Embolic Pneumopathy Induced by Oleic Acid

A Systematic Morphologic Study

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This paper presents a systematic study of acute and chronic pulmonary lesions resulting from a single intravenous injection of oleic acid and a new fibrosis lung model is proposed: pulmonary interstitial fibrosis is obtained by means of a number of oleic acid intravenous injections. Nineteen adult dogs received 0.045 g/kg or 0.09 g/kg of oleic acid. A systematic morphologic study was carried out after 1, 2, 3, 4, 6, 12, 24, and 48 hours and 1, 2 and 4 weeks. Eleven other adult dogs received weekly one injection of 0.09 g/kg of pure oleic acid over a period of 1 to 3 months. Examination of the lung was carried out by means of light and electron microscopy and morphometry. An early stage characterized by the formation of thrombosis and cellular necrosis was followed by a repair stage with the proliferation of Type 2 cells and fibrotic foci in the subpleural areas. Lipid staining with Sudan IV allowed the onset and disappearance of lipid-laden macrophages to be ascertained. The late stage showed pulmonary fibrosis. The extent of the lesions is related to the number of oleic acid injections. Since interstitial pulmonary fibrosis invariably appeared, and only 2 dogs out of 11 died, the model is satisfactory for pathologist and physiologist. (Am J Pathol 87:143–158, 1977)

IN RECENT YEARS, many experimental animal models simulating human pathologic conditions have been developed. Although they do not exactly reproduce what is observed in man, they enable a better understanding to be gained of the pathogenesis of the human lesions.

In neutral fat embolism, an initial stage of mechanical vascular obstruction is followed by a chemical stage related to the toxicity of the free fatty acids (FFA) liberated by the action of pulmonary lipase on neutral fats.[1] Intravenous injection of oleic acid, which represents more than 60% of the pool of fatty acids in mammals, is used by morphologists and physiologists to reproduce the clinical picture of the chemical stage in fat embolism.[2-4]

We present here a systematic study of acute and chronic pulmonary lesions resulting from a single injection of intravenous oleic acid. By sacrificing dogs at different times after oleic acid injection, we attempted to reconstitute the dynamic process of pulmonary lesions.

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The injected doses are such as to allow the animals to survive for a considerable period.

Since oleic acid led to pulmonary fibrosis in a late stage, in order to create a canine laboratory model of interstitial pulmonary fibrosis suitable for physiologic measurement, some animals were repeatedly injected with oleic acid. This new fibrosis lung model is studied by means of light and electron microscopy and morphometry.

Materials and Methods

Forty healthy adult dogs were used. In 8 fasting animals (each weighing between 6 and 8 kg), a single dose of 0.045 g/kg, and in eleven others 0.09 g/kg, of pure oleic acid was injected intravenously and was immediately followed by an injection of 20 ml of normal saline.

In both of groups, the animals were sacrificed at intervals of 1, 2, 4, 6, and 24 hours and 1, 2, and 4 weeks after injection. Three dogs injected with 0.09 g/kg oleic acid were killed at 3, 12, and 48 hours, respectively. Four control dogs received only one saline injection. Eight healthy dogs, weighing between 5 and 9 kg, received weekly one 0.1 ml/kg intravenous injection of pure oleic acid.

These injections continued over a period of 1 to 3 months. A control group of 6 dogs received normal saline injections. Physiologic and morphometric studies were undertaken in 6 normal dogs and in 3 dogs, weighing 15, 14, and 17 kg, which received the same oleic acid treatment for 1 ½ month, 2 months, and 3 months, respectively.

These animals were sacrificed 4 weeks after the last injection in order to avoid any acute lesions. During the course of the experiments they did not receive any antibiotics. Two animals died spontaneously after the first or the second injection.

In the first series (one single injection), lungs were prepared according to the method described by Weibel.[5] The dogs were deeply anesthetized by injection of Nembutal, 25 mg/kg. A cannula was introduced into the trachea; after the lungs were collapsed by pneumothorax, they were instilled with a 4% charcoal-purified glutaraldehyde solution buffered at pH 7.4 following the technique of Millonig, under a constant pressure of 25 cm water. After ligating the trachea, lungs were removed *en bloc*.

Samples of the upper and lower lobes and the ventral and dorsal sides were immersed for 2 hours in 4% glutaraldehyde at 4 C. About ten 1-cu mm blocks cut from these samples were rinsed overnight in Millonig's buffer complemented with 5.4 g/liter of glucose, postfixed in 2% osmium tetroxide in the same buffer for 90 minutes, dehydrated in graded alcohol, cleared in propylene oxide, and embedded in Epon.

Semi-thin sections cut with a LKB Ultrotome by means of a glass knife were stained with toluidine blue. Thin sections cut with a diamond knife were stained with uranyl acetate and lead citrate, coated with a carbon film, and examined with Siemens Elmiskop 1 and 101 microscopes at 80 kV.

For light microscopy, part of the lungs sampled were embedded in paraffin and stained with hematoxylin and eosin, and part was embedded in gelatin, cut with a freezing microtome, and stained with Sudan red IV.

In the second series (several injections), the lungs were handled in the same way but were fixed with cacodylate-buffered paraformaldehyde-glutaraldehyde adjusted to 550 mOsm/liter and pH 7.2.

The lungs were cut into slices 1 cm thick and grossly examined with a hand lens. Specimens of the pathologic areas were divided in two parts. One part, dehydrated in alcohol and embedded in paraffin, was sectioned at 5 μ and stained with hematoxylin and eosin, Masson trichrome for fibrosis, and aldehyde fuchsin for elastic tissue. Another part was cut into small blocks and processed for electron microscopy.

