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The Response of the Rat Tracheal Epithelium to Ozone Exposure

Injury, Adaptation, and Repair

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Although acute ozone (O_3) exposure injures tracheal epithelium, the response of the tracheal epithelium to prolonged O_3 exposure, and the degree of repair following cessation of exposure have not been previously reported. The purpose of this experiment was to characterize the morphologic response of rat tracheal epithelium to acute (3 days) and prolonged (60 days) exposure to 0.96 ppm O_3 as well as to evaluate repair in a 7- and 42-day post-60-day exposure period. Quantitative light- and electron-microscopic evaluation and thymidine labeling indices showed that after 3 days of O_3 exposure there was ciliary damage, cell necrosis, an increased density of intermediate cells, and an elevated thymidine labeling index. Following 60 days of exposure, the only major change from controls was the presence of ciliated cells with uniformly short cilia. Tracheal superoxide dismutase levels did not differ between control and 60-day exposure groups. Our findings suggest that the tracheal epithelium adapts to prolonged ozone exposure with the exception of cilia formation in ciliated cells. Complete epithelial recovery occurred by 42 days after exposure. (Am J Pathol 1988, 131:373–384)

OZONE is the major oxidant gas in photochemical smog. The centriacinar region is considered the focus for pulmonary damage by ozone. Short-term exposure (less than 7 days) to ozone results in injury to and necrosis of ciliated cells and degranulation of Clara cells.[1–3] Long-term (90 days or longer) exposure results in Clara cell hyperplasia in terminal bronchioles[3–5] and alteration of the centriacinar region to produce extensive respiratory bronchioles[5] in species with minimal respiratory bronchioles. In species with extensive respiratory bronchioles, Clara cell hyperplasia in the respiratory bronchioles is the main lesion.[6] After cessation of long-term exposure, the epithelial populations may remain altered for several months.[5,7]

The response of the upper respiratory tract to ozone exposure has been less extensively studied. Both short-term and long-term exposure cause ciliated cell necrosis, shortened cilia, and secretory cell hyperplasia in the nasal mucosa of monkeys.[8] In the tracheal epithelium of rats, monkeys, cats, and rabbits, short-term exposure to ozone causes damage primarily to ciliated cells.[1,9–13] Loss of cilia, increased ciliogenesis, necrosis of ciliated cells, alterations in secretory cells in some species, and increased cell turnover are the foremost lesions. The membranous portion of the trachea receives more ozone-induced injury than the cartilaginous portion.[11] The effect of long-term ozone exposure on the tracheal epithelium of rats or other species and the response to a postexposure period have not been previously evaluated.

In urban areas, humans are intermittently exposed to ozone for prolonged periods, and the question of whether or not adaptation to prolonged oxidant exposure occurs becomes important. In this sense, adaptation is meant as a response of cells to an altered envi-

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ronment so as to minimize potential harmful effects. Morphologic evidence of adaptation to ozone exposure has been derived from evaluation of the centriacinar region of the lung, where acute ozone induced injury is followed by reduced rates of cell division, decreased numbers of inflammatory cells, and lesser cytologic alterations in epithelial cells with continued exposure.[14,15] Numerous biochemical studies of lung tissue from rats and monkeys have shown acute degenerative changes such as sulfhydryl oxidation and inhibition of enzyme activities following acute exposures to high levels of ozone and, conversely, an adaptive enhancement of reducing compounds and an increase in enzyme activities following subacute low-level exposures.[16-23]

The potential for adaptation to occur in the tracheal epithelium, which has a complex epithelial population and serves as a model for the conducting airways, has not been previously explored. The purpose of this study was to characterize the morphologic response of the rat tracheal epithelium to acute and prolonged ozone exposure, as well as to prolonged ozone exposure followed by a postexposure period, with the aim of determining the cellular and population events leading to adaptation to prolonged oxidant exposure.

Materials and Methods

Animals

Disease-free male Sprague–Dawley rats weighing 234–263 g were purchased from Bantin and Kingman (Fremont, Calif), maintained on Purina Rodent Laboratory Chow No. 5001 and water *ad libitum* and serologically determined to be free of antibodies to pneumonia virus of mice, Reovirus 3, Theiler's virus, Sendai virus, Kilham rat virus, H-I virus, mouse adenovirus, lymphocytic choriomeningitis virus, rat corona virus/sialodacryoadenitis virus, and *Mycoplasma pulmonis* (Microbiological Associates, Bethesda, Md). Serologic monitoring of both exposed and control animals and complete necropsies of cohort sentinnel animals was done before the exposure began, and upon termination of each exposure or postexposure period. Chemicals for the assay of superoxide dismutase activity were obtained from Sigma Chemical Company (St. Louis, Mo).

Inhalation Exposures

Groups of 5 rats each were exposed to chemically and biologically filtered air (FA) or 0.96 ppm ozone (O_3) 8 hours/night for 3 days, 60 days, 60 days followed by a 7-day postexposure period in filtered air (PEPFA), or 60 days followed by a 42-day PEPFA. The exposures, ozone generation, and monitoring were conducted according to standard procedures of the inhalation exposure facility at the California Primate Research Center.[24] Upon completion of the assigned exposure or postexposure period, each rat was given an intraperitoneal injection of 0.5 mCi (0.5 ml) of tritiated thymidine (specific activity 6.7 Ci/mmol), euthanized 1 hour later by sodium pentobarbitol (300 mg/kg intraperitoneally) injection, the thorax opened, the proximal trachea cannulated, and the trachea and lungs fixed with 2% glutaraldehyde with 0.1 M cacodylic acid buffer (pH 7.4, 380 mOsm) by airway perfusion at 30 cm of water pressure. After 1 hour of fixation, the proximal trachea was ligated, and the trachea and lungs were immersed in fixative.

