

HEALTH EFFECTS INSTITUTE

Consequences of Prolonged Inhalation of Ozone on F344/N Rats: Collaborative Studies

Part VII: Effects on the Nasal Mucociliary Apparatus

Jack R. Harkema, Kevin T. Morgan, Elizabeth A. Gross,
Paul J. Catalano, and William C. Griffith

*Inhalation Toxicology Research Institute, Lovelace Biomedical and
Environmental Research Institute, Albuquerque, NM, Chemical Industry
Institute of Toxicology, Research Triangle Park, NC, and Dana-Farber
Cancer Institute, Boston, MA*

Research Report Number 65

November 1994

**Includes the Commentary of the Institute's
Health Review Committee**

HEI HEALTH EFFECTS INSTITUTE

The Health Effects Institute, established in 1980, is an independent and unbiased source of information on the health effects of motor vehicle emissions. HEI studies all major pollutants, including regulated pollutants (such as carbon monoxide, ozone, nitrogen dioxide, and particulate materials) and unregulated pollutants (such as diesel engine exhaust, methanol, and aldehydes). To date, HEI has supported more than 120 projects at institutions in North America and Europe.

Typically, HEI receives half its funds from the U.S. Environmental Protection Agency and half from 28 manufacturers and marketers of motor vehicles and engines in the United States. Occasionally, revenues from other public or private organizations either support special projects or provide resources for a portion of an HEI study. For this study, the Institute acknowledges the cooperation and support of the National Toxicology Program (NTP), which consists of four charter agencies of the U.S. Department of Health and Human Services. The NTP sponsored the inhalation component of this project as part of its studies on the toxicologic and carcinogenic effects of ozone. However, in all cases HEI exercises complete autonomy in setting its research priorities and in disbursing its funds. An independent Board of Directors governs the Institute. The Research Committee and the Review Committee serve complementary scientific purposes and draw distinguished scientists as members. The results of HEI-funded studies are made available as Research Reports, which contain both the Investigators' Report and the Review Committee's evaluation of the work's scientific and regulatory relevance.

HEI Statement

Synopsis of Research Report Number 65 Part VII

Prolonged Ozone Exposure Leads to Functional and Structural Changes in the Rat Nose

Background

Ozone, a common outdoor air pollutant is a highly reactive gas and a major component of smog. The U.S. Environmental Protection Agency (EPA) has set a National Ambient Air Quality Standard for ozone of 0.12 parts per million [ppm] that should not be exceeded for more than one hour, once per year. This standard is based largely on scientific data documenting the effects of short-term exposure on lung function in humans. The standard is currently being reevaluated by the EPA.

Because ozone can damage cells, prolonged or repeated exposures may be a risk factor for lung cancer. To assess this issue, the National Toxicology Program (NTP) conducted an animal bioassay to evaluate ozone's carcinogenicity in rodents. Another public health concern is that prolonged exposure to ozone may damage the cells that line the airways, leading to functional changes in the components of the respiratory tract.

The nose is the first line of defense against inhaled pathogens, dusts, and irritant gases; thus, changes induced by ozone in the normal functions of the nose could result in an increased susceptibility to respiratory infections and other diseases. Mucous flow is critical to a defense mechanism called mucociliary clearance. Inhaled pathogens or irritants are trapped in mucus, which is removed (or cleared) by the beating of cilia, which are tiny hair-like projections on cells that line the airways and extend into the mucous layer. Short-term exposure to high concentrations of ozone is known to damage the epithelial cells that line the nasal passages of laboratory rats; however, the effects of prolonged ozone exposures are not known, nor is there information on the impact of such exposures on nasal function. Dr. Jack Harkema's study, which was one of eight studies in the NTP/HEI Collaborative Ozone Project, was conducted to address these issues. Other studies in this project investigated possible changes in lung function, structure, and biochemistry, and are being published as other Parts of Research Report Number 65.

Approach

Drs. Harkema and Morgan and their colleagues used a video recording technique to measure the speed of mucous flow in different regions of the nasal cavities of rats exposed to 0, 0.12, 0.50, or 1.0 ppm ozone for six hours per day, five days per week, for 20 months. The investigators used specific stains and a technique called image analysis to determine the effect of ozone exposure on mucous content, and light and electron microscopy to study cellular changes in the epithelial cell layer.

