Acute Respiratory Bronchiolitis

An Ultrastructural and Autoradiographic Study of Epithelial Cell Injury and Renewal in Rhesus Monkeys Exposed to Ozone

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The pathogenesis of acute respiratory bronchiolitis was examined in rhesus monkeys exposed to 0.8 ppm ozone for 4–50 hours. Epithelial injury and renewal was qualitatively and quantitatively characterized by correlated techniques of scanning and transmission electron microscopy as well as by light-microscopic autoradiography following labeling with tritiated thymidine. Extensive degeneration and necrosis of Type 1 epithelial cells occurred on the respiratory bronchiolar wall during the initial 4–12 hours of exposure. Increased numbers of labeled epithelial cells were present in this region after 18 hours of exposure, and the highest labeling index (18%) was measured after 50 hours of exposure. Most (67–80%) of the labeled cells and all the mitotic epithelial cells (22) observed ultrastructurally were cuboidal bronchiolar epithelial cells. Of the labeled epithelial cells, 20–33% were Type 2 epithelial cells. After 50 hours of exposure the respiratory bronchiolar epithelium was hyperplastic. The predominant inflammatory cell in respiratory bronchiolar exudate was the alveolar macrophage. Monkeys that were exposed for 50 hours and allowed to recover in unozonized air for 7 days had incomplete resolution of respiratory bronchiolar epithelial hyperplasia. The results indicate that Type 1 epithelial cells lining respiratory bronchioles are the cell type most sensitive to injury and that both cuboidal bronchiolar epithelial cells and Type 2 epithelial cells function as stem cells in epithelial renewal. (Am J Pathol 1980, 98:811–840)

RESPIRATORY BRONCHIOLES are partially alveolarized airways interposed between terminal bronchioles and alveolar ducts. They are the first structure in the pulmonary acinus participating in gas exchange. This airway type is extremely sensitive to inhaled irritants such as cigarette smoke,[1] coal dust,[2] and ozone.[3,4,5] Centrilobular emphysema in man is associated with severe alteration of respiratory bronchiolar structure,[6] and respiratory bronchiolitis has been thought to be an important process in the pathogenesis of this disease.[1,6]

In man, respiratory bronchioles are developed to at least two or three generations.[2,7–10] Proximal generations of respiratory bronchioles are lined by nonciliated bronchiolar epithelial cells, ciliated cells, and Type 1 epithelial cells. Distal generations are lined by nonciliated cuboidal

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bronchiolar cells and Type 1 and Type 2 epithelial cells. Only a small number of laboratory animals are suitable models for use in studies on experimental respiratory bronchiolitis. Most commonly used rodents and lagomorphs lack well-developed respiratory bronchioles comparable to those in man.[11-14] Macaques have well-developed respiratory bronchioles that branch to form at least two to three generations.[15] The epithelium lining these airways is composed predominantly of nonciliated cuboidal bronchiolar epithelial cells and Type 1 epithelial cells. Small numbers of Type 2 cells are also present.

Rhesus and bonnet monkeys exposed intermittently for 7 days to concentrations of ozone as low as 0.2 ppm, a concentration frequently encountered by human populations, develop respiratory bronchiolitis characterized by intraluminal aggregations of alveolar macrophages and hyperplasia of cuboidal bronchiolar epithelial cells.[3-5] This bronchiolitis persists during exposures of 0.5 or 0.8 ppm for periods of up to 90 days.[16]

The present study was designed to examine basic processes of injury and repair in the respiratory bronchiole with the use of ozone-induced respiratory bronchiolitis in rhesus monkeys as an experimental model. The specific objectives were to determine 1) which epithelial cell types lining respiratory bronchioles are most susceptible to injury, 2) which cell types serve as stem cells in epithelial renewal, and 3) to what extent acutely induced lesions are reversible after a recovery period of 7 days.

Materials and Methods

Animals, Exposure, and Necropsy

Twenty-two male and female colony-bred rhesus monkeys 24 ± 8.1 months in age, weighing 3.2 ± 0.8 kg, were used in this study. They were identified as being free of disease by clinical examination, chest radiographs, hemograms, and blood chemistry.

Seven pairs of monkeys (14 monkeys total) were exposed to 0.8 ppm ozone continuously for durations of 4, 8, 12, 18, 26, 36, or 50 hours (Table 1). Two additional groups were exposed to ozone for 50 hours; one pair was allowed to recover in unozonized, filtered ambient air for 2 days, and the other pair recovered for 7 days. Four unexposed animals served as controls. Ozone exposures were conducted as previously described.[4,5] Ozone concentration in control and exposure chambers was monitored at least every 8 minutes with an ultraviolet ozone monitor (Model 1003 AH Ozone Monitor, DASIBI Corp., Glendale, Calif).

At the end of the exposure or postexposure period, the monkeys were lightly anesthetized with ketamine (50 mg intramuscularly), given intravenous injections of tritiated thymidine (1 mCi/kg, 6.7 Ci/mmol, New England Nuclear), and placed in metabolism cages for 1 hour. They were then further anesthetized with 100 mg ketamine intramuscularly and 60 mg sodium pentobarbital intravenously and killed by exsanguination. Complete gross and microscopic postmortem examinations were conducted. The lungs were fixed via the trachea at 30 cm pressure with modified Karnovsky's fixative.[17,18] Blocks of lung from cranial and caudal lung lobes were taken for routine paraffin sections.

Table 1—Exposure Data

	Hours exposure and postexposure	Ozone concentration*		Control† monkey
		Monkey 1	Monkey 2	
Group I	4 exposure	0.78 ± 0.046	0.78 ± 0.051	1
Group II	8 exposure	0.80 ± 0.031	0.78 ± 0.038	
Group III	12 exposure	0.81 ± 0.030	0.78 ± 0.032	
Group IV	18 exposure	0.79 ± 0.023	0.81 ± 0.026	
Group V	26 exposure	0.80 ± 0.022	0.81 ± 0.022	1
Group VI	36 exposure	0.79 ± 0.026	0.81 ± 0.021	
Group VII	50 exposure	0.79 ± 0.023	0.81 ± 0.024	1
Group VIII	50 exposure	0.78 ± 0.027	0.81 ± 0.024	
	48 postexposure	0	0	
Group IX	50 exposure	0.80 ± 0.016	0.80 ± 0.016	1
	168 postexposure	0	0	

* Ozone concentration expressed as mean ± SD.

† Control monkeys were housed under identical conditions during the same time period as exposed animals but did not receive ozone.

Transmission and Scanning Electron Microscopy

The cranioventral portion of the left caudal lobe was dissected. In each animal, 5–10 samples of first and second generation respiratory bronchioles were longitudinally bisected under a dissecting microscope. Complementary halves of the airway were prepared for scanning and transmission electron microscopy as previously described.[5,15] Respiratory bronchioles for light and transmission electron-microscopic analysis were embedded in Epon–Araldite as large (6×12×1.5mm) blocks, and 1.5-μ sections were cut with a Sorvall JB-4 microtome. After examination of 1.5-μ sections by light microscopy, 1.0–1.5-mm lengths of primary- and secondary-generation respiratory bronchioles were cut off the large blocks with razor blades and mounted with Epon–Araldite on size 00 epoxy blocks for thin sectioning. Thin sections from each block were supported on 150 mesh copper grids and on carbon-coated Formvar support films mounted on copper grids with a 1-mm hole. Thin sections were stained with uranyl acetate and lead citrate.

Montages of transmission electron micrographs were prepared from 4–5 blocks of respiratory bronchioles for each animal. The micrographs for montage construction were taken at a magnification of ×1556 and photographically enlarged to a final magnification of ×3890.

Quantitative Transmission Electron Microscopy

As a quantitative aid, each epithelial cell bounded by intercellular junctions and lining the bronchiolar wall and outpocketing alveoli was categorized as to cell type in analysis of the electron micrograph montages. Cells with attenuated cytoplasm (less than 1 μ thick) and covering a linear surface greater than 10 μ were classified as Type 1 epithelial cells. Cuboidal cells lining respiratory bronchioles and their alveoli were classified as one of four types according to the following criteria. Type 2 epithelial cells on bronchiolar walls and alveolar septums were identified on the basis of their distinct cytoplasmic osmiophilic lamellar inclusions. Cuboidal cells on bronchiolar walls that lacked osmiophilic lamellar inclusions and were with or without apical, homogeneously electron-dense secretory droplets were categorized as nonciliated cuboidal bronchiolar epithelial cells. They are referred to throughout this report as cuboidal bronchiolar cells. Cuboidal cells lacking lamellar inclusions in alveoli were categorized as cuboidal alveolar cells. Cells with cyto-

plasmic dense-cored vesicles (72–120 nm in diameter) were occasionally observed in bronchiolar walls. This last cell type has been described in macaques [15] and is similar to Kulchitsky cells or cells of neuroepithelial bodies found in man.[19,20] They are referred to throughout this report as Kulchitsky cells or K cells.

In order to determine indices of cell damage and hyperplasia as well as cell differentials, at least 100 respiratory bronchiolar epithelial cells were counted from each animal. We took measurements, beginning immediately distal to the abrupt termination of ciliated epithelium. We measured the length of the bronchiolar wall along the subepithelial basal lamina by superimposing a flexible cord. Similar measurements of basal-lamina perimeter and epithelial cell categories were made on alveolar septums of respiratory bronchioles. Only the alveolar surface facing the bronchiolar lumen was measured. Cell damage in exposed animals was categorized as either degeneration or necrosis by the following criteria. A cell was classified as necrotic if there was nuclear membrane rupture, pyknosis, or a uniform decrease in chromatin electron density. If the nucleus was not present in the plane of section, cytoplasmic changes such as large breaks in the plasma membrane with fragmentation of cytoplasmic elements were also used as criteria for classification as necrosis. A cell was classified as degenerated if there was moderate to severe swelling of mitochondria, endoplasmic reticulum, and nuclear envelopes. In instances where cellular debris was unidentifiable or only bare basal lamina was present, the involved length was recorded as bare basal lamina. From these data cell differentials (number of each cell type present per 100 cells), indices of epithelial cell damage (number of each cell type damaged per 100 cells on respiratory bronchiolar or alveolar walls), and cell density values (number of cells per millimeter length) were calculated.

