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## *In Vitro* Evidence of Cellular Adaptation to Ozone Toxicity in the Rat Trachea

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*In Vitro* Evidence of Cellular Adaptation to Ozone Toxicity in the Rat Trachea. NIKULA, K. J., WILSON, D. W., DUNGWORTH, D. L., AND PLOPPER, C. G. (1988) *Toxicol. Appl. Pharmacol.* 93, 394-402. Adaptation to prolonged ozone (O<sub>3</sub>) exposure occurs in the tracheal epithelium of rats and is marked by the presence of ciliated cells with uniform short cilia but is not accompanied by shifts in cell populations, altered characteristics of epithelial secretory cell products, increased cell turnover, or elevated tracheal superoxide dismutase activity. The purpose of this study was to test the hypothesis that adaptation is a result of alterations intrinsic to epithelial cells or to the cells and their matrix, and not due to systemic or neural influences. Rats were preexposed to either filtered air (FA) or 0.96 ppm O<sub>3</sub> for 8 hr/night for 60 days, and then their tracheae were removed and exposed to 3 ppm O<sub>3</sub> in an explant culture system where behavioral, nasal, exudative, and secretory product influences can be eliminated. After exposure to 3 ppm O<sub>3</sub> *in vitro*, quantitative electron microscopic evaluation demonstrated that the epithelia from the FA preexposure group had significantly more necrotic cells and sloughed cells and fewer ciliated cells than the epithelia from the O<sub>3</sub> preexposure group. Thus previous exposure to ozone *in vivo* induces a change in tracheal epithelium which confers resistance to ozone-induced injury in the explant exposure system. © 1988 Academic Press, Inc.

Ozone, the major oxidant in photochemical smog, is a significant air pollutant in many urban areas. There have been numerous studies of the biological effects of ozone exposure in man and experimental animals (Menzel, 1984). The effect of chronic intermittent exposure and the question of whether adaptation to prolonged oxidant exposure occurs are of particular concern relative to the human situation in urban areas.

Adaptation is a response to an altered environment so as to accommodate changes in workload or to minimize potential harmful effects. Previous morphologic evidence of adaptation to ozone exposure has been derived from evaluation of the centriacinar region of the lung in experimental animals and in-

cludes demonstration of acute ozone-induced injury followed by reduced rates of cell division, decreased numbers of inflammatory cells, and fewer cytologic alterations in epithelial cells with continued exposure (Boorman *et al.*, 1980; Eustis *et al.*, 1981). Numerous biochemical studies of lung tissue from rats, mice, and monkeys have shown acute degenerative changes such as oxidation of sulfhydryl-containing compounds and inhibition of enzyme activities due to acute high-level ozone exposures, and conversely an adaptive enhancement of reducing compounds and an increase in enzyme activities following subacute low level exposures (DeLucia *et al.*, 1972; Mustafa and Tierney, 1978; Mustafa *et al.*, 1982; Elsayed *et al.*, 1982;

Chow and Tappel, 1972; Chow *et al.*, 1974, 1979; Dubick and Keen, 1983).

A recent study in our laboratory (Nikula *et al.*, 1988) has shown adaptation (defined as diminution in cell damage during prolonged exposure) of tracheal epithelium in rats exposed to 0.96 ppm O<sub>3</sub> for 60 days. Possible mechanisms of adaptation include behavioral changes or alterations in the nasal cavity causing a decreased dose of ozone to the trachea, biochemical changes such as the induction of superoxide dismutase systems as has been reported in the lungs of oxygen-exposed (Crapo *et al.*, 1980; Freeman *et al.*, 1986) and ozone-exposed rats (Jackson and Frank, 1984), shifts in cell populations (Dungworth *et al.*, 1975), an increased epithelial turnover resulting in a new level of equilibrium between cell loss and replacement (Barry *et al.*, 1985), an increased amount or altered characteristic of secretory products, or exudation on the airway surface of albumin-rich fluid which might protect underlying tissues (Reasor *et al.*, 1979), and a decrease in the cellular surface area exposed to ozone.