Results and Implications

The investigators found that the effects of ozone on both nasal function and structure in laboratory rats were dependent on dose. Mucous flow rates decreased in nasal tissue isolated from rats exposed to 0.5 and 1.0 ppm ozone. Exposure to these concentrations of ozone also caused an increase in the number of cells that produce mucus and in the amounts of mucous components stored within the cells. No effects on nasal function or structure were observed after prolonged exposure to 0.12 ppm ozone. The investigators did not report the presence of carcinogenic cell transformations after exposure to any ozone concentration.

Although an increase in mucous production, such as was seen in this study, may reflect an adaptive mechanism that protects the underlying cells from injury induced by ozone, a decrease in mucous flow rate could lead to a reduction in mucociliary clearance. The agreement between threshold doses of ozone that caused injury by both functional and structural assessments strengthens the conclusions of this study regarding the toxicity of ozone to the nose in this strain of rat. These findings, while provocative, are difficult to extrapolate to humans because of marked differences in the nasal structures of rodents and humans.

This Statement, prepared by the Health Effects Institute and approved by its Board of Directors, is a summary of a research project sponsored by HEI from 1991 to 1994. The inhalation component of this project was supported by the National Toxicology Program as part of its studies on the toxicologic and carcinogenic effects of ozone. This study was conducted by Dr. Jack R. Harkema of the Inhalation Toxicology Research Institute. The following Research Report contains both the detailed Investigators' Report and a Commentary on the study prepared by the Institute's Health Review Committee.

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Library of Congress Catalog Number for the HEI Research Report Series: WA 754 R432.

The paper in this publication meets the minimum standard requirements of the ANSI Standard Z39.48-1984 (Permanence of Paper) effective with Report Number 21, December 1988, and with Report Numbers 25, 26, 32, 51, and 65 Part IV excepted. Reports 1 through 20, 25, 26, 32, 51, and 65 Part IV are printed on acid-free coated paper.

TABLE OF CONTENTS

Research Report Number 65 Part VII

Consequences of Prolonged Inhalation of Ozone on F344/N Rats: Collaborative Studies Part VII: Effects on the Nasal Mucociliary Apparatus

Jack R. Harkema, Kevin T. Morgan, Elizabeth A. Gross, Paul J. Catalano,
and William C. Griffith

| | |
|--|-----------|
| I. HEI STATEMENT Health Effects Institute. | i |
| The Statement, prepared by the HEI and approved by the Board of Directors, is a nontechnical summary of the Investigators' Report and the Health Review Committee's Commentary. | |
| II. INTRODUCTION The National Toxicology Program and Health Effects Institute Collaborative Ozone Project. | 1 |
| III. INVESTIGATORS' REPORT Jack R. Harkema et al. | 3 |
| When an HEI-funded study is completed, the investigators submit a final report. The Investigators' Report is first examined by three outside technical reviewers and a biostatistician. The Report and the reviewers' comments are then evaluated by members of the HEI Health Review Committee, who had no role in the selection or management of the project. During the review process, the investigators have an opportunity to exchange comments with the Review Committee, and, if necessary, revise the report. | |
| Abstract | 3 |
| Introduction | 3 |
| Specific Aims | 5 |
| Methods | 5 |
| Animals, Maintenance, and Exposures | 5 |
| Assessment of Nasal Mucociliary Function | 6 |
| Statistics for Mucous Flow Data | 7 |
| Morphologic and Morphometric Assessments of the Nasal Mucosa | 7 |
| Statistics for Morphometric Data | 10 |
| Results | 10 |
| Function of the Nasal Mucociliary Apparatus | 10 |
| Histopathology of the Nasal Mucosa | 12 |
| Morphometry of Intraepithelial Mucosubstances | 15 |
| Ultrastructural Morphology in Nasal Transitional Epithelium | 16 |
| Morphometry of Nasal Transitional Epithelium | 17 |
| Ultrastructural Morphology of Respiratory Epithelium in the Nasal Septum | 18 |
| Morphometry of Respiratory Epithelium of the Nasal Septum | 19 |
| Discussion and Conclusions | 19 |
| Acknowledgments | 23 |
| References | 23 |
| Appendix A. Identification of Specific Animals in Exposure Groups | 25 |
| About the Authors | 25 |
| Publications Resulting from This Research | 26 |
| Abbreviations | 26 |
| IV. COMMENTARY Health Review Committee | 27 |
| The Commentary on the Investigators' Report is prepared by the HEI Health Review Committee and staff. Its purpose is to place the study into a broad scientific context, to point out its strengths and limitations, and to discuss the remaining uncertainties and the implications of the findings for public health. | |
| Introduction | 27 |
| Regulatory Background | 27 |
| Scientific Background | 28 |
| Justification for the Study | 29 |
| Technical Evaluation | 29 |
| Implications for Future Research | 32 |
| Conclusions | 32 |
| Acknowledgments | 33 |
| References | 33 |
| V. RELATED HEI PUBLICATIONS | 35 |