Light-Microscopic Autoradiography

Epon–Araldite sections, 1.5 μ thick, were mounted on glass slides and coated with a 1:1 solution of Ilford L-4 emulsion and distilled water. They were dried and stored in light-tight boxes at 0–4 C for 4 weeks. Emulsions were then developed in 13.5% Microdol X for 10 minutes, fixed in 15% sodium thiosulfate for 10 minutes, and washed in water for 20 minutes.[21] Sections were stained with toluidine blue, and a coverslip was mounted. Five hundred respiratory bronchiolar nucleated epithelial cells were counted per animal. In addition, cells lining intervening alveoli were also counted. Each cell type was classified as a Type 1 or Type 2 cell, a cuboidal bronchiolar cell, or a cuboidal alveolar cell. Type 2 cells were differentiated from cuboidal cells on the basis of their lamellar inclusions, which appeared as vacuoles in 1.5-μ stained sections. Type 1 cells were identified by light microscopy on the basis of their flattened, fusiform nucleus and thin rim of perinuclear cytoplasm. In order for a cell to be classified as labeled, a minimum of five silver grains superimposed on the nucleus was required.

Results

Control Animals

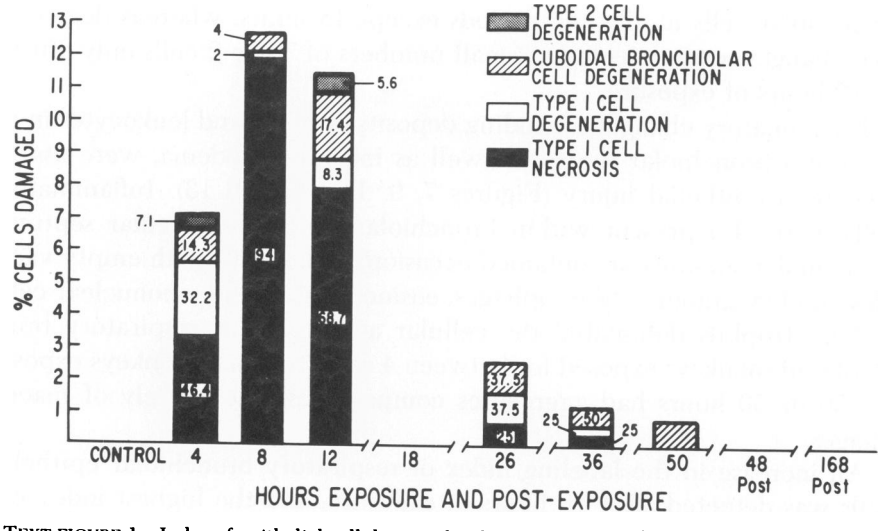
The ultrastructure of respiratory bronchioles in rhesus monkeys has been previously described.[4,15] Ciliated epithelium in control monkeys usually ended abruptly at the end of terminal bronchioles or in the proximal portion of first-generation respiratory bronchioles. Respiratory bronchioles had alveolar outpocketings that became more numerous in successively more distal generations (Figures 1–3). The differential of epithelial cells lining respiratory bronchiolar walls was: cuboidal bronchiolar cells,

54.7 ± 16.6% (mean ± SD); Type 1 cells, 36.8 ± 6.3%; Type 2 cells, 8.0 ± 11.1%; and K cells, 0.5 ± 0.8% (Figures 4–6). The cuboidal bronchiolar cells were closely comparable in ultrastructure to the nonciliated bronchiolar cells that were found in small numbers in distal portions of terminal bronchioles. These cells occasionally contained homogeneously electron-dense secretory droplets near the apical plasma membrane and within microvilli (Figure 4). Respiratory bronchiolar submucosa contained small numbers of mast cells.

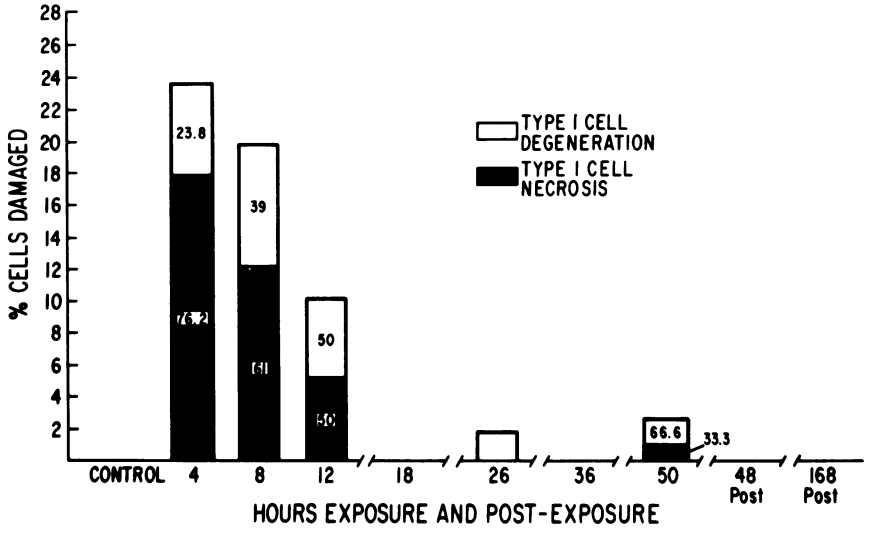
Alveoli opening out of respiratory bronchioles had septums lined by Type 1 epithelial cells (69.7 ± 15.9%) and Type 2 epithelial cells (21.1 ± 9.0%). Small numbers of cuboidal alveolar cells (9.1 ± 7.3%) were present that were identical to cuboidal bronchiolar cells.

Animals Exposed to Ozone

Severe epithelial damage was observed on bronchiolar walls and associated alveolar septums after 4, 8, and 12 hours of exposure (Text-figures 1 and 2). Necrosis and degeneration of Type 1 epithelial cells accounted for the major proportion of cell damage observed between 4 and 36 hours of exposure (Figures 7 and 8). Type 1 cells were the only cells showing changes compatible with necrosis. Bare basal lamina was present between 4 and 36 hours of exposure (Figure 9). The maximum amount of bare basal



TEXT-FIGURE 1—Index of epithelial cell damage for the respiratory bronchiolar wall. Mean values are expressed by the height of the bar for each group in all of the following figures. The number in or near the bar in each figure indicates the percentage that each cell type contributes to the total bar height.



TEXT-FIGURE 2—Index of epithelial cell damage for alveolar septums of respiratory bronchioles.

lamina was observed on bronchiolar walls and associated alveolar septums after 12 hours of exposure (17% and 14% of the linear distances measured, respectively). Mild degenerative changes were observed in cuboidal bronchiolar cells and Type 2 cells (Text-figure 1; Figure 10). Swelling of mitochondria and endoplasmic reticulum was observed in cuboidal bronchiolar cells in all time periods except 18 hours, whereas degenerative changes were detected in small numbers of Type 2 cells only after 4 or 12 hours of exposure.

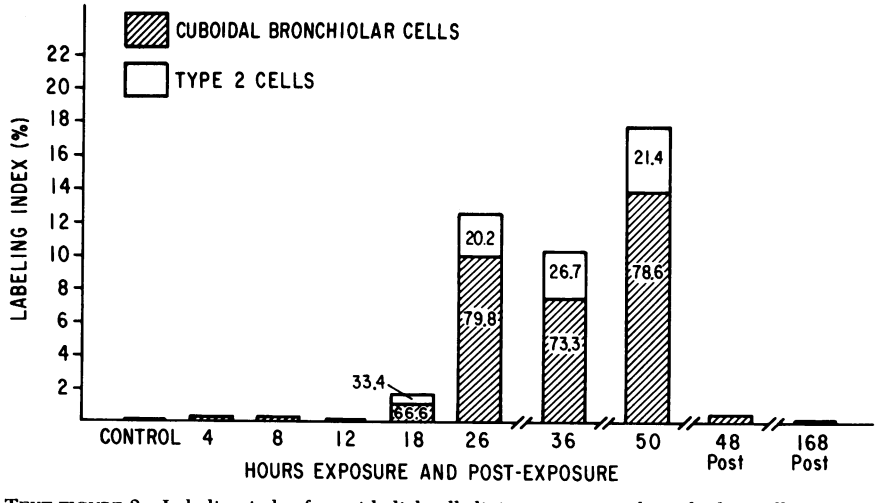
Inflammatory changes, including deposits of fibrin and leukocytes in alveoli and bronchiolar lumens as well as intramural edema, were associated with epithelial injury (Figures 7, 9, 11, 12, and 13). Inflammatory cells were also present within bronchiolar walls and alveolar septums. Bronchiolar submucosa contained occasional mast cells with empty vacuoles and few granules. Macrophages, eosinophils, small mononuclear cells, and neutrophils dominated the cellular aggregates in respiratory bronchioles of monkeys exposed for between 4 and 26 hours. Monkeys exposed for 36 or 50 hours had aggregates composed almost entirely of macrophages.

An increase in the labeling index of respiratory bronchiolar epithelial cells was detected after 18 hours of exposure, and the highest index was measured in the group exposed for 50 hours (Text-figure 3). Of the labeled cells, 67–80%, by light-microscopic criteria, were cuboidal bronchiolar cells (Figure 14). The remainder of labeled cells (20–33%) were Type 2

epithelial cells (Figure 15). No labeled Type 1 cells were observed. Twenty-two respiratory bronchiolar epithelial cells in mitosis were observed ultrastructurally. All were cells with ultrastructural features of cuboidal bronchiolar cells (Figure 16). Occasional mitotic figures and labeled nuclei were observed in mural and luminal small mononuclear cells and macrophages (Figure 17).

During ozone exposure there was an alteration in the epithelium lining respiratory bronchiolar walls (Table 2; Text-figures 4 and 5). There was a marked decrease in the number of Type 1 epithelial cells, as compared with control animals, and an increase in cuboidal bronchiolar cells and Type 2 epithelial cells. This increased number of cuboidal bronchiolar cells was easily recognized in animals exposed for 36 and 50 hours because of the continuous lining and occasional stratification of these cells on the bronchiolar wall (Figures 16, 18, and 19). Included occasionally in these hyperplastic cell clusters were K cells containing dense-cored vesicles (Figures 21 and 22). These cells constituted almost 9% of the epithelial lining in monkeys exposed for 36 hours but were not observed in monkeys exposed for 50 hours.