Results of our recent studies indicate that adaptation of the rat tracheal epithelium 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 (Nikula *et al.*, 1988). The most striking change induced by 60 days of ozone exposure was the presence of ciliated cells with uniform short cilia. These altered ciliated cells may represent an adaptive decrease in membrane surface per ciliated cell exposed to ozone. The purpose of this study was to test the hypothesis that adaptation is a result of alterations intrinsic to epithelial cells or to the cells and their matrix, and not due to systemic or neural influences. To test this hypothesis, rats were preexposed to either filtered air or 0.96 ppm ozone for 8 hr/night for 60 days, and then their tracheae were removed and exposed to 3 ppm ozone in an explant exposure

system. In the explant exposure system, the presence or absence of local cellular adaptation can be tested because behavioral, nasal, exudative, and secretory product influences are eliminated.

## METHODS

*Animals.* Disease-free male Sprague-Dawley rats weighing 232–268 g were purchased from Bantin and Kingman (Fremont, CA), and maintained on Purina rodent laboratory chow No. 5001 and water *ad libitum*. They were serologically determined to be free of antibodies to pneumonia virus of mice, reovirus 3, Theiler's virus, Sendai virus, Kilham rat virus, H-1 virus, mouse adenovirus, lymphocytic choriomeningitis virus, rat corona virus/sialodacryoadenitis virus, and *M. pulmonis* (Microbiological Associates, Bethesda, MD). Serological monitoring of both ozone-exposed and control animals was done before and after the inhalation exposures.

*Inhalation exposures.* Ten rats were exposed to chemically and biologically filtered air (FA) for 60 days (FA preexposure group) while 10 rats were exposed to FA plus 0.96 ppm ozone (O<sub>3</sub>) 8 hr/night for 60 days (O<sub>3</sub> preexposure group). The animal housing, exposures, ozone generation, and monitoring were conducted according to standard procedures at the California Primate Research Center inhalation exposure facility (Last *et al.*, 1983). The rats were maintained with a 10-hr light and 14-hr dark cycle.

*Explant exposures.* After 60 days of either FA preexposure or O<sub>3</sub> preexposure, the rats were deeply anesthetized and their tracheae were removed and placed in exposure vessels mounted on a rocking platform in a tracheal culture system where they were intermittently exposed to an atmosphere of 95% oxygen, 5% carbon dioxide, and either 0 or 3 ppm O<sub>3</sub> (Nikula and Wilson, 1986). Three ppm O<sub>3</sub> was chosen as the test concentration in this experiment because this concentration had been shown to cause readily detectable morphologic damage (ciliated cell injury, necrosis, and sloughing of cells) to tracheal epithelium which resembled lesions induced by ozone exposure *in vivo* (Nikula and Wilson, 1986). The explant exposures were conducted with 10 tracheal explants in each of two 24-hr trials (Table 1). After the 24-hr explant exposures, the tracheae were placed in 2% glutaraldehyde fixative and 0.1 M cacodylic acid buffer (pH 7.4, 385 mOsm).

*Microscopic analysis.* Transverse sections of trachea that included cartilage ring 10 (counting rings from the carina proximally) were washed in Zetterquist's wash (Pease, 1964) and postfixed in 1% OsO<sub>4</sub> Zetterquist's solution for 2 hr at 25°C. The tissues were washed and block stained with 2% uranyl acetate overnight. The specimens

TABLE 1

TRACHEAL EXPLANT EXPOSURE TRIALS FOLLOWING EITHER OZONE OR FILTERED AIR PREEXPOSURE *IN Vivo*

Trial	60-Day preexposure <i>in vivo</i> (ppm O <sub>3</sub> )	<i>n</i> <sup>a</sup>	24-hr explant exposure (ppm O <sub>3</sub> )
I	0	3	3
	0.96	2	3
	0	3	0
	0.96	2	0
II	0	2	3
	0.96	3	3
	0	2	0
	0.96	3	0

<sup>a</sup> *n* = number of tracheae.

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 were mounted on Formvar-coated slotted copper grids. Specimens were coded and viewed randomly with a Zeiss Model EM-10 transmission electron microscope, and montages were prepared by photographic enlargement to 2720× on 5 × 7-in. prints. Cell counts were estimated by classifying the cell type of the central 100 nuclear profiles in the specimen. The numerical density (nuclei/mm) was determined by measuring the length of the corresponding basement membrane using a planimeter (MOP-3, Zeiss, Inc.).

