Morphologic Alterations Induced by Short-Term Cigarette Smoking

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Pulmonary fibrosis and emphysema were produced in beagle dogs by their direct inhalation of cigarette smoke over a relatively short period of time (2–7 cigarettes daily for 2–4 months). One dog was sacrificed after having smoked 172 cigarettes, one after 282 cigarettes, and the others after 480 and 534 cigarettes, respectively. Examination of the lungs by scanning and transmission electron microscopy showed a range of response from the presence of numerous smoker's macrophages to extensive alterations, including destruction and enlargement of alveolar ducts and varying degrees of enlargement of alveolar spaces. Interalveolar pores were enlarged, and marked fenestration leading to destruction of the alveolar walls became apparent. These features were accompanied by interstitial fibrosis of the interalveolar septa. Light- and electron-microscopic examination showed no evidence of bronchitis and/or bronchiolitis or of physical obstruction to the terminal airways in the early development of fibrosis and emphysema. (Am J Pathol 1983, 111:11–20)

CIGARETTE SMOKING has been used as a method of experimentally producing emphysema in beagle dogs and has been the subject of several reports from our laboratory.[1–3] In these studies the dogs had smoked large numbers of cigarettes over long periods of time. The light-microscopic findings of extensive fibrosis and emphysema in these dogs showed the early changes to be fibrous thickening and subsequent rupture of the alveolar septa. These characteristics were similar to those found in human beings who had been heavy cigarette smokers for many years.[4] Study of these lesions in dogs by transmission electron microscopy showed that the septal thickening was due mainly to the presence of increased amounts of collagen and that in many areas interalveolar capillaries were rare or nonexistent. Emphysema in these cases was too far advanced and too extensive for the determination of early events that had led to the destruction of the alveolar walls.

To determine the early structural alterations which can be attributed to cigarette smoking, we have now examined the lungs of dogs who smoked relatively few cigarettes over a short period of time. The specimens were examined with the scanning electron microscope. This approach has been of particular value in this study, because large amounts of tissue surface were examined at low and high magnification with greater depth of focus, resolution, and ease than could be accomplished by light microscopy. Use of scanning electron microscopy also eliminated many of the difficulties in interpretation that may be caused by sampling problems inherent in transmission electron microscopy.

Materials and Methods

The animals used in this study were 12 pedigreed male beagle dogs that ranged in age from 1.7 to 2.1 years, with a mean age of 1.8 years. They ranged in weight from 11.1 to 13.3 kg, with a mean weight of 11.8 kg.

Tracheostomies were performed on each dog; the

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stoma was kept open with a hollow Teflon tracheostomy tube and allowed to heal. An ADL I smoking machine fabricated by Arthur D. Little Inc. (Cambridge, Mass) was used to facilitate the inhalation of smoke by the dogs. These machines were operated by air pressure and mechanical springs with an electrically operated switching mechanism. One cigarette was smoked at a time, and the machine was adjusted to deliver a 35.0-ml puff volume in 2 seconds with a puff interval every 20 seconds to the tracheostomy opening. The cigarettes used were of a research type (National Cancer Institute, Code 26), were nonfiltered, and delivered 27.0 mg of tar and 3.25 mg of nicotine when smoked to a 23-mm butt length.[5]

During 7 consecutive days, we gave the dogs unlighted cigarettes and had them sham-smoke to condition them to the experimental procedure. After conditioning, 10 dogs were assigned to actual smoking and the remaining 2 to sham-smoking. Starting on Day 1 of smoking, dogs smoked or sham-smoked one cigarette in the morning and one cigarette in the afternoon 7 days a week. Cigarettes were gradually increased during the course of the experiment until a level of 7 per day was reached at 110 days.

At the stated intervals the dogs were killed by intravenous injection of pentobarbital (Table 1). The abdomen was then opened and the diaphragm punctured. The lungs were fixed *in situ* for 1 hour by intratracheal perfusion with 2.5% paraformaldehyde in 0.15 M cacodylate buffer adjusted to 305 mOsM at 25 cm of water pressure. The lungs were removed *en bloc* and placed in a fixative bath at the same perfusion pressure for an additional 72 hours.

Three adjacent blocks of lung tissue (1 × 1 × 0.5 cm) were taken from 16 different sites for light microscopy (LM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (Figure 1). The samples for LM were dehydrated in ethanol, cleared in xylene, embedded in paraffin, and stained with hematoxylin and eosin. Samples for TEM were postfixed in 1.0% OsO_4, processed in tannic acid,[6] dehydrated in ascending concentrations of ethanol and propylene oxide, and embedded in Epon 812.[7] Thin sections were doubly stained with uranyl magnesium acetate and lead citrate[8] and examined with a Siemens Elmiskop I. Specimens for SEM were rendered conductive by either the thiocarbohydrazide method of Kelly et al[9] or the tannic acid procedure of Sweney and Shapiro,[10] dehydrated in ascending concentrations of ethanol and critical-point-dried after removal from absolute alcohol. All specimens were coated with gold-palladium with a magnetically assisted sputtering device and examined with an AMR-1200 at 5 or 10 kv at a 10–20-degree tilt.