In alveoli of respiratory bronchioles, there was also a rise in the labeling index of epithelial cells, which reached the highest measured level in animals exposed for 50 hours (Text-figure 6). Of the labeled cells, 70–100% were Type 2 cells. A smaller percentage of cuboidal alveolar cells were labeled in monkeys exposed for 26, 36, and 50 hours. In animals exposed for

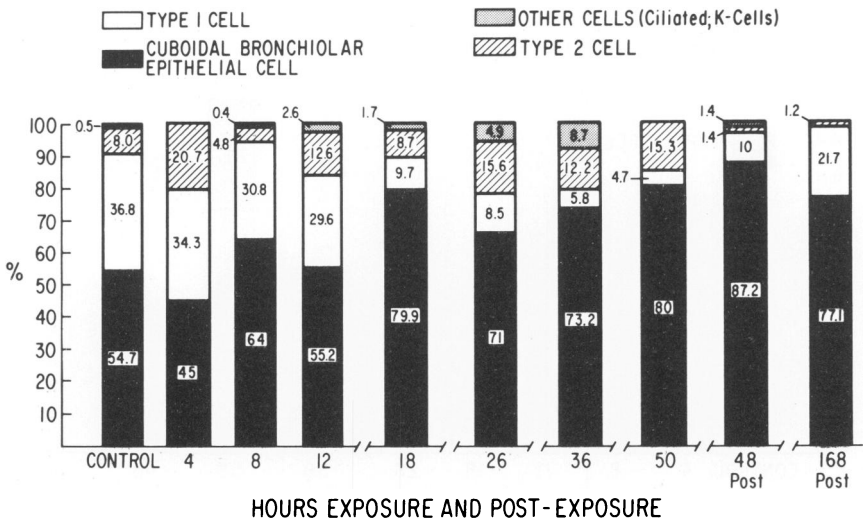


TEXT-FIGURE 3—Labeling index for epithelial cells lining respiratory bronchiolar walls. Percentages of the total for the two cell types are indicated in the bars. Cuboidal bronchiolar cells were the only cells observed labeled except in monkeys exposed between 18 and 50 hours.

Table 2—Epithelial Cells Lining Respiratory Bronchiolar Walls

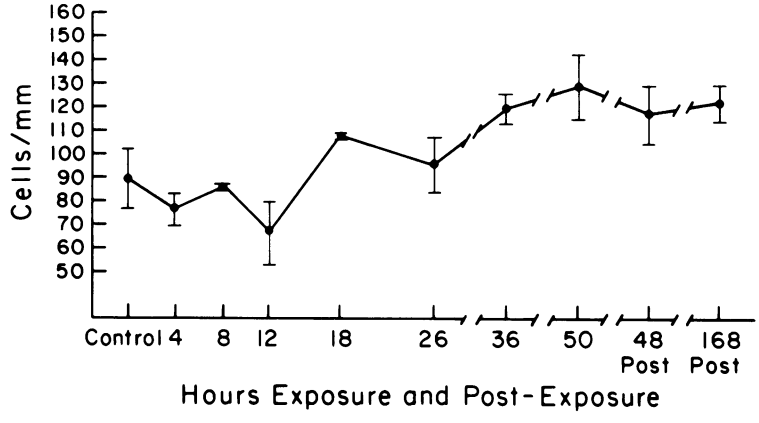
Hours exposure and postexposure	Number of cells per mm length of respiratory bronchiole						
	Cuboidal bronchiolar cells		Type 1 cells	Type 2 cells	K cells	Ciliated cells	Total cells per mm
	Total	Secretory*					
Control	49.2 ± 19.1† (54.7)‡	2.2 ± 1.8	32.3 ± 4.8† (36.8)‡	6.8 ± 9.2 (8.0)	0.4 ± 0.7 (0.5)	0	88.6 ± 13.1
4 exposure	34.3 ± 1.8 (45.0)	0.2 ± 0.4	26.6 ± 11.6 (34.3)	15.5 ± 6.5 (20.7)	0	0	76.4 ± 6.9
8 exposure	54.2 ± 14.4 (64.0)	0.7 ± 1.0	26.0 ± 11.3 (30.8)	4.1 ± 2.4 (4.8)	0	0.4 ± 0.5 (0.4)	84.6 ± 0.1
12 exposure	37.1 ± 12.3 (55.2)	0	19.9 ± 6.3 (29.6)	7.9 ± 3.1 (12.6)	1.4 ± 2.0 (2.6)	0	66.3 ± 13.4
18 exposure	85.8 ± 9.8 (79.9)	5.7 ± 3.5	10.4 ± 4.2§ (9.7)	9.4 ± 5.7 (8.7)	1.0 ± 1.5 (0.9)	0.8 ± 0.4 (0.8)	107.4 ± 1.3
26 exposure	69.5 ± 33.7 (71.0)	0	7.9 ± 3.1§ (8.5)	14.0 ± 12.0 (15.6)	4.3 ± 6.0 (4.9)	0	95.6 ± 12.5
36 exposure	86.8 ± 4.0 (73.2)	9.3 ± 4.4	7.0 ± 4.2§ (5.8)	14.4 ± 4.5 (12.2)	8.8 ± 5.2§ (7.3)	1.5 ± 2.2 (1.4)	118.6 ± 6.6§
50 exposure	101.4 ± 4.3§ (80.0)	4.6 ± 3.5	5.8 ± 2.6§ (4.7)	20.6 ± 20.7 (15.3)	0	0	127.8 ± 13.8§
48 postexposure	101.4 ± 14.2§ (87.2)	3.4 ± 0.2	11.5 ± 2.2§ (10.0)	1.6 ± 0.7 (1.4)	1.8 ± 2.6 (1.4)	0	116.2 ± 13.6
168 postexposure	93.0 ± 3.4§ (77.1)	0.3 ± 0.4	26.3 ± 5.6 (21.7)	1.4 ± 0.9 (1.2)	0	0	120.8 ± 8.1§

* Number of cuboidal bronchiolar cells containing apical secretory droplets.
† Mean value ± SD for control or exposure group.
‡ Mean percentage value in parentheses.
§ Indicates a significant difference between the exposure and control groups by the Student *t* test ($P < 0.05$).

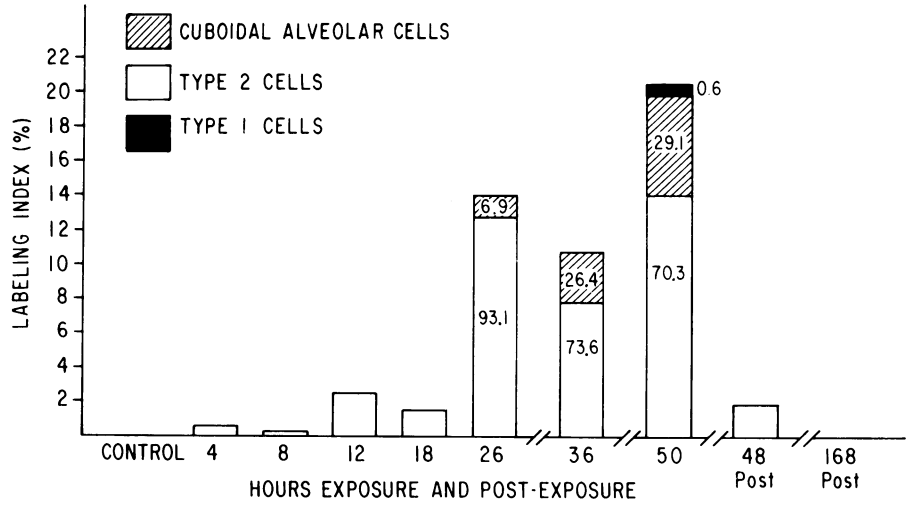


TEXT-FIGURE 4—Changes in percentages of epithelial cell types lining respiratory bronchiolar walls during ozone exposure and recovery.

50 hours, a small number of nuclei from cells with light-microscopic features of Type 1 cells were also labeled. Three epithelial cells in mitosis were observed ultrastructurally at 36 and 50 hours. All were Type 2 cells with osmiophilic lamellar inclusions. Monkeys exposed to ozone for 4 to 36 hours had smaller percentages of Type 1 cells lining respiratory bronchiolar alveolar septums and higher percentages of Type 2 and cuboidal alveolar cells (Text-figure 7; Figure 20) when compared with controls.



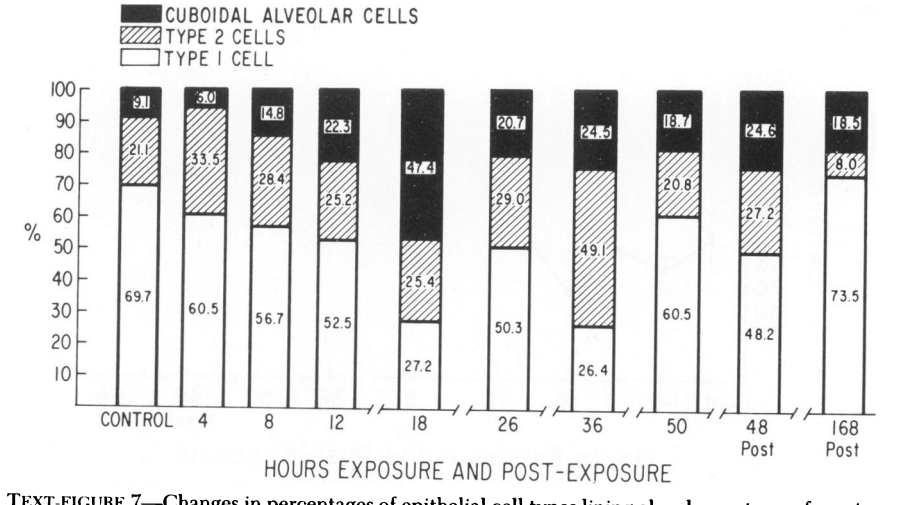
TEXT-FIGURE 5 —Changes in epithelial cell density on respiratory bronchiolar walls during ozone exposure and recovery. The line connects values, and bars indicate standard deviations.



