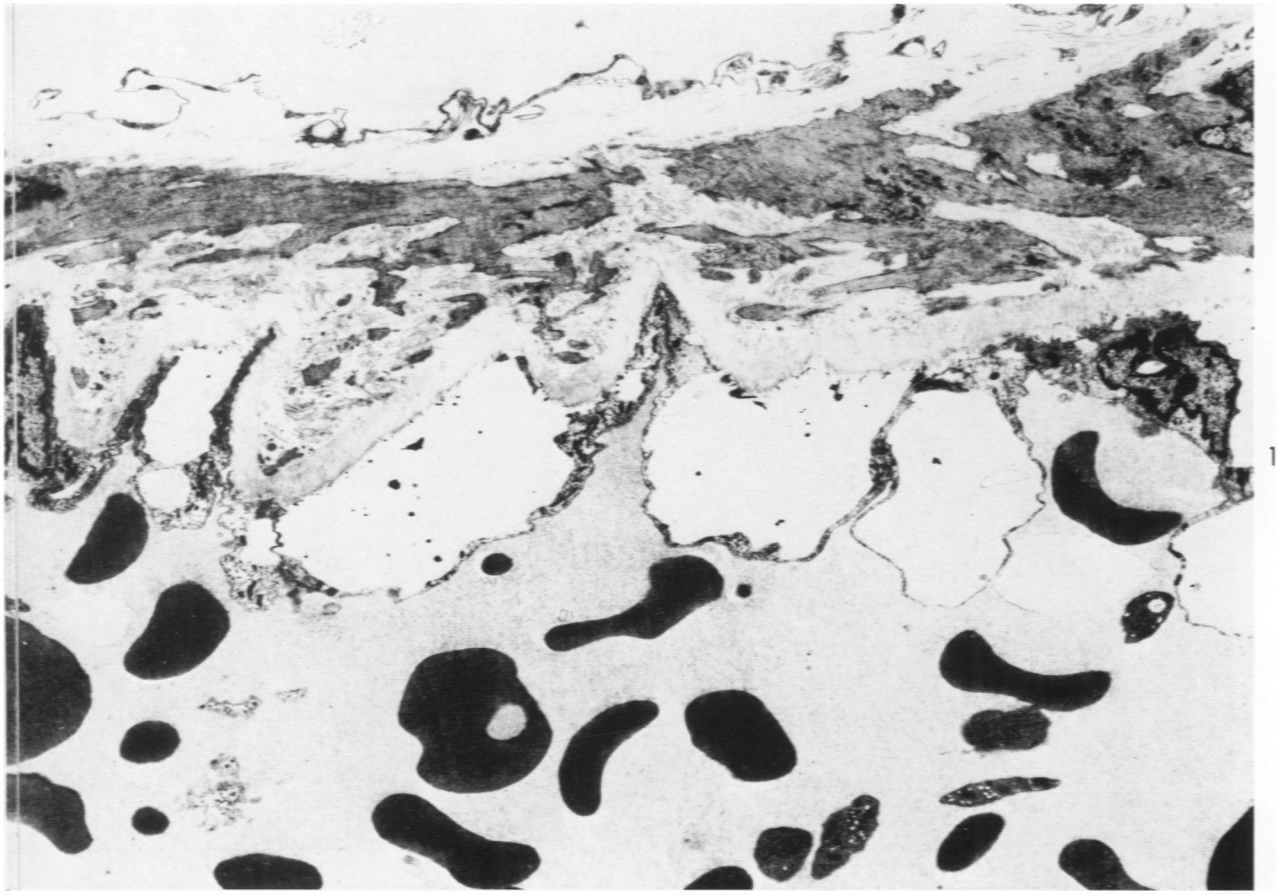
Fig 10—Peribronchiolar region 2 weeks after 4 weeks administration of bleomycin. Alveoli *(A)* are lined by flattened cells some of which are ciliated. Numerous lymphocytes and plasma cells are seen beneath the bronchiolar epithelium *(BR)* (H&E, × 1000).

Fig 11—Alveolus *(A)* after 4 weeks bleomycin. Intraalveolar aggregate of fibrin *(F)* surrounded by collagen-secreting fibroblasts *(FB)* (× 10,500).

Fig 12—Alveoli *(A)* 2 weeks after 4 weeks administration of bleomycin. Intraalveolar fibroblasts with collagen *(Co)* secretion and some fibrous thickening of alveolar septa (× 4000).

Fig 13—Lung 2 weeks after 4 weeks administration of bleomycin. Extensive intraalveolar and septal fibrosis (Silver methenamine, × 350).

Fig 14—Lung 6 weeks after 4 weeks administration of bleomycin. Extensive interstitial and intraalveolar fibrosis accompanied by subpleural distention and disruption of air sacs (Silver methenamine, × 130).



