Epithelial Injury and Interstitial Fibrosis in the Proximal Alveolar Regions of Rats Chronically Exposed to a Simulated Pattern of Urban Ambient Ozone[1]

Ling-Yi Chang,* Ying Huang,† Barbara L. Stockstill,* Judith A. Graham,‡ Elaine C. Grose,‡ Margaret G. Menache,* Frederick J. Miller,* Daniel L. Costa,‡ and James D. Crapo*

*Duke University Medical Center, Box 3177, Durham, North Carolina 27710; †The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8; and ‡U.S. Environmental Protection Agency, Research Triangle Park, North Carolina 27711

Epithelial Injury and Interstitial Fibrosis in the Proximal Alveolar Regions of Rats Chronically Exposed to a Simulated Pattern of Urban Ambient Ozone. Chang, L.-Y., Huang, Y., Stockstill, B. L., Graham, J. A., Grose, E. C. Menache, M. G., Miller, F. J., Costa, D. L., and Crapo, J. D. (1992). Toxicol. Appl. Pharmacol. 115, 241–252.

Electron microscopic morphometry was used to study the development of lung injury during and after chronic (78 weeks) exposure to a pattern of ozone (O_3) designed to simulate high urban ambient concentrations that occur in some environments. The daily exposure regimen consisted of a 13-hr background of 0.06 ppm, an exposure peak that rose from 0.06 to 0.25 ppm, and returned to the background level over a 9-hr period, and 2-hr downtime for maintenance. Rats were exposed for 1, 3, 13, and 78 weeks. Additional groups of rats exposed for 13 or 78 weeks were allowed to recover in filtered clean air for 6 or 17 weeks, respectively. Rats exposed to filtered air for the same lengths of time were used as controls. Samples from proximal alveolar regions and terminal bronchioles were obtained by microdissection. Analysis of the proximal alveolar region revealed a biphasic response. Acute tissue reactions after 1 week of exposure included epithelial inflammation, interstitial edema, interstitial cell hypertrophy, and influx of macrophages. These responses subsided after 3 weeks of exposure. Progressive epithelial and interstitial tissue responses developed with prolonged exposure and included epithelial hyperplasia, fibroblast proliferation, and interstitial matrix accumulation. The epithelial responses involved both type I and type II epithelial cells. Alveolar type I cells increased in number, became thicker, and covered a smaller average surface area. These changes persisted throughout the entire exposure and did not change during the recovery period, indicating the sensitivity of these cells to injury. The main response of type II epithelial cells was cell proliferation. The accumulation of interstitial matrix after chronic exposure consisted of deposition of both increased amounts of basement membrane and collagen fibers. Interstitial matrix accumulation underwent partial recovery during follow-up periods in air; however, the thickening of the basement membrane did not resolve. Analysis of terminal bronchioles showed that short-term exposure to O_3 caused a loss of ciliated cells and differentiation of preciliated and Clara cells. The bronchiolar cell population stabilized on continued exposure; however, chronic exposure resulted in structural changes, suggesting injury to both ciliated and Clara cells. We conclude that chronic exposure to low levels of O_3 causes epithelial inflammation and interstitial fibrosis in the proximal alveolar region and bronchiolar epithelial cell injury. © 1992 Academic Press, Inc.

Experimental acute exposures of humans to ambient concentrations of ozone (O_3) that occur in some urban environments causes decrements in pulmonary function and subjective symptoms of cough, pain on deep inspiration, and shortness of breath. Lung functional changes include increases in airway resistance and respiratory rate and decreases in forced expiratory volume at 1 sec (FEV1), forced vital capacity (FVC), total lung capacity, and tidal volume (Bates et al., 1972; Becket et al., 1985; Folinsbee et al., 1984; McDonnell et al., 1983). These observations are consistent with animal studies (Raub et al., 1982; Costa et al., 1983).

While the toxicity of high levels of O_3 is well established, there are few studies which address whether long-term exposures to the levels of O_3 that occur in ambient, urban environments cause lung injury. Recent field studies on the effects of subchronic exposures to ambient O_3 on respiratory function in active, normal children have revealed O_3-related decreases in FVC and FEV1 (Lioy et al., 1985; Spektor et al., 1988). An important question is what the effects of O_3 on the lungs of these children will be after a lifetime of breathing O_3 polluted air. Studies with repeated human or animal exposure to O_3 (Horvath et al., 1981; Tepper et al., 1989) as well as the study of children in summer camps,

have found that the initial decrement in lung function attenuates with succeeding exposures. However, Tepper *et al.* (1989), using rats exposed to 0.5 ppm O_3 for 5 days (2.25 hr/day), reported epithelial damage and inflammation in the terminal bronchiolar regions that persisted after the functional alterations diminished. The type of bronchiolar lesion reported by Tepper *et al.* (1989) is similar to those described by a number of previous studies (Castleman *et al.*, 1980; Fujinaka *et al.*, 1985; Lum *et al.*, 1978).

A number of animal studies using subchronic exposure to low levels of O_3 have found epithelial thickening and inflammation in the region of the bronchiolar–alveolar duct junction (Barry *et al.*, 1985; Boorman *et al.*, 1980; Plopper *et al.*, 1978). Barry *et al.* (1985) reported that rats exposed to 0.12 ppm O_3 for 6 weeks (12 hr/day) had thickened alveolar epithelium in the proximal alveolar region. It is not certain whether and how these changes progress with chronic exposure and whether chronic exposures to these levels of O_3 lead to focal fibrotic or emphysematous lesions.

To address these questions and to determine whether the patterns of lung injury that occur after chronic low level O_3 exposure are reversible, the Inhalation Toxicology Division of the Environmental Protection Agency's Health Effects Research Laboratory conducted an 18-month O_3 exposure study and coordinated a series of studies designed to evaluate the progression of health effects associated with chronic low-level O_3 exposure. We carried out detailed electron microscopic morphometric studies on animals from these exposures to characterize alveolar cell and tissue reactions.

METHODS

Animals. Male, 60-day-old F-344 rats (CDF [F-344] Crl BR, VAF + animals, Charles River Breeding Laboratories, Inc., Kingston, NY) were weighed, ear tagged, and randomly assigned to either the air- or O_3-exposure groups. They then were transferred to individual wire cages within a barrier-maintained exposure facility with food (Purina Rodent Lab Chow, St. Louis, MO) and water available *ad libitum* for 10 days prior to initiation of exposure. Separate groups of rats were purchased for the 1-, 3-, 13-, and 78-week studies. Ozone-exposed rats and matched air-exposed controls were studied at six time points after the onset of exposure. These time points were 1 week, 3 weeks, 13 weeks, 13 weeks plus 6 weeks recovery in clean air (19 weeks), 78 weeks, and 78 weeks plus 17 weeks recovery in clean air (95 weeks). Twelve animals from each exposure group were used. Serum was obtained on arrival from 5% of rats in each shipment and from sentinel rats exposed along with test rats. Sera were tested for and found to be free of antibodies to the following viruses: reovirus Type 3, pneumonia virus of mice, encephalomyelitis, Sendai, mouse adenovirus, sialodacryo adenitis, Toolan H-1, Kilham rat virus, lymphocytic choriomeningitis, and rat coronavirus. In addition, lung washes were tested for and found to be free of *Mycoplasma pulmonis,* and nothing unusual appeared in bacterial cultures from the nasopharynx, trachea, and gut. Rats were also monitored and found to be free of ectoparasites and endoparasites. Air- and O_3-exposed animals were handled identically at all times.

Exposure regimen and facility. The exposure was carried out in facilities operated by the Inhalation Toxicology Division of the U.S. Environmental Protection Agency's Health Effects Research Laboratory (Research Triangle Park, NC) and by ManTech Environmental Technology, Inc. (RTP, NC).