TEXT-FIGURE 6—Labeling index for epithelial cells lining alveolar septums of respiratory bronchioles during ozone exposure and recovery.

Recovery Monkeys

In the monkeys allowed to recover in filtered room air for 2 days after 50 hours of ozone exposure, there was a decrease in the density of alveolar macrophages present in respiratory bronchioles, compared with that found in animals killed after exposure for 50 hours (Figure 23). Ultrastructural features of cell injury were not seen in recovery animals (Text-fig-



TEXT-FIGURE 7—Changes in percentages of epithelial cell types lining alveolar septums of respiratory bronchioles.

ures 1 and 2). Labeling indices were similar to or minimally higher than those of control monkeys (Text-figures 3 and 6). Flattening of cuboidal bronchiolar cells and Type 2 cells was observed in recovery monkeys, and there was often cytoplasmic overlapping in these more flattened cells (Figure 24). By 7 days after exposure, there was a marked reduction of alveolar macrophages in respiratory bronchioles (Figure 25). Small clusters of macrophages, however, were still present. Labeling indices returned to control values. On the bronchiolar wall, epithelial cell density was higher than that in control animals, although a return to a mixture of Type 1 cells and cuboidal cells was observed in areas (Table 2 and Figures 26 and 27). The number of Type 1 cells did not completely return to that found in control animals, and the number of cuboidal bronchiolar cells remained higher. In alveoli of respiratory bronchioles, there was a return of the epithelium to a mixture of Type 1 cells and cuboidal cells similar to that found in control animals. A greater percentage of cuboidal alveolar cells relative to Type 2 cells was observed in recovery monkeys than in control animals (Text-figure 7).

Discussion

Previous studies from this laboratory have characterized the inflammatory lesions that are present in respiratory bronchioles of macaques after exposure to ozone for 7 days, 8 hours/day, at concentrations from 0.2 to 0.8 ppm.[3–5] The present study examined the pathogenesis of respiratory bronchiolitis induced by ozone and especially focused on events of epithelial cells in order to provide basic information on the patterns of epithelial injury and renewal. The results demonstrate that severe epithelial injury occurs between 4 and 12 hours of continuous exposure to 0.8 ppm ozone and that reparative division by epithelial stem cells increases at 18 hours and reaches a high level after 50 hours of exposure. Type 1 epithelial cells lining walls and alveolar septums of respiratory bronchioles are the cell type most susceptible to injury. This finding is consistent with the general concept that Type 1 cells are extremely sensitive to the direct or indirect effects of inhaled or ingested pulmonary toxins [22–28] and possibly respiratory viruses.[29] The basis of this cellular sensitivity to ozone has not been precisely determined. Since ozone produces many of its toxic effects on cells through oxidation and peroxidation of membrane lipids,[30] it could be that the large membrane surface that Type 1 cells present to ozone enhances the susceptibility of these cells to injury. The generally low enzymatic activity of these cells [31,32] and possibly low activity of protective antioxidant enzyme systems [33,34] could also heighten their sensitivity.

Mild damage to cuboidal bronchiolar cells as indicated by swelling of

mitochondria and endoplasmic reticulum occurred in monkeys at most exposure periods. A small percentage of Type 2 cells showed similar degenerative changes in monkeys exposed for 4 or 12 hours. Only 2 monkeys were examined at each time period, but these results suggest that Type 2 cells lining respiratory bronchioles could be more resistant to ozone-induced damage than are cuboidal bronchiolar cells. It is also possible, however, that the small number of Type 2 cells observed with degenerative changes reflects a sampling error due to their proportionately lower percentage on the respiratory bronchiolar wall as compared with cuboidal bronchiolar cells. No cell degeneration or necrosis was detected in monkeys exposed to ozone for 18 hours. Since a small percentage of damaged cells were observed at later time periods, the absence of detectable damage at 18 hours could be related to a sampling error in addition to a marked decrease in cell injury after 12 hours.

Cuboidal bronchiolar cells appeared to function as principal stem cells for epithelial renewal. Between 67% and 80% of the labeled epithelial cells on the bronchiolar wall in monkeys exposed for 18–50 hours were classified as cuboidal bronchiolar cells by light-microscopic criteria. In addition, all epithelial cells in mitosis observed ultrastructurally on respiratory bronchiolar walls were cuboidal bronchiolar cells. It is possible that the nonciliated cells observed in mitosis or labeled with tritiated thymidine were Type 2 cells that had extruded their osmiophilic lamellar inclusions. We think this is unlikely, since Type 2 cells dividing on alveolar septums of respiratory bronchioles did not extrude their inclusions, which were easily recognized within labeled cells in light-microscopic autoradiographs and within dividing cells by transmission electron microscopy. In addition, Evans et al [21] found that following exposure of rats to nitrogen dioxide, alveolar Type 2 cells observed ultrastructurally in mitosis or labeled with tritiated thymidine in electron-microscopic autoradiographs retained their lamellar inclusions. In terminal bronchioles of rats, nonciliated bronchiolar cells are the primary stem cell for renewal of epithelium following injury by inhaled oxidants.[35,36] The cuboidal bronchiolar cells in respiratory bronchioles of primates probably function similarly following various types of injury. Type 2 epithelial cells also functioned as stem cells on the bronchiolar wall in the present study. Their role in epithelial repair, however, appeared to be less than that of cuboidal bronchiolar cells, since fewer Type 2 cells were labeled.

Studies with rodents indicate that alveolar Type 2 epithelial cells can differentiate into Type 1 cells in as little time as 2 days [21,26,37] and that nonciliated bronchiolar cells in terminal bronchioles are capable of differentiating into ciliated cells.[35] There are several interrelationships that

could exist between cells lining respiratory bronchioles of primates. In a previous study [15] we reported that cuboidal bronchiolar cells in rhesus monkeys did not have secretory droplets. Small numbers of cuboidal cells in the present study contained apical secretory droplets, suggesting that they can assume at least a minor secretory function similar to that of nonciliated bronchiolar (Clara) cells of man and other species. Cuboidal bronchiolar cells were indistinguishable from the cuboidal alveolar cells, except for their location, and probably represent the same cell type. Since the only reliable criterion for separating cuboidal bronchiolar cells without apical secretory droplets from Type 2 cells was the presence or absence of osmiophilic lamellar inclusions, the possibility that these cell types are closely related and might be capable of differentiating into one another should be considered. In monkeys exposed to ozone and allowed to recover for 2 or 7 days, there was an increase in the number of Type 1 epithelial cells toward control values. Flattened cells with cytoplasmic characteristics between those of cuboidal bronchiolar cells and those of Type 1 cells were observed. It is possible that the increased number of Type 1 cells that were present in recovery animals were derived from both cuboidal bronchiolar cells and Type 2 cells. Although pulse-labeling studies would be required to examine critically these potential interrelationships of cell types, it appears that the spectrum of differentiation of stem cells within the pulmonary acinus is much broader than was previously recognized.[21,26,37,38]

Epithelial cell density in monkeys allowed to recover for 7 days did not return to that found in control animals. This hypercellularity was primarily due to the large numbers of cuboidal bronchiolar cells that were present in recovery monkeys. It is likely that epithelial density would have returned to control levels if the monkeys had been allowed to recover for longer periods; however, studies with mice and dogs have demonstrated the phenomenon of hyperplastic bronchiolar nonciliated cells persisting for long periods, even after there has been resolution of hyperplasia of alveolar Type 2 epithelial cells.[39,40] This slower or incomplete resolution of epithelial hyperplasia in bronchioles, as compared with that in alveoli, could be related to inherently slower cell turnover times or microenvironmental factors influencing rates of cell senescence and necrobiosis.

Another interesting observation was that of large clusters of K cells present in respiratory bronchioles of monkeys exposed to ozone for 26 or 36 hours. We have observed occasional small clusters of these cells in control animals from this and other studies.[15] The K cell clusters in control monkeys were never as densely cellular or as heavily granulated as those in monkeys exposed to ozone. Kulchitsky cells have been described in

many mammalian species [19,41–43] and are thought to be the cell of origin for bronchial and peripheral carcinoid tumors [19,44] as well as for oat cell carcinomas in man.[45,46] It is unclear whether the increased numbers of K cells we have seen in the present study are the result of increased granulation of preexisting cells or whether these cells are dividing in response to ozone exposure. The presence of K cells within stratified clusters of hyperplastic nonciliated bronchiolar cells suggests the latter mechanism. We did not, however, identify K cells ultrastructurally in mitosis, and they could not be differentiated reliably from cuboidal bronchiolar cells in light-microscopic autoradiographs.

Most of the inflammatory cells in respiratory bronchioles at all exposure periods were macrophages and small mononuclear cells, although small to moderate numbers of eosinophils and fewer neutrophils were present in inflammatory exudates between 4 and 36 hours. The aggregates of macrophages that were present in respiratory bronchioles were at least partially derived from locally dividing macrophages, since mitotic figures and labeled nuclei were observed in bronchiolar macrophages of exposed animals. Other factors important in the accumulation of these cells could be local generation of chemotactic factors and ozone-induced inhibition of motility in macrophages normally leaving the lung via bronchioles. In another study,[47] alveolar macrophages that had been lavaged from rhesus monkeys after exposure to 0.8 ppm ozone for 7 days had decreased random migration when tested in an underagarose migration system.

This study has examined the pathogenesis of acute, ozone-induced respiratory bronchiolitis in rhesus monkeys and has focused particularly on processes of epithelial injury and repair. The results indicate that Type 1 cells lining respiratory bronchioles are most susceptible to injury and that cuboidal bronchiolar cells and Type 2 cells are responsible for epithelial renewal. Important objectives for future work in respiratory bronchiolar pathobiology will be: 1) to determine whether mild chronic respiratory bronchiolitis can lead to airway distortion (narrowing or ectasia) and physiologic obstruction; 2) to evaluate the duration of recovery required for resolution of epithelial hyperplasia; and 3) to define the differentiating capacity of dividing cuboidal bronchiolar cells and Type 2 cells on the bronchiolar wall.