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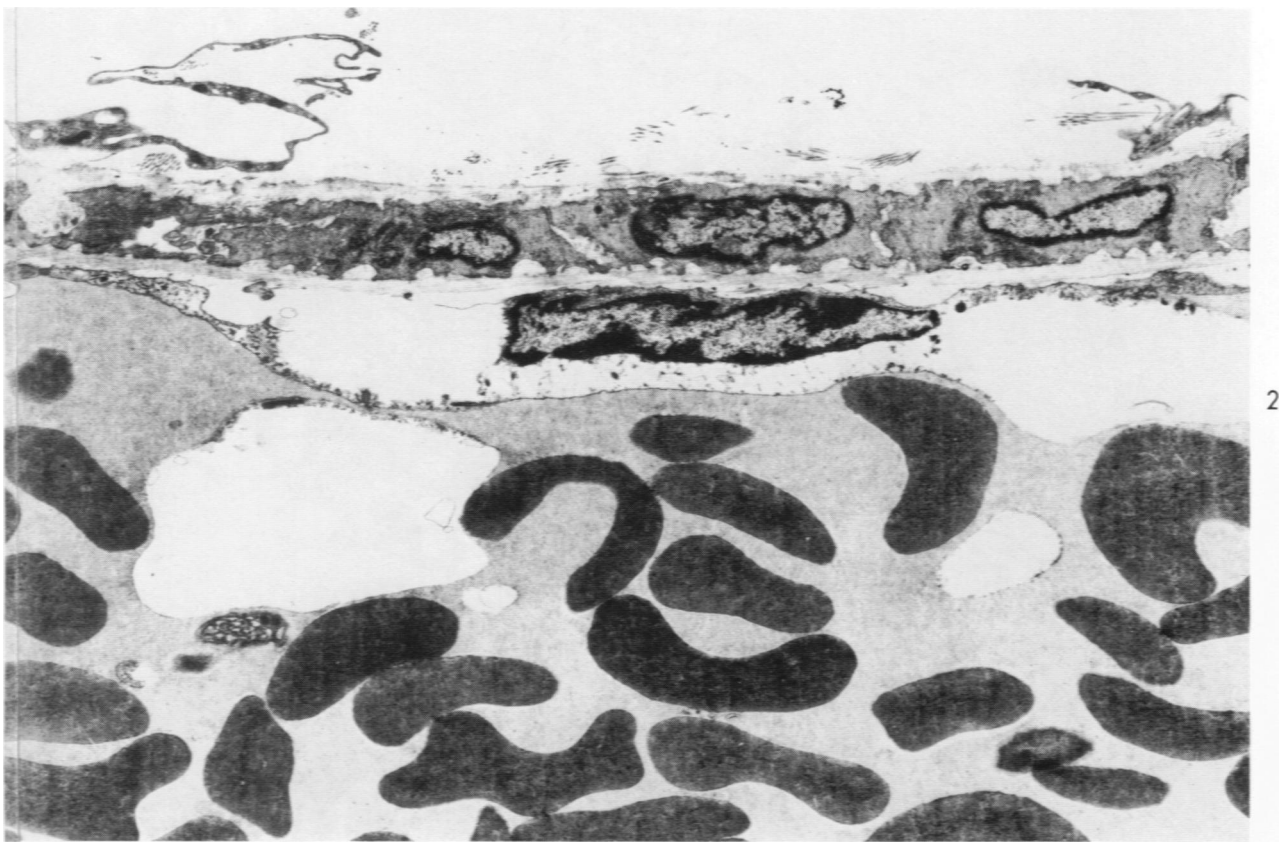

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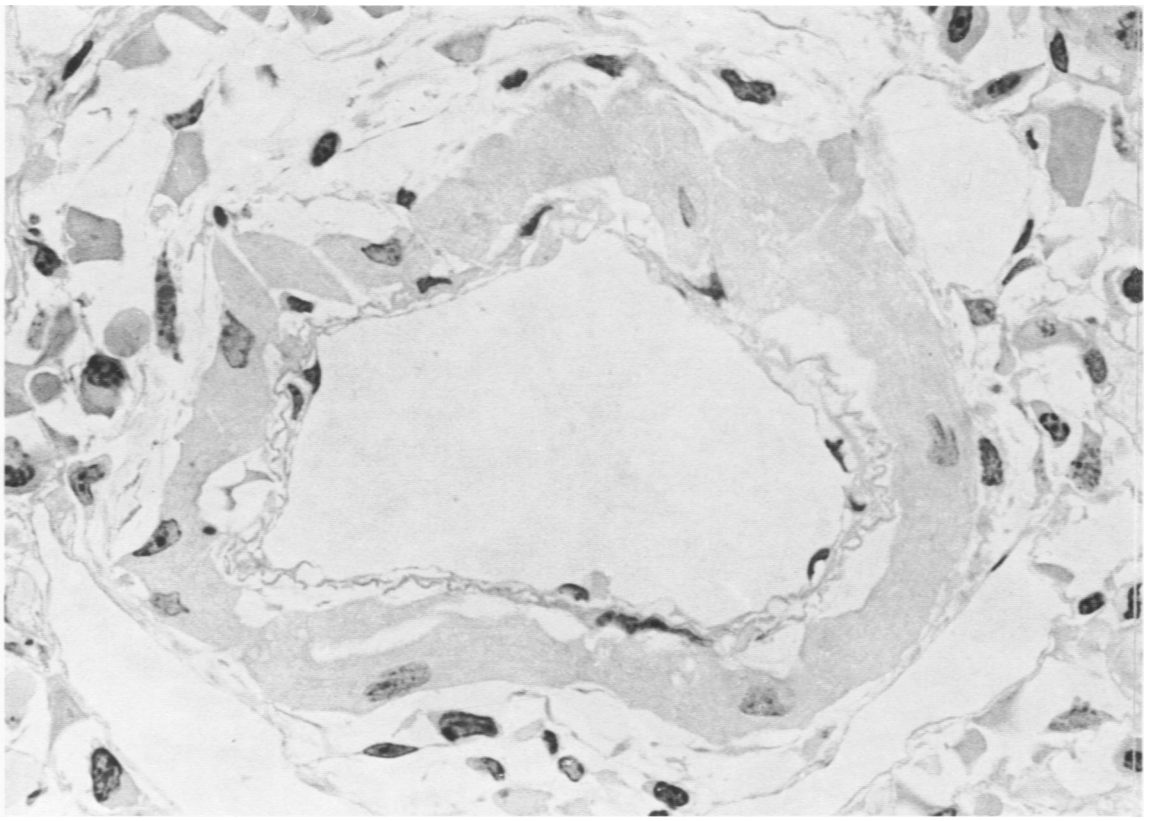