A complete description of the automated exposure facility has been reported by Davies *et al.* (1987). Briefly, animals were exposed in identical walk-in 14.2 m³ environmental rooms. Computer-interfaced mechanical systems monitored and controlled exposure concentration, temperature ($74 \pm 3°F$), humidity ($60 \pm 10\%$), chamber air flow, and lighting. Ozone was generated by passing cylinder-supplied oxygen (National Specialty Gas Co.) through a silent arc O_3 generator (Ozone Research and Equipment Corporation, Model 05V-0), and was monitored using continuous chemiluminescent analyzers (Bendix Model 8002) (RF0A-0176). These analyzers were calibrated every 2 weeks using a uv standard. The O_3 exposure consisted of a continuous background level of 0.06 ppm. During the 5-day work week, a 9-hr "ramped spike" (maximum concentration, 0.25 ppm; integrated concentration, 0.19 ppm) was added to the background exposure (Fig. 1). Animals and equipment were serviced daily during a 2-hr shutdown. On weekends the rats were exposed for 22 hr per day at the background level (0.06 ppm). The standard deviation for the peak part of the exposure was less than 4% of its mean at all times and that for the background portion did not exceed 2% of its mean concentration. During the 18-month exposure, a total of 40 hr of O_3 exposure was missed due to mechanical problems.

Histology. Rats were removed from the chambers and euthanized within 4 hr after their exposure was terminated. Lungs were fixed by infusing the fixative (2% glutaraldehyde in 0.085 M sodium cacodylate buffer, pH 7.4) through a tracheal cannula at a pressure of 20 cm H_2O. The lungs were removed from the chest after 10 min of fixation inside the chest wall. They were immersed in the 2% glutaraldehyde fixative for 2 weeks before being trimmed free of other tissue. Measurement of lung volumes by fluid displacement was then performed. Three 2-mm thick slices were cut from the left lobe in a manner that divided the rest of the lobe into four pieces approximately equal in width (cranial to caudal direction). Each slice was cut into large blocks with approximately 4×4-mm faces. The blocks were mixed and then six to nine blocks from each slice were arbitrarily selected. They were embedded in Epox 812 with large molds with a random orientation. The random orientation of terminal bronchioles allowed them to be cut in a random direction with respect to the central axis of each terminal bronchiole.

Microdissection. Three blocks of epox-embedded lung tissue were selected arbitrarily, softened with mild heat, and cut into slices 0.3–0.5 mm thick. These slices were sequentially inspected under a dissecting microscope. Small airways were followed until they led into terminal bronchioles and proximal alveolar ducts. All the terminal bronchioles in one tissue block that were perpendicularly cut in cross section were isolated. One terminal bronchiole from each block was randomly selected for study. We defined the proximal alveolar region (PAR) as the alveolar tissue surrounding the alveolar ducts beginning at the bronchiolar–alveolar duct junction and ending
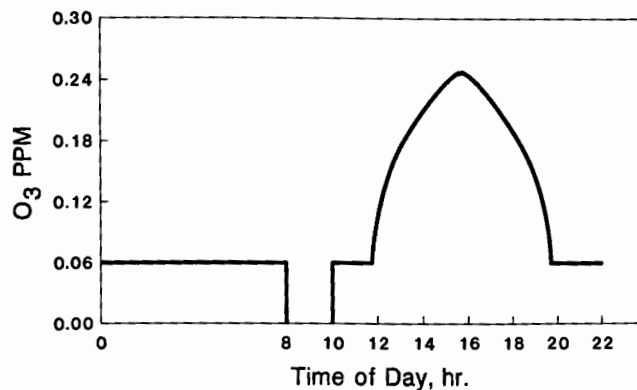

FIG. 1. Exposure regimen for the simulated ambient pattern of ozone. Rats were exposed to this regimen for 5 days each week. On weekends, the exposure consisted only of a 22 hr/day 0.06 ppm background.

TABLE 1
Primary Variable Groupings for Multivariate Analyses of Variance: Proximal Alveolar Region

Volume	Surface density	Surface area	Tissue thickness	Cell characteristics for each cell type[a]
Whole tissue	Alveolar BM[b]	Type 1 BM	Epithelium	Number
Epithelium	Capillary surface	Smooth type 2 cell surface	Interstitium	Mean volume
Interstitium	Epithelial type I cell BM	Capillary surface	Endothelium	Mean surface area
Endothelium	Smoothed type 2 cell surface			
Alveolar macrophages				

[a] Type I epithelial cells, type II epithelial cells, interstitial fibroblasts, endothelial cells, and alveolar macrophages.
[b] Basement membrane.

at the second alveolar duct bifurcation. Samples were taken at the approximate center of this region by taking them just distal to the proximal edge of the first alveolar duct bifurcation. We required that cross sections of the two first alveolar ducts be present in the section. All proximal alveolar regions contained in the series of embedded tissue slices that satisfied the definitions given above were isolated. Three terminal bronchioles and three proximal alveolar regions were used from each animal. Methods for microdissection have been described in detail by Chang and Crapo (1990).

Electron microscopy. Blocks of PARs were trimmed to include only the alveolar septa surrounding the alveolar ducts. Ultrathin sections (~60 nm) were cut and stained with uranyl acetate and lead citrate. Sections were examined with a Zeiss CM 10 electron microscope (EM) at 2000×. Photographs were taken at the upper left and lower right corner of each grid square, and the entire section was photographed. A montage of the entire bronchiolar epithelium was made for each terminal bronchiole. Micrographs were printed on 11 × 14 inch paper at a magnification of 8500× using a calibration grid (Barry and Crapo, 1985).

EM morphometry. The parameters measured in proximal alveolar regions in this study included the volumes of type I epithelium, type II epithelium, interstitial cells, interstitial matrix, and endothelium as well as alveolar cell characteristics (cell number, mean cell size, and mean cell-surface area). Because the absolute volume of proximal alveolar tissue cannot be easily measured, tissue volumes were expressed as the ratio of tissue volume density to surface density of the alveolar epithelial basement membrane (Barry and Crapo, 1985). We have found that although the thickness of the basement membrane may change, its surface density was not altered significantly during these chronic O_3 exposures (Table 4). It can be used as a

TABLE 2
Primary Variable Groupings for Multivariate Analyses of Variance: Terminal Bronchiole

Cell number	Volume density	Cell characteristics
Ciliated cells	Ciliated cells	Number
Clara cells	Clara cells	Mean cell volume
Preciliated cells	Preciliated cells	Mean cell luminal surface area
Brush cells	Brush cells	Mean cell BM surface area
Unknown cells	Unknown cells	

reliable denominator for normalization. Increases in values of the volume to surface area ratio indicate increases of tissue volumes. Since type I epithelial cells cover >95% of the alveolar surface, the normalized volume density is nearly equivalent to the arithmetic mean thickness of type I cells. Detailed methods for the determinations of volume densities, surface densities, numerical densities, and the calculation of cell characteristics have been reported previously (Barry and Crapo, 1985; Chang and Crapo, 1990).

Morphometric studies of the terminal bronchioles included measurement of the thickness of the bronchiolar epithelium and mean cell characteristics of the bronchiolar epithelial cells. The major terminal bronchiolar cells studied were ciliated cells, Clara cells, preciliated cells, and brush cells. All unidentified cell profiles were classified as "other" cells. Methods for the morphometry of terminal bronchiolar cells and morphological criteria used to identify them have been reported by Barry *et al.* (1985) and Chang *et al.* (1986).

Statistical analyses. A large number of morphometric analyses were conducted on the terminal bronchiolar and proximal alveolar regions. In order to perform the statistical analyses on the most meaningful biological endpoints and to minimize the need for correcting results for multiple comparisons, primary variable groupings were defined a priori. The critical variables identified for changes in volumes, surface densities, surface areas, tissue thickness, and the characteristics of the major cell types are given in Tables 1 and 2. These sets of primary variables were analyzed using multivariate analysis of variance (MANOVA). The remaining variables which had not been identified in multivariate groupings as being primary, were analyzed only in univariate analysis of variance (ANOVA).

The factors of interest in the statistical analyses were pollutant exposure (air, O_3) and length of exposure including recovery, if done, (1, 3, 13, 19, 78, and 95 weeks). A two-factor ANOVA examined the effects of pollutant and length of exposure in a 2 × 6 design. For the primary variables, ANOVA tests were only examined if a significant MANOVA effect due to pollutant or to an interaction with pollutant was found. Significant ANOVA effects were further examined by using the ANOVA mean square error to test the least squares means via t tests with application of a Bonferroni correction for multiple comparisons, as appropriate.