Table 1—Smoking Schedule

Number of dogs	Number of cigarettes*	Number of days smoked
2	0	Sham
1	172	64
1	282	87
1	480	116
7	534	123

* Unfiltered: tar, 27.0 mg; nicotine, 3.25 mg.

Results

The normal appearance of the canine lung by TEM and SEM has been described previously.[3,11,12] Briefly, the alveoli of the sham-smoked dog lung present a uniform honeycomb appearance under the SEM (Figure 2). They are deep, uniform in size, and approximately 100 μ in diameter (Figure 3). A striking feature of these alveoli is their sievelike appearance, attributable to the numerous round to oval interalveolar pores (pores of Kohn) (Figure 4). Pores range in size from 3.0 to 10.0 μ in their largest axis and there are approximately 20 per exposed alveolar surface. Alveolar ducts are numerous and uniformly round and when viewed in profile with accompanying alveoli, have a symmetrical pattern (Figure 5).

On gross examination the lungs of all dogs in the present study appeared normal, with the exception of 1 dog that had smoked 534 cigarettes. In this dog white areas varying in size from 3 to 5 cm were found on all lobe surfaces. Cross-sections of these areas showed the presence of dilated air spaces (approximately 1 mm), which gave the lung a spongy appearance.

Changes within the lung parenchyma, as observed by LM, SEM, and TEM, occurred in all smoking dogs. The extent of change varied from that of subpleural enlargement of air spaces to widespread air space enlargement with evidence of tissue destruction. All lobes were affected, and no preferential distribution was seen in any lobular region. Extensive changes were present in a dog that smoked the least number of cigarettes, and less extensive air space enlargement was noted in some dogs that had smoked a greater number of cigarettes (Figures 6, 7, and 12). In addition, a wide range in the degree of air space alterations was found between one site and another within the lung of an individual dog (Figures 8–11). The degree and extent of alteration did not appear to be dose-related.

The presence of numerous large macrophages on alveolar surfaces was found consistently in all smok-

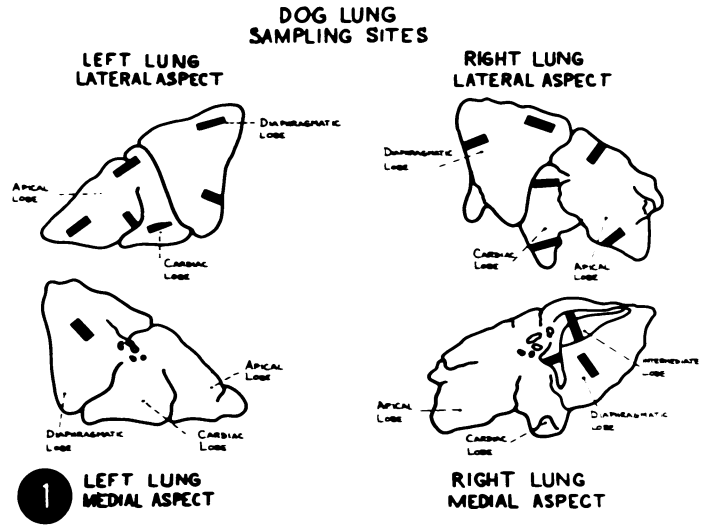

Figure 1 – Three adjacent blocks of lung tissue for LM, SEM, and TEM were selected from the indicated sites on the diagram (*darkened areas*).

ing dogs. However, their numbers were greater in less affected areas (Figures 13 and 14) and diminished in areas of greater alteration (Figures 9–11). TEM examination of such macrophages showed them to contain large pleomorphic cytoplasmic inclusions, sometimes with crystalloid material (Figure 15). Interstitial fibrosis of the interalveolar septa was also a consistent finding in all smoking dogs (Figure 16).

The alveolar ducts were enlarged with effacement of interalveolar septa (Figures 6–9). Some alveolar ducts were distorted and bridged by trabeculae composed of fibrotic tissue or avascular alveolar walls (Figures 10 and 11). The interalveolar pores were enlarged (fenestrae) and often coalesced at the expense of alveolar wall tissue (Figures 18–20 and 25). Large fenestrae subdivided by strands of tissue (Figures 19–21) were frequently seen. TEM examination of such strands showed them to be fibrotic interalveolar septa and/or collapsed capillaries devoid of blood cells (Figure 22). A proliferation of Type II alveolar cells (Figures 21 and 25) was frequently seen in areas of marked fenestration. TEM examination of such areas confirmed their identity as Type II alveolar cells.

Light-microscopic findings of detached or loose pieces of tissue, pathognomonic of emphysema,[13] were consistently present (Figures 23 and 26). Attachment sites of the strands were clearly demonstrated by SEM (Figures 10 and 11) and have been previously reported.[14]

Generally, enlargement of alveolar ducts was not associated with fenestration. However, areas of fenestration were occasionally found together with enlarged alveolar ducts (Figure 11). More frequently fenestrations were found distal to sites of alveolar duct enlargement. The converse, normal-sized ducts with fenestration were found in specimens that appeared otherwise normal (Figure 20).