INTRODUCTION

The National Toxicology Program and Health Effects Institute Collaborative Ozone Project

The NTP/HEI Collaborative Ozone Project was a four-year project with many investigators that was organized to evaluate the effects of prolonged ozone exposure on lung injury in animals. The ozone exposures were conducted by the National Toxicology Program (NTP) at Battelle Pacific Northwest Laboratories. The individual investigators' studies, which addressed the pathologic and physiologic consequences of prolonged ozone exposure, were supported by the Health Effects Institute (HEI). A full description of the NTP/HEI Collaborative Ozone Project and the exposure protocol can be found in the Introduction and Supplement to Research Report Number 65 Part I. This information also will be published in Part VI of Research Report Number 65 that describes the exposure and distribution of the animals.

Briefly, in 1987, the Health Effects Institute entered into a partnership with the National Toxicology Program to evaluate the effects of chronic ozone exposure in rats. The NTP, consisting of four agencies of the U.S. Department of Health and Human Services, coordinates the nation's testing of potentially toxic and hazardous chemicals. The Health Effects Institute, an independent research organization supported by both government and industry, provides unbiased information on the health effects of motor vehicle emissions.

Because of the widespread exposure to ozone and concerns about its potential health effects, HEI and the California Department of Health and Human Services nominated ozone for carcinogenicity and toxicity testing by the NTP. The NTP, recognizing that cancer was only one of the chronic diseases of concern, included additional animals for HEI-supported studies of the pathologic and physiologic consequences of prolonged ozone exposures. The HEI animals were housed in cages that would otherwise have been empty. By developing a partnership, the HEI and NTP were able to leverage their funds to develop a comprehensive research program that extended beyond carcinogenicity endpoints; the HEI-sponsored research focused on the relation between long-term ozone exposure and the pathogenesis of chronic lung diseases, such as asthma, emphysema, and fibrosis. The Health Effects Institute would not have been able to undertake such an expensive project, which requires special facilities and trained personnel, without the NTP's support of the inhalation component and the cooperation of the NTP's contractor, Battelle Pacific Northwest Laboratories.

For the HEI component of the Project, eight studies were selected for funding from proposals submitted in response to the Request for Applications (RFA) 90-1, Health Effects of Chronic Ozone Inhalation: Collaborative National Toxicology Program-Health Effects Institute Studies, Part A: Respiratory Function Studies, and Part B: Structural, Biochemical, and Other Alterations. Because of the complexity of a project with many investigators and many endpoints, the HEI Health Research Committee also funded a Biostatistical Advisory Group to provide assistance with experimental design, animal allocation, and data analyses. Figure 1 presents a diagram of the studies in the NTP/HEI Collaborative Ozone Project and their relations to each other. They include those studies that were part of the NTP bioassay, the eight HEI-funded studies, and the biostatistical study. In addition, HEI engaged Battelle Pacific Northwest Laboratories to provide support services for the HEI-sponsored investigators.

Starting at six to seven weeks of age, male and female F344/N rats were exposed to 0, 0.12, 0.5, or 1.0 parts per million (ppm) ozone, six hours per day, five days per week. These concentrations were selected to include the maximum concentration the animals would tolerate (1.0 ppm), the current National Ambient Air Quality Standard (NAAQS) for ozone (0.12 ppm), and an intermediate concentration. The NTP's carcinogenicity bioassay consisted of a two-year study and a lifetime study in rats and mice, and a study of male rats exposed to 0.5 ppm ozone and two levels of a human pulmonary carcinogen, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK). The design of the HEI studies was directed, to some extent, by the constraints of the NTP protocol. These included ozone exposure concentrations that were set by the NTP, a limit on the sample size (164 rats) to the number of available exposure chambers, and quarantine restrictions that did not allow reentry of animals into the exposure chambers once they had been removed, thus eliminating the possibility of conducting serial tests.