RESULTS

Morphometric Analysis of the Proximal Alveolar Region

Table 3 lists the body weights and lung volumes as well as the cumulative exposure concentrations of the groups of rats exposed for 13 weeks, 13 weeks plus recovery, 78 weeks, and 78 weeks plus recovery to air and O_3. Cumulative ex-

CHANG ET AL.

TABLE 3
Animal and Exposure Characteristics

	13 weeks		13 weeks + recovery		78 weeks		78 weeks + recovery	
	Control	Ozone	Control	Ozone	Control	Ozone	Control	Ozone
Body weight (g)	331 ± 7	340 ± 3	342 ± 11	347 ± 7	407 ± 10	407 ± 9	400 ± 5	422 ± 8
Lung volume (cm³)	9.2 ± 0.3	9.3 ± 0.3	9.6 ± 0.2	9.5 ± 0.2	11.5 ± 0.3	11.8 ± 0.2	11.1 ± 0.2	11.9 ± 0.3
Cumulative dose (ppm·hr)[a]	0	196	0	196	0	1180	0	1180

Note. All data are means ±SE from an *n* of 12 for each group. None of the differences in body weight or lung volumes between matched exposure and control groups were statistically significant for these exposure times.

[a] This is the product of concentration and time summed over the entire exposure period.

posure was computed using both peak and background exposure levels, the latter of which comprised approximately 30% of the total dose. Analysis of variance showed that O_3 exposure had no effect on body weights and lung volumes, but time-related changes were seen. Because these growth-related structural changes were expected, a separate age-

matched control group was studied with each O_3 exposure group. The tissue volumes, surface areas, and cell numbers (all normalized to basement membrane surface area) of proximal alveolar tissue of O_3- and air-exposed rats from the four exposure times mentioned above are listed in Table 4. Changes in total tissue volume in the PAR showed a biphasic

TABLE 4
Morphometrically Determined Characteristics of Tissue in Proximal Alveolar Regions after Chronic Exposure to a Simulated Ambient Pattern of Ozone

	13 weeks		13 weeks + recovery		78 weeks		78 weeks + recovery	
	Control	O_3	Control	O_3	Control	O_3	Control	O_3
n	12	12	12	12	12	12	12	12
Basement membrane surface density (cm²/cm³)	436 ± 26	418 ± 22	415 ± 21	450 ± 31	418 ± 15	407 ± 15	401 ± 35	383 ± 18
Volumes (V/SEPMB) (μm³/μm²)								
Total tissue	0.863 ± 0.040	1.025 ± 0.040*	0.905 ± 0.040	0.954 ± 0.040	0.964 ± 0.040	1.186 ± 0.040*	1.142 ± 0.040	1.160 ± 0.040
Epithelium	0.228 ± 0.015	0.307 ± 0.015*	0.246 ± 0.015	0.260 ± 0.015	0.233 ± 0.010	0.310 ± 0.015*	0.314 ± 0.015	0.289 ± 0.015
Type I	0.169 ± 0.009	0.228 ± 0.009*	0.175 ± 0.009	0.191 ± 0.009	0.184 ± 0.009	0.216 ± 0.009	0.221 ± 0.009	0.235 ± 0.009
Type II	0.053 ± 0.010	0.075 ± 0.010	0.067 ± 0.010	0.067 ± 0.010	0.048 ± 0.010	0.091 ± 0.010*	0.091 ± 0.010	0.051 ± 0.010*
Interstitium	0.398 ± 0.025	0.462 ± 0.025	0.420 ± 0.025	0.460 ± 0.025	0.473 ± 0.025	0.619 ± 0.025*	0.531 ± 0.025	0.580 ± 0.025
Cellular	0.153 ± 0.012	0.174 ± 0.012	0.144 ± 0.012	0.153 ± 0.012	0.149 ± 0.012	0.179 ± 0.012	0.171 ± 0.012	0.168 ± 0.012
Noncellular	0.245 ± 0.017	0.288 ± 0.017*	0.276 ± 0.017	0.306 ± 0.017	0.325 ± 0.017	0.440 ± 0.017*	0.360 ± 0.017	0.410 ± 0.017
Endothelium	0.206 ± 0.011	0.234 ± 0.011	0.217 ± 0.011	0.204 ± 0.011	0.216 ± 0.011	0.212 ± 0.011	0.260 ± 0.011	0.244 ± 0.011
Macrophages	0.032 ± 0.009	0.022 ± 0.009	0.022 ± 0.009	0.031 ± 0.009	0.041 ± 0.009	0.045 ± 0.009	0.037 ± 0.009	0.049 ± 0.009
Surface area (% total)								
Type I	98.2 ± 0.2	98.3 ± 0.2	98.0 ± 0.5	98.6 ± 0.3	98.8 ± 0.3	97.5 ± 0.5*	98.4 ± 0.4	98.9 ± 0.3
Type II	1.7 ± 0.2	1.7 ± 0.2	1.3 ± 0.2	1.4 ± 0.3	1.2 ± 0.2	2.4 ± 0.5*	1.6 ± 0.4	1.0 ± 0.2
Cell number (×10⁶/mm²)								
Alveolar type I cells	111 ± 6	156 ± 16	134 ± 17	187 ± 16*	123 ± 13	180 ± 16*	103 ± 10	137 ± 14
Alveolar type II cells	180 ± 17	213 ± 9	169 ± 15	173 ± 12	118 ± 13	191 ± 17*	170 ± 14	143 ± 11
Interstitial fibroblasts	322 ± 27	325 ± 16	237 ± 23	239 ± 20	213 ± 18	274 ± 31	267 ± 18	331 ± 22
Endothelial cells	815 ± 64	854 ± 41	780 ± 55	716 ± 40	644 ± 37	685 ± 41	664 ± 48	730 ± 82
Alveolar macrophages	40 ± 9	37 ± 7	33 ± 6	37 ± 5	24 ± 4	37 ± 6	38 ± 6	51 ± 12

Note. All data are means ± SE. Volumes and cell numbers are normalized to the surface density of epithelial basement membranes (SEPBM).

* $p < 0.05$ when compared to matched control based on Bonferroni adjusted *t* test using least squares means and the mean square error from the ANOVA.
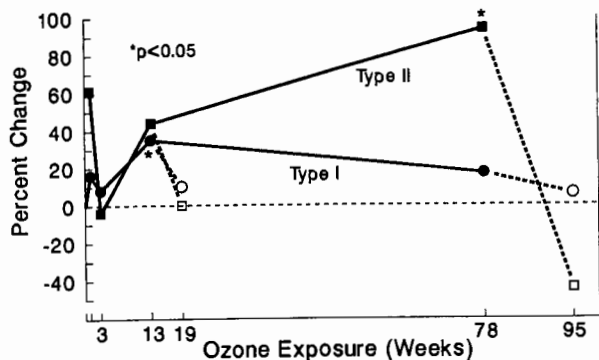
FIG. 2. Changes in the volumes of type I and type II epithelium during chronic exposure to the simulated ambient pattern of O_3. Solid line and closed symbols, O_3 exposure; dotted line and open symbols, clean air recovery.
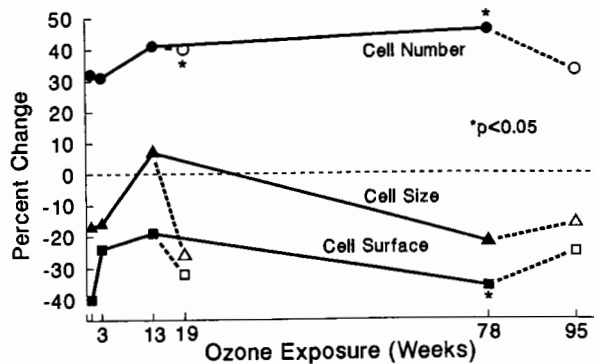

FIG. 3. Changes in cell characteristics of the alveolar type I cells in the proximal alveolar regions during O_3 exposure. Solid line and closed symbols, O_3 exposure; dotted line and open symbols, clean air recovery.

pattern. An acute increase in tissue volume after 1 week of exposure (+32%) returned toward baseline at three weeks (+4%), and then entered a phase of progressive increase through 13 (+19%) and 78 weeks (+23%) of exposure. Many of the changes induced by the subchronic and chronic exposures were reversed when rats were placed in clean air. Epithelium and interstitium were the major compartments of the septal wall tissue involved in these reactions. Analysis of variance indicated that there was a significant pollutant ($p < 0.01$) and time ($p < 0.01$) effects in the volumes of epithelium and interstitium.