Light-microscopic examination of bronchi and bronchioles showed no evidence of bronchitis or bronchiolitis (Figure 26), nor did SEM show any mechanical obstruction in the terminal airways.

Discussion

Studies on lungs of human cigarette smokers have demonstrated a clear relationship between the number of cigarettes smoked and the duration of smoking to the degree of fibrosis and emphysema.[15,16] Fibrosis and emphysema in these instances were too far advanced to permit a clear view as to the early lesions which ultimately led to the extensive destruction seen in the lungs of long-term cigarette smokers. It should also be noted that fibrosis and emphysema were present in the dogs that died from cardiovascular complications very early in the previous experiments designed for studying the long-term effects of cigarette smoking.[1,2]

Examination of the lungs of the short-term cigarette-smoking dogs in this study has permitted us to describe two morphologic patterns of alterations leading to the development of fibrosis and emphysema. These alterations are similar to those described in other studies. One is the enlargement of interalveolar pores into fenestrations and their coalescence.[12,13,17–22] The other is the dilatation of respiratory bronchioles and alveolar ducts resulting in enlarged alveoli and shortened interalveolar septa.[12,20–23] Zwicker et al[24] have also reported "alveolar emphysema" by LM in 3 of 12 dogs that had smoked

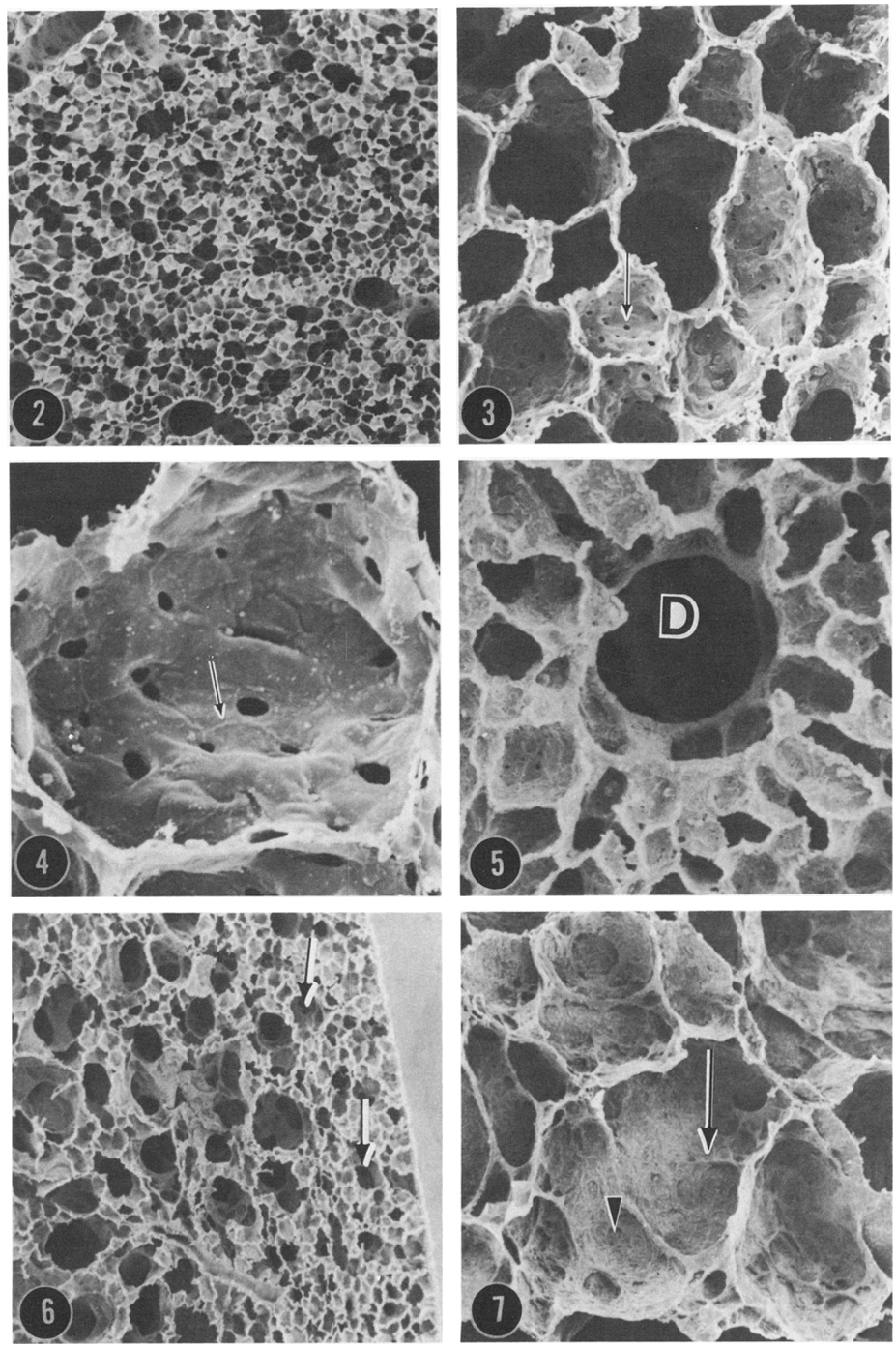

Figure 2 – Lung from a dog that sham-smoked. Alveoli and alveolar ducts present a uniform honeycomb appearance. (×20) **Figure 3** – Alveoli from lung from a dog that sham-smoked are deep and uniform in size, with numerous interalveolar pores (*arrow*). (×200) **Figure 4** – Higher magnification of an alveolus from Figure 3 shows the numerous round to oval interalveolar pores characteristic of the canine lung. Epithelial junctions can be seen converging at the pores (*arrow*). (×1000) **Figure 5** – An alveolar duct (*D*) from a dog that sham-smoked. Note the symmetrical pattern formed by the alveoli surrounding the duct and the uniformity of the alveolar duct wall. (×100) **Figure 6** – Lung from a dog that smoked 172 cigarettes. Compare the enlarged irregular alveolar ducts with those which appear normal beneath the pleura (*arrows*). (×20) **Figure 7** – Higher magnification of enlarged alveolar ducts; the major portion of the walls appears to be relatively smooth. Some contain shortened interalveolar septa (*arrow*) and shallow alveoli (*arrowhead*). (×50)