The methods and results of the physiologic studies have been published elsewhere.[6]

Morphometry was carried out following Weibel's methods.[7] Lung volumes were measured through water displacement; 68 samples were randomly taken from each lung, the number for one lobe depending on its volume. Fibrosis volume and alveolar volume were measured by the point-counting method; vessels and peribronchial and bronchial fibrosis were excluded.

Results

When sacrificed, the animals looked quite normal and none showed respiratory distress.

Gross Aspects

In the early stage, macroscopically hemorrhagic lesions were disseminated in both pulmonary fields, mainly in the ventral and subpleural areas.

In the late stage, yellow-tan patches were scattered over the pleura. Lesions were most frequent in lower lobes, chiefly on the ventral sides. The cut section revealed normal lung interspersed with irregular confluent patches of gray areas of consolidation irregularly distributed but prominent in the subpleural and the perivascular areas (Figure 1). Large microcysts measuring about 1 to 2 nm were found in the peripheral portions of lobe near these consolidation zones.

Light Microscopy

The lungs of dogs injected with a single dose of oleic acid showed damaged areas interspersed with normal lung (Table 1). The two groups (small and large dose) differed only in the extent of diseased zones and will be described together.

The lungs of the dogs killed 1 hour after injection exhibited irregularly distributed edematous areas; the alveolar capillaries were diffusely congested.

At 2 and 3 hours, edema and capillary congestion became more marked; polymorphonuclear leukocytes had infiltrated the alveolar septa. Sudan staining revealed many intravascular fat emboli (Figure 2).

At 4 hours, histologic pictures were identical to the last one described, but numerous sudanophilic macrophages had infiltrated the perivascular connective tissue (Figure 3).

The picture after 6 and 12 hours revealed maximal congestion, edema, hemorrhage, and septal necrosis (Figure 4).

After 24 hours, congestion, edema, hemorrhage, and septal necrosis decreased. Such edema as remained was prominent in the perivascular areas. Many sudanophilic macrophages were to be found in the zones of sclerosis.

Table 1—Summary of Lesions in Light and Electron Microscopy

Type of lesion	Time after injection										
	1 hr	2 hrs	3 hrs	4 hrs	6 hrs	12 hrs	24 hrs	48 hrs	1 wk	2 wks	1 mon
Light microscopy											
Capillary congestion	1+	2+	2+	2+	3+	3+	2+	1+	±	±	−
Alveolar edema	1+	2+	2+	2+	3+	3+	2+	1+	−	−	−
Alveolar hemorrhage	1+	2+	2+	2+	3+	3+	2+	1+	−	−	−
Fat embolism	3+	3+	3+	1+	−	−	−	−	−	−	−
Septal necrosis	1+	1+	2+	2+	3+	3+	2+	1+	−	−	−
Macrophages	−	−	−	1+	2+	2+	3+	3+	2+	2+	1+
Fibrosis	−	−	−	−	−	−	−	−	1+	2+	3+
Electron microscopy											
Recent thrombi	2+	2+	1+	−	−	−	−	−	−	−	−
Interstitial edema	2+	2+	2+	2+	2+	2+	1+	1+	−	−	−
Endothelial cell necrosis	3+	3+	3+	3+	2+	2+	1+	1+	−	−	−
Type 1 cell necrosis	3+	3+	3+	3+	2+	2+	1+	1+	−	−	−
Polymorphonuclear leukocytes in vascular lumen	−	2+	2+	2+	2+	1+	1+	−	−	−	−
Inclusions in endothelial cells	−	−	−	1+	2+	2+	3+	3+	3+	3+	3+
Type 2 cell hyperplasia	−	−	−	−	−	−	−	−	1+	2+	2+

At 48 hours the picture was the same, but the acute lesions were less intensive.

After 1 week, all acute lesions, with the exception of a moderate edema, had disappeared. Seldomly, some fibrotic areas disseminated in the lungs were observed, but they did not destroy the normal alveolar pattern. The thickened alveolar walls contained macrophages with foamy cytoplasm and collagen fibers, and were lined by a cuboidal or flattened epithelium.

Similar changes were seen at 2 weeks and 1 month, but fibrosis was more extensive and macrophages less numerous.

In the second series (several injections), changes leading to interstitial fibrosis (Figure 5) were so extensive that the normal alveolar pattern had been modified: alveoli were shrunken and sometimes no more alveolar lumen were recognizable. The thickened septa were infiltrated not only by numerous macrophages but also by less numerous mast cells and lymphocytes. The alveolar capillaries decreased in number. The adjacent alveolar ducts were normal.

In less affected zones, alveolar septa were thick and infiltrated with fibroblasts and newly synthesized collagen fibers. This repairing process displaced capillaries which were normal in appearance, leading to honeycombing. In none of the treated dogs were inflammatory cells seen. The deep parts of the lung were in most cases normal.

The 2 dogs that died spontaneously showed acute alveolitis, with edema and large hemorrhagic foci.

None of the changes described above were seen in the control groups.

Morphometry

The fibrosis volume is represented like a percentage of the respiratory zone volume, i.e., a percentage of the alveolocapillary membrane volume but, above all, of air alveolar volume.

Pulmonary lesions were most severe in those dogs which had received the longest oleic acid treatment. The dog which had received injections over 1½ months exhibited 3% fibrosis; the second 6.7%; and the last one, 9% in volume.

Electron Microscopy

One hour after injection of oleic acid, capillaries were obstructed by recent thrombi formed of fibrin, platelets, and cell debris (Table 1). Endothelial and Type 1 cells were necrotic and were separated from their basal membrane. In the vicinity of these acute lesions, some endothelial cells were normal and some were swollen; the connective tissue of the alveolar septum was edematous.

After 2 hours, a few images of erythrophagocytosis and many polymorphonuclear leukocytes were observed in the vessels.