*Statistical analysis of data.* The numerical density 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 (Kossack and Henschke, 1975; Numbercruncher statistical systems, version 3.1, Keysville, UT).

## RESULTS

Seventeen morphologic categories of normal or damaged cells were distinguished (Table 2). The undamaged ciliated, serous, basal, intermediate, brush and migratory cells were identified by standard criteria (Jeffery and Reid, 1975; Plopper *et al.*, 1983; Pearsall *et al.*, 1984). Damaged ciliated cells were cate-

gorized as those with short or damaged cilia or as degenerate, or necrotic cells. Short-cilia cells were cells with cilia of uniform but less than 2.9  $\mu$ m length, with no other evidence of injury. There were no apparent differences in the density or basal body structure of these cilia compared to 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 which measured greater than 1  $\mu$ m in observed cross section. They contained electron-dense material and resembled autophagosomes, multivesicular bodies, or residual bodies. Cells with electron-dense, condensed nuclei and extremely electron-dense or vacuolated and lucent staining 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. Necrotic cells which were located along the luminal surface of the epithelium, but not within the epithelium, were counted separately as sloughed cells. Many of these sloughed cells had features (cilia, basal bodies, nuclear characteristics) which identified them as ciliated cells, but not all sloughed cells could be identified as to cell type. After the 24-hr explant exposures no material resembling secretory product was seen still attached to the luminal surface.

The epithelia of the tracheal explants from the O<sub>3</sub> preexposure group exposed to 3 ppm O<sub>3</sub> in culture had a ciliated surface and the majority of ciliated cells were undamaged (Fig. 1). Occasional ciliated cells had uniformly short cilia, and lesser numbers of ciliated cells had irregular, broken cilia or evidence of degenerative changes. Necrotic cili-

TABLE 2

COMPARISON OF THE NUMERICAL DENSITY OF TRACHEAL EPITHELIAL CELLS IN EXPLANTS EXPOSED TO 3 ppm O<sub>3</sub> FOLLOWING PREEXPOSURE TO 0.96 ppm O<sub>3</sub> OR FILTERED AIR (FA) *IN VIVO*

Category of cell	Numerical density (cells/mm) exposure regimen			
	O <sub>3</sub> exposure <i>in vivo</i> followed by explant 3 ppm O <sub>3</sub> exposure	FA exposure <i>in vivo</i> followed by explant 3 ppm O <sub>3</sub> exposure	O <sub>3</sub> exposure <i>in vivo</i> followed by explant 0 ppm O <sub>3</sub> exposure	FA exposure <i>in vivo</i> followed by explant 0 ppm O <sub>3</sub> exposure
Total cells	213.8 ± 4.8 <sup>a</sup>	216.8 ± 18.8	203.8 ± 5.6	265.2 ± 17.1 <sub>x</sub>
Total ciliated cells	53.9 ± 5.8	10.0 ± 5.7*	38.6 ± 8.9	47.8 ± 17.4
Normal (undamaged)	46.3 ± 6.6	4.6 ± 3.2*	23.2 ± 4.9	47.0 ± 17.2
Short-cilia	3.5 ± 1.1	0.0 ± 0.0†	10.7 ± 2.4	0.0 ± 0.0 <sub>xx</sub>
Damaged-cilia	1.8 ± 0.8	4.1 ± 2.1	3.1 ± 1.6	0.0 ± 0.0
Degenerate	1.3 ± 0.8	1.2 ± 0.8	0.9 ± 0.9	0.8 ± 0.5
Necrotic	0.9 ± 0.6	0.0 ± 0.0	0.9 ± 0.9	0.0 ± 0.0
Intermediate cells	0.0 ± 0.0	0.4 ± 0.4	0.0 ± 0.0	0.4 ± 0.4
Basal cells	44.8 ± 1.6	56.7 ± 5.0	50.3 ± 2.4	50.9 ± 4.5
Total serous cells	101.8 ± 6.7	127.9 ± 14.4	104.9 ± 7.2	146.7 ± 24.3
Normal	100.4 ± 6.9	122.5 ± 14.9	102.5 ± 6.3	146.7 ± 24.3
Degenerate	1.4 ± 1.0	3.2 ± 1.5	2.4 ± 1.2	0.0 ± 0.0
Necrotic	0.0 ± 0.0	2.1 ± 0.9†	0.0 ± 0.0	0.0 ± 0.0
Brush cells	3.1 ± 0.5	0.4 ± 0.4	0.4 ± 0.4	4.0 ± 0.9
Total migratory cells	6.5 ± 3.2	14.6 ± 4.2	9.3 ± 4.8	10.9 ± 2.2
Normal	5.6 ± 2.7	11.7 ± 3.4	8.8 ± 4.6	10.9 ± 3.9
Degenerate	0.4 ± 0.4	1.2 ± 0.8	0.0 ± 0.0	0.0 ± 0.0
Necrotic	0.4 ± 0.4	1.6 ± 0.8	0.4 ± 0.4	0.0 ± 0.0
Necrotic (unidentified)	1.3 ± 0.5	5.3 ± 0.9*	0.8 ± 0.5	1.8 ± 1.1
Unidentified cells	1.4 ± 0.6	1.5 ± 1.5	0.4 ± 0.4	1.8 ± 1.1
Sloughed cells	0.0 ± 0.0	9.8 ± 2.9*	0.0 ± 0.0	0.0 ± 0.0