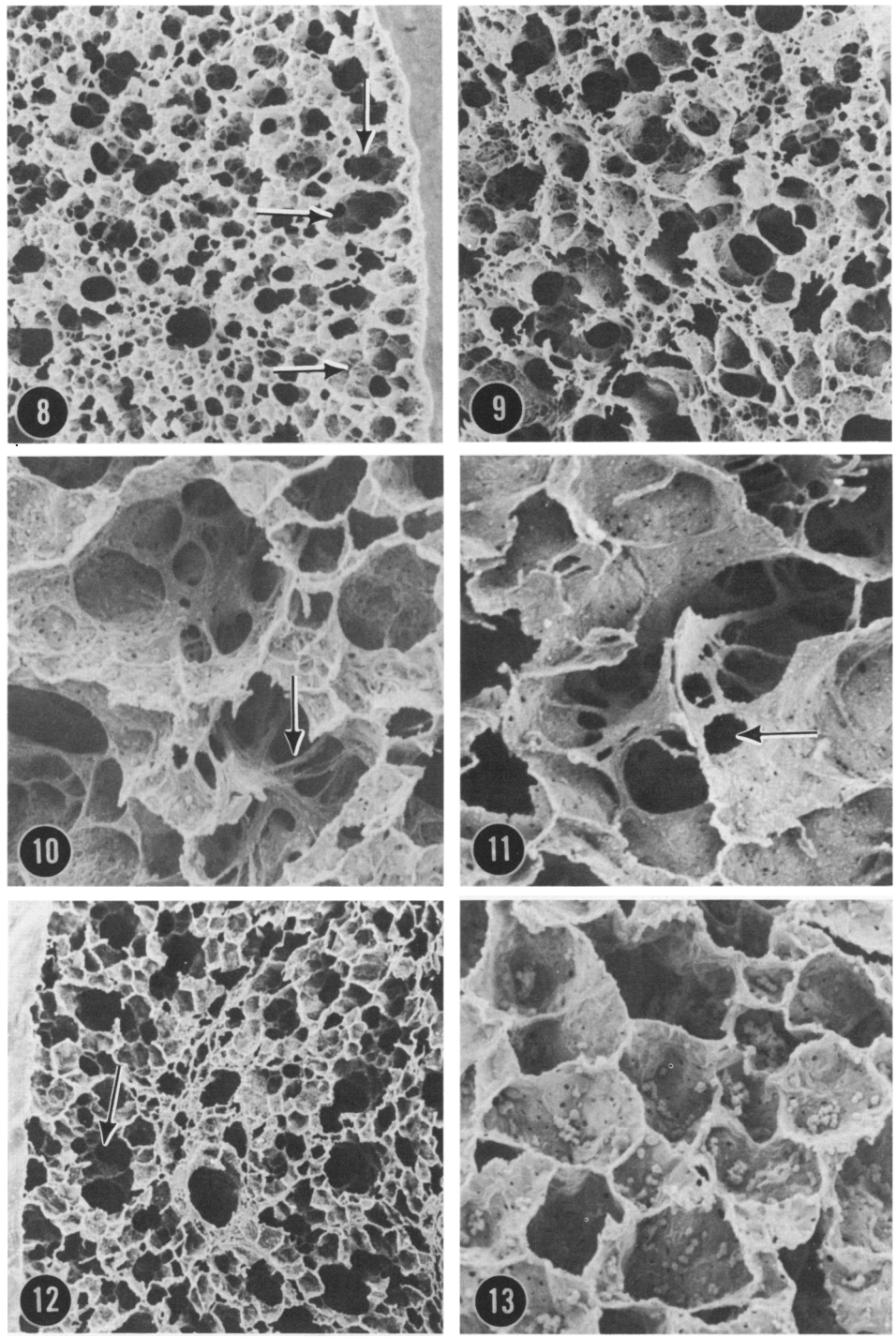

Figure 8 — Lung from a dog that smoked 480 cigarettes. Enlargement of air spaces appears principally in the subpleural areas in this lobe (*arrows*). (×20) **Figure 9** — Lung from the same dog as in Figure 8 but taken from another site of the same lobe shows widespread enlargement of the alveolar ducts. (×20) **Figure 10** — A cluster of enlarged and distorted alveolar ducts from an area adjacent to that in Figure 9. The associated alveoli are shallow and irregular. One alveolar duct (*arrow*) is bridged by a trabecula. (×100) **Figure 11** — Widespread destruction of alveolar walls leaves only trabeculae bridging enlarged alveolar ducts. Alveoli are enlarged and shallow, and show some fenestrations (*arrow*). (×100) **Figure 12** — Lung from a dog that smoked 282 cigarettes. Enlargement of the air spaces is seen predominantly in the subpleural area (*arrow*). (×20) The remaining samples showed essentially the same pattern. **Figure 13** — Lung from a dog that smoked 534 cigarettes. This site is adjacent to an area similar to that seen in Figure 9. The alveoli are slightly enlarged and shallow and contain numerous macrophages. (×200)