As early as the fourth hour, capillary obstruction had disappeared. The endothelial cells showed cytoplasmic modifications. In some cells, large pinocytotic vacuoles were found, while in others myelin-like figures and lipid droplets were found (Figure 6). These changes persisted in all the animals injected, and their frequency increased during the first 24 hours. The control animals showed no similar lesions. At this stage, Type 2 cells were normal.

At 48 hours, the pictures were similar.

After 1 week, the most striking change was the proliferation of Type 2 cells which became more numerous than in the control animals (Figure 7). These cells showed irregular limits which were often difficult to identify; they were concentrated in certain places and were connected at their apical poles by tight cellular junctions. They lay on a collagen-rich basement membrane. Their apical microvilli were small, and their nuclei were irregular in shape. The cytosomes were numerous, large, often anastomosed together and contained abnormal lipid inclusions (Figure 8). The interstitial edema had disappeared. The basement membrane was invariably normal. In the alveolar spaces, large cells were found with many osmiophilic inclusions and phagocytized material (Figure 9).

Two and 4 weeks later, a strikingly persistant concentration of Type 2 cells was still to be found: the alveolar septa were thickened by the proliferation of collagen fibers (Figure 10). No inflammatory cells were seen.

More specific for the second series (several injections) was the infiltration of alveolar walls by hypertrophied septal cells, fibroblasts, some lymphocytes, plasma cells, and mast cells (Figure 11), and collagen fibers (Figure 12). This displaced capillaries of normal appearance.

The fibroblasts showed short cytoplasmic pseudopodia, increased rough endoplasmic reticulum, and dilated cisternae. Around these cells, there could be seen periodic and nonperiodic fine collagen fibers, which appeared newly synthesized (like fine fibrillar material)

The alveolar lumen were often filled with large vacuolated cells bearing many delicate pseudopodia and containing numerous lamellar bodies.

Only a few endothelial cells contained myelin-like figures; sometimes this material was expulsed in the vascular lumen.

Discussion

Fat embolism is a clinical entity which can be found in such widely different circumstances as fracture of long bones, orthopedic manipulation, injection of oily fluids, hyperlipemia, and diabetes.[8]

In the event of fat embolism, the pulmonary reactions will clearly be much more complex than those arising in the case of injection of a free fatty acid. A number of authors simulate pulmonary fat embolism by injecting perinephric fat [9] or by means of musculoskeletal trauma.[10] These models are certainly much more akin to reality, but it is unfortunately impossible to identify the role played by the various embolized components involved.

In our study, the aim has not been to simulate exactly the clinical fat embolism syndrome, but to study the development of pulmonary pathoanatomic lesions following the toxic action of one component.

Oleic acid was chosen from among the FFA because lesions are identical to those of fat embolism in the chemical stage [2,4,11,12] and because oleic acid constitutes more than 60% of the fatty acid pool in mammals.[13]

A number of arguments corroborate Peltier's chemical theory:[1] first, intravenous injection of FFA gives a clinical picture identical to that in the fat embolism syndrome; second, following injection of neutral fats, there is an increase in both circulating and pulmonary lipase.[13]

The toxicity of FFA is well known. These acids are directly toxic to pulmonary cells; this leads to rupture of the alveolocapillary wall.[4] They inhibit surfactant activity, leading thereby to edema, hemorrhage, and alveolar collapse.[11] Emboli create a mechanical obstruction which leads to impaired diffusion.[14] Embolized fat droplets cluster around platelets with the result that platelets and fibrinogen are consumed and intravascular fibrin is deposited, confirming that disseminated intravascular coagulation is a consequence of the experimental model.[15] Lastly, FFA bring about hemolysis and a resultant diminution in oxygen transfer.[1]

Among these aggravating factors, disseminated intravascular coagulation is doubtless one of the prime agents responsible; patients presenting a serious clinical picture are precisely those who have not received the correct heparinizing treatment.[16]

Our fibrosis model fulfils Carrington's criteria:[17] a) mixed cellular exudate in interstitium, b) protein exudate in air spaces with leukocytes in alveolar spaces, c) proliferation of lining epithelium, d) gradual progression to fibrosis and honeycombing slow enough for several physiologic studies, e) continuing activity even when partly fibrotic, and f) diffuse distribution, but may have skip zones.

Various experimental approaches, such as radiation pneumonitis,[18] bleomycin-induced pneumopathy,[19] and *N*-nitroso-*N*-methylurethane (NNMU) poisoning,[20] were used in attempts to produce interstitial fibrosis. Only NNMU and oleic acid–induced fibrosis complied sufficiently with Carrington's criteria.

For Spencer,[21] damage to the alveolar epithelium is the *primum*

movens of interstitial fibrosis. Mast cells were also seen in fibrotic septa. The role of mast cells in producing fibrosis was noted not only in hepatic fibrosis [22] but also after ionizing irradiation-induced diffuse fibrosis.[23] In our model the mechanism of fibrosis is compatible with these two mechanisms.

The canine experimental model was suitable for many reasons: the dog's size allows physiologic measurements to be carried out easily; the animals tolerate the treatment quite well (of 11 dogs, only 2 died); they did not need special care, such as antibiotics or intensive nursing; and above all, interstitial pulmonary fibrosis invariably appeared. As shown by the morphometric results, the extent of the lesions is in relation to the number of oleic acid injections.