<sup>a</sup> Each value represents the mean ± 1 standard error, *n* = 5 animals per group.\* FA preexposure and explant 3 ppm O<sub>3</sub> exposure different from O<sub>3</sub> preexposure and explant 3 ppm O<sub>3</sub> exposure.*p* < 0.01† FA preexposure and explant 3 ppm O<sub>3</sub> exposure different from O<sub>3</sub> preexposure and explant 3 ppm O<sub>3</sub> exposure.*p* < 0.05<sub>x</sub> FA preexposure and explant 0 ppm O<sub>3</sub> exposure different from O<sub>3</sub> preexposure and explant 0 ppm exposure.*p* < 0.05<sub>xx</sub> FA preexposure and explant 0 ppm O<sub>3</sub> exposure different from O<sub>3</sub> preexposure and explant 0 ppm O<sub>3</sub> exposure.*p* < 0.01

ated cells were very rare, and necrotic cells of any type were only occasionally present. In contrast, ciliated cells were rare in the epithelia of the explants from the FA preexposure group exposed to 3 ppm O<sub>3</sub> in culture, and approximately 50% of the ciliated cells had irregular broken cilia or degenerative changes. These epithelia also exhibited more frequent necrotic cells and sloughed cells

were present only in this group (Fig. 2). No short cilia were present in these epithelia.

Quantitation of cell types and cell damage revealed several significant differences between the FA preexposure group and the O<sub>3</sub> preexposure group in the response of the tracheal epithelium to O<sub>3</sub> exposure *in vitro* (Table 2). Two of the most striking differences were in the number of necrotic cells (Fig. 3)

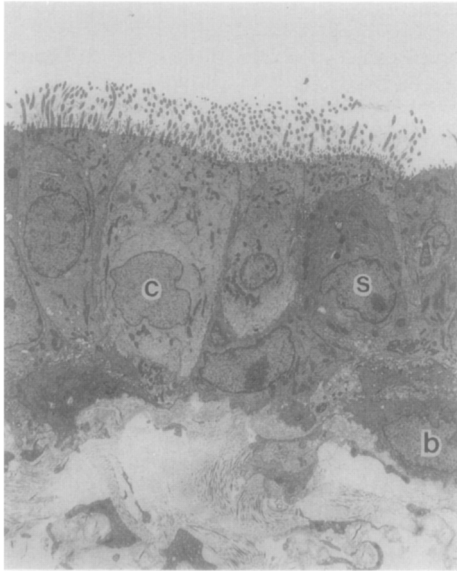


FIG. 1. Representative transmission electron micrograph of epithelium from rat preexposed to 0.96 ppm  $O_3$  for 60 days followed by explant exposure to 3 ppm  $O_3$ .  $\times 1600$ . Ciliated cells (c), serous cells (s), and basal cells (b). This epithelium is indistinguishable from that of rats preexposed to filtered air for 60 days followed by explant exposure to 0 ppm  $O_3$ . The surface is ciliated and there is no evidence of ciliary or epithelial damage.