In a separate experiment, 9 rats were exposed to FA for 60 days, and 8 rats were exposed to 0.96 ppm O_3 8 hours/night for 60 days followed by euthanasia and removal of their tracheae for determination of superoxide dismutase (SOD) activity. Individual tracheae were homogenized in isotonic saline by a Polytron (Brinkman Instrument Inc., Westbury, NY). The homogenate was centrifuged at 12,000*g* at 4 C for 20 minutes. SOD activity of the supernate was measured using the inhibition of the autoxidation of epinephrine assay.[25] The production of adrenochrome in reaction mixtures containing 3.3×10^{-4} M epinephrine, 1×10^{-4} M EDTA, and 0.05 M sodium bicarbonate at pH 10.2 and 30 C was followed at 480 nm in a spectrophotometer (Cary 219, Varian). This assay measures both copper-zinc and manganese forms of SOD. A 50% inhibition in the reduction of adrenochrome production was taken as one unit of SOD activity. Protein content of the supernatant was determined[26] with a bovine serum albumin standard.

Microscopic Analysis and Autoradiography

The tracheal segment consisting of the distal seven cartilage rings and associated membranous portion was prepared for scanning electron microscopy by dehydration in a graded series of ethanol and amyl acetate, then critical point-dried with CO_2 and gold-coated by use of a Denton Vacuum Sputter Coater. Mounted tracheae were coded, randomized, and examined by a stratified sampling method on an ETEC scanning electron microscope. In order to evaluate unbiased samples of the tracheal surface, we photographed the entire specimen surface at ×20; then the length of the membranous portion was measured and subdivided, so that five equally spaced locations along the center of the membranous portion were selected

and viewed at ×800. The surface morphology was assessed with the ×800 micrographs.

Transverse sections of trachea that included cartilage rings 8 and 9 (counting rings from the carina proximally) were dehydrated in ethanol and embedded in glycol-methacrylate. Consecutive serial 1-μ sections were mounted on glass slides and stained with toluidine blue, periodic acid–Schiff (PAS), alcian blue (AB), pH 2.5,[27] or high iron diamine (HID)[28] or processed for autoradiography. Sections for autoradiography were dipped in Ilford L4 photographic emulsion mixed 1:1 with water at 40 C. They were allowed to dry, placed in light-tight boxes containing desiccant, and kept at 4 C for 4 weeks. Autoradiographs were developed in Microdol-X, fixed with 15% sodium thiosulfate, and stained with toluidine blue. A labeling index for the membranous portion was determined by counting all intraepithelial nuclei in the membranous portion of the tracheal transverse section and determining the number of labeled nuclei/100 nuclei counted. Labeled nuclei were identified as to cell type whenever possible. Due to the limitations of light-microscopic autoradiography, it was not always possible to distinguish basal cells, serous cells, intermediate cells, and migratory cells. When nuclei could not be precisely identified, they were categorized as being in the upper or lower half of the epithelium. Thus, nuclei in the lower half of the epithelium could potentially include those of basal cells, serous cells, intermediate cells, or migratory cells, whereas those in the upper half could include all but the basal cells. The staining characteristics of the central 100 stained secretory cells in the membranous portion were determined by use of the PAS-, AB-, and HID-stained transverse sections. These cells were scored as containing PAS-positive, AB-positive, or both PAS- and AB-positive secretory material, and as HID-positive or -negative.

Transverse sections of trachea that included cartilage ring 10 were washed in Zetterquist's wash[29] and postfixed with 1% OsO_4 Zetterquist's solution for 2 hours at 25 C. The tissues were washed and block-stained in 2% uranyl acetate overnight. The specimens were dehydrated in a graded series of ethanols followed by propylene oxide and embedded in Epon–Araldite. The membranous portion was reembedded in Beem capsules for ultrathin sectioning. Thin sections (approximately 90 nm) were made with a Sorvall MT2B ultramicrotome and a diamond knife and mounted on Formvar-coated slotted copper grids. Specimens were coded and viewed in a random sequence with a Zeiss model EM-10 transmission electron microscope at 60 kv. A montage of each coded section was prepared with photographs of the entire section at a final magnification of ×3400. Cell counts