Epithelium

Both alveolar type I and type II epithelia were involved in the response to O_3. The volume of type I epithelium increased 16, 8, 35, and 17% after 1, 3, 13, and 78 weeks of exposure (Fig. 2), respectively. Ozone exposure had an overall effect on mean type I cell number per unit area of basement membrane ($p < 0.01$). Increased type I cell numbers of 28, 41, and 46% at 1, 13, and 78 weeks, respectively, were observed (Fig. 3). These cells each covered a smaller alveolar surface area on the average. Although there was an apparent recovery in the total volume of type I epithelium at 3 weeks, the type I epithelium still consisted of more cells with less average surface area per cell. Chronic O_3 exposure (78 weeks) caused an increase in the volume of type II epithelium ($p < 0.01$) with increases of 61, 42, and 91% after 1, 13, and 78 weeks of exposure (Fig. 2), respectively. These increases were primarily the result of type II epithelial cell hyperplasia ($p < 0.01$). Type II cell number per unit area of basement membrane increased 29, 18, and 62%, respectively, after these exposures. The epithelial cell responses diminished when the animals were placed in clean air after either 13 weeks (19 weeks) or 78 weeks (95 weeks) of O_3 inhalation.

Interstitium

A biphasic response occurred in the interstitium after exposure to O_3 (Fig. 4). There was an overall pollutant effect

on the volume of interstitium ($p < 0.01$) that involved both interstitial fibroblasts and interstitial matrix ($p < 0.01$). The volume of interstitial cells increased 53, 12, 14, and 21% after 1, 3, 13, and 78 weeks of exposure. The number and volume of interstitial macrophages increased approximately 60% but these changes were not statistically significant. The volume of interstitial matrix—which includes collagen, elastin, basement membrane, and the acellular ground substances—increased ($p < 0.01$) after 1, 13, and 78 weeks (33, 18, and 36%, respectively) of exposure to O_3 (Fig. 4). The changes manifested by interstitial cells largely resolved after a period of recovery in clean air. There was also a reduction of the increases in interstitial matrix during the recovery periods.

Capillaries and Endothelium

Capillary endothelium showed no structural changes in response to O_3 at any time during the chronic exposure. The volumes of the capillary lumen and endothelial cells were not changed. Endothelial cells demonstrated neither statistically significant changes in mean cell volume or number
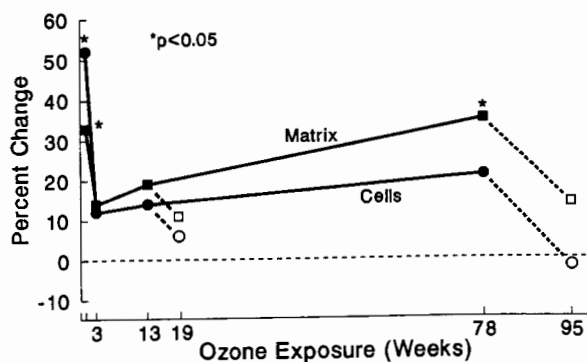

FIG. 4. Changes in the volumes of interstitial cells and interstitial matrix during chronic exposure to O_3. Solid line and closed symbols, O_3 exposure; dotted line and open symbols, clean air recovery.
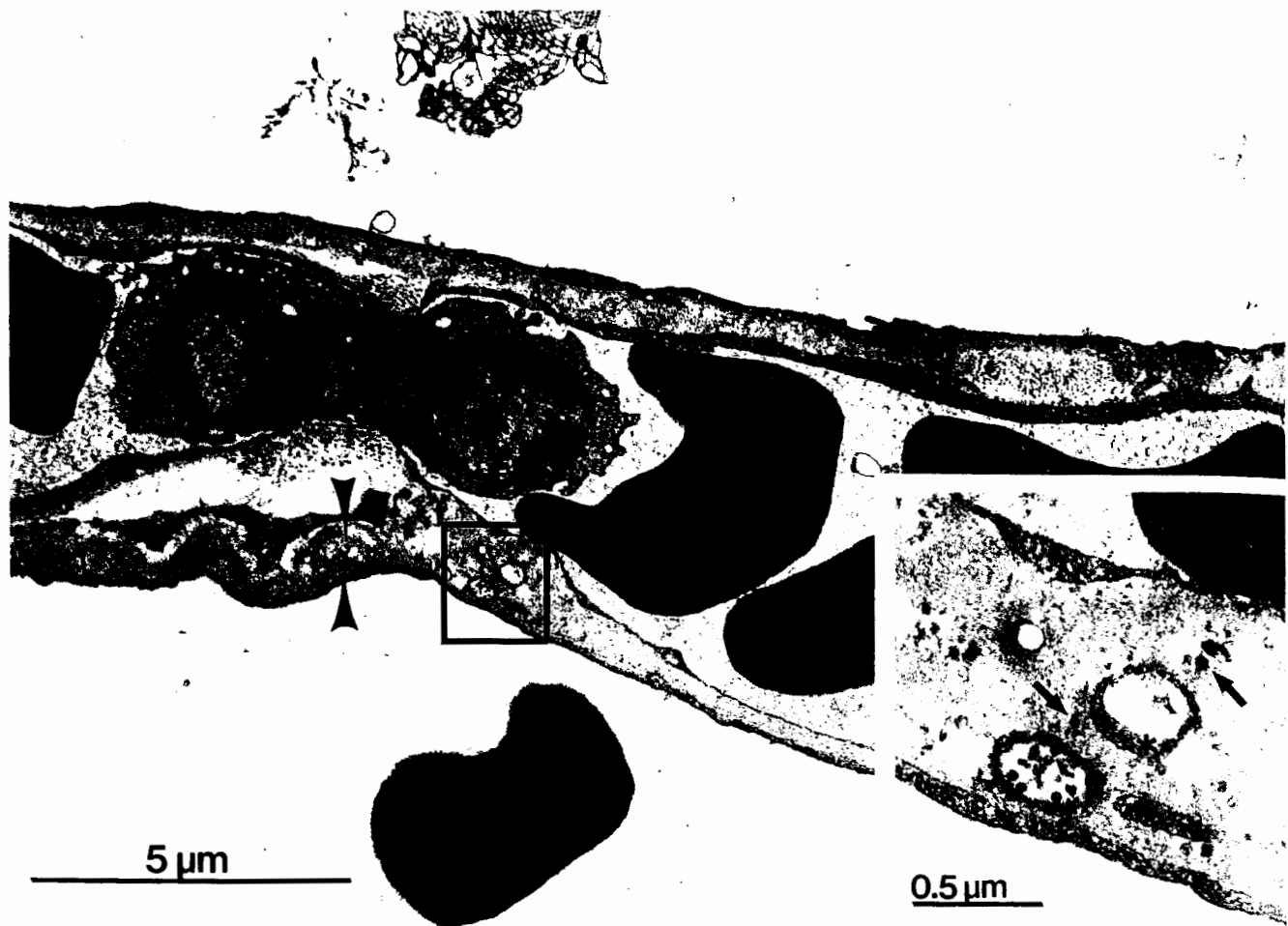
FIG. 5. Thickened basement membrane. Alveolar septa in the proximal alveolar region of rats exposed to O_3 for 78 weeks displayed thickened basement membrane (arrowheads). Inset, dense crystaline deposit (arrows) and cellular extensions (*).

nor a consistent pattern of change during the chronic exposure.

Alveolar Macrophages

A significant pollutant effect was found in the number of alveolar macrophages but there were no differences in the size of the cells. The only statistically significant change observed was a 108% increase in mean number per unit area of proximal alveolar surface after 1 week of exposure to O_3. Few alveolar macrophages were found in the PAR after 3 and 13 weeks of exposure. A 49% increase in the number of alveolar macrophages was found after the 78-week exposure, but the change was not statistically significant.