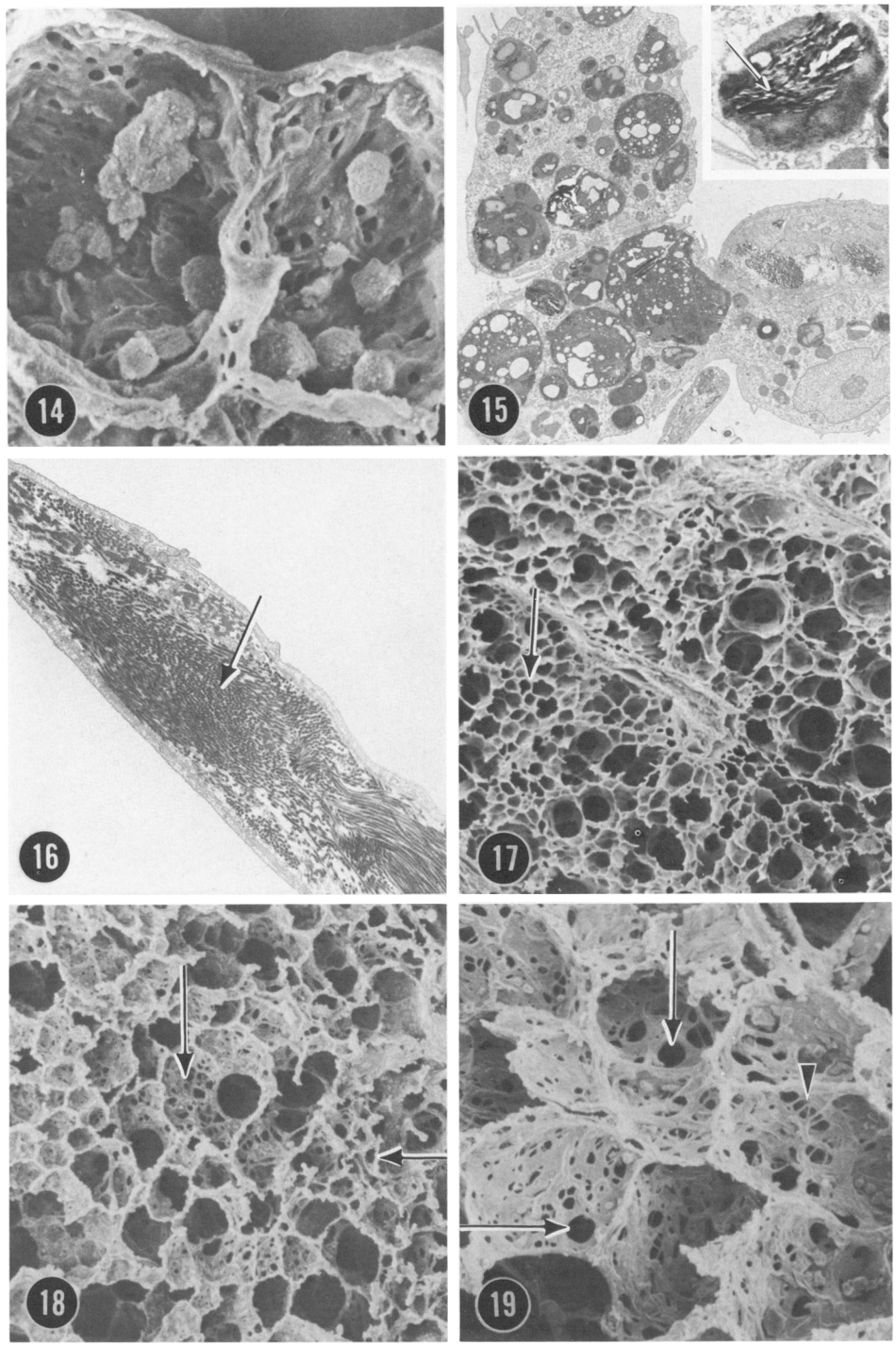

Figure 14 — A higher magnification of macrophages as commonly seen in the smoking dog. (× 500) **Figure 15** — A transmission electron micrograph of typical smoker's macrophages containing large pleomorphic cytoplasmic inclusions. (× 3000) **Inset** — Crystalloid material is present in some of the inclusions (*arrow*). (× 13,000) **Figure 16** — A transmission electron micrograph of an interalveolar septum of a dog that smoked 534 cigarettes. The interstitium is filled by collagen fibers (*arrow*). Note the decreased vascularity. (× 7500) **Figure 17** — Lung from another dog that smoked 534 cigarettes. The major portion of the lung is occupied by greatly enlarged alveolar ducts. Some areas contain normal-appearing alveoli (*arrow*). (× 20) **Figure 18** — Lung from the same dog as in Figure 17 but from a different site of the same lobe. Note "lacy-appearing" areas (*arrows*). (× 50) Figures 18–22 are all from this sample of lung. **Figure 19** — Higher magnification of a lacy-appearing area shows distorted alveoli containing numerous fenestrations (*arrows*). Some of these are bridged by delicate strands of tissue (*arrowhead*). (× 200)