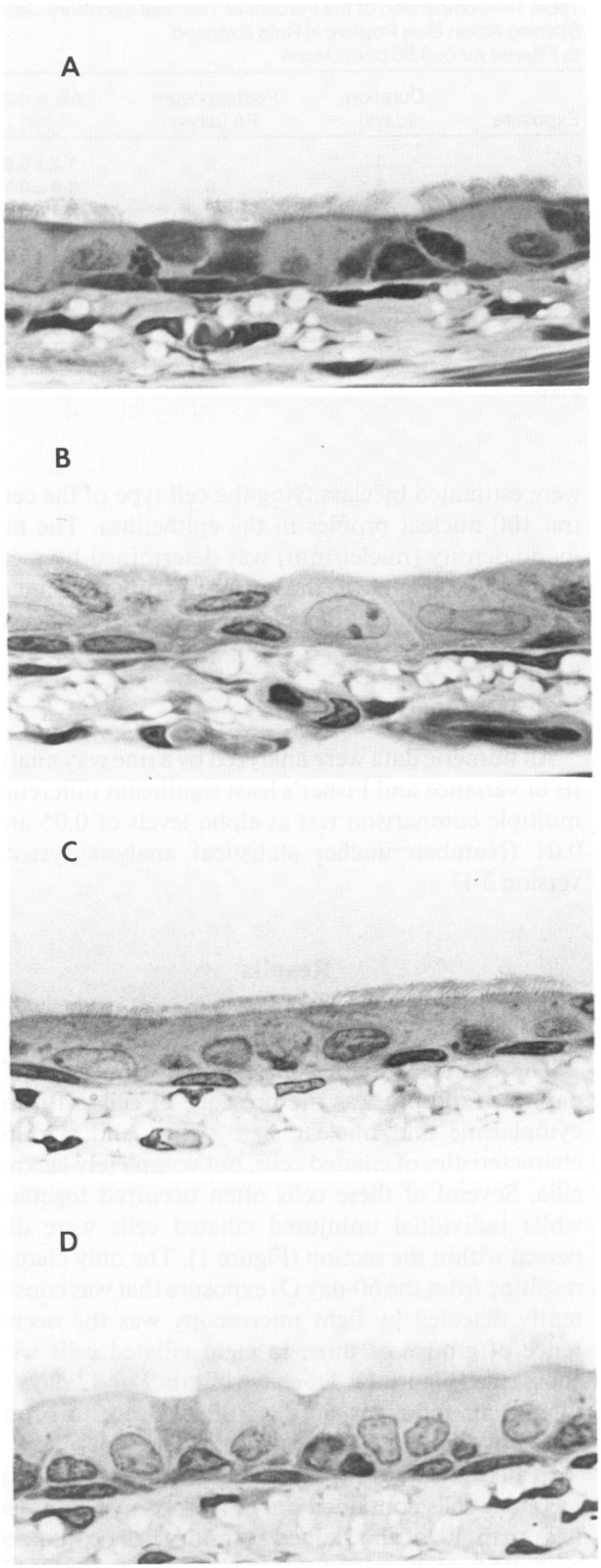

Figure 1—Representative light micrographs of tracheal epithelium. (Toluidine blue, ×361) **A**—Control (3-day filtered air exposure). **B**—Three-day 0.96 ppm O_3 exposure. Ciliated cells lack cilia. **C**—Sixty-day 0.96 ppm O_3 exposure. Ciliated cells with uniform dense short cilia. **D**—Sixty-day 0.96 ppm O_3 exposure followed by 42-day postexposure period in filtered air. No evidence of ciliary damage.

Table 1—Comparison of the Percent of Tracheal Secretory Cells Staining Alcian Blue Positive in Rats Exposed to Filtered Air or 0.96 ppm Ozone

Exposure	Duration (days)	Postexposure FA (days)	AB + cells (%)
FA	3	0	1.2 ± 0.6*
O_3	3	0	1.0 ± 0.5
FA	60	0	2.4 ± 0.9
O_3	60	0	0.6 ± 0.2
FA	60	7	1.4 ± 0.9
O_3	60	7	1.0 ± 0.4
FA	60	42	0.6 ± 0.2
O_3	60	42	1.8 ± 0.9

* Each value represents the mean ± 1 standard error, n = 5 animals per group.

were estimated by classifying the cell type of the central 100 nuclear profiles in the epithelium. The numeric density (nuclei/mm) was determined by measuring the length of the corresponding basement membrane by means of a planimeter (MOP-3, Zeiss).

Statistical Analysis of Data

All numeric data were analyzed by a one way analysis of variance and Fisher's least significant difference multiple comparison test at alpha levels of 0.05 and 0.01 (Numbercruncher statistical analysis system, version 3.1).

Results

Light Microscopy

The characteristic lesion resulting from acute (3-day) O_3 exposure was the presence of cells with the cytoplasmic and nuclear size, shape, and staining characteristics of ciliated cells, but completely lacking cilia. Several of these cells often occurred together, while individual uninjured ciliated cells were dispersed within the section (Figure 1). The only change resulting from the 60-day O_3 exposure that was consistently detected by light microscopy was the occurrence of groups of three to eight ciliated cells with short cilia (Figure 1). Tracheae of rats 7 or 42 days in filtered air following 60 days of exposure to ozone were not different from controls (Figure 1).

In all exposure groups, the majority of the epithelial secretory cells contained only PAS-positive granules. Less than 3% of the stained secretory cells contained both PAS-positive and AB-positive granules (Table 1). No cells contained only AB-positive granules, and no HID-positive secretory granules were present in the surface epithelium, although HID-positive staining was noted in secretory cells of submucosal glands. Neither PAS, AB, nor HID revealed significant differences in the staining characteristics of serous cell granules associated with exposure to ozone at 3 or 60 days or following either postexposure period.

Surface Morphology

In the control animals the majority of the surface of the membranous portion of the trachea was covered by cilia, and the cilia were oriented parallel to one another, uniform, long, fine, and dense (Figure 2). There were occasional irregular patches or ridges where most of the cells were nonciliated. Also, among the ciliated cells, there were rare cells with short cilia. After 3 days of ozone exposure, the most striking lesions were the large areas of the trachea which were not covered by cilia and the variability in the appearance of the cilia (Figure 2). The cilia lacked uniformity and regular orientation one to another. The length and density of the cilia varied from frequent areas with extremely short and sparse cilia to occasional areas with cilia of normal length and density. After 60 days of ozone exposure, and after 60 days of ozone exposure with a 7-day PEPFA, the majority of the surface of the membranous portion was covered by cilia similar in density to the corresponding controls, but the cilia had a uniform short appearance, or there were areas where uniform short cilia were mixed with cilia of normal length and rare, extremely short cilia (Figure 2). The tracheae from the animals exposed to O_3 for 60 days followed by a 42-day PEPFA were indistinguishable from the controls (Figure 2).

Ultrastructural Changes and Cell Densities (Table 2)

Sixteen morphologic categories of normal or damaged cells were distinguished (Table 2, Figure 3). The undamaged ciliated, serous, basal, brush, and migratory cells were identified by standard criteria.[30–32] Damaged ciliated cells were categorized as short-cilia, damaged-cilia, degenerate, or necrotic cells. Short-cilia cells were cells with cilia of uniform but less than 2.9-μ length, with no other evidence of injury. There were no apparent differences in the density or basal body structure of these cilia, compared with those of undamaged ciliated cells. Damaged-cilia cells had sparse, broken, irregular cilia. Cells with apical basal bodies, arranged either regularly or irregularly, but lacking cilia were also placed in this category. Degenerate cells were those with several membrane-bound cytoplasmic vacuoles that measured more than 1 μ in observed cross-section. They contained dark condensed material resembling autophagosomes, multivesicular bodies, or residual bodies. Cells lacking cilia