Morphological Changes in the Proximal Alveolar Regions

Electron microscopic examination of the epithelium in the PAR showed that damage to the plasma membrane of the type I epithelial cells, such as ruffling and blebbing, occurred throughout the exposure. A thickened type I epithe-

lium was observed after longer exposure durations (13 and 78 weeks). The ultrastructure of the type II epithelium appeared to remain normal. Areas of type II cell hyperplasia were observed at all time points, but were most evident after 78 weeks of exposure.

Interstitial edema was observed after 1 week of exposure to O_3, but was resolved by 3 weeks. Although areas of collagen fiber deposition were found in rat lungs exposed for 13 weeks, the accumulation of collagen was not readily apparent until after 78 weeks of O_3 treatment. Large bundles of collagen were observed frequently in the interstitium of alveolar duct bifurcations, or in the septum bordering alveolar ducts. The basement membrane, although not changed in surface area density, was found to be thickened after 13 and 78 weeks of exposure. Both the epithelial and the endothelial basement membranes were increased in thickness (Fig. 5). Crystalline deposits were found in the thickened membrane (Fig. 5, inset). The thickened basement membrane was still present after the 4-month recovery period in clean air.

Morphometric Analysis of Terminal Bronchioles

Table 5 lists the mean diameters and mean epithelial thicknesses of terminal bronchioles. It also gives the numbers of terminal bronchiolar cells per square millimeter basement membrane and the mean cell luminal surface areas of ciliated and Clara cells. Terminal bronchioles had an average diameter of approximately 200 μm. The mean thickness of the epithelium was around 7.0 μm. A total of approximately 16,000 to 19,000 cuboidal epithelial cells were found per square millimeter of basement membrane in terminal bronchioles. These characteristics were not affected by the chronic exposure to O_3; however, the mean surface area of cilia on ciliated cells ($p < 0.05$) and mean luminal surface area of Clara cells ($p < 0.01$) were affected by both the pollutant and length of the exposure (Fig. 6).

Overall, the number of ciliated cells in terminal bronchioles decreased after chronic exposure to O_3, but none of the differences at the individual time points were statistically significant. The average surface area of cilia per cell was reduced 27% after 78 weeks exposure (Fig. 6). The average size of ciliated cells was found to range from 300 to 375 μm^3 in all exposure groups, and no statistically significant changes in mean cell volume between exposed and control groups were observed.

The number of nonciliated (Clara) cells in terminal bronchioles was not changed by exposure to O_3. The average cell volume of Clara cells ranged from 480–670 μm^3. There was no difference between control and O_3-exposed animals. The mean luminal surface area of Clara cells was decreased throughout the entire exposure. After exposure to the sim-
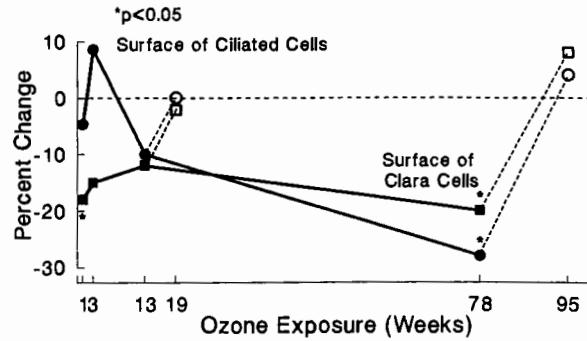

FIG. 6. Major changes in mean cell surface areas of terminal bronchiolar cells. The surface area of cilia and the dome surface area of Clara cells were reduced after 78 weeks of exposure to O_3. Solid line and closed symbols, O_3 exposure; dotted line and open symbols, clean air recovery.

ulated ambient pattern of O_3 for 1, 3, 13, and 78 weeks, the luminal surface area was found to be 18, 15, 10, and 20% less than control values, respectively (Fig. 6). The basement membrane surface of the Clara cell increased during O_3 exposure ($p < 0.05$). However, except for an increase of 14% after 3 weeks of exposure, the changes were less than 10% above control values and were not statistically significant.

Preciliated cells are a rare cell type in the terminal bronchiole (Barry et al., 1988). They constitute less than 1% of the total cell population. Increased cell number and mean cell volume (air, 307 ± 22 μm^3; O_3, 469 ± 31 μm^3) were observed in preciliated cells after 1 week of O_3 exposure. These cell characteristics returned to normal by 3 weeks of exposure. Because the population size of these cells is small,

TABLE 5
Characteristics of Terminal Bronchioles after Chronic Exposure to a Simulated Ambient Pattern of Ozone

	13 weeks		13 weeks + recovery		78 weeks		78 weeks + recovery	
	Control	O_3	Control	O_3	Control	O_3	Control	O_3
n	12	12	12	12	12	12	12	12
Average terminal bronchiole diameter (μm)	219 ± 7	216 ± 5	217 ± 7	208 ± 6	206 ± 8	214 ± 6	205 ± 5	224 ± 6
Average epithelial thickness (μm)	7.2 ± 0.3	7.4 ± 0.2	6.9 ± 0.3	7.0 ± 0.3	6.3 ± 0.1	6.4 ± 0.1	6.7 ± 0.2	6.5 ± 0.2
Cells per unit area of basement membrane (/mm^2)								
Total	18600 ± 600	18300 ± 300	17700 ± 400	17300 ± 400	16600 ± 400	16100 ± 300	16700 ± 400	15700 ± 300
Ciliated cells	13300 ± 400	12900 ± 300	12700 ± 400	12000 ± 200	12400 ± 400	11900 ± 300	12200 ± 400	11400 ± 300
Clara cells	4240 ± 170	4410 ± 120	4100 ± 140	4180 ± 180	3270 ± 100	3370 ± 190	3620 ± 100	3450 ± 140
Preciliated cells	123 ± 31	98 ± 21	60 ± 17	79 ± 25	27 ± 10	42 ± 14	39 ± 20	57 ± 21
Mean cell surface area (μm^2)								
Ciliated cells[a]	303 ± 15	270 ± 15	271 ± 16	269 ± 18	246 ± 12	178 ± 9*	216 ± 14	224 ± 9
Clara cells[a]	122 ± 6	110 ± 6	124 ± 3	123 ± 6	148 ± 7	118 ± 5*	133 ± 4	143 ± 7

[a] SA is airway luminal surface which includes surface for all cilia of ciliated cells and the dome surface area for Clara cells. All data are means ± SE.

* $p < 0.05$ for comparison to own control based on t test using least square means and the mean square error from the MANOVA.

small changes in preciliated cell characteristics indicated large percentages of changes in effects, for example, the number of preciliated cells after 78 weeks of exposure was 56% higher than that of air-exposed animals, the change was not statistically significant.

Very few structural alterations were observed in terminal bronchiolar cells of O_3-exposed rats. After 78 weeks of exposure to the simulated ambient pattern of O_3, ciliated cells displayed fewer cilia and Clara cells were found to have smaller dome protrusions.

DISCUSSION

Electron microscopic morphometric analyses were conducted on groups of rats exposed to a simulated ambient pattern of O_3 for up to 78 weeks. In addition to the progression of tissue and cell injury with long-term exposure, the effect of recovery in clean air was evaluated. Because of the low, realistic levels of O_3 involved and the long duration of the study, the results are particularly relevant to the health effects of environmental O_3. The proximal alveolar region was the major site of tissue injury. Cells in terminal bronchioles were also affected, but to a lesser degree. A biphasic response was found in the PAR which involved both the epithelial and interstitial compartments. Accumulation of interstitial matrix, primarily collagen and basement membrane, was detected after 78 weeks of exposure. While most cell reactions subsided when the animals were returned to clean air for substantial amounts of time, the changes in basement membrane did not resolve. Not all bronchiolar alveolar junctions in the rat lung were affected equally by the O_3 exposure. Modeling of reactive gas uptake predicts ~5% of the ventilatory units in the lung have a twofold greater and ~5% have a twofold less than average O_3 exposure per unit surface area in the PAR (Mercer et al., 1991). Our random sampling procedure may lead to undersampling of hot or cold spots. Nevertheless, when sufficient numbers of animals are studied, random sampling provides an accurate estimate of mean tissue responses.