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[*Illustrations follow*]

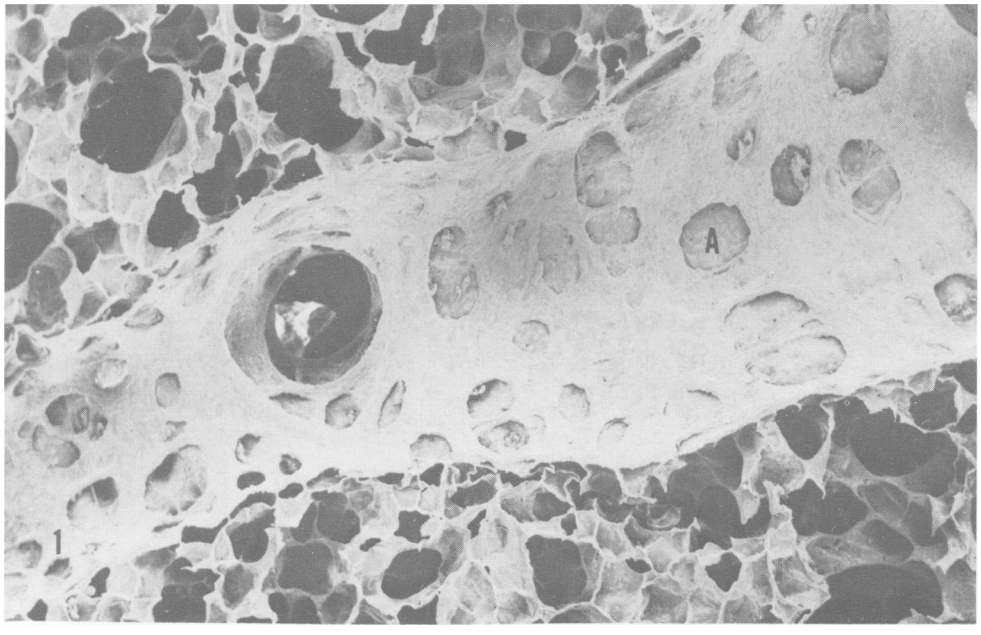
Figure 1—Scanning electron micrograph (SEM) of a respiratory bronchiole from a control monkey. There are numerous alveolar outpocketings (*A*) in the wall (×100)

Figure 2—Paraffin section of control respiratory bronchiole. Alveoli in the wall contain an occasional macrophage. (H&E, ×84)

Figure 3—Section of control respiratory bronchiolar wall and an outpocketing alveolus (*A*). The wall is lined by cuboidal nonciliated bronchiolar epithelial cells (*N*) and a Type 1 epithelial cell that has an attenuated nucleus (*arrow*). Epon–Araldite section. (Toluidine blue, ×800)

Figure 4—Cuboidal bronchiolar cell lining a respiratory bronchiole of a control monkey. Homogenously electron-dense secretory droplets (*arrow*) are present in apical microvilli. (Transmission electron micrograph (TEM, ×6885)

Figure 5—Type 2 epithelial cell lining a respiratory bronchiolar wall in a control monkey. Osmiophilic lamellar inclusions are present in the basilar cytoplasm. (TEM, ×6502) (Figures 1–5 with a photographic reduction of 3%)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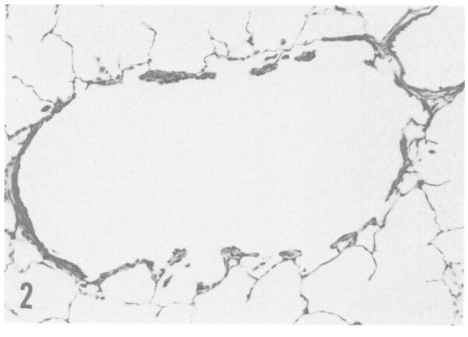
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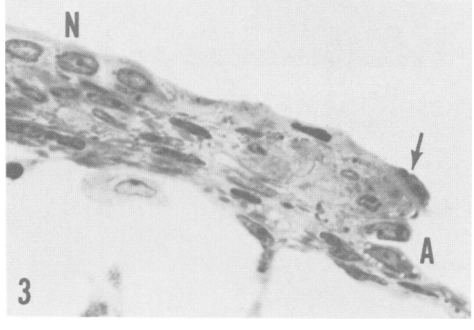
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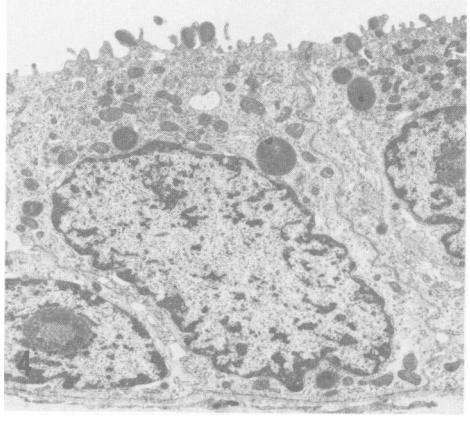

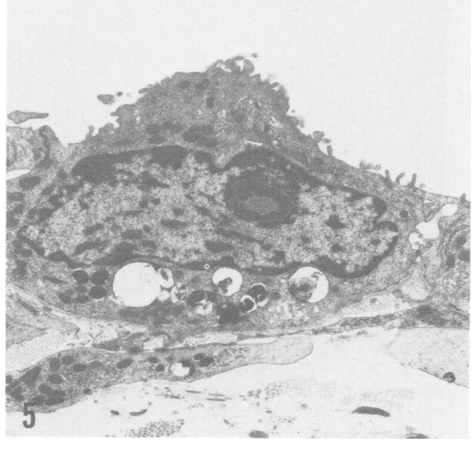
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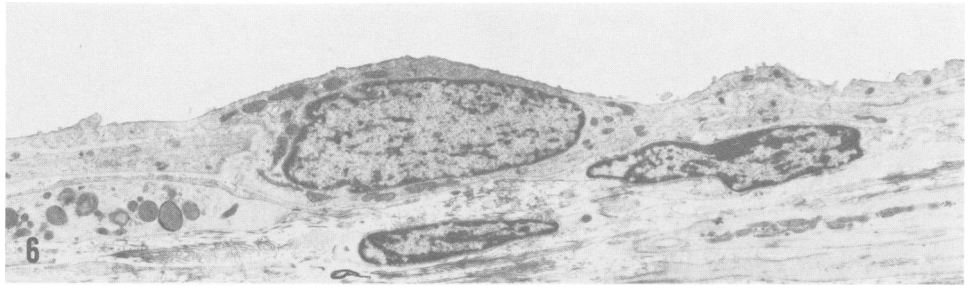
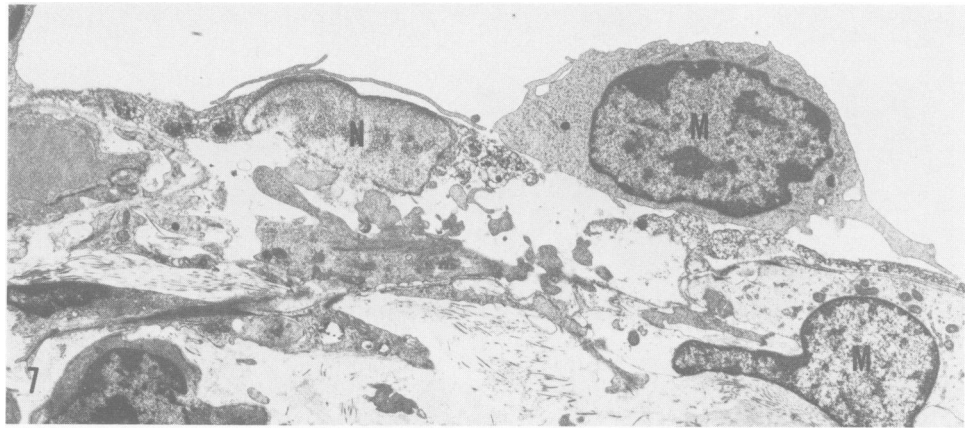




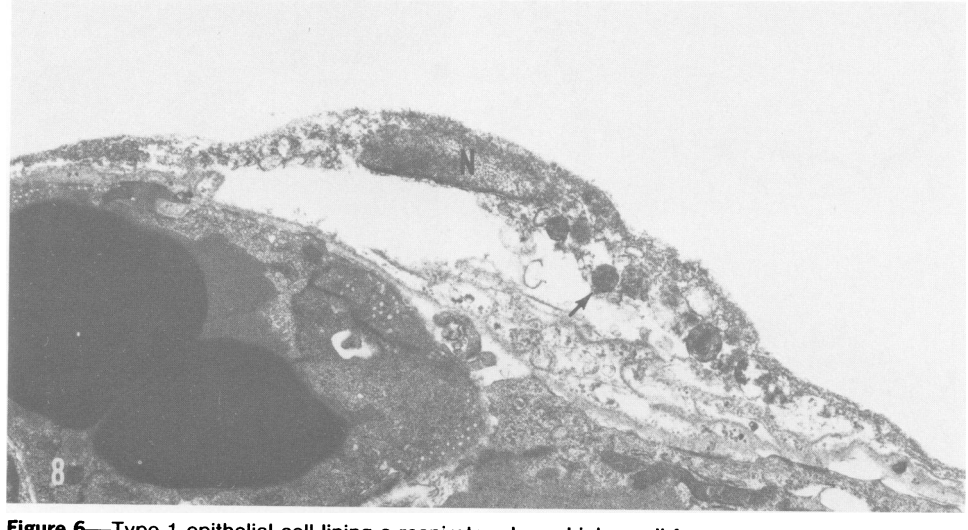
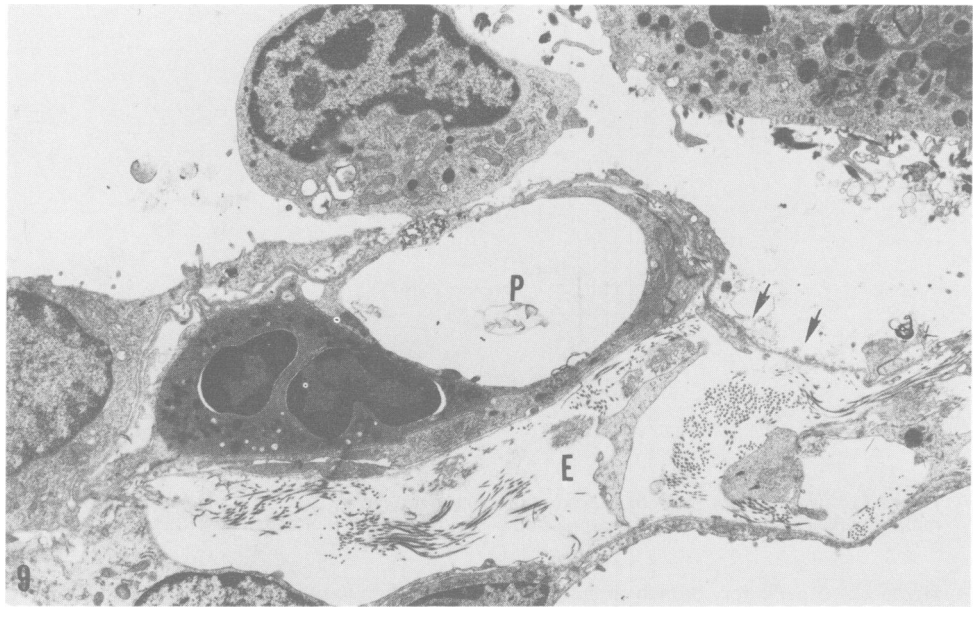