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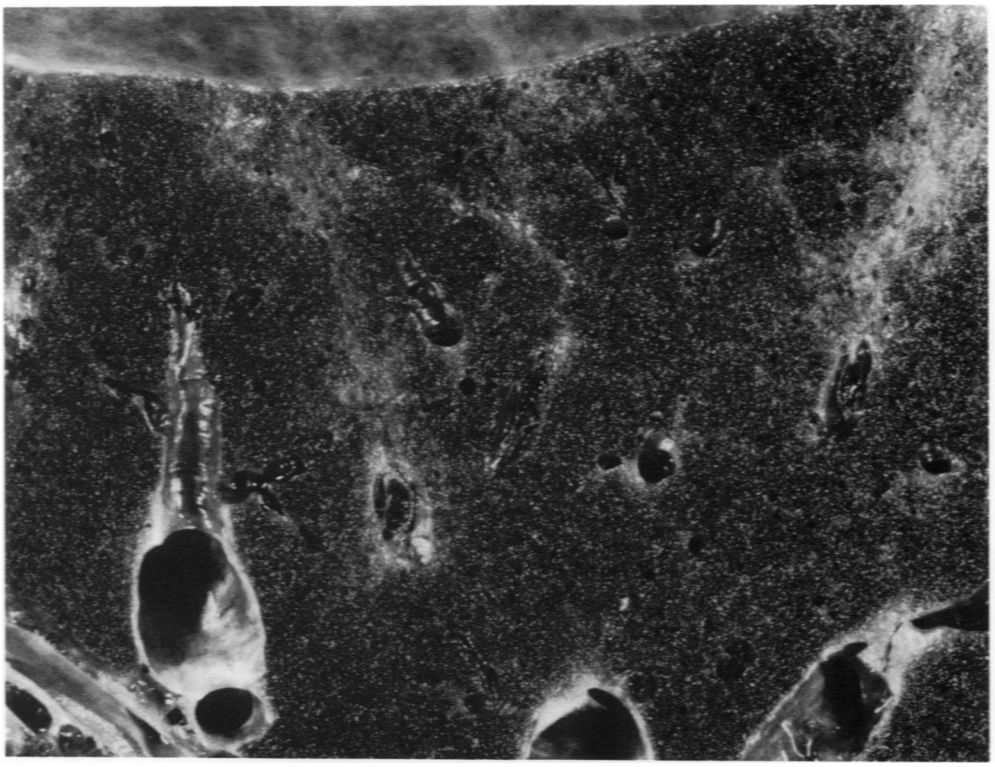
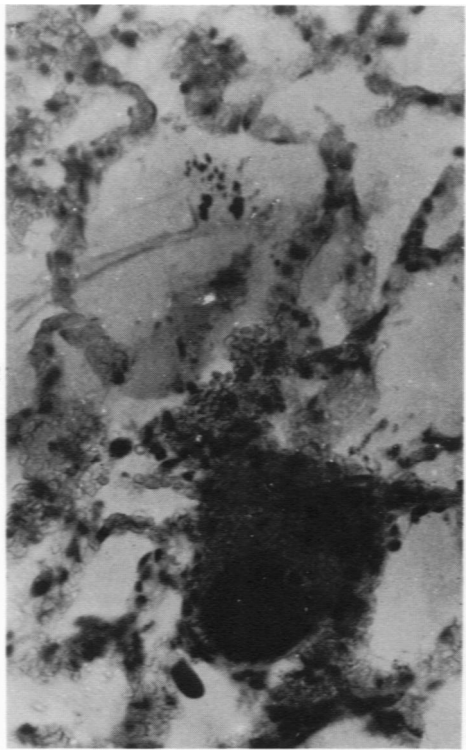
[*Illustrations follow*]





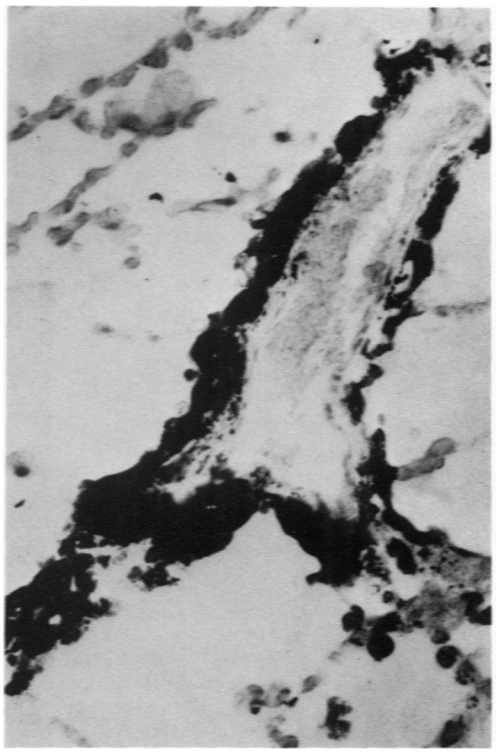


Figure 1—Gross aspect: whitish patches in the subpleural and the perivascular areas. Deep parts of the lung remain normal. **Figure 2**—Two hours after oleic acid injection. Capillary obstruction by lipid droplet. (Frozen section, Sudan staining, × 500) **Figure 3**—Four hours after oleic acid injection. Accumulation of sudanophilic macrophages in the perivascular connective tissue. (Frozen section, Sudan staining, × 300)