Changes induced in animals by brief exposures to O_3 at concentrations from 0.2 to 1.0 ppm have been well documented (Barr et al., 1988; Barry et al., 1985; Boorman et al., 1980; Evans et al., 1985; Fujinaka et al., 1985; Schwartz et al., 1976; Stephens et al., 1974; Plopper et al., 1978, 1979). They include influx of alveolar macrophages, thickening of the type I epithelium, hyperplasia of type II epithelial cells, and increases in the volume of interstitial matrix in the proximal alveolar region. These previous observations on relatively high levels of O_3 or on low levels of O_3 for relatively short periods of time led to the design of the current study which utilized a low-level chronic exposure to determine whether the tissue changes are progressive and whether they can be reversed after a chronic exposure is stopped. F344 rats were used since it is the standard strain used in chronic

studies by the National Cancer Institute and the National Toxicology Program and because they exhibit relatively little lung growth during their adult life, thus providing a stable model for studies of chronic lung injury. It has been suggested that the F-344 rats may be less sensitive to O_3 than are other commonly used rat strains (such as Sprague–Dawley or Wistar) and nonhuman primates (Plopper et al., 1991; Costa et al., 1992). If F344 rats are more resistant to O_3 than are humans the injury responses found with F344 rats may occur in humans at even lower O_3-exposure concentrations.

The epithelium is perhaps the most immediate target of O_3 insult since O_3 is highly reactive. Increased numbers of both type I and type II cells and a thickened type I epithelium were found after the chronic low-level exposure to O_3. We noted that the magnitude of the epithelial cell response during the acute phase of the reactions appeared to be associated with the presence or absence of alveolar macrophages. This correlates with reports of epithelial cell injury and proliferation induced by proteases and inflammatory mediators released by macrophages (Hurst and Coffin, 1971; Leslie et al., 1986; King et al., 1990). Alternately, the apparent recovery of epithelial injury after 3 weeks of exposure may be related to increases in epithelial cell antioxidant defenses following the initial exposure (Mustafa and Lee, 1976; Plopper et al., 1979). Studies of antioxidant enzymes in whole lung homogenates and bronchoalveolar lavage fluids of another cohort of rats in the same chronic study have shown increases in glutathione-related enzyme activities and/or ascorbate and tocopherol levels at 12 and 18 months of exposure (Jaskot et al., 1989; Norwood et al., 1989).

The alveolar interstitium was another major site of O_3-induced tissue reactions. The acute responses consisted of an influx of interstitial macrophages, fibroblast hypertrophy, and interstitial edema. Deposition of increased amounts of interstitial matrix was found by 13 weeks of exposure. The matrix deposition included basement membranes and most likely also interstitial collagen. Epithelial cells, including type II cells, have been shown to synthesize the components of basement membrane in vitro (Zimmerman et al., 1985). Whether the thickened basement membrane was synthesized by differentiating type II cells as they replaced injured type I epithelium or whether the process also involved type I epithelial cells is unknown.

Morphological detection of collagen fibers in the PAR after O_3 exposure has been reported in a number of studies (Boorman et al., 1980; Plopper et al., 1978, 1979; Schwartz et al., 1976; Barry et al., 1985). An increase in total lung collagen content and in the rate of collagen synthesis was reported in rats exposed to >0.5 ppm O_3 (Last and Greenberg, 1980; Last et al., 1983, 1984; Bhatnagar et al., 1983; Wright et al., 1988) as well as in rats exposed to as low as 0.1 ppm O_3 (Warren and Last, 1987). In our study, an increased volume of interstitial matrix was not found until 13 weeks of exposure. EM morphological examination of the matrix sug-
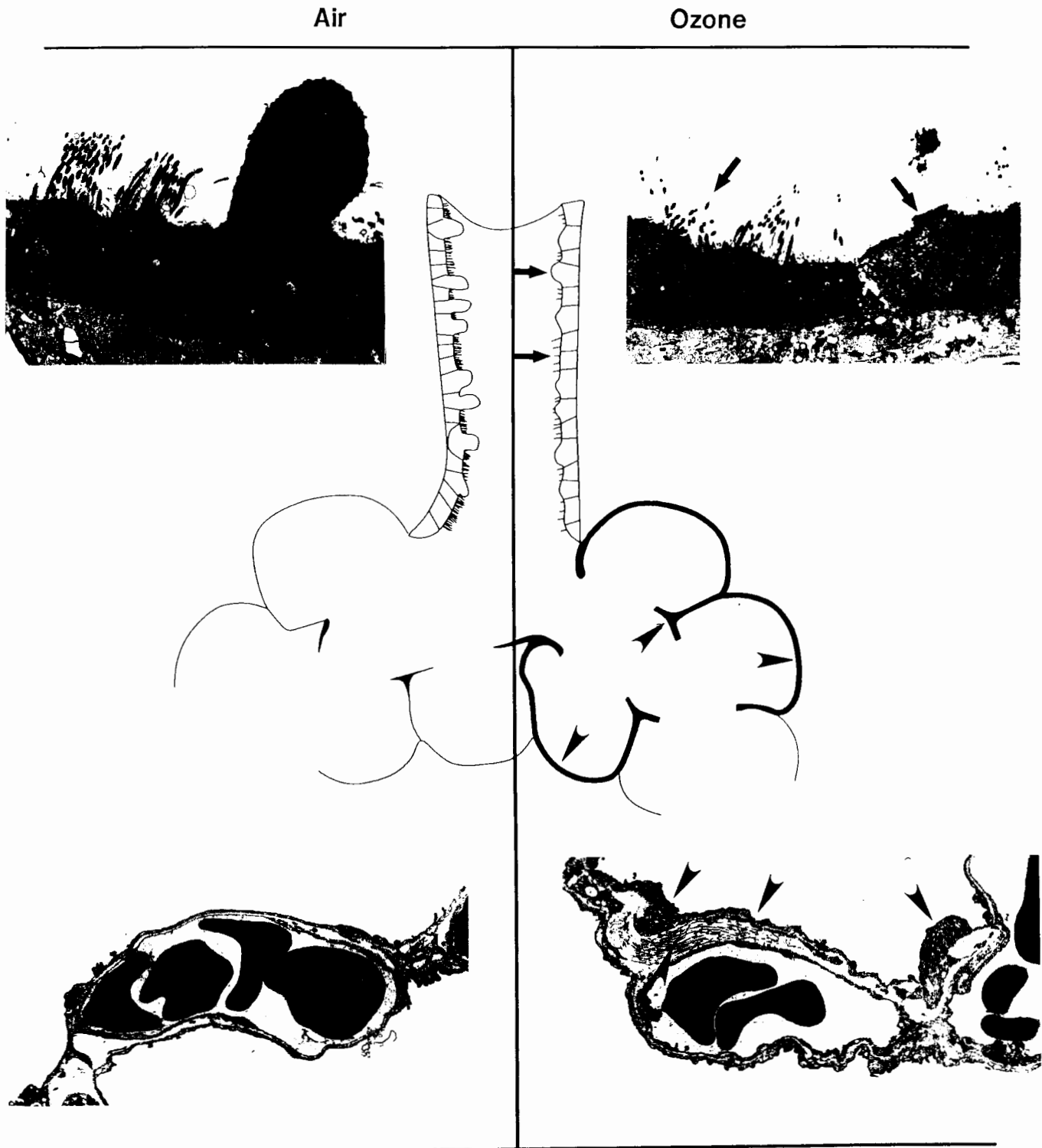
Air   Ozone

FIG. 7. A summary of lung injuries following chronic exposure to the simulated ambient pattern of ozone. In the terminal bronchiole the sizes of the dome of Clara cells become smaller and the number of cilia is reduced (arrows). In the proximal alveolar region the epithelium becomes thicker and accumulation of collagen fibers occurs (arrowheads).

gests that substantial collagen deposition did not occur at this time. By 78 weeks the accumulation of collagen could be discerned qualitatively.

Fibroblast proliferation and collagen production are thought to be caused mainly by cytokines released by inflammatory cells such as alveolar macrophages (King *et al.*,

1990). In the present study, increased numbers of macrophages were present only at the beginning and disappeared 3 weeks into the exposure, long before the increase in interstitial matrix was detected. Active involvement of alveolar macrophage in the development of the localized fibrosis seemed unlikely. Recently bronchiolar epithelial cells have been shown to express, intensely, the mRNA message of transforming growth factor (Pelton et al., 1991), an important growth factor that regulates extracellular matrix synthesis (Fine and Goldstein, 1987). We have found a hyperplastic and hypertrophic epithelium occurring in combination with matrix accumulation during chronic O_3 exposure. The O_3-induced interstitial injury and repair may be regulated in part by epithelium-derived cytokines or growth factors in the absence of activation by alveolar macrophages. It has been known that epithelial–interstitial interactions exist in the developing lung (Brody, 1985). Tissue injury and repair often involve mechanisms similar to those occurring in growth (King et al., 1990).