and in the number of sloughed cells. The other major change was the significant decrease in the numerical density of ciliated cells in the epithelia from the FA preexposure group. In order to express the degree of ciliated cell damage relative to the population at risk, the ratio of abnormal ciliated cells (short-cilia, damaged-cilia, degenerate, and necrotic ciliated cells) to the total ciliated cell population was calculated (Fig. 4). This ratio is significantly greater in the FA preexposure group compared to the  $O_3$  preexposure group. The large proportion of ciliated cells damaged by the explant  $O_3$  exposure in the FA preexposed group is especially apparent when the existence of the short-cilia cells in the  $O_3$  preexposed epithelia is taken into account (Fig. 4).

The results of the two control group exposures ( $O_3$  preexposure followed by explant O

ppm  $O_3$  exposure and FA preexposure followed by explant O ppm  $O_3$  exposure) showed no significant difference between these two groups in the total number of ciliated cells, but there was a significant number of short-cilia cells in the  $O_3$  preexposure group. Occasional damaged-cilia cells were present, but their numbers were low relative to the total population of ciliated cells. Necrotic cells of any type were rare in both of these groups, and there were no sloughed cells.

## DISCUSSION

The decreased numerical density of ciliated cells in the epithelia from the FA preexposure group exposed to 3 ppm  $O_3$  in culture is probably due to necrosis and sloughing of ciliated cells. The degenerate changes in ciliated cells, the increased density of unidentified necrotic cells, the presence of sloughed cells only in this group, and the lack of increase in unidentified cells support this conclusion. If the decreased density was simply due to the ciliated cells losing their cilia thus becoming difficult to identify, then the unidentified population would be expected to increase. The fact that the total number of sloughed cells does not equal the total decrease in ciliated cells is not surprising since the majority of exfoliated cells would be lost into the culture medium rather than remaining adherent to the epithelial surface.

Several factors, both local and systemic, have been proposed to account for the resistance to ozone-induced injury which follows extended ozone exposure. In this experiment, the tracheal epithelium from animals previously exposed to ozone or filtered air was isolated from the whole animal, thus removed from systemic and neural influences, and then exposed to ozone. The results suggest that previous exposure to ozone induced a change in the tracheal epithelium which confers cellular resistance to ozone-induced