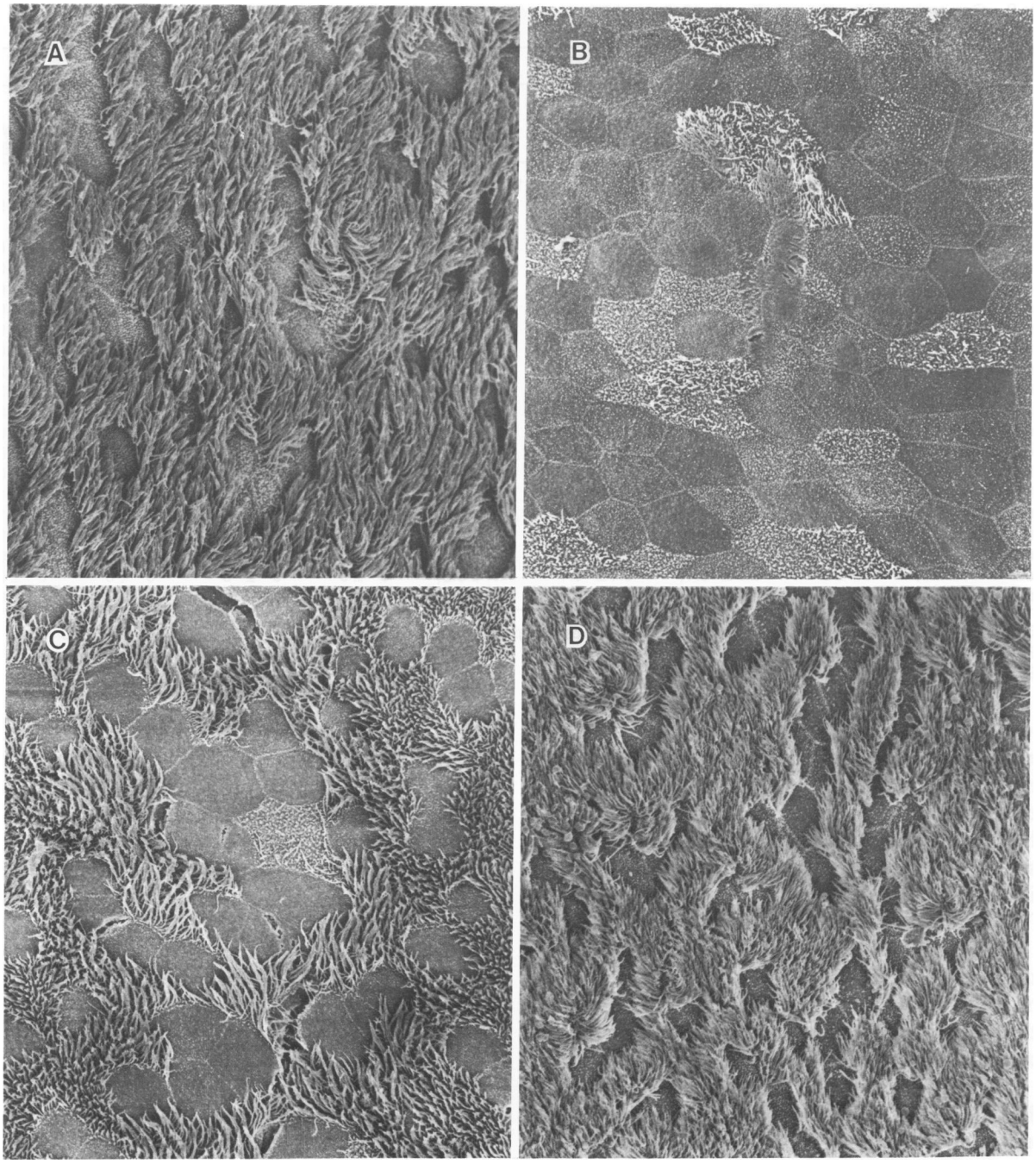

Figure 2—Representative scanning electron micrographs. (×920) **A**—Control (60-day filtered air exposure). Uniform long cilia. **B**—Three-day 0.96 ppm O_3 exposure. Ciliated cells with extremely short, sparse, and irregular length cilia. **C**—Sixty-day 0.96 ppm O_3 exposure. Several ciliated cells with uniform short dense cilia, one cell with extremely short cilia (*center*), and other cells with long cilia. **D**—Sixty-day 0.96 ppm O_3 exposure followed by a 42-day postexposure period in filtered air. Uniform long cilia.

or basal bodies but with a nuclear shape and stippled chromatin pattern resembling those of ciliated cells, and with cytoplasmic staining similar to, but slightly darker than, that of ciliated cells, were labeled intermediate cells. These cells did not have secretory granules. They contained more profiles of rough endoplasmic reticulum than mature ciliated cells of controls, but fewer than serous cells. Some of these cells contained apical fibrogranular aggregates (basal body precursors).[30] The intermediate cells in this study are similar to those observed previously in rat respiratory epithelium and appear to represent immature cells, probably preciliated cells.[30,33] Cells with dense shrunken nuclei and extremely electron-dense stain-

Table 2—Comparison of the Numeric Density (Cells per Millimeter) of Tracheal Epithelial Cells in Rats Exposed to Air or 0.96 ppm O_3 Categorized by Cell Type