Figure 6—Type 1 epithelial cell lining a respiratory bronchiolar wall from a control monkey. (TEM, ×5122) **Figure 7**—Necrosis of a Type 1 epithelial cell lining a respiratory bronchiole from a monkey exposed to 0.8 ppm ozone for 8 hours. The cytoplasm is fragmented, and there is a break in the nuclear membrane (*N*). Chromatin is of low electron density. Small mononuclear inflammatory cells (*M*) are on the airway surface and in the wall immediately subjacent to the basal lamina. (TEM, ×5270) **Figure 8**—Type 1 epithelial cell lining a respiratory bronchiolar wall from a monkey exposed to 0.8 ppm ozone for 8 hours. The cell has separated from the subjacent basal lamina and has breaks in the plasma membrane and markedly condensed mitochondria (*arrow*). Part of the nucleus (*N*) is in the section. (TEM, ×9900) (Figures 6–8 with a photographic reduction of 3%)



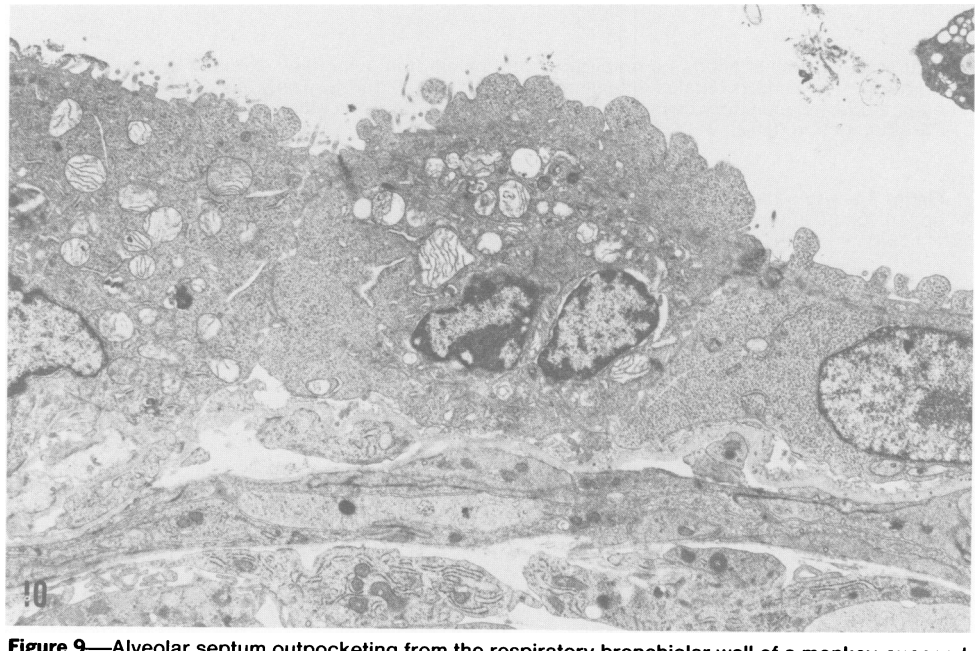


Figure 9—Alveolar septum outpocketing from the respiratory bronchiolar wall of a monkey exposed to ozone for 4 hours. There is edematous dispersion of septal collagen fibers (*E*). Also present is an edematous perivascular pocket (*P*). A neutrophil is present in intravascular location. Bare basal lamina is present on the septal surface that faces the bronchiolar lumen (*arrows*). Macrophages are present in the lumen with electron-dense strands of fibrin. (TEM, ×6820) **Figure 10**—Cuboidal bronchiolar cells lining a respiratory bronchiole of a monkey exposed to ozone for 50 hours. There is swelling of mitochondria with increased electron-lucency of inner compartments. The nucleus has two lobes in the section. (TEM, ×7440) (Figures 9 and 10 with a photographic reduction of 2%)

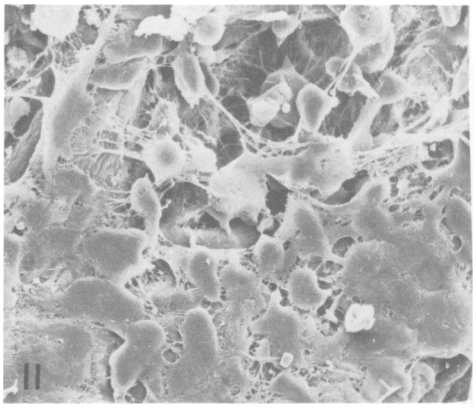
Figure 11—Respiratory bronchiole from a monkey exposed to ozone for 12 hours. Inflammatory cells with fine, interlacing pseudopodia cover the bronchiolar surface. (SEM, ×980)

Figure 12—Respiratory bronchiole from a monkey exposed to ozone for 50 hours. Aggregates of inflammatory cells are on the wall and in alveoli. The epithelial surface is visible between the clusters of inflammatory cells. (SEM, ×280)

Figure 13—Cross-section of a respiratory bronchiole from a monkey exposed to ozone for 26 hours. The lumen contains inflammatory cells and cell debris. The wall is thickened and edematous. Inflammatory cells are present in the wall and in the adjacent perivascular area. Paraffin section. (H&E, ×168)

Figure 14—Two cuboidal bronchiolar cells lining a respiratory bronchiole of a monkey exposed for 50 hours are labeled with tritiated thymidine. (Autoradiograph, toluidine blue, ×1764)