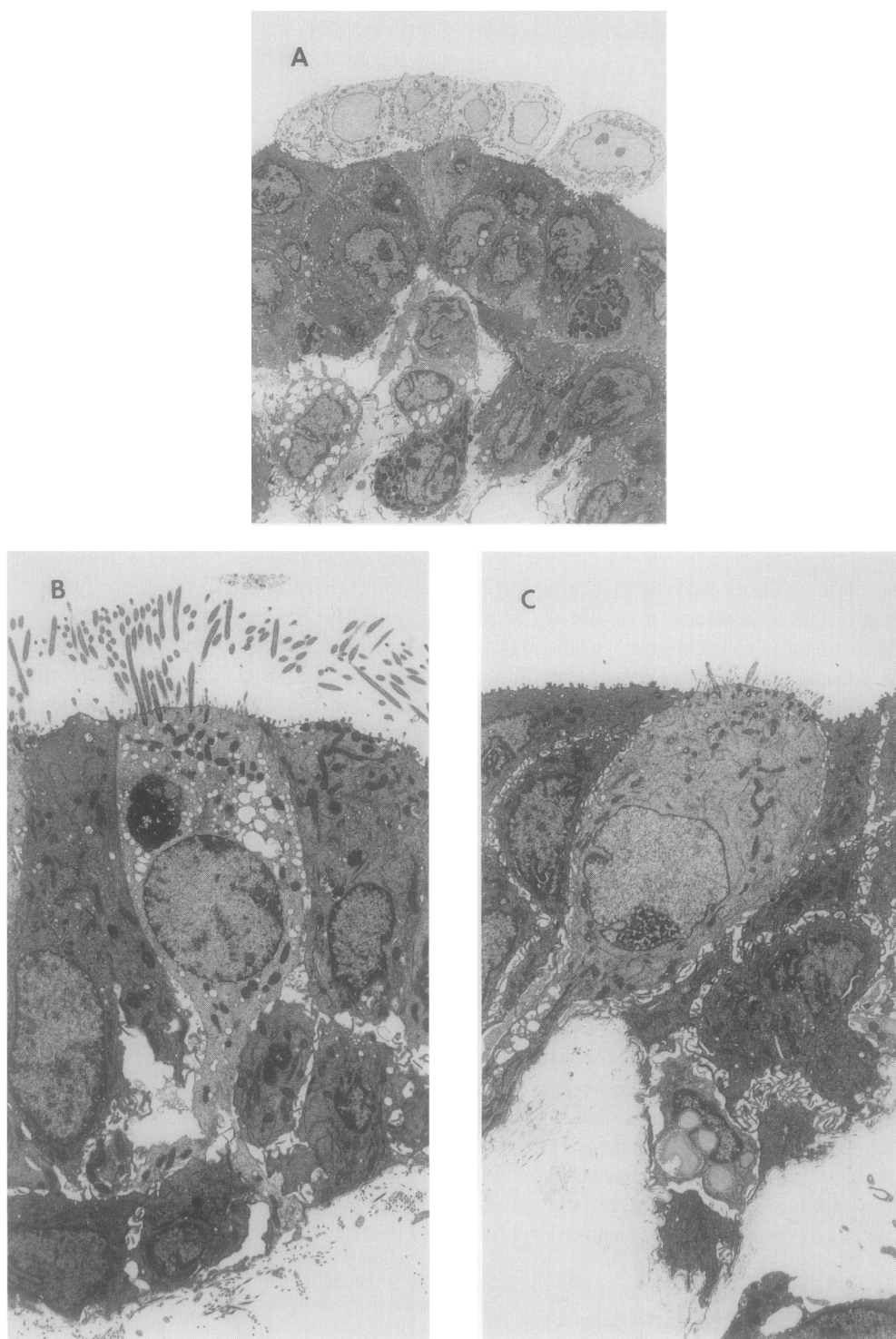


FIG. 2. Representative transmission electron micrographs of epithelia from rats preexposed to filtered air for 60 days followed by explant exposure to 3 ppm  $O_3$ . (A) The epithelium lacks ciliated cells. Most of the epithelial cells are serous cells. There are six sloughed cells on the luminal surface.  $\times 1600$ . (B) Severely degenerated ciliated cell (center).  $\times 2944$ . (C) Damaged-cilia cell (center).  $\times 2944$ .

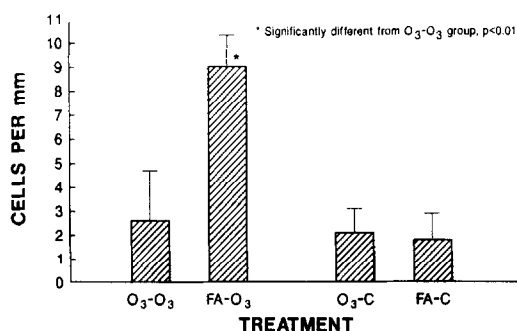


FIG. 3. Total necrotic cells per millimeter. Bar graph represents mean + 1 standard error,  $n = 5$  tracheae per exposure group. O<sub>3</sub>-O<sub>3</sub>: O<sub>3</sub> preexposure followed by explant O<sub>3</sub> exposure. FA-O<sub>3</sub>: Filtered air preexposure followed by explant O<sub>3</sub> exposure. O<sub>3</sub>-C: O<sub>3</sub> preexposure followed by control explant (O ppm O<sub>3</sub>) exposure. FA-C: Filtered air preexposure followed by control explant (O ppm O<sub>3</sub>) exposure.