	Exposure regimen							
Category	FA 3 days	O_3 3 days	FA 60 days	O_3 60 days	FA 60 days +7 PEFA	O_3 60 days +7 PEFA	FA 60 days +42 PEFA	O_3 60 days +42 PEFA
Total cells	104.6 ± 7.9*	124.1 ± 12.1	167.9 ± 11.6	153.2 ± 8.9	159.1 ± 7.4	141.7 ± 12.5	150.3 ± 9.8	140.1 ± 1.8
Total ciliated cells	16.4 ± 4.2	9.0 ± 2.1	53.6 ± 4.3	65.8 ± 3.9	39.8 ± 8.9	39.1 ± 6.9	45.6 ± 3.2	51.3 ± 5.6
Normal (undamaged)	14.1 ± 3.8	2.9 ± 1.2	52.7 ± 4.5	23.5 ± 4.8†	37.7 ± 8.7	31.9 ± 5.1	44.1 ± 2.8	49.3 ± 6.3
Short-cilia	0.6 ± 4.2	0.4 ± 0.4	0 ± 0	37.1 ± 3.1†	1.2 ± 0.7	5.9 ± 2.0‡	0.6 ± 0.6	1.2 ± 0.7
Damaged-cilia	0.7 ± 0.3	3.5 ± 1.0†	0.9 ± .05	4.3 ± 0.6†	0.6 ± 0.4	1.4 ± 0.7	0.6 ± 0.4	0.8 ± 0.8
Degenerate cells	0.9 ± 0.2	1.4 ± 0.9	0 ± 0	0.8 ± 0.5	0.3 ± 0.3	0 ± 0	0.3 ± 0.3	0 ± 0
Necrotic cells	0 ± 0	0.7 ± 0.4‡	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Intermediate cells	0.4 ± 0.3	7.6 ± 2.4†	0 ± 0	0.9 ± 0.4	0.9 ± 0.4	0.6 ± 0.4	0.4 ± 0.4	0.3 ± 0.3
Basal cells	25.3 ± 4.5	29.9 ± 4.1	34.7 ± 3.1	25.7 ± 2.4	38.0 ± 2.2	36.4 ± 5.6	28.9 ± 3.0	25.7 ± 2.5
Total serous cells	53.5 ± 4.8	65.1 ± 8.6	69.7 ± 4.5	53.5 ± 5.1	69.5 ± 7.1	57.8 ± 11.0	63.9 ± 4.9	53.5 ± 3.9
Normal	53.3 ± 4.9	64.2 ± 7.9	68.2 ± 4.3	53.5 ± 5.1	69.5 ± 7.1	57.8 ± 6.9	63.9 ± 4.9	53.5 ± 3.9
Degenerate	0.2 ± 0.2	0.9 ± 0.9	1.4 ± 0.6	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Necrotic	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Brush cells	1.0 ± 0.4	1.1 ± 0.3	1.7 ± 1.0	1.1 ± 1.1	3.1 ± 0.5	1.9 ± 0.7	0.6 ± 0.4	1.9 ± 0.7
Total migratory cells	7.1 ± 1.6	9.4 ± 2.3	8.4 ± 1.5	5.5 ± 1.5	6.1 ± 1.4	6.1 ± 1.7	7.5 ± 2.0	7.1 ± 1.7
Normal	7.1 ± 1.6	7.7 ± 2.6	8.4 ± 1.5	5.2 ± 1.4	6.1 ± 1.4	6.1 ± 1.4	7.5 ± 2.0	6.8 ± 1.8
Degenerate	0 ± 0	0.9 ± 0.6	0 ± 0	0.3 ± 0.3	0 ± 0	0 ± 9	0 ± 0	0.3 ± 0.3
Necrotic	0 ± 0	0.8 ± 0.6	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Necrotic (unidentified)	0 ± 0	2.6 ± 0.4†	0.4 ± 0.4	1.1 ± 0.5†	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Unidentified cells	0.7 ± 0.4	1.2 ± 0.4	1.0 ± 0.7	1.6 ± 0.5	2.0 ± 0.6	0.9 ± 0.4	0.7 ± 0.4	0.8 ± 0.3

* Each value represents the mean ± 1 standard error, n = 5 animals per group.
† Exposed significantly different from control, $P < 0.01$.
‡ Exposed significantly different from control, $P < 0.05$.

ing cytoplasm were identified as necrotic cells and categorized according to cell type, unless they lacked discernible identifying features, in which case they were labeled unidentified necrotic cells. Occasional cells with only a small nuclear profile, little or no cytoplasm, and lacking distinguishing features were categorized as unidentified cells.

There was a significant increase in the numeric density of damaged-cilia cells, necrotic ciliated cells, intermediate cells, and unidentified necrotic cells after 3 days of ozone exposure. The response of the tracheal epithelium to 60 days of ozone exposure was characterized primarily by a significant increase in the density of short-cilia cells. The magnitude and character of the ciliated cell alteration is best appreciated when expressed as the ratio of abnormal ciliated cells relative to the total ciliated cell population at risk (Figure 4). After 3 days of ozone exposure, the abnormal ciliated cell population is composed of damaged-cilia, degenerate, and necrotic ciliated cells, with a negligible contribution by short-cilia cells. After 60 days of ozone exposure, the proportion of the ciliated cell population characterized as abnormal is almost as great as after 3 days, but short-cilia cells account for 88% of the abnormal ciliated cells.

There was also a significant increase in the damaged-cilia cell density; but only 10% of the abnormal ciliated cells fit this category, compared with 58% after 3 days of ozone exposure. The number of necrotic ciliated cells was not increased above control after 60 days of ozone exposure. The unidentified necrotic cell population was significantly increased, but not to the same extent as after 3 days' O_3 exposure. Comparison of the total density of necrotic cells (Figure 5) or the density of unidentified necrotic cells shows significantly more necrotic cells after 3 days than after 60 days of ozone exposure. After 60 days of ozone exposure and a 7-day postexposure period, the only significant change within the epithelial cell populations was in the density of short-cilia cells. However, although the density of short-cilia cells was significantly increased from the controls, the density was also significantly less than after 60 days of O_3 exposure. There were no significant changes amongst the epithelial cell populations after 60 days O_3 exposure followed by 42 days' PEPFA, compared with the corresponding controls.

Labeling Indices (Table 3)

Labeled cells were always basal cells or cells with nuclei in the lower half of the epithelium, except in the 3-day O_3 exposure group (Figure 6). In this group, 17% of the labeled cells were identified as serous cells, and nuclei in the upper half of the epithelium were also labeled. There was a significant increase in the