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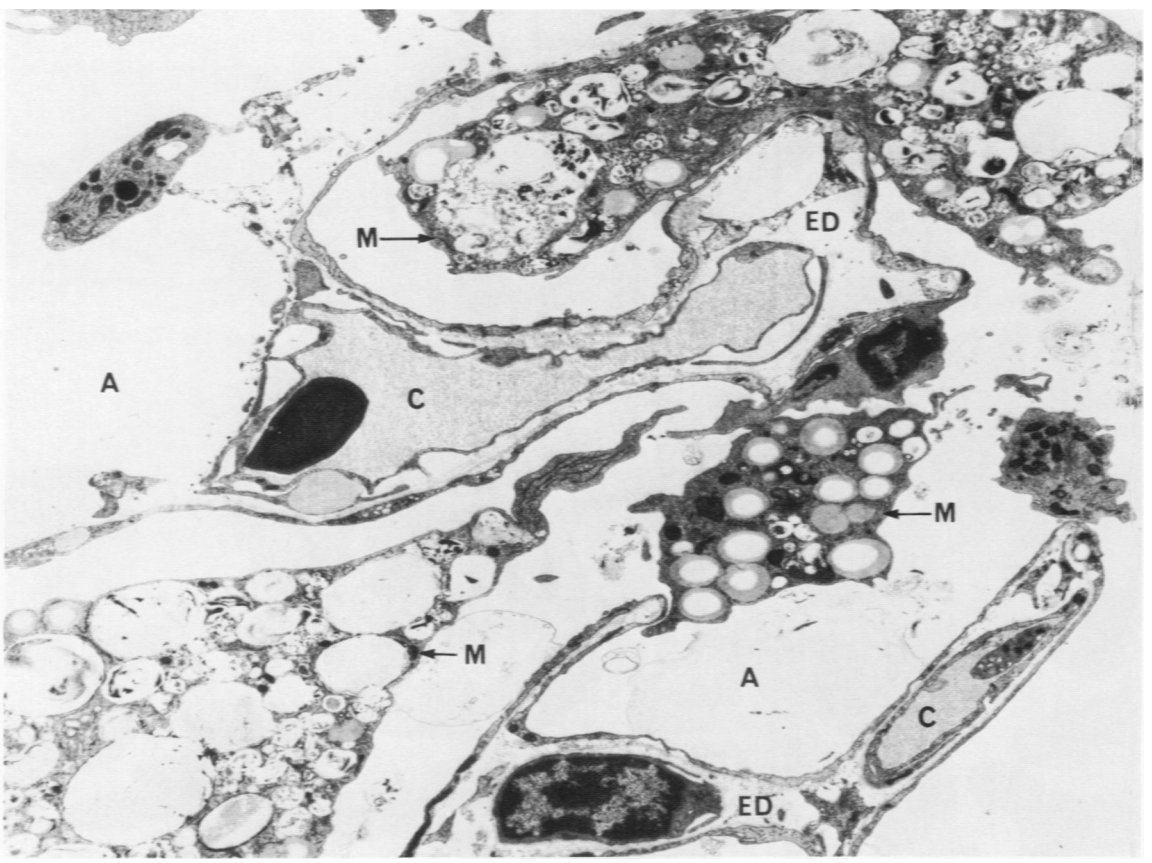