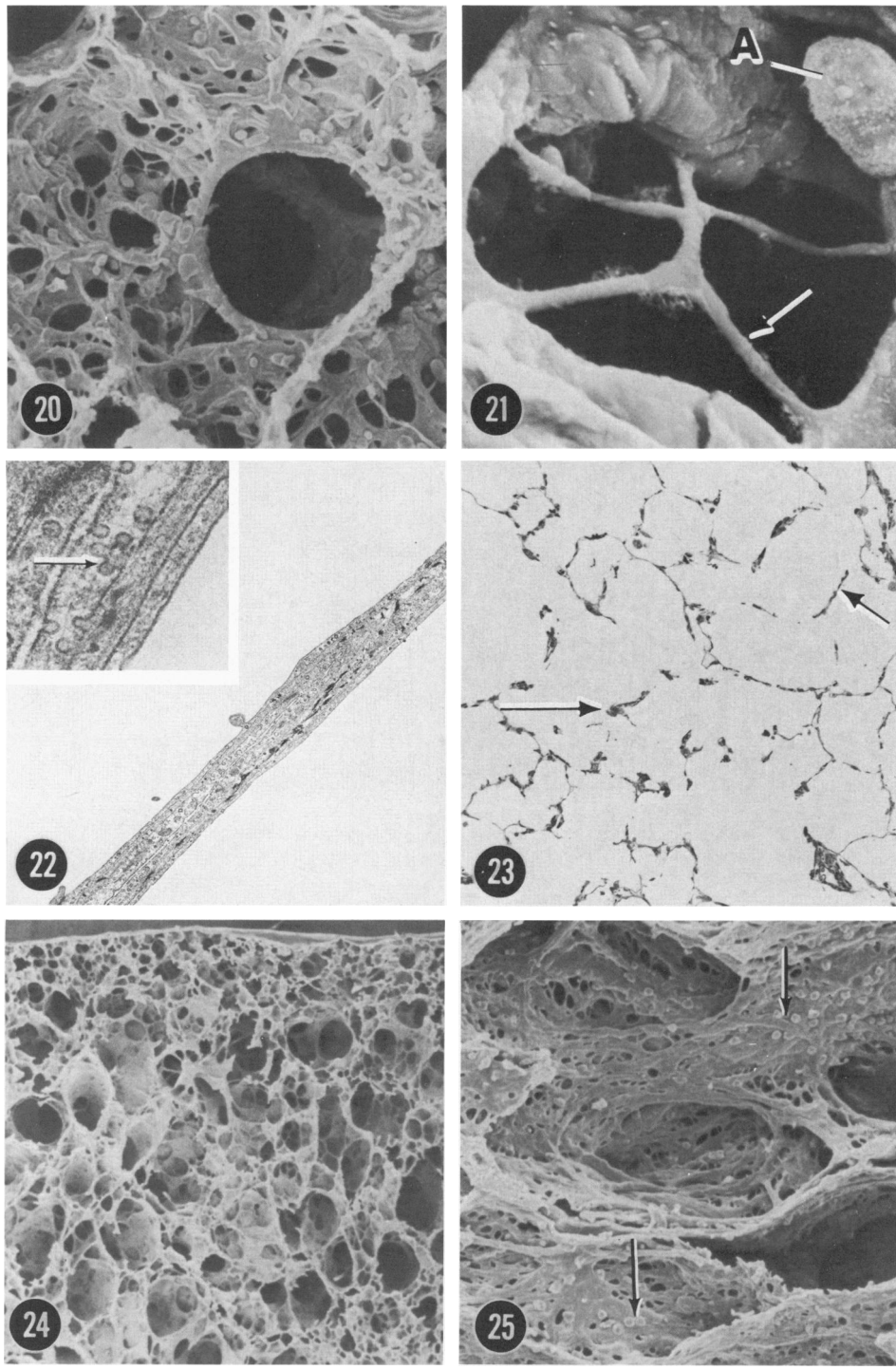

Figure 20 – Individual alveoli that normally surround alveolar ducts (see Figure 5) are not present here because of the enlargement and coalescence of the numerous fenestrations of the alveolar walls. (×200) **Figure 21** – A large fenestration from Figure 20 is subdivided into five segments by delicate strands of tissue (*arrow*). Such images show all that remains following enlargement and coalescence of the fenestrations in the destruction of the alveolar walls. *A*, Type II alveolar cell. (×2000) **Figure 22** – Transmission electron micrograph of a strand of tissue such as that seen in Figure 21 shows it to be a thin interalveolar septum with collapsed capillaries. (×6000) **Inset** – Endothelial cell with micropinocytotic vesicles. (×24,000) **Figure 23** – Light micrograph of a section adjacent to that seen in Figure 18, which shows pieces of tissue (*arrows*) apparently lying free in enlarged air spaces, an accepted hallmark of