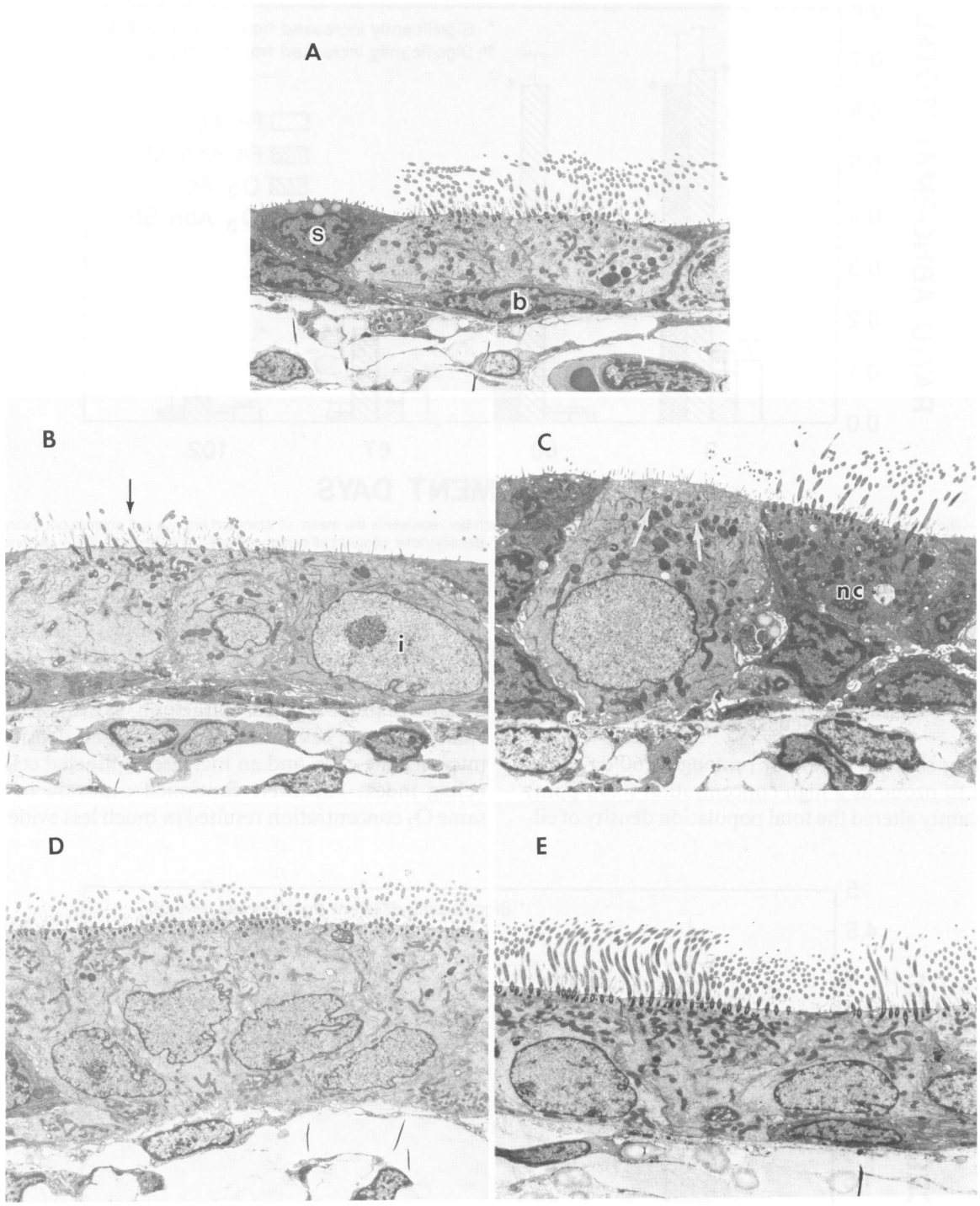

Figure 3—Representative transmission electron micrographs of tracheal epithelium. **A**—Control (3-day filtered air exposure). (×2000) Ciliated cells with long cilia, serous cell (*s*), and basal cell (*b*). **B**—Three-day exposure to 0.96 ppm O_3. (×2000) Ciliated cell with sparse broken cilia (*arrow*) and intermediate cell (*i*). **C**—Three-day exposure to 0.96 ppm O_3. (×2380) Intermediate cell with apical fibrogranular aggregates (*arrows*). Necrotic ciliated cell (*nc*). **D**—Sixty-day exposure to 0.96 ppm O_3. (×2000) Ciliated cells with uniform dense short cilia. **E**—Sixty-day exposure to 0.96 ppm O_3 followed by a 42-day postexposure period in filtered air. (×2000) Ciliated cells with long cilia.

labeling index after 3 days of ozone exposure but no significant difference after 60 days of exposure or after either of the 60-day O_3 exposures followed by either 7- or 42-day postexposure periods.

Superoxide Dismutase Activity (Table 4)

SOD activity was detected in the whole tracheas assayed. After 60 days of exposure to filtered air or O_3,

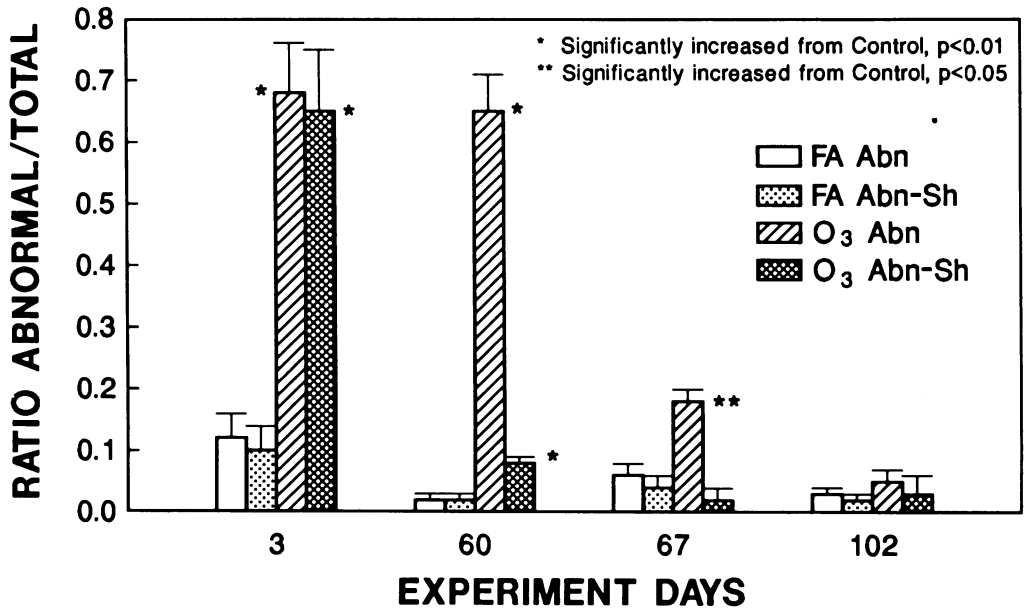

Figure 4—Bar graph of the ratio of abnormal ciliated cells/total ciliated cells. Each bar represents the mean +1 standard error, n = 5 animals per exposure group. Abn = (short-cilia + damaged-cilia + degenerate + necrotic ciliated cell density)/total ciliated cell density. Abn-sh = (damaged-cilia + degenerate + necrotic ciliated cell density)/total ciliated cell density.

there was no significant difference in the SOD activities between the two groups of tracheas.