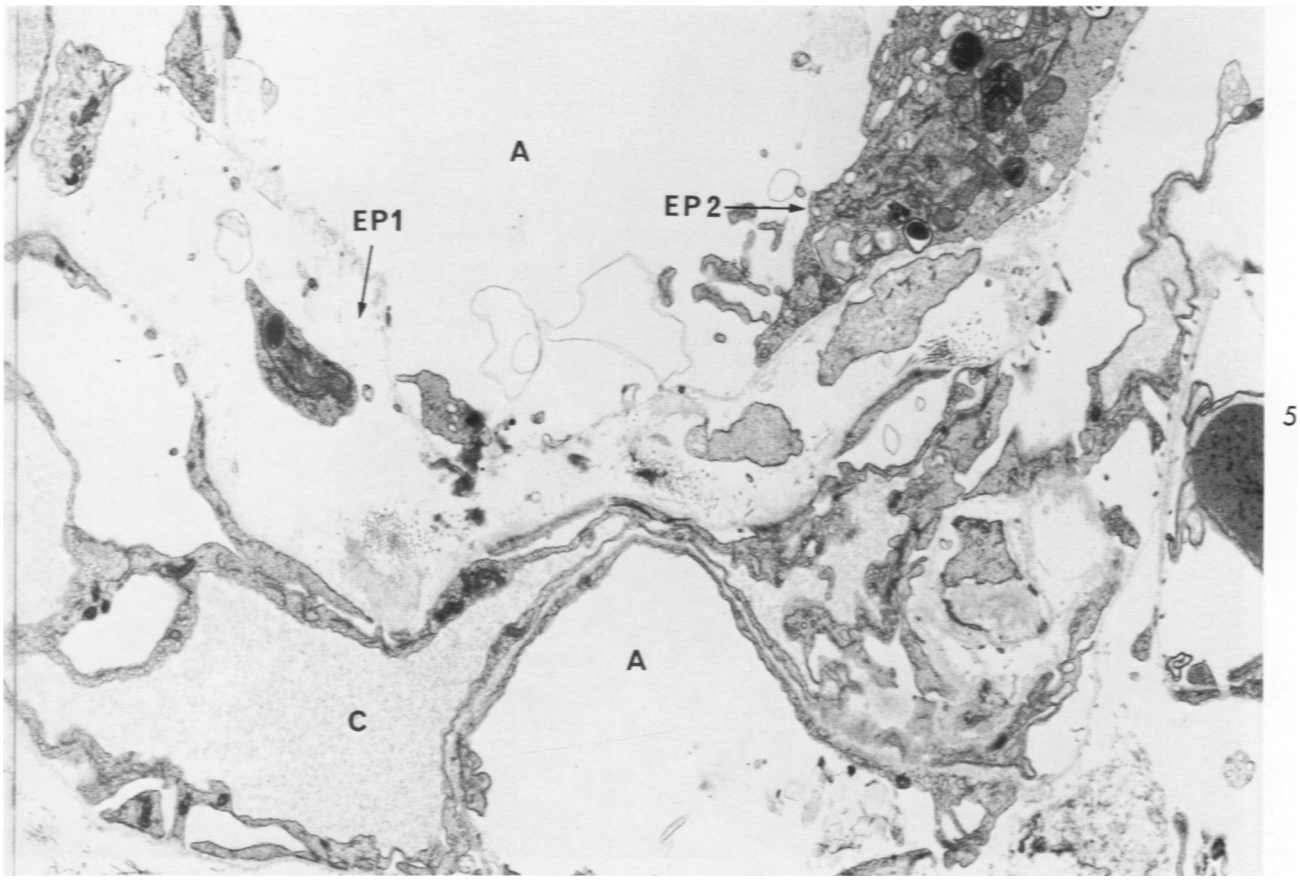

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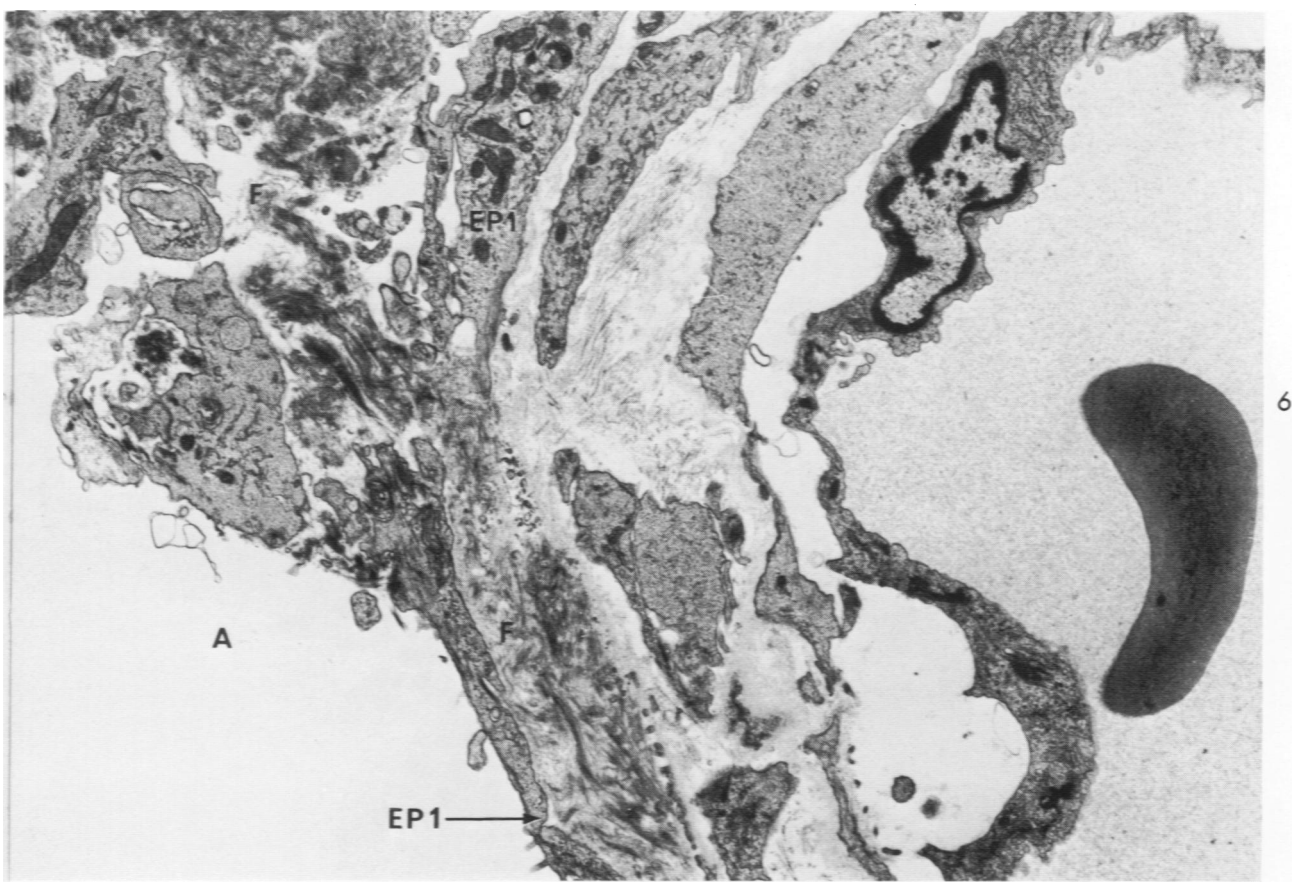

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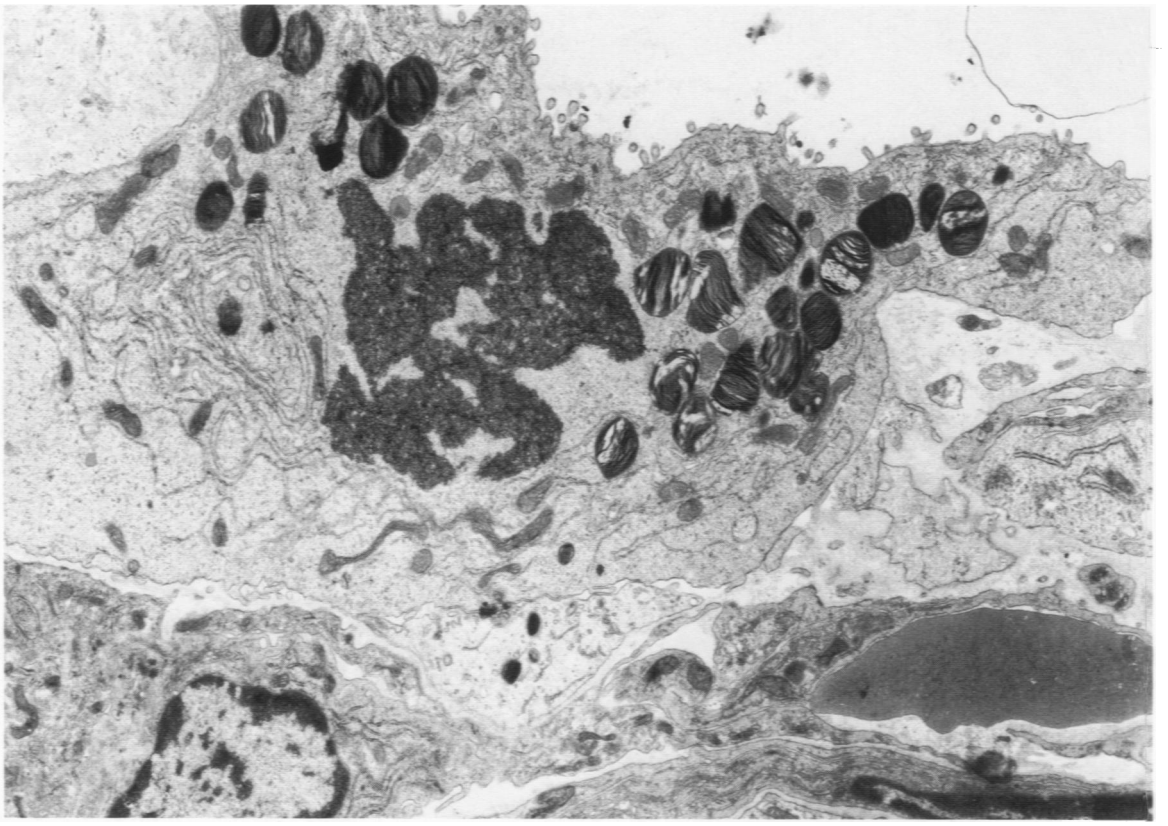