The Biostatistical Advisory Group developed a sample allocation scheme that allowed several researchers to obtain measurements on tissue samples from the same subset of study animals, providing the maximum overlap of animals and tissues among the eight studies while ensuring balance with respect to dose, gender, and time of death. When the ozone exposure of the HEI animals ended (at 20

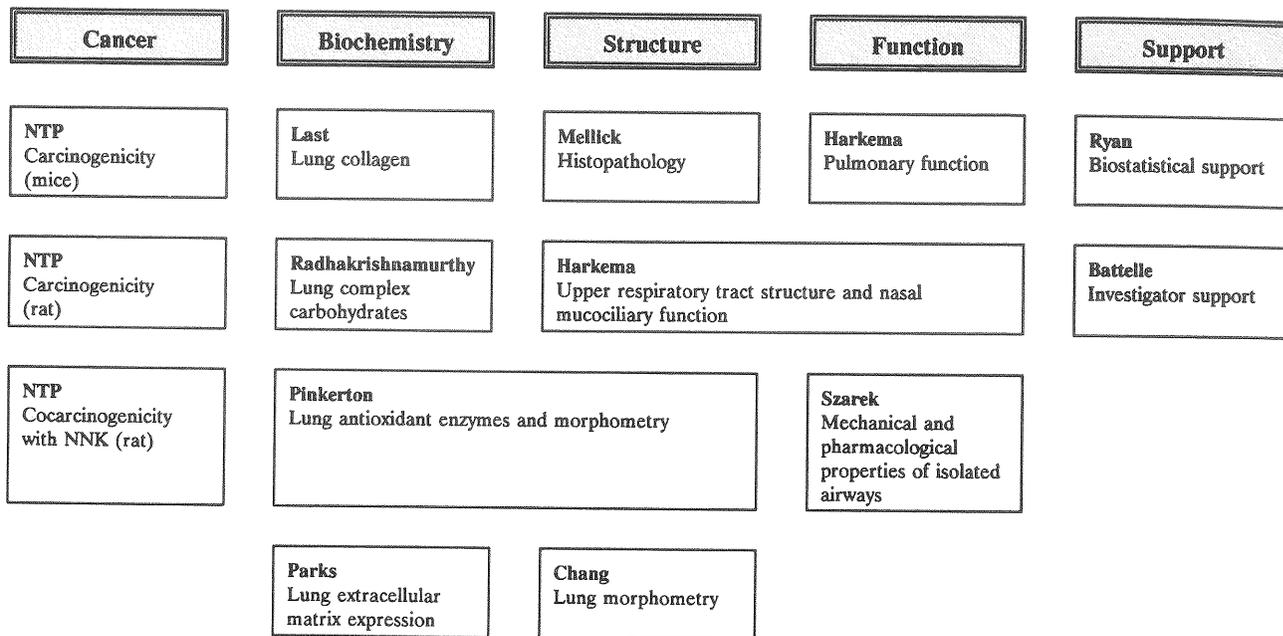


Figure 1. The NTP/HEI Collaborative Ozone Project: individual studies.

months), several investigators traveled to Battelle Pacific Northwest Laboratories to conduct their assays or to obtain samples on site. Battelle personnel prepared the tissues for off-site investigators and shipped them directly to their laboratories.

Because the studies varied in duration from six months to two years, HEI is publishing the reports for each individual study after the Institute's review process for each study is complete. Each Investigator's Report and a forthcoming Integrative Summary Report will be Parts of Report Number 65 of the HEI Research Report series. The present study by Dr. Jack Harkema and colleagues of the effects of long-term ozone exposure on the nasal mucociliary apparatus is Part VII. Other investigators in the Collaborative Ozone Project examined the effects of ozone on lung function (Harkema), airway reactivity (Szarek), and structural (Pinkerton, Chang) or biochemical (Last, Radhakrishnamurthy, and Parks) alterations.

Although some conclusions can be drawn on the basis of the results from each individual study, the interpretation of Dr. Harkema's findings will be strengthened when those data are correlated with the outcomes of the other investigators.