Discussion

Neither short (3 days) nor prolonged (60 days) exposure to ozone at a high concentration (0.96 ppm) significantly altered the total population density of ciliated cells, serous cells, basal cells, brush cells, migratory cells, or unidentified cells in the tracheal epithelium of rats. Acute exposure to 0.96 ppm O_3 resulted in damage to the tracheal epithelium, as evidenced by ciliary damage, cell necrosis, an increased density of intermediate cells, and an increased epithelial cell labeling index. However, continued exposure to the same O_3 concentration resulted in much less evidence

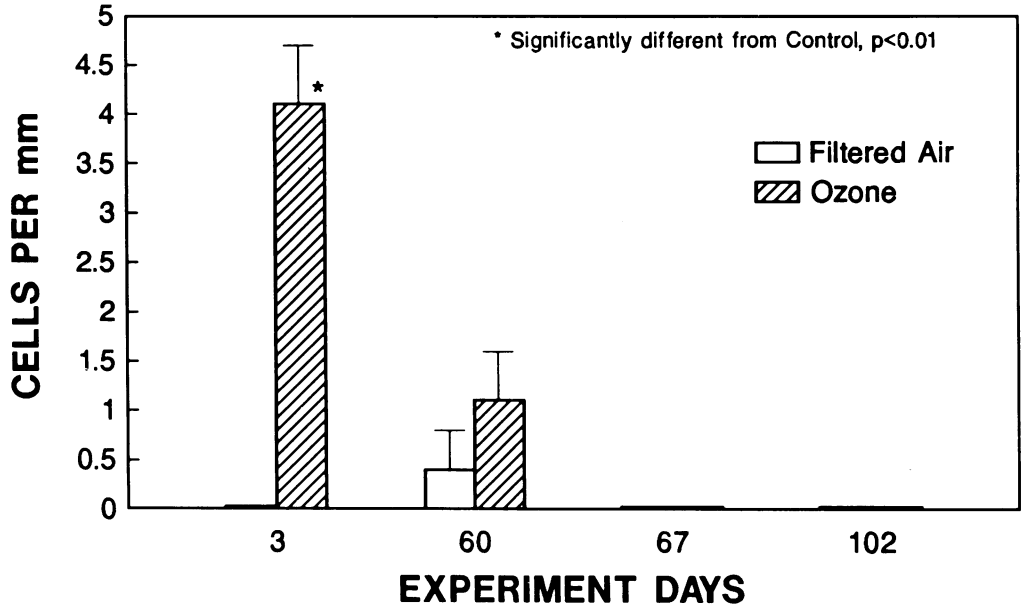

Figure 5—Bar graph of the total necrotic cells per millimeter. Each bar represents the mean + 1 standard error, n = 5 animals per exposure group. Mean total necrotic cell density equals 0 for the filtered air exposure at 3 days and for both filtered air and ozone exposures at 67 and 102 experiment days.

Table 3—Comparison of Tracheal Epithelial Cell Labeling Indices in Rats Exposed to Filtered Air or 0.96 ppm O_3

Exposure	Duration (days)	Postexposure FA (days)	Labeling index (%)
FA	3	0	0.3 ± 0.1*
O_3	3	0	2.6 ± 0.9†
FA	60	0	0.3 ± 0.1
O_3	60	0	0.1 ± 0.6
FA	60	7	0.4 ± 0.2
O_3	60	7	0.6 ± 0.2
FA	60	42	0.3 ± 0.1
O_3	60	42	0.1 ± 0.1

* Each value represents the mean ± 1 standard error, n = 5 animals per group.

† Significant difference from FA control, $P < 0.01$.

of injury. The same epithelial cell types and densities as present in the animals exposed to filtered air were reestablished even as the O_3 exposure continued. With continued exposure, the major alteration was the uniform shortness of cilia. In view of this finding, it is not surprising that during the postexposure period the only alteration was the disappearance of the short cilia, and there was no change in epithelial cell types or densities. If the manifestation of adaptation to ozone exposure is defined as a diminution in cell damage during prolonged exposure, then the decrease in necrosis and thymidine labeling index after 60 days of exposure, compared with the acute exposure, indicates that adaptation to prolonged O_3 exposure does occur in the tracheal epithelium of rats.

Possible mechanisms of adaptation include alterations in the nasal cavity causing a decreased dose of ozone to the trachea; biochemical changes such as the induction of superoxide dismutase systems reported in the lungs of oxygen-[34] and ozone-[35] exposed rats; shifts in cell populations[9]; an increased epithelial turnover resulting in a new level of equilibrium between cell loss and replacement[36]; an increased or altered secretory product; exudation onto the airways surface of albumin-rich fluid which might protect underlying tissues[37]; or a decrease in luminal surface area of cells exposed to ozone.

Significantly increased levels of both cytosolic and mitochondrial SOD activity have been reported in the lungs of rats[35,38] and mice[23] exposed to ozone. When the activity is expressed per gram of lung[23] or per milligram DNA[35] there is no increase in SOD activity. These findings have been interpreted to mean that the increased SOD level in the lung of ozone-exposed animals is due to increased cellularity and not due to increased enzyme activity per cell. Our finding that tracheas from rats exposed to O_3 for 60 days did not have increased levels of SOD activity supports this interpretation and indicates that increased SOD activity per