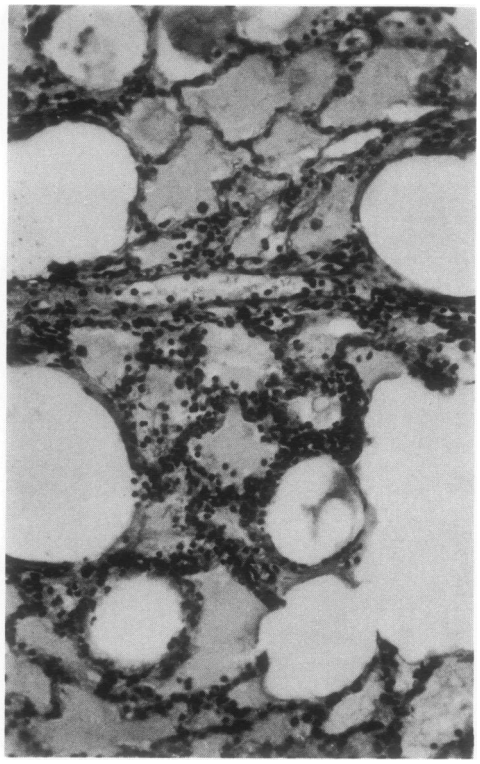


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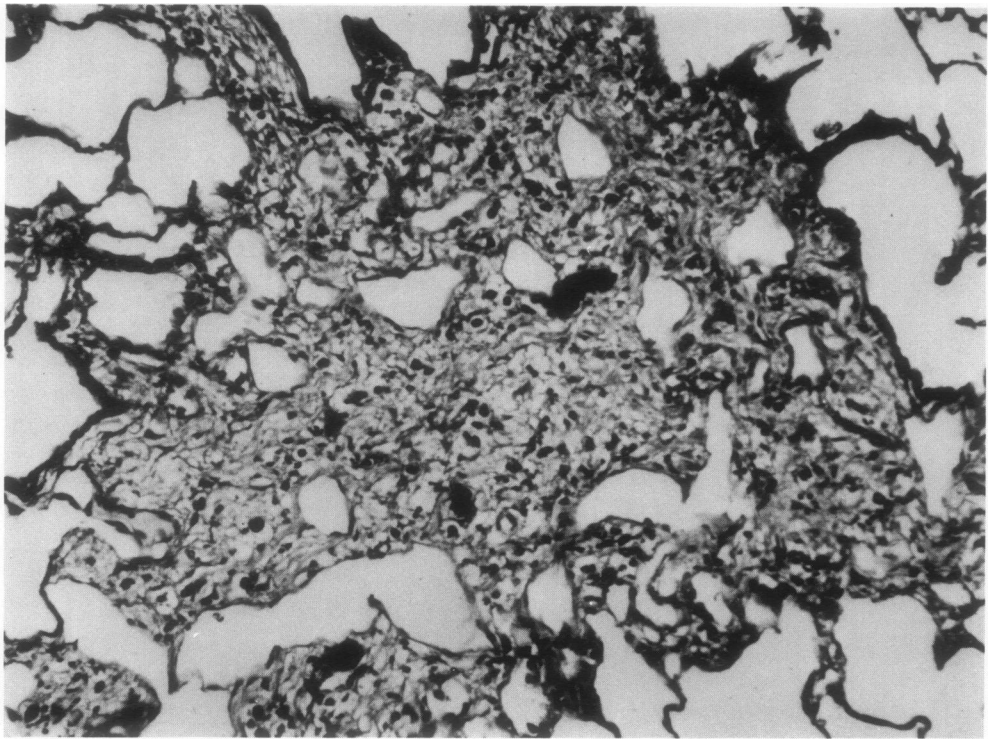
Figure 4—Twelve hours after oleic acid injection. Septal necrosis which is infiltrated with polymorphonuclear leukocytes. (H&E, × 250) **Figure 5**—Advanced stage showing interstitial fibrosis and destruction of the lung; sometimes no alveolar lumen can be seen (H&E, × 140).



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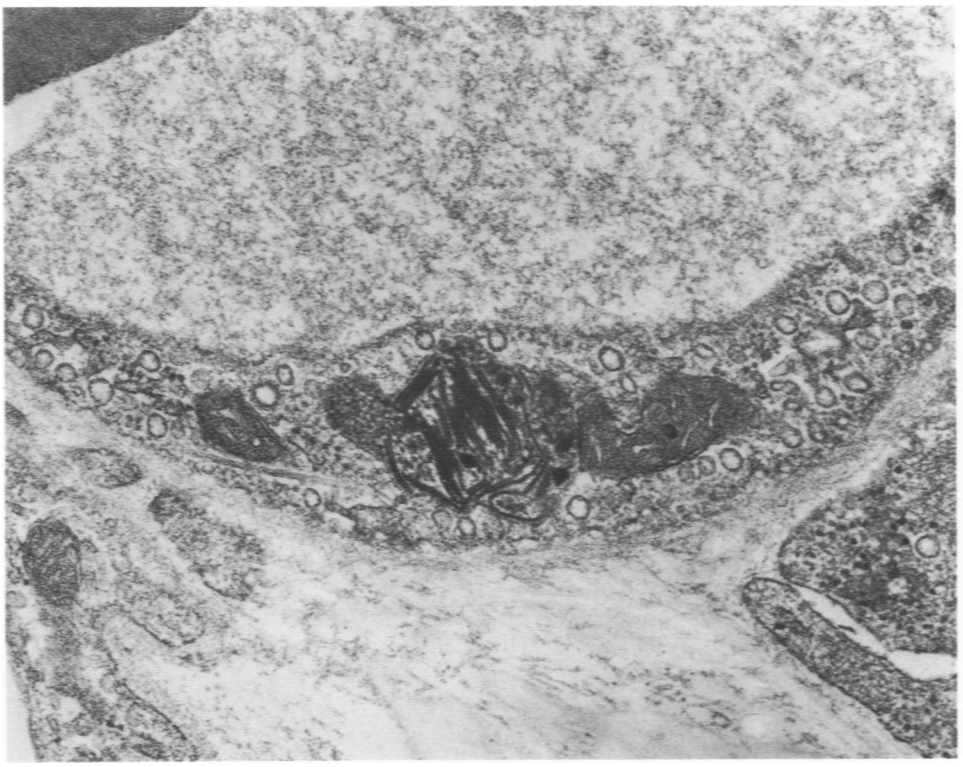