The importance of the collaborative NTP and HEI chronic ozone exposure studies is that they provide an unparalleled opportunity to examine the effects of prolonged ozone exposure using a variety of scientific approaches. The interaction of a number of methods to analyze the pathologic and physiologic consequences of chronic ozone exposure is one of this project's unique features. The results of these studies will provide new information about the threshold effects of ozone exposure on lung injury and the type and extent of damage in a well-established animal model. These results may be helpful for evaluating current standards of ozone exposure as they apply to human health and for designing future animal and human studies.

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ABSTRACT

Besides the centriacinar region of the lung, the nose is a principal target for ozone toxicity. Acute exposures to concentrations of ozone in ambient air induce secretory cell metaplasia in the nasal transitional epithelium of rats. This study examined the effects of chronic ozone exposure on the structure and function of the nasal mucociliary apparatus of the rat. Male and female F344/N rats were exposed to ozone concentrations of 0.0 (controls), 0.12, 0.5, or 1.0 parts per million (ppm)*, six hours per day, five days per week, for 20 months. All rats were killed seven or eight days after the end of the exposure. Immediately after death, mucous flow rates throughout the nasal passages were determined using in vitro video motion analysis. Following assessment of mucociliary function, the nasal tissues were processed for light microscopy and stained with Alcian blue (pH 2.5)/periodic acid-Schiff to detect intraepithelial mucus. Image analysis was used to quantitate the amount of mucus within the nasal transitional epithelium. In rats exposed to 0.5 or 1.0 ppm ozone, mucous flow rates were markedly slower over the lateral wall and turbinates of the proximal third of the nasal airways than they were in rats exposed to 0.0 or 0.12 ppm ozone. These intranasal regions in the rats exposed to 0.5 or 1.0 ppm ozone contained marked mucous cell metaplasia and 25 to 300 times

more mucus in nasal transitional epithelium than was found in control rats. In addition, male and female rats exposed to 0.5 or 1.0 ppm ozone had marked epithelial hyperplasia in nasal transitional epithelium, increases in eosinophilic globules in the surface epithelium lining the distal nasal airways, and a mild to moderate inflammatory cell influx in the nasal mucosa in the proximal and middle nasal passages. Male rats also had conspicuous bony atrophy in maxilloturbinates and nasoturbinates. There were no significant decreases between the mucous flow rates of rats exposed to 0.12 ppm ozone and those of control rats. There were, however, mild increases in various flow rates in some areas of the nasal airways in rats exposed to 0.12 ppm ozone compared with control rats. No significant morphologic alterations were evident in the rats exposed to 0.0 or 0.12 ppm ozone. The results of this study indicate that rats chronically exposed to 0.5 or 1.0 ppm ozone have significant alterations in the function and structure of the nasal mucociliary apparatus. Though there was a mild increase in mucous flow rates in a few nasal regions of some rats exposed to 0.12 ppm ozone, this functional change was interpreted as a physiologic, rather than a pathologic, response to ozone at this relatively low concentration.

INTRODUCTION

Ozone is an irritating oxidant gas and the principal deleterious agent in photochemical smog. Among the major air pollutants for which National Ambient Air Quality Standards (NAAQS) have been designated under the Clean Air Act, ozone currently presents the most pervasive problem (Steinfeld 1991). The U.S. Environmental Protection Agency has estimated that 13 million healthy adults are exposed to ozone at concentrations in excess of the NAAQS (0.12 ppm) for at least one hour each week during the summer (Paul et al. 1987). The episodic high concentrations of ozone in large metropolitan areas like Los Angeles, CA, and Mexico City, Mexico, pose significant threats to the health of their inhabitants. The current NAAQS of 0.12 ppm ozone is being questioned because documented studies indicate that pulmonary function is impaired in exercising children and adults exposed to ozone concentrations

* A list of abbreviations appears at the end of the Investigators' Report.

This Investigators' Report is one section of Part VII of Health Effects Institute Research Report Number 65, which also includes an Introduction to the NTP/HEI Collaborative Ozone Project, a Commentary by the Health Review Committee on the Investigators' Report, and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. Jack Harkema, Room A47, VMC, Department of Pathology, Michigan State University, East Lansing, MI 48824-1314.

This study was supported by HEI funds from the U.S. Environmental Protection Agency and the motor vehicle industry. The inhalation component of this project was sponsored by the National Toxicology Program as part of its studies on the toxicologic and carcinogenic effects of ozone.