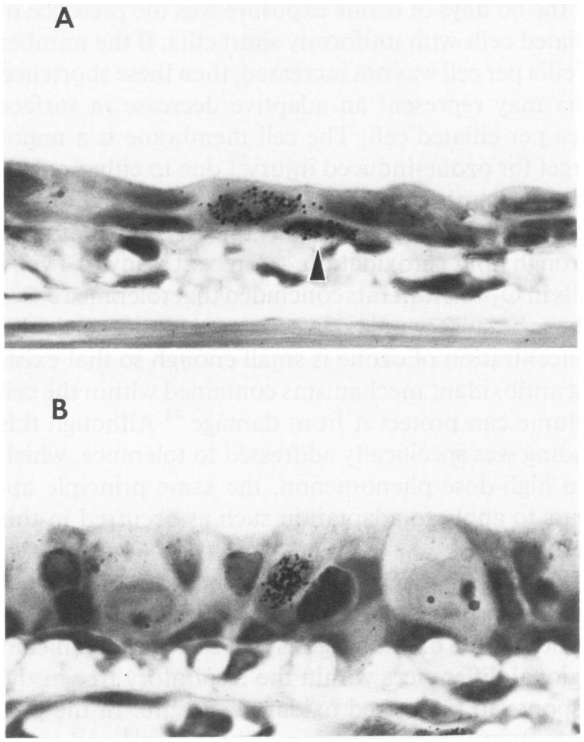

Figure 6—Autoradiographs of epithelia from rats exposed to 0.96 ppm O_3 for 3 days. (Toluidine blue, ×361) **A**—Labeled basal cell (*arrowhead*) and labeled cell in suprabasal position. **B**—Labeled serous cell (*center*).

cell is not a mechanism of tracheal adaptation to ozone exposure. The quantitation of epithelial cell density and the results of the PAS, AB, and HID staining indicate that neither shifts in cell populations nor altered characteristics of secretory products account for the adaptation. Although the numeric density of secretory cells did not increase, the possibility that there was an increased amount of secretory product cannot be absolutely eliminated, since neither the volume density of stored secretory product nor its rate of secretion was quantitated. Increased epithelial cell turnover can be eliminated as a mechanism of tracheal adaptation to ozone exposure, because there was no increase in thymidine labeling in the tracheas from rats exposed to O_3 for a prolonged period. Among the various tracheobronchial cell types, ciliated cells are commonly considered the most sensitive to ozone-in-

Table 4—Tracheal Superoxide Dismutase Activity After 60 days' Exposure to Filtered Air or 0.96 ppm O_3

	Filtered Air	O_3
Activity in U/mg protein	7.83 ± 0.87 (9)*	8.51 ± 0.93 (8)
Activity in U/g tissue	350.1 ± 26.9 (9)	340.4 ± 15.5 (8)

* Each valve represents the mean ± SE of the number of animals shown in parenthesis.

duced injury,[9,39] and the most striking change induced by the 60 days of ozone exposure was the presence of ciliated cells with uniformly short cilia. If the number of cilia per cell was not increased, then these shortened cilia may represent an adaptive decrease in surface area per ciliated cell. The cell membrane is a major target for ozone-induced injury[39] due to either oxidation of membrane lipids,[40] proteins,[41] or the formation of free radicals either directly or indirectly through lipid peroxidation.[42] A recent study of Type I cells in O_3-tolerant rats concluded that tolerance exists when the surface area of a cell exposed to a particular concentration of ozone is small enough so that existing antioxidant mechanisms contained within the cell volume can protect it from damage.[43] Although this finding was specifically addressed to tolerance, which is a high-dose phenomenon, the same principle appears to apply to adaptation such as occurred in this study.

The finding that prolonged ozone exposure did not alter the proportion or density of epithelial cell types in the trachea of rats suggests that there are significant regional differences within the respiratory tree in the response to prolonged oxidant exposure. In the pulmonary centriacinar region of both rats[14] and monkeys[15] chronically exposed to ozone, there was proliferation of Type II alveolar epithelial cells and cuboidal nonciliated cells. There was no increase in the total epithelial cell density in the anterior nasal cavity of monkeys chronically exposed to ozone, but there was an increased proportion of secretory cells.[8]

The lack of change in the number of secretory cells and the lack of change in the staining characteristics of the secretory material in the ozone-exposed rats differ from the results reported for exposures to tobacco smoke,[44] marijuana smoke,[45] and sulfur dioxide.[46] Exposure of rats to tobacco smoke and sulfur dioxide resulted in an increase in total goblet cells in the tracheal epithelium and a shift from PAS-positive to AB-positive goblet cells. Exposure to marijuana smoke resulted in an increase in the volume density of glycoprotein in the secretory cells and an increased numeric density of secretory cells stained with AB-PAS, and a shift in the secretory mucin from PAS positive to production of mixtures of PAS positive and alcian blue positive glycoproteins. At the same time, there was a decrease in the volume density of total secretory cells and the number of unstained secretory cells. The differences in results among these experiments may indicate a difference in response to ozone exposure as opposed to these other irritants. Differences in the location sampled, techniques employed, and terminology (goblet cells versus serous cells) must also be considered.

The thymidine labeling index was markedly increased after 3 days of exposure to O_3, and labeling occurred in all four categories of nuclei (basal cell nuclei, serous cell nuclei, unidentified cells with nuclei in the lower or upper half of the epithelium). The labeling index was not elevated after 60 days of exposure or in either postexposure period, and like the filtered-air controls, the labeled nuclei were either basal cell nuclei or nuclei in the lower half of the epithelium. Studies in rats and monkeys suggest that secretory cell division may be important in repair of tracheal,[47,48,13] bronchial,[49] and bronchiolar[50–52] epithelium. The finding that a decreased proportion of the labeled nuclei belonged to basal cells and an increased proportion belonged to serous cells and unidentified cells in the upper and lower half of the epithelium is in accord with recent studies of renewal of bronchial epithelium[49] that combined both light- and electron-microscopic autoradiography to allow more precise identification of labeled cells. They found that while both basal and secretory cells were labeled in control animals, the secretory cells were the ones stimulated to divide after nitrogen dioxide-induced damage.

The results of this study indicate that acute ozone-induced injury to rat tracheal epithelium is followed by adaptation to prolonged ozone exposure, and the ozone-induced epithelial change is completely repaired during a 42-day postexposure period. The adaptation to prolonged ozone exposure is not due to enhanced activity of superoxide dismutase, altered numbers or proportions of epithelial cells, an increased turnover of epithelial cells, or changes in secretory cell products. Instead, adaptation may be accomplished via a morphologic alteration in a sensitive cell population.

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