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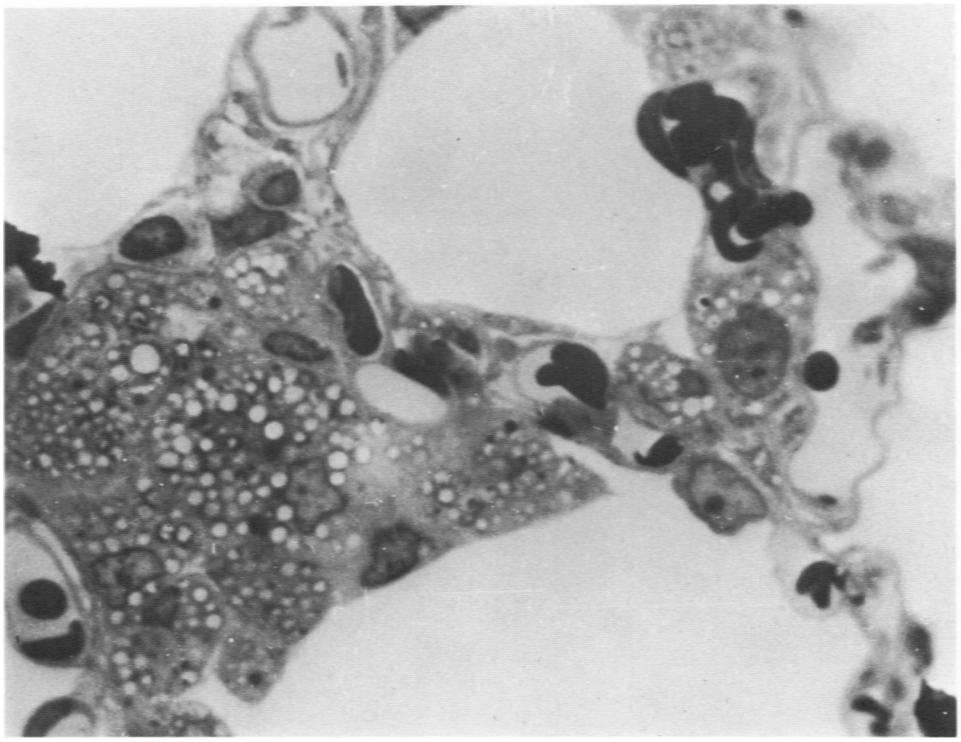

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Figure 6—Four hours after oleic acid injection. A capillary endothelial cell containing myelin figures (× 45,000) **Figure 7**—One week after oleic acid injection. Cluster of Type 2 cells lying in an alveolus. (Semithin section, Epon embedded, × 1000)

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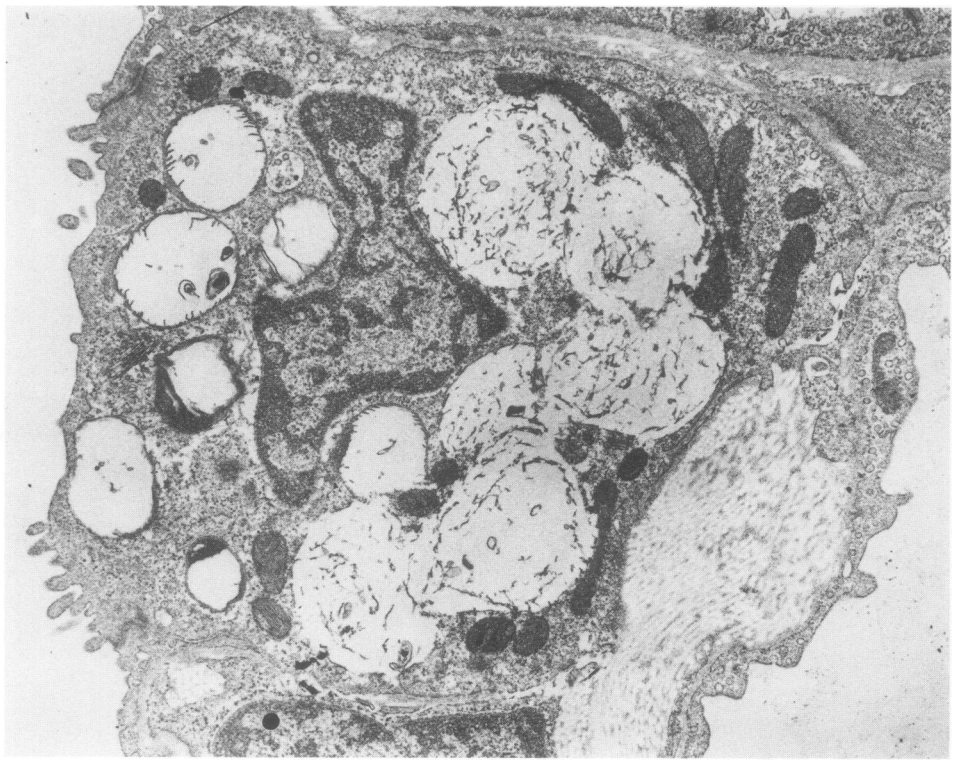