Terminal bronchioles have been reported to be a major site of O_3-induced lesions. Injuries described include loss of cilia, reduction of the height of Clara cells, proliferation of Clara cells, and remodeling of the terminal airways or bronchiolization (Boorman et al., 1980; Freeman et al., 1973; Schwartz et al., 1976; Barr et al., 1988; Lum et al., 1978). Similar changes were observed in the present study, except we did not find significant bronchiolization. The initial exposure caused loss of cilia and changes in Clara cell surface area. That Clara cells displayed a reduction in dome surface after 3 weeks of exposure while ciliated and preciliated cells returned to normal suggests either that ciliated cells adapt to O_3 toxicity while Clara cells do not, or that there was continued cell loss and differentiation but the cell population balance was restored. With chronic exposure, a significant loss of cilia on ciliated cells was noted. Ozone-induced cell injury in the terminal bronchioles was reversible upon return to clean air.

Figure 7 summarizes the changes that were found after 78 weeks of exposure to a simulated ambient pattern of O_3. In the terminal bronchioles, cilia were lost and the size of the domes of Clara cells was reduced. In the proximal alveolar region, epithelium was thickened and deposition of interstitial matrix occurred. An important question is whether or not these structural alterations are likely to result in significant functional impairment. Parallel lung function studies have revealed a significant O_3-induced increase in expiratory resistance particularly after 78 weeks of exposure (Tepper et al., 1991). Small but significant decreases in lung volume and enhanced nitrogen washout kinetics were also found (Costa, personal communication) that are consistent with the fibrotic lesions reported herein. Local and systemic immune functions were apparently unaffected by the chronic O_3 exposure (Selgrade et al., 1990). Pinkerton et al. (1989) reported that rats exposed to the same simulated ambient pattern of O_3 for 6 weeks followed by a 5-hr exposure to asbestos fibers retained three times as much fibers in their lungs as did control rats exposed only to the asbestos fibers. Warren and Last (1987) had demonstrated that exposure to O_3 and sulfuric acid aerosol simultaneously produced synergistic effects on the rate of collagen synthesis. The impact of even minor O_3 effects should not be underestimated because O_3 may interact with other airborne pollutants such as cigarette smoke, silica, and particulates to produce additive or synergistic injuries. It should be noted that human beings are commonly exposed for far longer periods than were the animals in this study, and that the relative sensitivity of humans in comparison to F-344 rats remains uncertain. Tyler et al. (1987) found that lung growth and development in young rats exposed to O_3 for 42 nights were altered and that these changes persisted after O_3 inhalation was stopped. The effects of O_3 on lung structure described in this study appear to be adverse and suggest that long-term functional impairment and lung disease could result from inhalation of air polluted with O_3.

ACKNOWLEDGMENTS

REFERENCES

Barr, B. C., Hyde, D. M., Plopper, C. G., and Dungworth, D. L. (1988). Distal airway remodeling in rats chronically exposed to ozone. Am. Rev. Respir. Dis. 137, 124–138.

Barry, B. E., and Crapo, J. D. (1985). Application of morphometric methods to study diffuse and focal injury in the lung caused by toxic agents. CRC Crit. Rev. Toxicol. 14, 1–32.

Barry, B. E., Miller, F. J., and Crapo, J. D. (1985). Effects of 0.12 and 0.25 parts per million ozone on the proximal alveolar region of juvenile and adult rats. Lab. Invest. 53, 692–704.

Barry, B. E., Mercer, R. R., Miller, F. J., and Crapo, J. D. (1988). Effects of inhalation of 0.25 ppm ozone on the terminal bronchioles of juvenile and adult rats. Exp. Lung Res. 14, 225–245.

Bates, D. V., Bell, G. M., and Burnham, C. D. (1972). Short-term effects of ozone on the lung. J. Appl. Physiol. 32, 176–181.

Beckett, W. S., McDonnell, W. F., Horstman, D. H., and House, D. (1985). Role of the parasympathetic nervous system in acute lung responses to ozone. J. Appl. Physiol. 58, 1879–1885.

Bhatnagar, R. S., Hussain, M. Z., Sorensen, K. R., Mustafa, M. G., Uan Dohlen, F. M., and Lee, S. D. (1983). Effect of ozone on lung collagen biosynthesis. In International Symposium on the Biomedical Effects of Ozone and Related Photochemical Oxidants (S. D. Lee, M. G. Mustafa, and M. A. Mehlman, Eds.), pp. 311–321. Princeton Scientific Publishers, Inc., Princeton, NJ.

Boorman, G. A., Schwartz, L. W., and Dungworth, D. L. (1980). Pulmonary effects of prolonged ozone insult in rats: Morphometric evaluation of the central acinus. Lab. Invest. 43, 108–115.

Brody, J. S. (1985). Cell to cell interactions in lung development. Pediatr. Pulmonol. 1, 542–548.

astleman, W. L., Dungworth, D. L., Schwartz, L. W., and Tyler, W. S. (1980). Acute respiratory bronchiolitis: An ultrastructural and autoradiographic study of epithelial cell injury and renewal in rhesus monkeys exposed to ozone. *Am. J. Pathol.* **98,** 811–840.

hang, L., Graham, J. A., Miller, F. J., Ospital, J. J., and Crapo, J. D. (1986). Effects of subchronic inhalation of low concentrations of nitrogen dioxide. I. The proximal alveolar region of juvenile and adult rats. *Toxicol. Appl. Pharmacol.* **83,** 46–61.

hang, L., and Crapo, J. D. (1990). Quantitative evaluation of minimal injuries. In *Models of Lung Disease: Microscopy and Structural Models* (J Gil, Ed.), pp. 597–640. Dekker, New York.

osta, D. L., Katzman, R. S., Lehmann, J. R., Popenoe, E. A., and Drew, R. T. (1983). A subchronic multiple dose ozone study in rats. In *International Symposium on the Biomedical Effects of Ozone and Related Photochemical Oxidants* (S. D. Lee, M. G. Mustafa, and M. A. Mehlman, Eds.) pp. 369–393. Princeton Scientific Publishers, Princeton, NJ.

osta, D. L., Tepper, J. R., Devlin, R., Madden, M., Hatch, G. E., Koren, H., Boere, J., and Rombout, P. J. A. (1992). Differential sensitivity to acute ozone: A study of lung dysfunction and inflammation in 3 rat strains. *Toxicologist* **12,** 614.

Davies, D. W., Walsh, L. C., Hiteshew, M. E., Menache, M. G., Miller, F. J., and Grose, E. C. (1987). Evaluating the toxicity of urban patterns of oxidant gases. I. An automated chronic gaseous animal inhalation exposure facility. *J. Toxicol. Environ. Health* **21,** 89–97.

Evans, M. J., Stephens, R. J., and Freeman, G. (1976). Cell renewal in the lungs of rats exposed to low levels of ozone. *Exp. Mol. Pathol.* **24,** 70–83.

Evans, M. J., Dekker, N. P., Cabral-Anderson, L. J., and Shami, S. G. (1985). Morphological basis of tolerance to ozone. *Exp. Mol. Pathol.* **42,** 366–376.

Fine, A., and Goldstein, R. H. (1987). The effect of transforming growth factor-beta on cell proliferation and collagen formation by lung fibroblasts. *J. Biol. Chem.* **262,** 3897–3902.

Folinsbee, L. J., Bedi, J. F., and Horvath, S. M. (1984). Pulmonary function changes after 1 hr continuous heavy exercise in 0.21 ppm ozone. *J. Appl. Physiol.* **37,** 984–988.

Freeman, G., Stephen, R. J., Coffin, D. L., and Stara, J. F. (1973). Changes in dogs lungs after long term exposure to ozone light and electron microscopy. *Arch. Environ. Health* **26,** 209–216.