Figure 15—A Type 2 epithelial cell lining a respiratory bronchiolar wall of a monkey exposed to ozone for 50 hours is labeled with tritiated thymidine. Cytoplasmic vacuoles corresponding to lamellar inclusions are visible. (Autoradiograph, toluidine blue, ×1764) (Figures 11–15 with a photographic reduction of 3%)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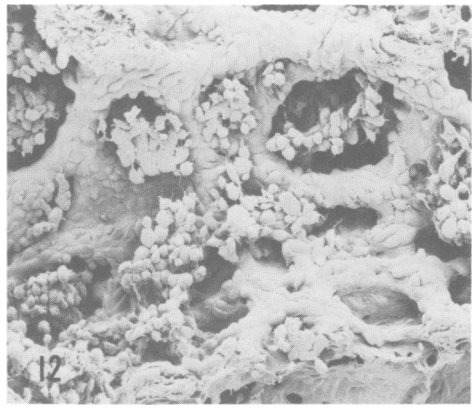
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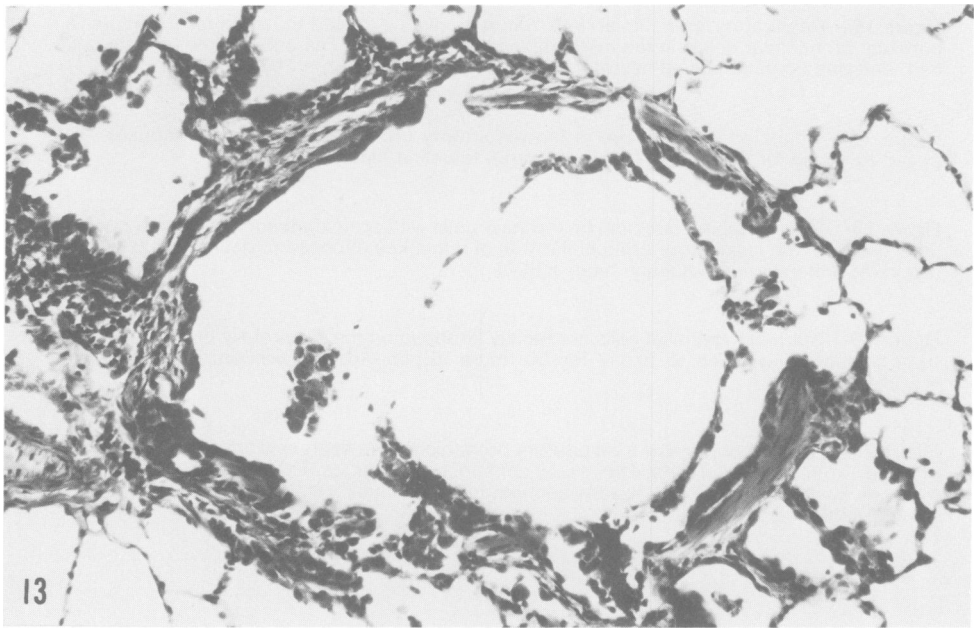
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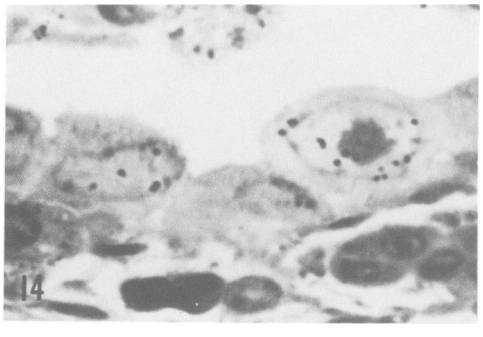
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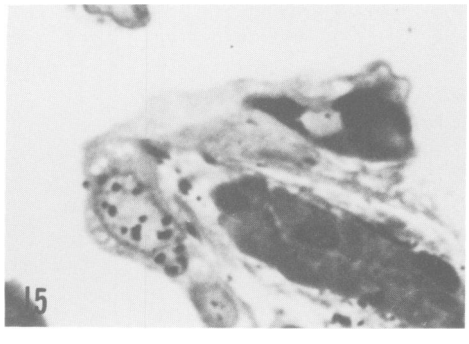
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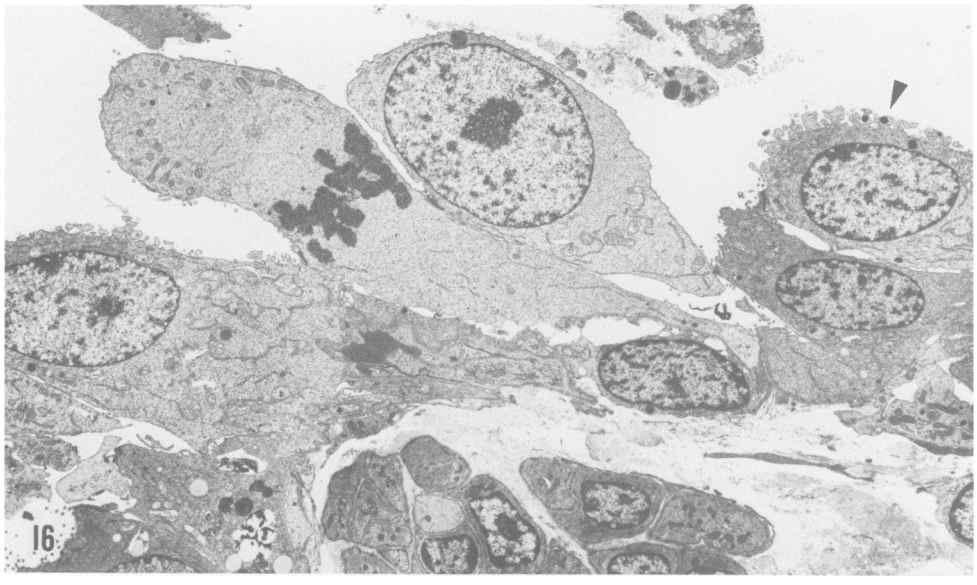
Figure 16—Respiratory bronchiolar wall from a monkey exposed to ozone for 36 hours. A cuboidal bronchiolar cell is in the metaphase stage of mitosis. The cuboidal cells are stratified, and one contains apical secretory droplets (*arrow*). (TEM, ×2956)

Figure 17—A labeled macrophage in the respiratory bronchiolar lumen of a monkey exposed to ozone for 50 hours. (Autoradiograph, toluidine blue, ×1764)

Figure 18—Hypertrophied cuboidal bronchiolar cells with prominent nucleoli form a continuous lining on the respiratory bronchiolar wall of a monkey exposed to ozone for 36 hours. (Epon–Araldite section, toluidine blue, ×800)

Figure 19—Cuboidal epithelial cells are focally stratified on the respiratory bronchiolar wall from a monkey exposed to ozone for 50 hours. (Epon–Araldite section, toluidine blue, ×800)

Figure 20—Alveolar septum of a respiratory bronchiole. The septum is lined by a collection of three Type 2 epithelial cells. The alveoli contain macrophages. The monkey was exposed to ozone for 50 hours. (Epon–Araldite section, toluidine blue, ×800) (Figures 17–20 with a photographic reduction of 3%)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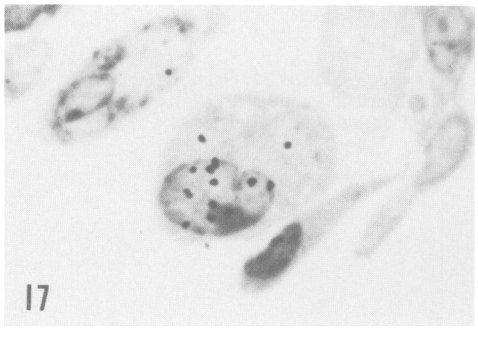
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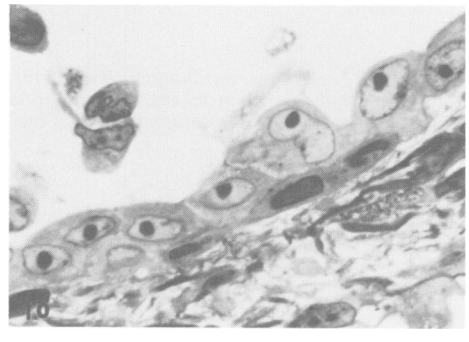
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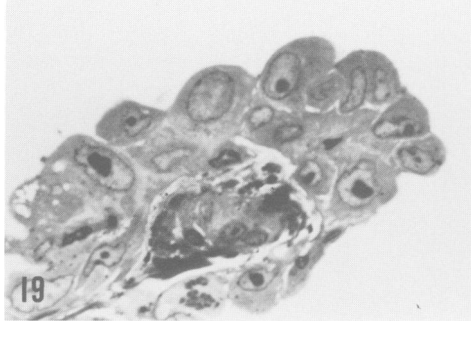
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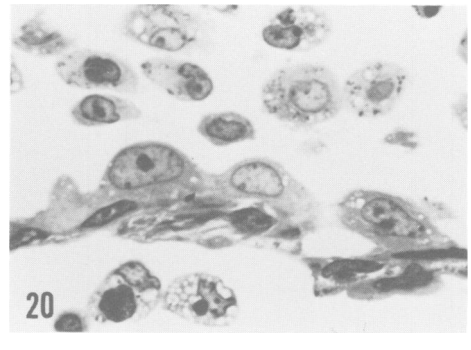
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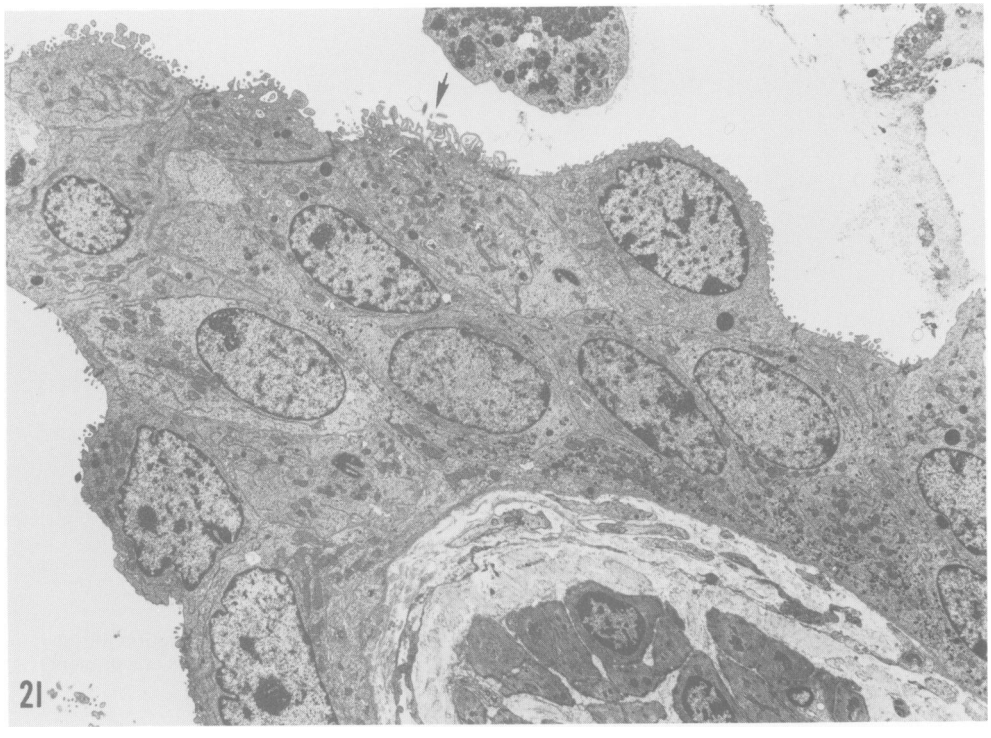
Figure 21—Transmission electron micrograph of stratified epithelial cells similar to those illustrated in Figure 19. Nonciliated cuboidal cells and one ciliated cell (*arrow*) are present in the nodule, in addition to Kulchitsky cells. The monkey was exposed to ozone for 36 hours. (×2645)

Figure 22—High-power magnification of the field illustrated in Figure 21. Granulated K cells are present. Nerve processes (*arrow*) are present subjacent to the basal lamina. (TEM, ×6510) **Inset**—TEM demonstrates that the granules are dense-cored vesicles. (×117,072)