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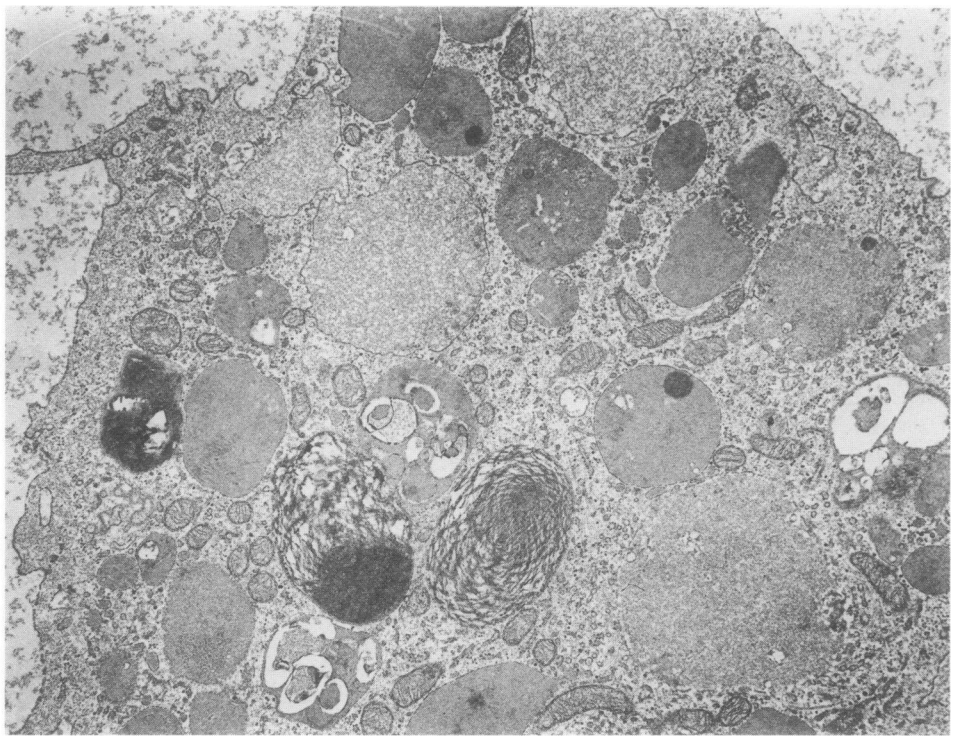
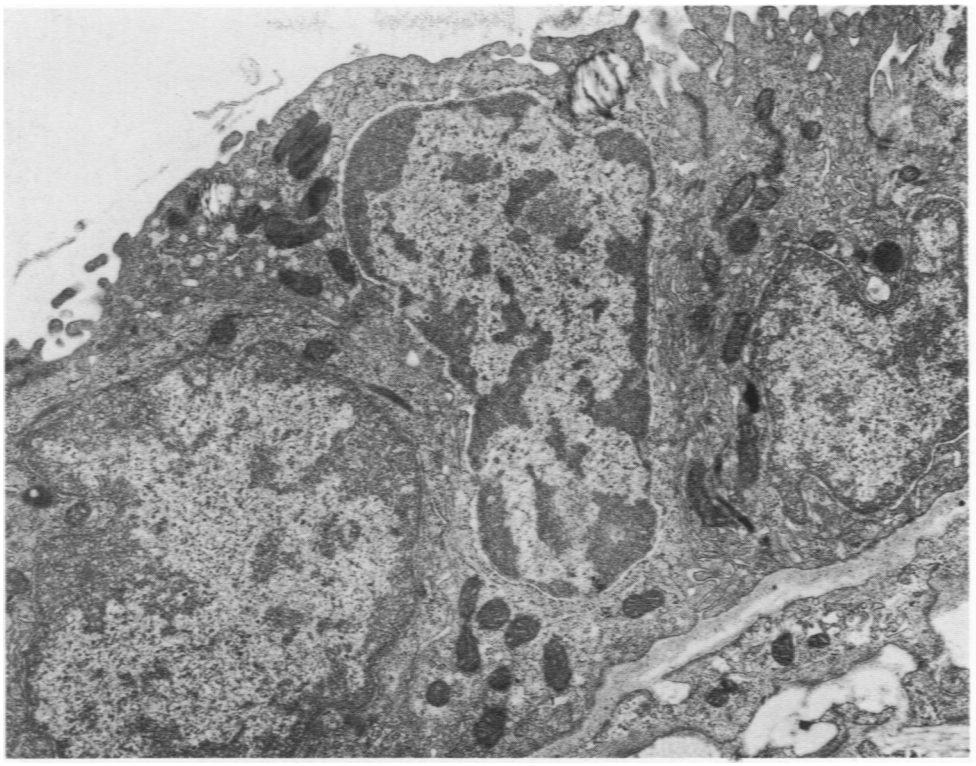


Figure 8—One week after oleic acid injection. A Type 2 cell containing large confluent cytosomes. (× 18,000) **Figure 9**—One week after oleic acid injection. An alveolar macrophage containing numerous phagocytized material. (× 14,000)



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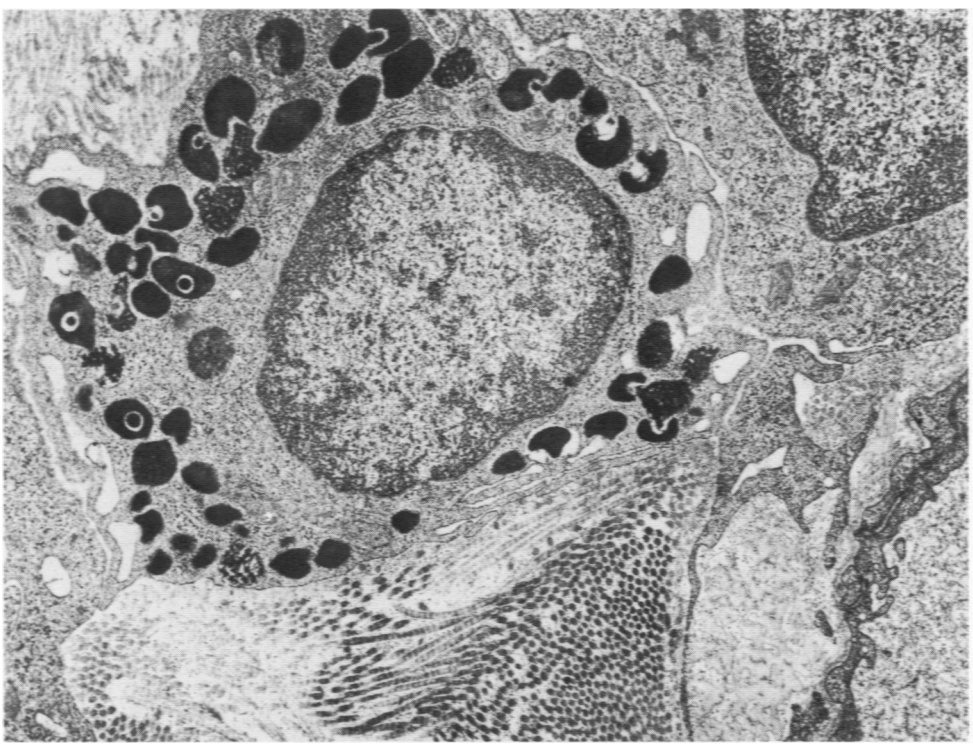