emphysema. (H&E, ×100) **Figure 24** – Lung of another dog that smoked 534 days. The greatly enlarged air spaces are predominately alveolar ducts. Enlargement of pores or the presence of numerous fenestrations is not a feature in this sample. (×20) **Figure 25** – Lung from the same dog as in Figure 24, but from a different lobe. The alveoli are enlarged and shallow and contain many enlarged pores and some fenestrations. Proliferation of Type II cells is commonly seen in such areas (*arrows*). (×200)

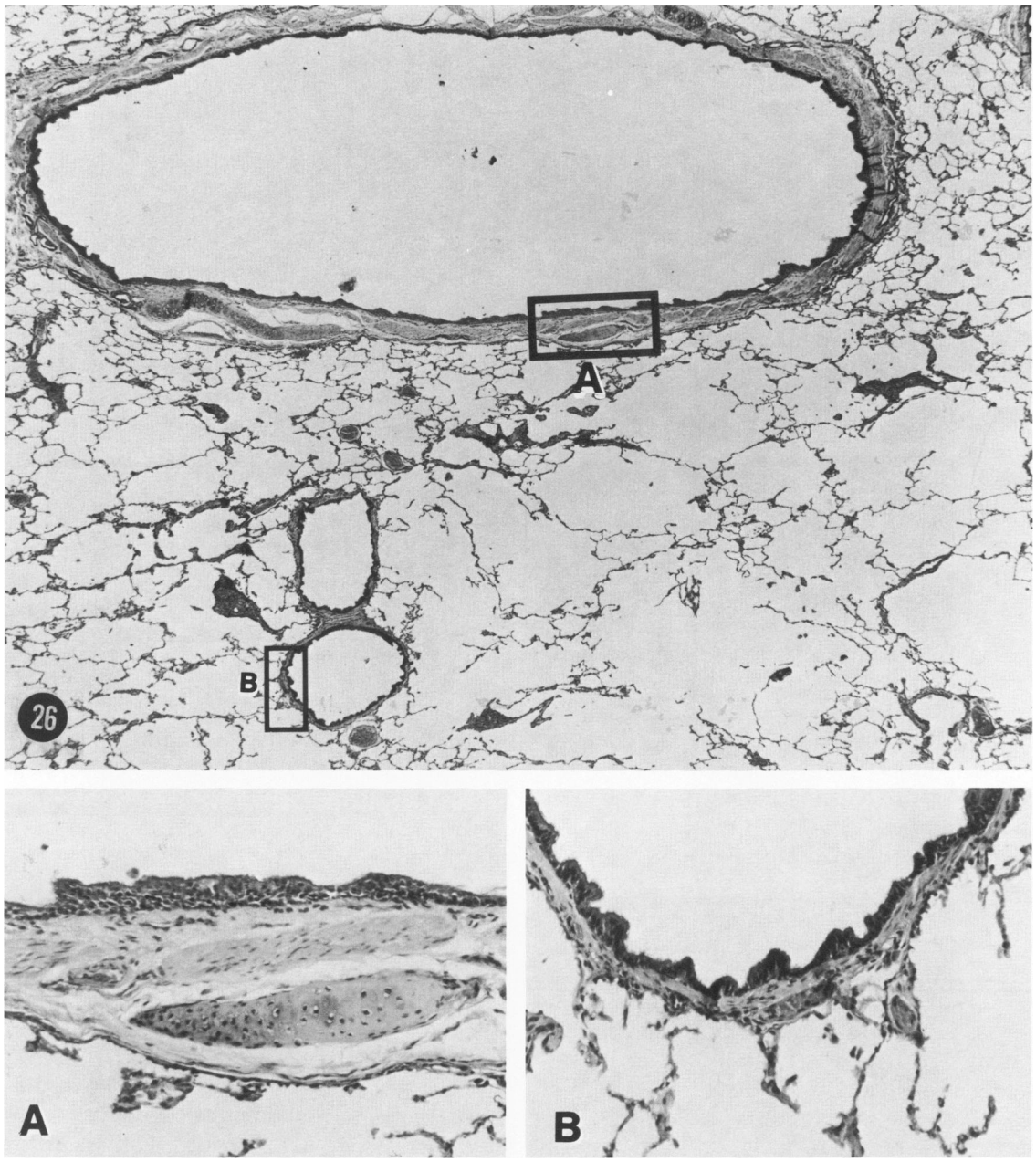

Figure 26 – Light micrograph of the lung of a dog that smoked 534 cigarettes shows a moderate degree of fibrosis and emphysema. (H&E, ×30) The walls of a bronchus (**A**) and bronchiole (**B**) are thin and lack evidence of cellular inflammatory infiltration. (×150)

824–1721 cigarettes over a period of 5 months. TEM demonstrated thickened alveolar septa, reactive hyperplasia of type II cells, lipid containing macrophages, and increased amounts of collagen.