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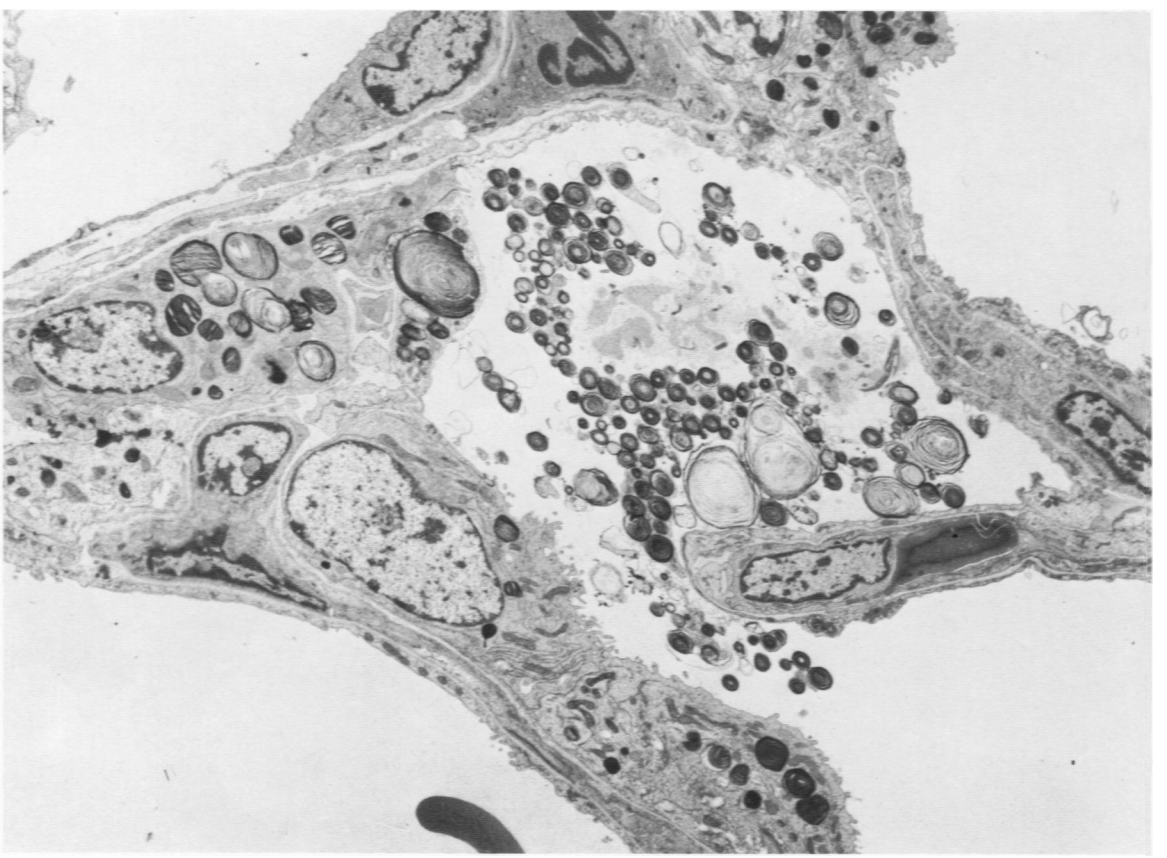