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Figure 10—Two weeks after oleic acid injection. Alveolar wall lined by Type 2 cells joined by tight junctions at their apical poles. (× 16,000) **Figure 11**—High power of an alveolar septum showing a mast cell lying between a proliferation of collagen fibers (× 12,800).

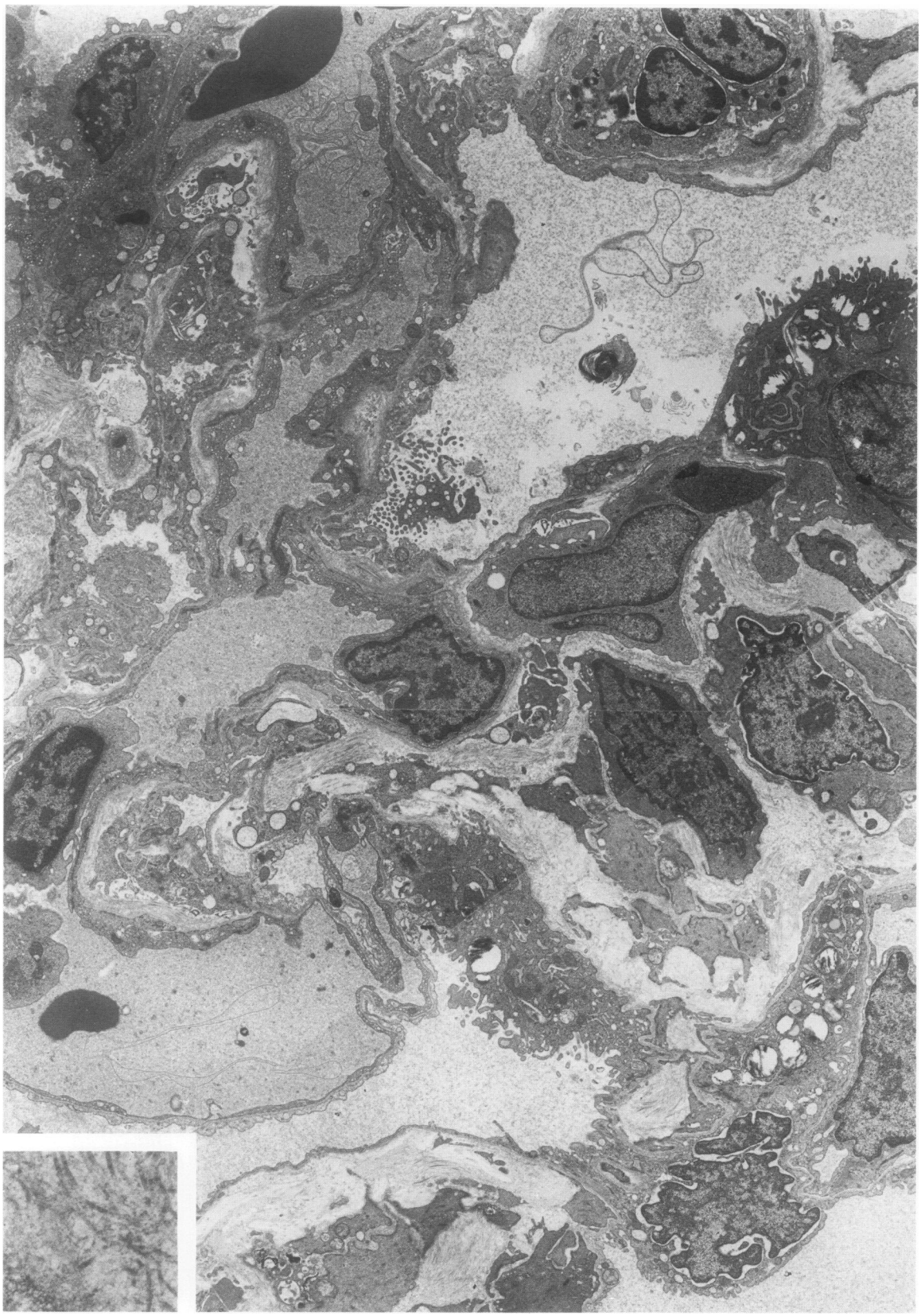

Figure 12—Two months after beginning of treatment. Alveolar septa are thick, rich in smooth muscular cells and collagen fibers. The alveolar spaces are filled with edema fluid. The alveolar walls are mainly lined with Type 2 cells. Note displacement, by the fibrosis process, of capillaries that remain intrinsically normal. (× 5600) **Inset**—Newly synthesized collagen fibers (× 55,000).