injury. The explant exposures exclude the possibilities that adaptation of the tracheal epithelium is due to behavioral modification by the animal, altered nasal absorption of O<sub>3</sub>, or changes in inflammatory mediators or exudates from more distal airways. The adaptive response must be a local phenomenon intrinsic to the epithelium, the glands, or associated connective tissue. Integration of the results of our recent inhalation exposure study and this combined inhalation and explant exposure study allows several conclusions to be drawn concerning adaptation of the tracheal epithelium to prolonged ozone exposure. Tracheae from rats exposed to O<sub>3</sub> for 60 days *in vivo* did not have evidence of increased epithelial turnover detectable by thymidine labeling, nor was there increased superoxide dismutase activity. Carbohydrate histochemistry and quantitation of epithelial cell density indicate that neither shifts in cell populations nor altered characteristics of secretory products account for adaptation (Nikula *et al.*, 1988). The results of this explant exposure study suggest that increased amounts or altered characteristics of secretory material do not account for adaptation, since it is likely that, due to the culture condi-

tions, secretory material would be washed-off the tracheal epithelium and diluted by the culture medium, and thus would not cover and protect the underlying epithelium. A similar argument can be used to refute the hypothesis that adaptation is mainly due to a protein-rich exudate from the submucosa, which coats the epithelium.

Among the various tracheobronchial cell types, ciliated cells are commonly considered the most sensitive to ozone-induced injury (Dungworth *et al.*, 1975; Menzel, 1984), and the cell membrane is a major target for ozone-induced injury (Menzel, 1984) either due to oxidation of membrane lipids (Menzel, 1970), proteins (Mudd and Freeman, 1977), or due to the formation of free radicals either directly or indirectly through lipid peroxidation (Pryor *et al.*, 1983). The most striking change in the rat tracheal epithelium induced by 60 days of ozone exposure *in vivo* was the presence of ciliated cells with uniform short cilia (Nikula *et al.*, 1988). If the number of cilia per cell did not increase, then these altered ciliated cells may represent an adaptive decrease in surface area per ciliated cell. The cellular adaptation could also be due to enhanced antioxidant mechanisms, particu-

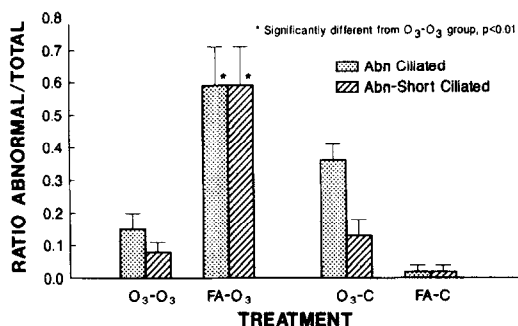


FIG. 4. Ratio of abnormal ciliated cells/total ciliated cells. Bar graph represents mean + 1 standard error,  $n = 5$  animals per exposure group. Abn ciliated = (short-cilia + damaged-cilia + degenerate + necrotic ciliated cell density)/total ciliated cell density. Abn-short ciliated = (damaged-cilia + degenerate + necrotic ciliated cells)/total ciliated cell density. O<sub>3</sub>-O<sub>3</sub>, FA-O<sub>3</sub>, O<sub>3</sub>-C, FA-C as for Fig. 3.



larly glutathione and the glutathione peroxidase enzyme system. Glutathione (GSH) has been shown to be important in the defense of cultured alveolar type II cells (A549 cell-line) against the toxic action of ozone during short-term (2.5 hr) exposures (Rietjens *et al.*, 1985). In rats exposed to O<sub>3</sub> for several days, increased activities of lung GSH peroxidase, GSH reductase, and glucose-6-phosphate (G-6-P) dehydrogenase have been observed, leading to the proposal that the increased activity of GSH peroxidase catabolizes lipid peroxides formed during ozone exposure, while the increased activities of GSH reductase and G-6-P dehydrogenase provide increased levels of GSH and NADPH, respectively (Chow and Tappel, 1972). The association between the short cilia, which are the morphologic correlate of tracheal adaptation to ozone exposure, and the antioxidant enzyme systems and perhaps alterations in the glycocalyx or matrix need to be further explored.

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