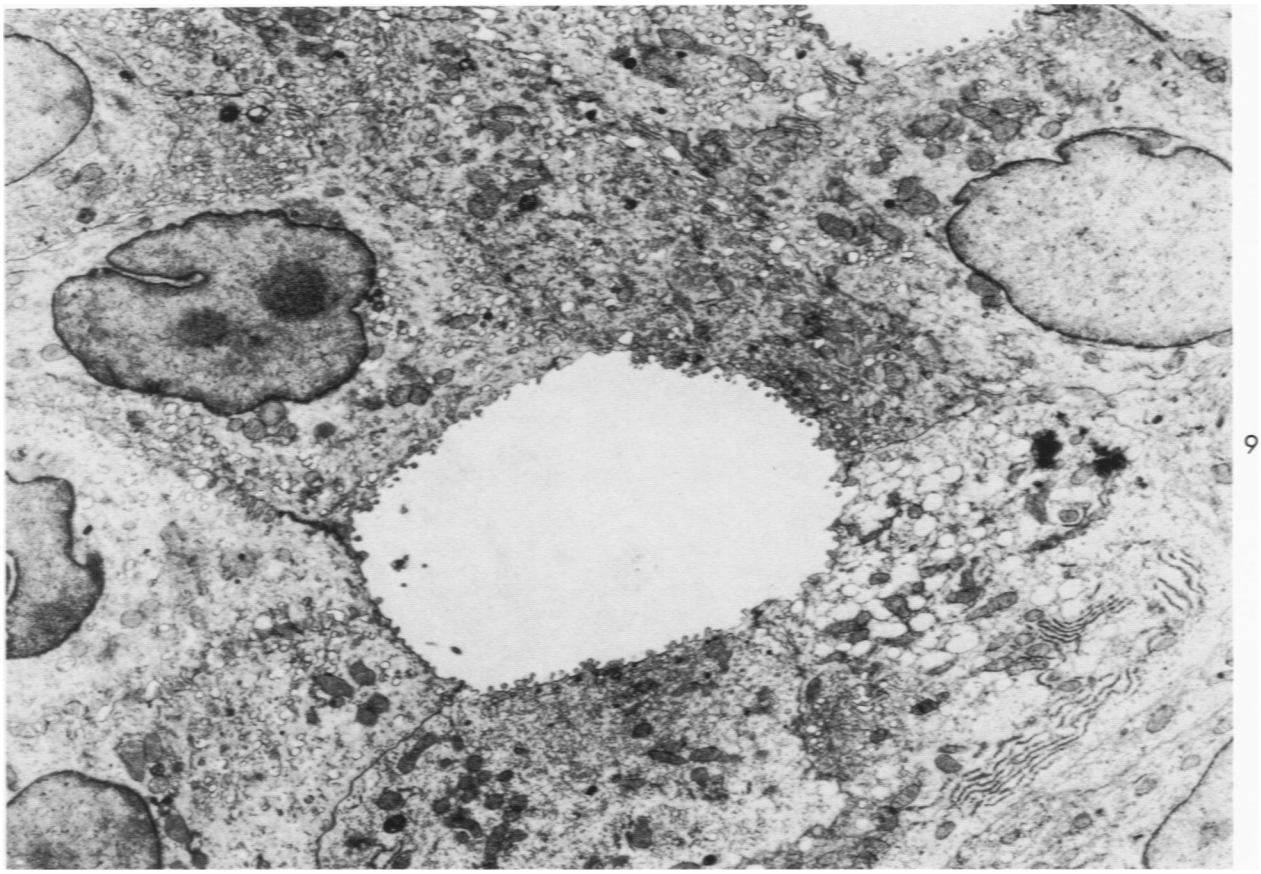

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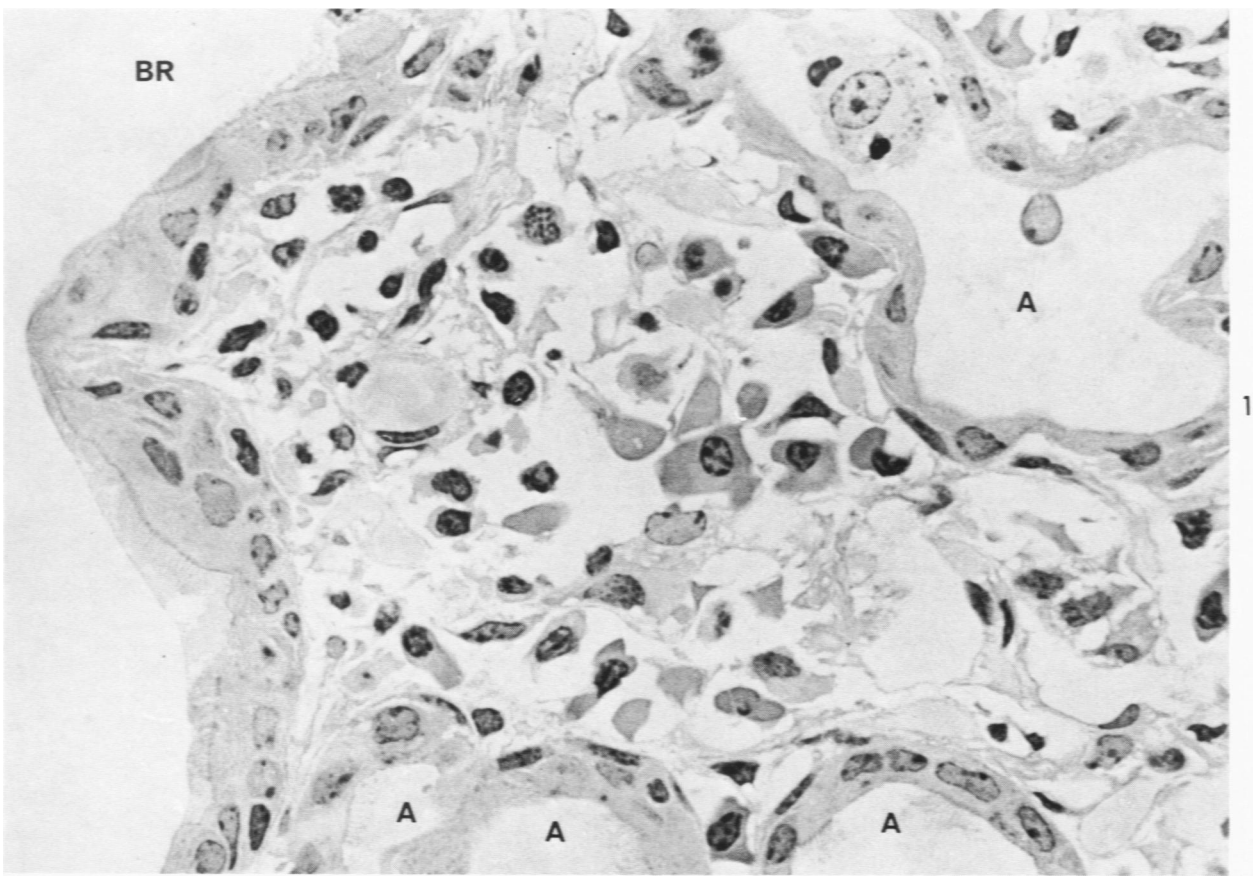