Although this document was produced with partial funding by the U.S. Environmental Protection Agency under Assistance Agreement 816285 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the view of the Agency, and no official endorsement should be inferred. The contents of this document also have not been reviewed by private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

below or slightly above this standard (Lippmann et al. 1983; McDonnell et al. 1983; Avol et al. 1984, 1985; Gibbons and Adams 1984; Kulle et al. 1985; Liroy et al. 1985; Gong et al. 1986; Linn et al. 1986; Spektor et al. 1988a,b). Furthermore, laboratory animals have structural lesions in the upper and lower airways after repeated exposures to low ambient concentrations (0.12 to 0.3 ppm) of this oxidant gas (Plopper et al. 1979; Boorman et al. 1980; Barry et al. 1985; Harkema et al. 1987a,b, 1989, 1993).

Numerous studies have characterized the ozone-induced lesions in the lungs of several laboratory animal species (Dungworth et al. 1975; Schwartz et al. 1976; Plopper et al. 1979; Boorman et al. 1980; Castleman et al. 1980; Barry et al. 1985; Fujinaka et al. 1985; Moffatt et al. 1987; Barr et al. 1988; Harkema et al. 1993), but only recently have studies been designed specifically to examine the effects of ozone on the upper airways (Harkema et al. 1987a,b, 1989; Hotchkiss et al. 1989; Johnson et al. 1990). In both the rat (Harkema et al. 1989) and the monkey (Harkema 1987a,b), the nasal airway epithelium is a principal target for ozone toxicity after acute and subchronic exposures.

Harkema and colleagues (1987a) demonstrated that monkeys exposed to ambient concentrations of ozone for 6 or 90 days, eight hours per day, had damage to epithelial cells in the nasal transitional epithelium and the respiratory epithelium in the proximal aspects of the nose. Nasal epithelial lesions were characterized by a marked increase of mucous cells in both the nasal transitional epithelium (a nonciliated, stratified, cuboidal epithelium that normally has few mucous cells) and the respiratory epithelium (a ciliated, pseudostratified epithelium with numerous mucous cells). The degree of this change did not depend on the concentration of the ozone (that is, 0.15 versus 0.3 ppm), but on the duration of the exposure (there were more mucous cells after 90 days of exposure than after 6 days of exposure). Ozone-induced epithelial alterations were also evident in the respiratory bronchioles within the lungs of these same monkeys (Harkema et al. 1993). In the nasal transitional epithelium of ozone-exposed monkeys, there were 15% to 20% more nonciliated cells with secretory granules than were in that of air-exposed (0 ppm ozone) control monkeys. In addition, the amount of mucosubstances within the nasal transitional epithelium increased dramatically after ozone exposure. For example, there was 300% more mucous material in the nasal transitional epithelium of monkeys exposed to 0.15 ppm ozone for six days (eight hours per day) than in that of air-exposed controls (Harkema et al. 1987b). Accompanying the increase of mucous cells in the nasal passages of ozone-exposed monkeys were ciliated cell necrosis (after 6 and 90 days of exposure),

attenuation of ciliary length (after 6 and 90 days of exposure), and inflammatory cell influx (only after 6 days of exposure; acute rhinitis).

These nasal epithelial alterations induced by ozone may indicate alterations in normal physiologic functions (for example, mucociliary clearance) that are important upper respiratory tract mechanisms for defending the lungs against excessive burdens of harmful agents. This type of nasal damage could also increase susceptibility to acute infections of the upper respiratory tract. The findings of this study had added importance because of their presence in a species of nonhuman primate whose nasal airways resemble, at gross and microscopic levels, those of humans (Tyler 1983).