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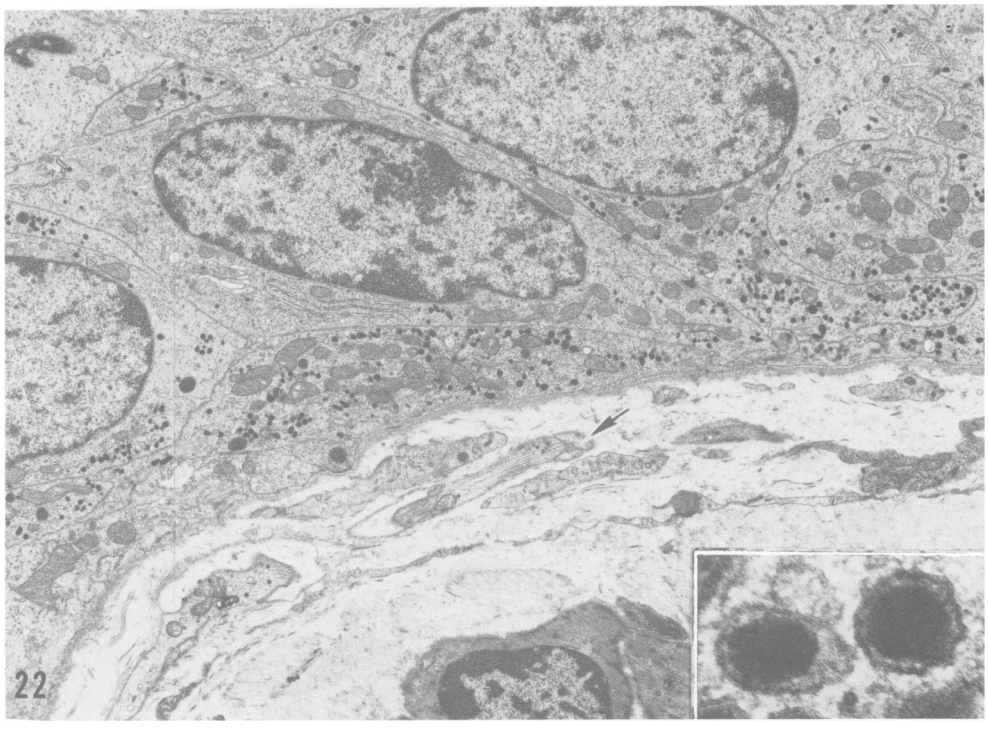

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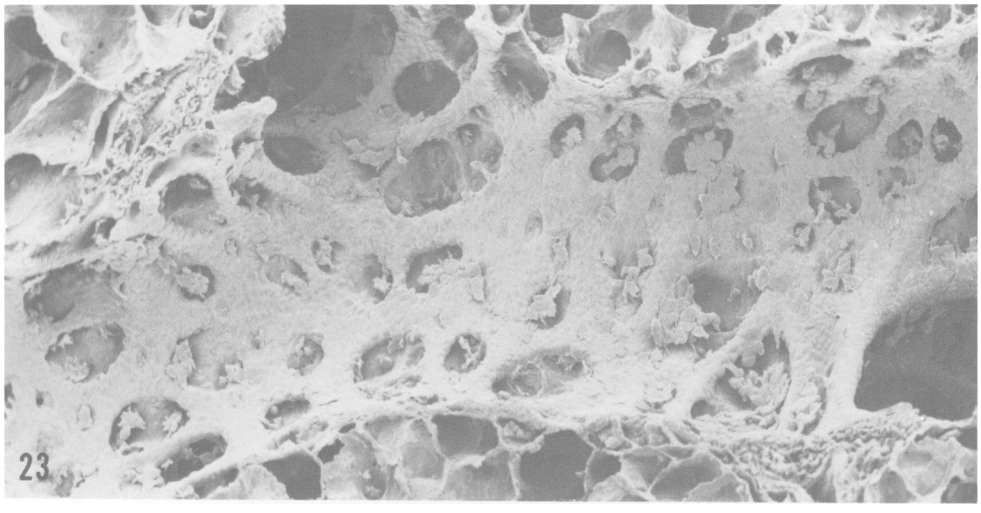
Figure 23—Respiratory bronchiole from a monkey exposed to ozone for 50 hours and allowed to recover in filtered room air for 2 days. Small clusters of macrophages are present on the wall and in alveoli. (SEM ×144)

Figure 24—Transmission electron micrograph of the respiratory bronchiolar wall from a monkey exposed to ozone for 50 hours and allowed to recover for 7 days. An epithelial cell (*N*) with thin and elongated cytoplasm covers a capillary (*C*) and another epithelial cell (*E*). (×6820)

Figure 25—Respiratory bronchiole from ozone-exposed monkey allowed to recover for 7 days. The surface and alveoli contain scattered small numbers of macrophages. (SEM, ×147)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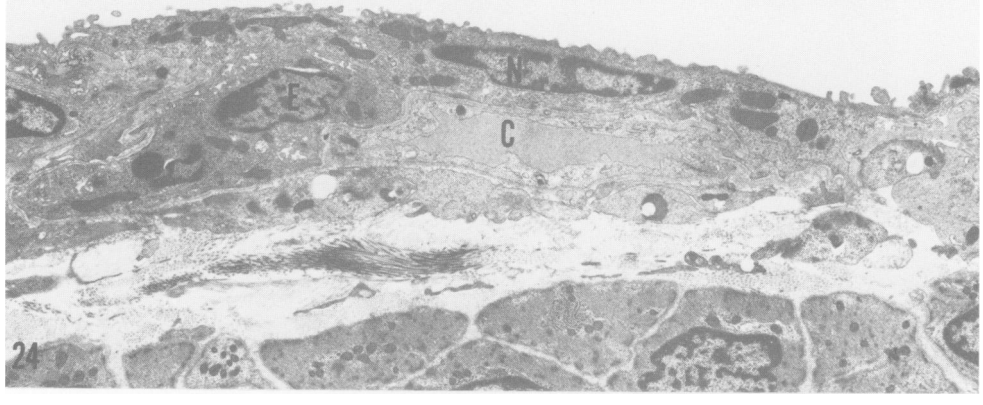
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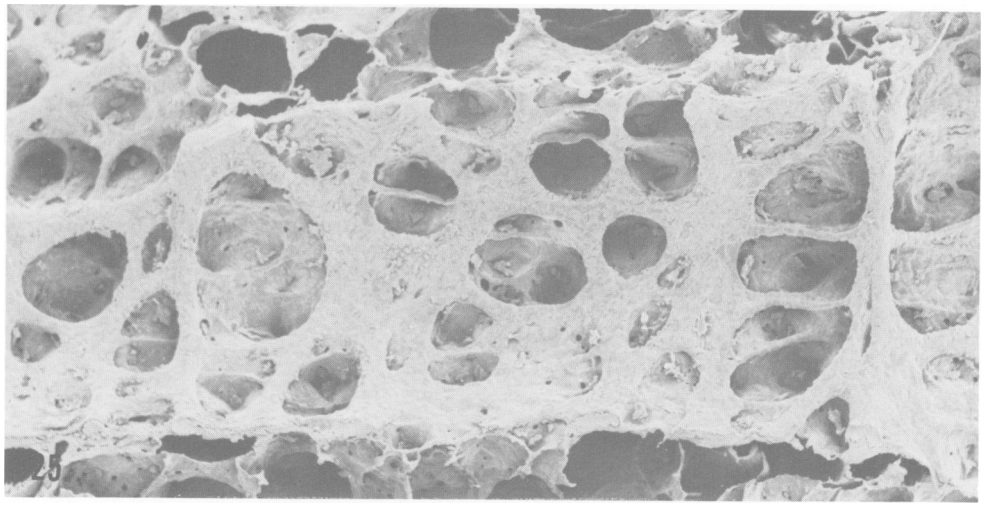
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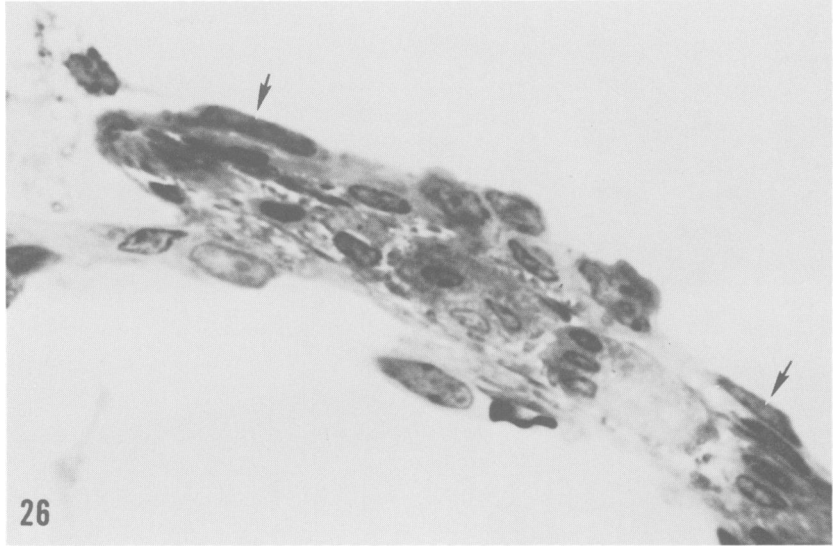

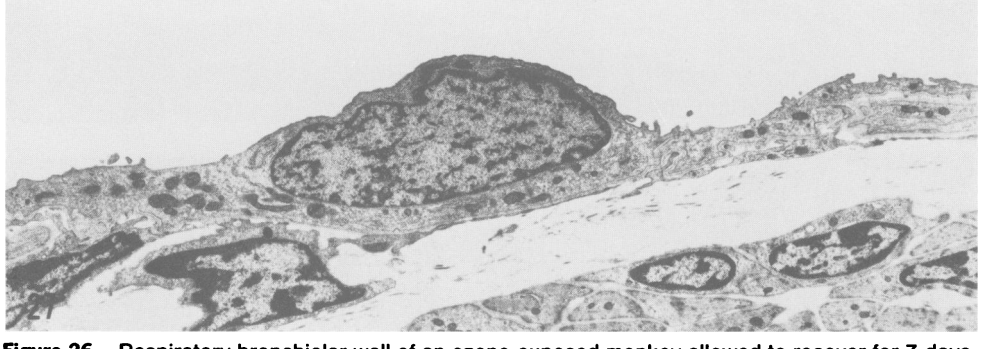

Figure 26—Respiratory bronchiolar wall of an ozone-exposed monkey allowed to recover for 7 days. There is a mixture of Type 1 cells (*arrows*) and cuboidal bronchiolar cells. (Epon–Araldite section, toluidine blue, ×1440) **Figure 27**—Transmission electron micrograph of a Type 1 epithelial cell lining a respiratory bronchiole of an ozone-exposed monkey allowed to recover for 7 days. (×6200) (Figures 26 and 27 with a photographic reduction of 3%)