Fujinaka, L. G., Hyde, D. M., Plopper, C. G., Tyler, W. S., Dungworth, D. L., and Lollthi, L. O. (1985). Respiratory bronchiolitis following long term ozone exposure in Bonnet monkeys: A morphometric study. *Exp. Lung Res.* **8,** 167–190.

Horvath, S. M., Gliner, J. A., and Folinsbee, L. J. (1981). Adaptation to ozone: Duration of effect. *Am. Rev. Respir. Dis.* **123,** 496–499.

Hurst, D. J., and Coffin, D. L. (1971). Ozone effect on lysosomal hydrolases of alveolar macrophages *in vitro. Arch. Intern. Med.* **127,** 1059–1063.

Jaskot, R. H., Grose, E. C., Richards, J. H., and Stead, A. G. (1989). Pulmonary biochemical changes in the rat following a chronic ozone exposure. *Toxicologist* **9,** 174.

King, R. J., Jones, M. B., and Minoo, P. (1990). Regulation of lung cell proliferation by polypeptide growth factors. *Am. J. Physiol. Lung Cell Mol. Physiol.* **258,** L23–L38.

Last, J. A., and Greenberg, D. B. (1980). Ozone-induced alterations in collagen metabolism of rat lungs. II. Long-term exposures. *Toxicol. Appl. Pharmacol.* **55,** 108–114.

Last, J. A., Gerriets, J. E., and Hyde, D. M. (1983). Synergistic effects on rat lungs of mixtures of oxidant air pollutant (ozone or nitrogen dioxide) and respirable aerosols. *Am. Rev. Respir. Dis.* **128,** 539–544.

Last, J. A., Reiser, K. M., Tyler, W. S., and Rucker, R. B. (1984). Long-term consequences of exposure to ozone. I. Lung collagen content. *Toxicol. Appl. Pharmacol.* **72,** 111–118.

Leslie, C. L., McCormick-Shannon, K., Cook, J. L., and Mason, J. R. (1986). Macrophage stimulate DNA synthesis in rat alveolar type II cells. *Am. Rev. Respir. Dis.* **132,** 1246–1252.

Lioy, D. J., Vollmuth, T. A., and Lippmann, M. (1985). Persistence of peak flow decrement in children following ozone exposure exceeding the national ambient air quality standard. *J. Air Pollut. Control Assoc.* **35,** 1068–1071.

Lum, H., Schwartz, L. W., Dungworth, D. L., and Tyler, W. S. (1978). A comparative study of cell renewal after exposure to ozone or oxygen: Response of terminal bronchiolar epithelium in the rat. *Am. Rev. Respir. Dis.* **118,** 335–345.

McDonnell, W. F., Horstman, D. H., and Hazucha, M. J. (1983). Pulmonary effects of ozone exposure during exercise. *J. Appl. Physiol.* **54,** 1345–1352.

Mercer, R. R., Anjilvel, S., Miller, F. J., and Crapo, J. D. (1991). Inhomogeneity of ventilatory unit volume and its effects on reactive gas uptake. *J. Appl. Physiol.* **70,** 2193–2205.

Mustafa, M. G., and Lee, S. D. (1976). Pulmonary biomedical alterations resulting from ozone exposure. *Ann. Occup. Hyg.* **19,** 17–26.

Norwood, J., Crissman, K., Slade, R., and Hatch, G. (1989). The effect of chronic ozone exposure on bronchoalveolar lavage supernatant, cell and whole lung antioxidants. *Toxicologists* **9,** 177.

Pelton, R. W., Johnson, M. D., Perkett, E. A., Gold, L. I., and Moses, H. L. (1991). Expression of transforming growth factor-β_1, -β_2 and -β_3 mRNA and protein in the murine lung. *Am. Rev. Respir. Cell Mol. Biol.* **5,** 522–530.

Pinkerton, K. E., Brody, A. R., Miller, F. J., and Crapo, J. D. (1989). Exposure to low levels of ozone results in enhanced pulmonary retention of inhaled asbestos fibers. *Am. Rev. Respir. Dis.* **140,** 1075–1081.

Plopper, C. G., Chow, C. K., Dungworth, P. L., Brammer, M., and Nemeth, T. J. (1978). Effect of low levels of ozone on rat lung. II. Morphologic responses during recovery and re-exposure. *Exp. Mol. Pathol.* **29,** 400–411.

Plopper, C. G., Chow, C. K., Dungworth, D. L., and Tyler, W. S. (1979). Pulmonary alterations in rats exposed to 0.2 and 0.1 ppm ozone: A correlated morphological and biochemical study. *Arch. Environ. Health* **34,** 390–395.

Plopper, C. G., Harkema, J. R., Last, J. A., Pinkerton, K. E., Tyler, W. S., St. George, J. A., Wong, V. J., Nishio, S. J., Weir, A. S., Dungworth, D. L., Barry, B. E., and Hyde, D. M. (1991). The respiratory system of nonhuman primates responds more to ambient concentrations of ozone than does that of rats. In *Tropospheric Ozone and the Environment* (R. L. Berglund, D. R. Lawson, and D. J. McKee, Eds.), pp. 137–150. Air and Waste Management Association, Pittsburgh, PA.

Raub, J. A., Miller, F. J., and Graham, J. A. (1982). Effects of low level ozone in adult and neonatal rats. In *International Symposium on Biomedical Effects of Ozone and Related Photochemical Oxidants* (S. D. Lee, M. G. Mastafa, and M. A. Mehlman, Eds.), pp. 363–367. Princeton Scientific Publishers, Princeton, NJ.

Schwartz, W., Dungworth, D. L., Mustafa, M. G., Tarkington, B. K., and Tyler, W. S. (1976). Pulmonary responses of rats to ambient levels of ozone: Effects of 7 day intermittent or continuous exposures. *Lab. Invest.* **34,** 565–578.

Selgrade, M. J. K., Daniels, M. J., and Grose, E. C. (1990). Acute, subchronic, and chronic exposure to a simulated urban profile of ozone: Effects on extrapulmonary natural killer cell activity and lymphocyte mitogenic responses. *Inhalation Toxicol* **2,** 375–389.

Smith, B. T., and Post, M. (1989). Fibroblast pneumocyte factor. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **257,** L174–L178.

Spektor, D. M., Lippmann, M., Lioy, P. L., Thurston, D., Citak, K., James, D. J., Bock, N., Seizer, F. E., and Hayes, C. (1988). Effects of ambient ozone on respiratory function in active normal children. *Am. Rev. Respir. Dis.* **137,** 313–320.

Stephens, R. J., Sloan, M. T., Evans, M. J., and Freeman, G. (1974). Alveolar type I cell response to exposure to 0.5 ppm O_3 for short periods. *Exp. Mol. Pathol.* **20,** 11–23.

Tepper, J. S., Costa, D. L., Lehmann, J. R., Weber, M. F., and Hatch, G. E. (1989). Unattenuated structural and biochemical alterations in the rat lung during functional adaptation to ozone. *Am. Rev. Respir. Dis.* **140,** 493–501.

Tepper, J. S., Wiester, M. J., Weber, M. F., Fitzgerald, S., and Costa, D. L. (1991). Chronic exposure to a simulated urban profile of ozone alters ventilatory responses to carbon dioxide challenge in rats. *Fundam. Appl. Toxicol.* **17,** 52–60.

Tyler, W. S., Tyler, N. K., Last, J. A., Barstow, T. J., Magliano, D. J., and Hinds, D. M. (1987). Effects of ozone on lung and somatic growth: Pair fed rats after ozone exposure and recovery periods. *Toxicology* **46,** 1–20.

Warren, D. L., and Last, J. A. (1987). Synergistic interactions of ozone and respirable aerosols on rat lungs. III. Ozone and sulfuric acid aerosol. *Toxicol. Appl. Pharmacol.* **88,** 203–216.

Wright, E. S., Kehrer, J. P., White, D. M., and Smiler, K. L. (1988). Effects of chronic exposure to ozone on collagen in rat lung. *Toxicol. Appl. Pharmacol.* **92,** 445–452.

Zimmermann, B., Barrach, H., Merker, H., and Hinz, N. (1985). Basement membrane formation and lung cell differentiation *in vitro. Eur. J. Cell. Biol.* **36,** 66–73.