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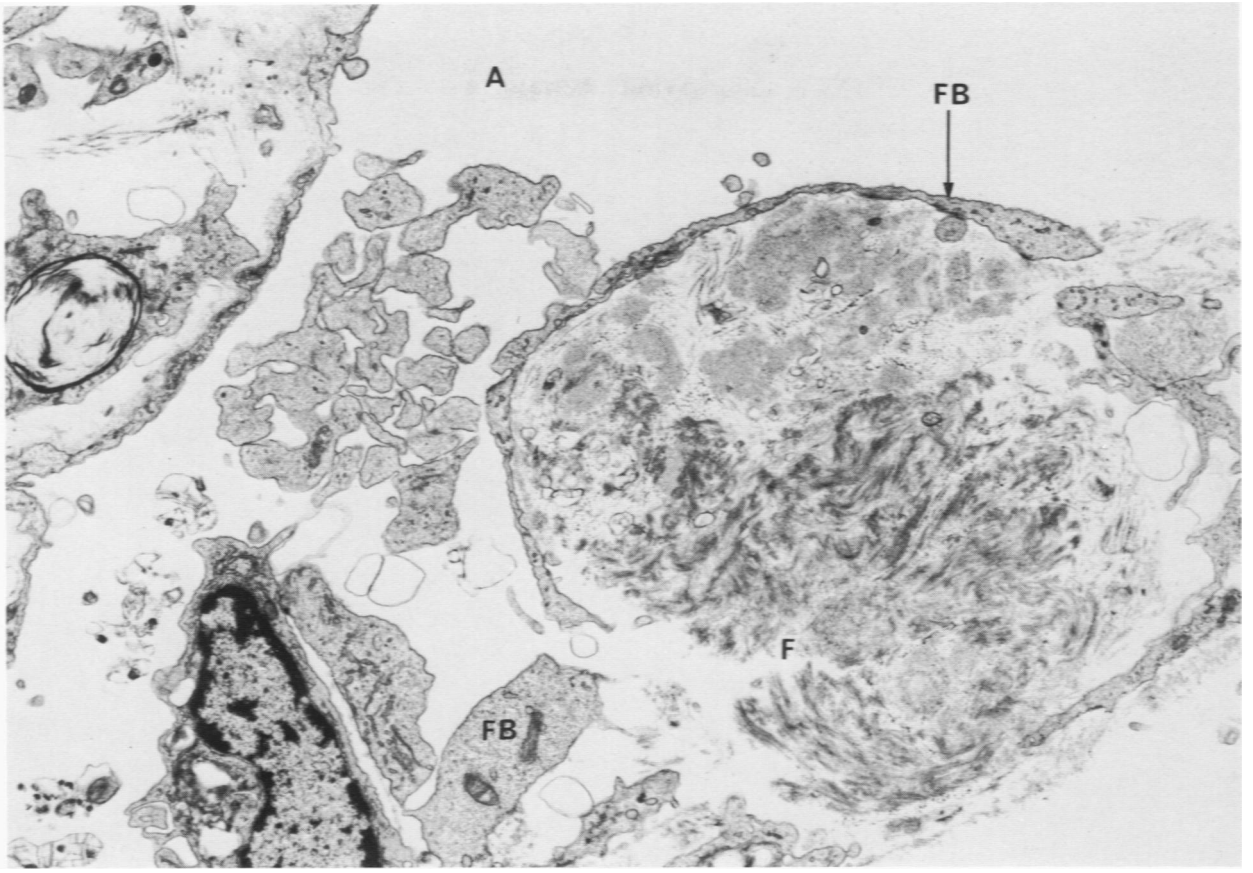

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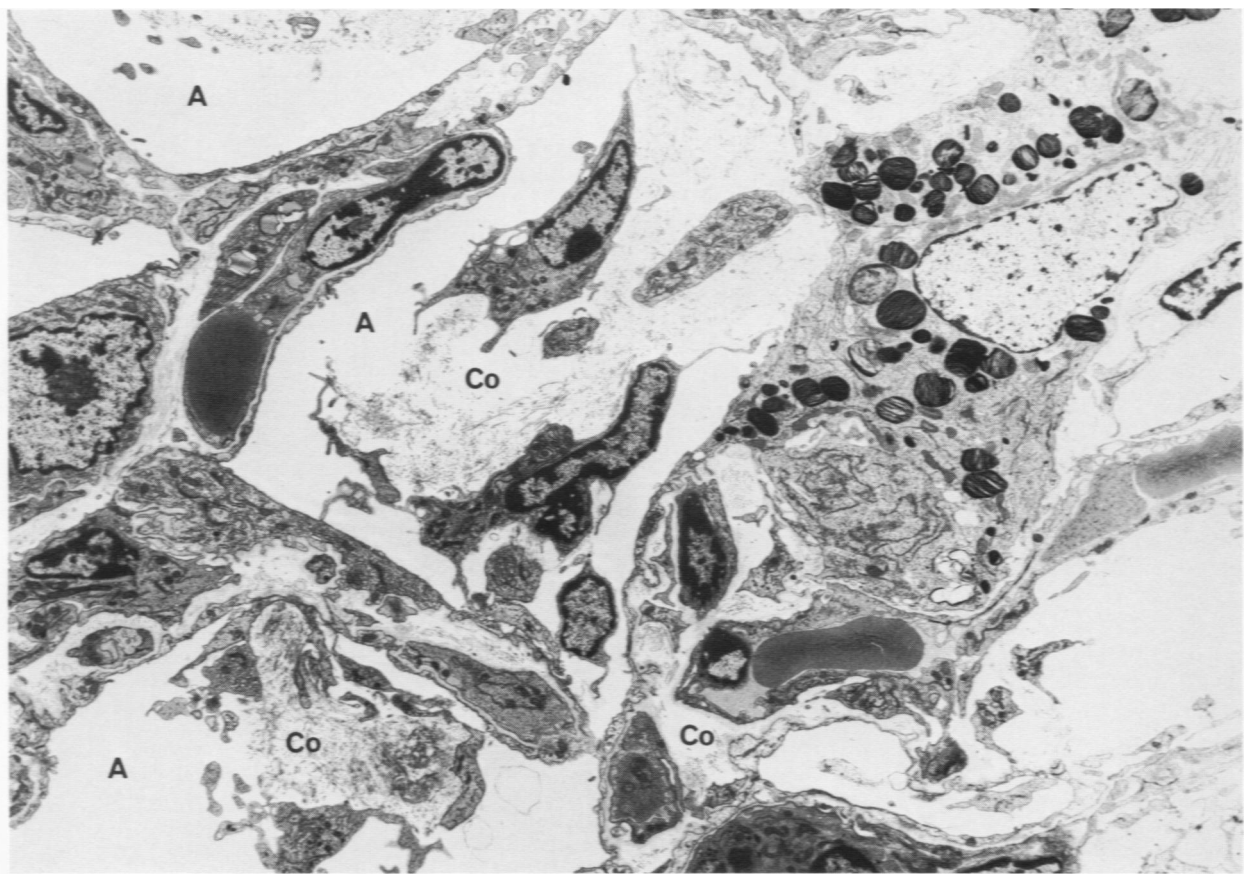