The findings in our study show that after only 172 cigarettes were smoked there are early morphologic alterations similar to those found in the above-mentioned experiments, in which exogenous proteases and air pollutants were studied, and also as described in equine pulmonary emphysema. Macrophages are present in areas of early change and have been described by others.[12,18–20,22] In this experiment macrophages were not present in great numbers in the more advanced lesions. This may be due to sequestration of the damaged lung from cigarette smoke, resulting in a decrease in the stimulation and thus a decreased number.

The presence of macrophages make a complete double-blind approach to this study impossible due to their relative absence in control animals and their

universal abundance and unique contents in the lungs of the smoking dogs. Descriptions of the content of the smoker's macrophages and of the fibrosis of the interalveolar septa are the same as previously reported.[3]

It is now generally accepted that lung injury produces proliferation of Type II alveolar cells, and this has been found in smoking dogs and in dogs exposed to papain.[22,24,25] It is also accepted that Type I alveolar cells are derived from Type II alveolar cells.[26] The abundance of Type II cells in numerous areas of fenestration of the alveolar walls in this experiment suggests that they may play a role in repair by providing a source of Type I alveolar cells.

The extent of alterations found in this study does not appear to be directly related to the extent of cigarette smoke exposure. This could be due to individual vulnerability or possibly to the fact that some dogs can synchronize their breathing with the machine cycle, making the inhalation of smoke more shallow than in nonsynchronized breathing. However, all dogs that smoked did show abnormal morphology. We feel that sampling as a source of error is minimized by the use of SEM. The degree of human pulmonary fibrosis and emphysema also varies from individual to individual and may also vary in anatomic distribution.

Earlier studies have demonstrated the advantages of SEM in the study of lung diseases, particularly emphysema.[14,19] Correlation with light and transmission electron microscopy enabled us to evaluate the internal structure of macrophages and of interalveolar septa, thus showing the surface and internal alterations of emphysema. The scanning electron microscope permitted the examination of alveolar surfaces, interalveolar pores, and fenestrae with such ease that large samples and extensive areas could be documented with confidence. Morphometry has not been applied in evaluating these changes because the small number of animals in this study would negate the value of such an approach. Observation of the types of early abnormal morphologic changes rather than the degree of such changes was the object of this study.

The similarity of morphologic changes in the development of emphysema in the smoking dog to that of other experimental approaches using proteases[12,20,22] and air pollutants[21] suggests that regardless of the etiologic agent, the mechanism may be similar. A comparative study of emphysema in man with elastase-induced emphysema in hamsters by SEM was interpreted by Kuhn and Travassoli[20] as demonstrating two possible morphogenetic processes leading to emphysema. The two alterations are alveolar duct enlargement with retraction of alveolar walls and destruction of alveolar surfaces by enlargement and coalescence of interalveolar pores (fenestrations). Parra et al[22] conducted experiments on papain-induced emphysema in dogs and found both processes present, which led them to believe that the two mechanisms could be different manifestations of a single process. The studies of Hyde et al[21] on the effects of air pollutants on dog lungs showed that enlargement of air spaces with loss of interalveolar septa could develop in the absence of alveolar fenestration. However, they did observe a concurrence between enlarged air spaces and fenestrations in dogs exposed to high nitrogen oxides and sulfur oxides.

The repeated observations in our study of fenestrations in both normal-sized alveolar ducts and in ducts that are enlarged and bridged by strands of tissue support the concept of there being a single process that leads to different morphologic manifestations. The greater number of interalveolar pores in the dog lung makes the fenestration portion of the process easier to interpret. The hamster has relatively few interalveolar pores per alveolus, compared with other animals.[12] It would seem that this would tend to diminish the number of fenestrations produced and make the process more difficult to document.

The removal or damage to the alveolar surface-active material covering the interalveolar pores has been thought to be responsible for their enlargement.[22,27] In the present study the observation of large fenestrations subdivided by attenuated interalveolar septa containing collapsed capillaries suggests that increased surface tension forces in these areas may have interfered with the capillary circulation.[17] Whether the initial lesion in the development of emphysema in cigarette-smoking dogs is the removal of or damage to surface-active material lining the alveolar surface has not been determined. Additional experiments and fixation of the lung by vascular perfusion utilizing surface tension studies are needed to determine the role of the surface-active material in the development of the initial lesion of pulmonary emphysema.

Airflow obstruction and/or chronic bronchitis are not likely to be etiologic factors in the pathogenesis of emphysema.[13] Our failure to find morphologic evidence of bronchitis or bronchiolitis or physical obstruction to the terminal airways, but with emphysematous alterations already present, supports the view that these factors are not involved in the early development of emphysema.

The functional state of the terminal airways was not evaluated in this study. However, if the tissue destruction is extensive in the alveolar structures sup-

porting the terminal airways, it appears likely that luminal narrowing with accompanying airflow obstruction during expiration could occur.[13]

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