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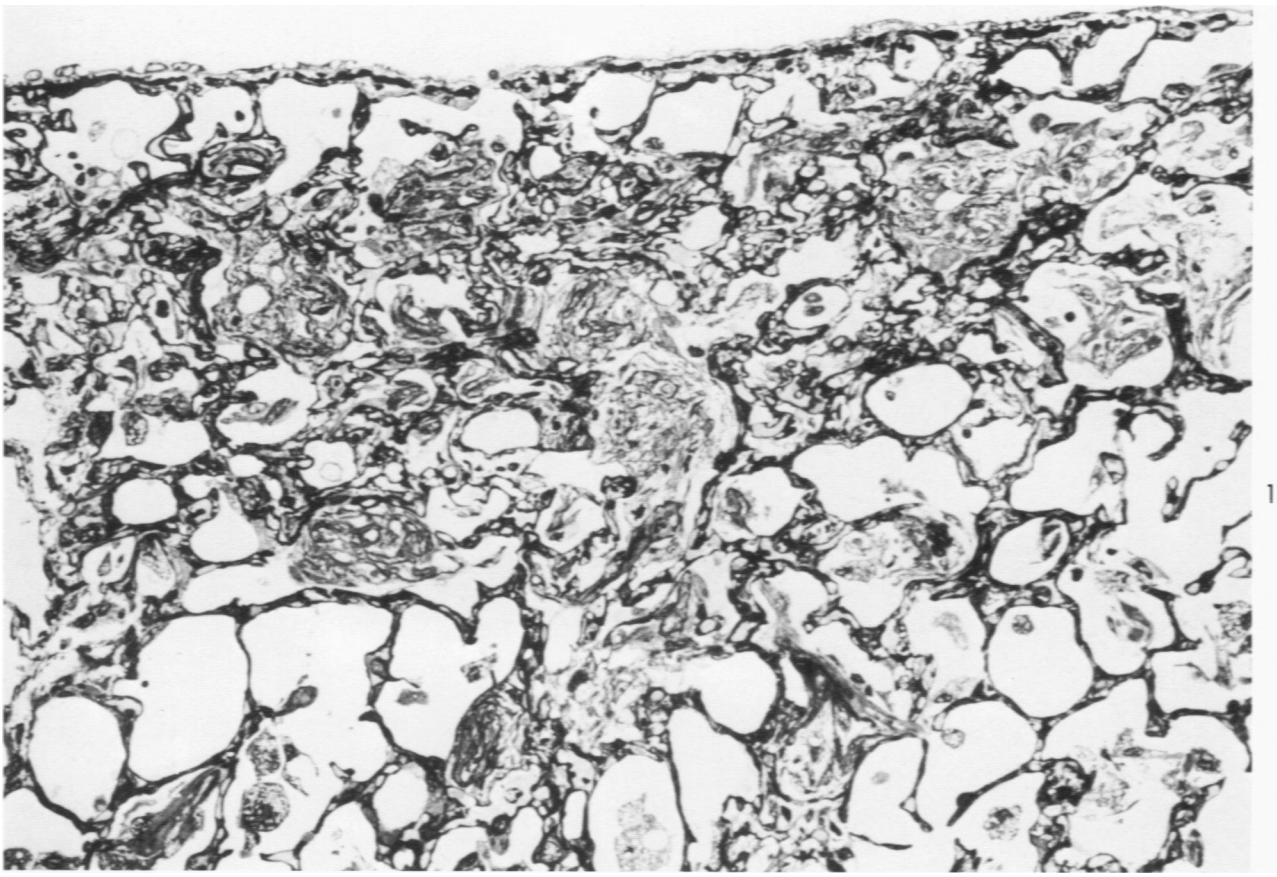

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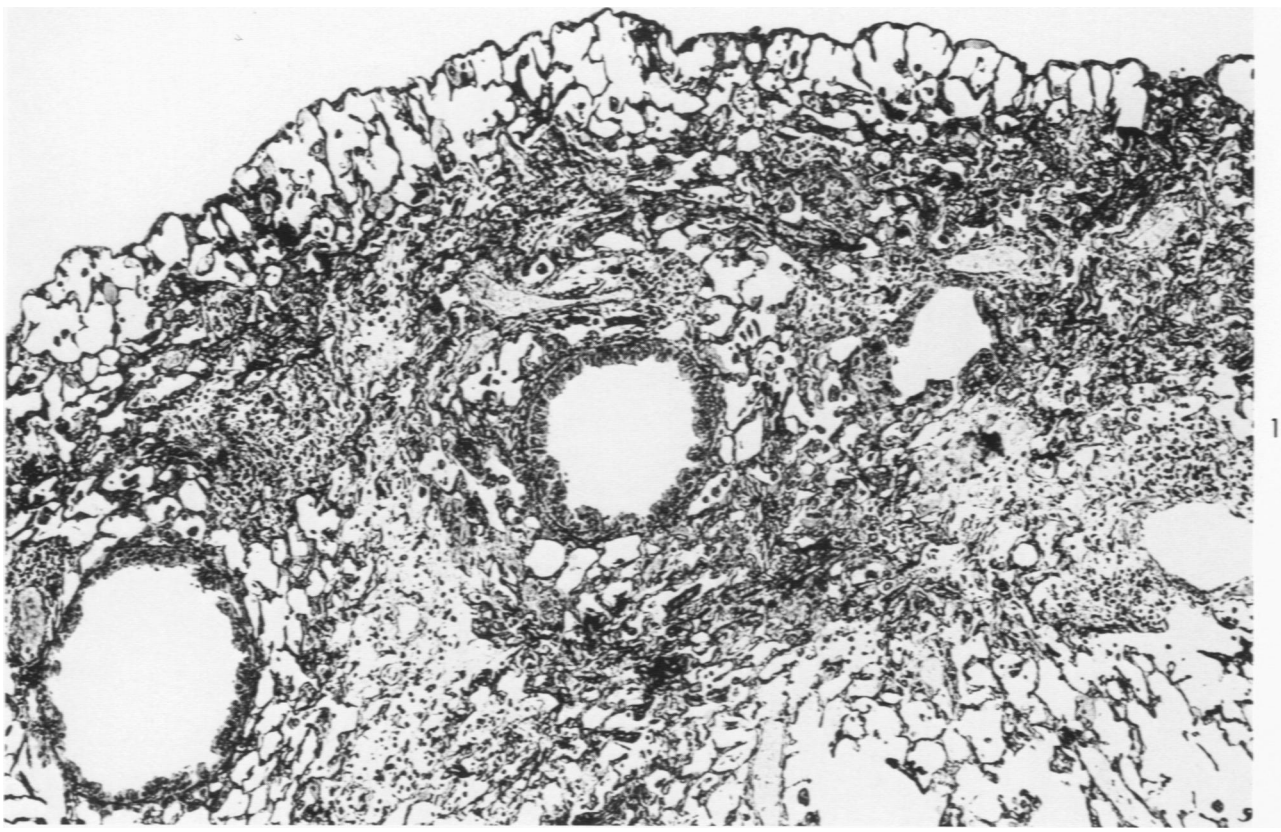

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