In 1989, Harkema and associates reported that F344/N rats exposed for seven days to 0.8 ppm ozone, six hours per day, developed mucous cell metaplasia (numerous mucous cells in an epithelium normally devoid of these cells) in the nasal transitional epithelium lining the maxilloturbinates, lateral wall, and lateral aspects of the nasoturbinates in the proximal nasal passages. The ozone-induced alterations in the F344/N rats resembled those that were experimentally induced in the nasal airways of bonnet monkeys. There were, however, some noticeable differences. The increase in mucous cells in the rat nasal airways was restricted to the nasal transitional epithelium, which in the rat is only one to two cells thick and usually contains no secretory cells and only a few widely scattered ciliated cells (Harkema 1991). In the monkey, however, the nasal transitional epithelium is stratified (four to six cells thick), with few ciliated cells, widely scattered mucous cells, and other secretory cells (Harkema et al. 1987c). Therefore, the ozone-induced alteration of nasal epithelium in the rat was a true metaplastic response that differed from the hyperplastic response (increase in the number of mucous cells in an epithelium that normally contains some mucous cells) induced in monkeys after ozone exposure. Furthermore, no epithelial alterations were evident in the nasal respiratory epithelium of the rat, unlike that of the monkey. Finally, no metaplastic alterations were evident in the nasal transitional epithelium of rats exposed to 0.12 ppm ozone, six hours per day, for seven days. This was in contrast to the conspicuous mucous cell hyperplasia that was induced in monkeys after exposure to 0.15 ppm ozone for six days (eight hours per day). These results suggest that the rat nasal transitional epithelium may not be as sensitive to acute ozone-induced injury as the monkey nasal transitional epithelium.

Though there is now substantial information on the effects of acute exposures to ozone on the nasal mucosa of

rats and monkeys, nasal alterations induced by long-term exposures (for example, months to years) have not been determined. Recent histopathologic studies of nasal airways in people who live in Mexico City, which has high ambient air concentrations of ozone and other air pollutants, suggest that these urban dwellers have substantially more lesions in their nasal mucosa than do people of similar age and gender living in rural Mexico, where there is little air pollution (Calderon-Garciduenas et al. 1992). A better understanding of the potential long-term effects of ozone on nasal airways would be gained by studies of animals repeatedly exposed for most of their life span to various concentrations of ozone.

Primarily on the basis of previous inconclusive data from mice suggesting that ozone may act as a carcinogen or cocarcinogen (Witschi 1988), the National Toxicology Program (NTP) sponsored a bioassay of chronic ozone inhalation using rats and mice. The Health Effects Institute (HEI) worked with NTP to complement the standard bioassay by adding collaborative studies focused on various health effects endpoints. These studies made multiple use of a limited number of rats exposed for 20 months to ozone.

The present study was included in the joint NTP/HEI Collaborative Ozone Project to determine the structure and function of the nasal mucociliary apparatus of the rat after 20 months of exposure to ozone at three concentrations, including the current NAAQS of 0.12 ppm. The function of the nasal mucociliary apparatus of the rat was studied using video motion analysis, and the structure of the rat nasal mucosa was analyzed by light and transmission electron microscopy. The ability to assess the function of the nasal mucociliary apparatus by video motion analysis is well developed (Morgan et al. 1984a), and this assay has proved sensitive to toxicant-induced nasal injury (Morgan et al. 1986a,b). Methods for morphometric analysis of the rat nasal mucosa after acute ozone exposures are also well established (Harkema et al. 1987a,b, 1989). The purpose of the present study was to determine whether or not chronic exposure to ozone would significantly alter the normal morphology of the nasal mucosa in rats, to characterize the extent and severity of the toxicant-induced lesions, and to determine whether or not ozone-induced structural alterations coexist with deleterious changes in the function of the nasal mucociliary apparatus in these same rats.

SPECIFIC AIMS

This research was conducted in response to the HEI Request for Applications No. 90-1 Part B, "Health Effects of Chronic Ozone Inhalation: Collaborative NTP/HEI Studies: Structural, Bio-

chemical, and Other Alterations." This request solicited collaborative studies of the health effects of 20-month exposures of rats to ozone, aimed at complementing the standard evaluations included in an NTP-sponsored cancer bioassay.

The objectives of this research were to determine the nature and magnitude of the morphologic alterations in the rat nasal mucosa that occur after chronic ozone inhalation. We qualitatively and quantitatively characterized ozone-induced morphologic and ultrastructural alterations of the nasal epithelium using state-of-the-art image analysis and morphometric techniques. We also characterized the effects of chronic ozone exposure on the function of the nasal mucociliary apparatus and correlated these functional alterations with the ozone-induced structural changes in the nasal surface epithelium. The effects of chronic ozone exposure on the function of the nasal mucociliary apparatus, including patterns and rates of mucous flow in different intranasal regions, were determined, as mentioned above, using video motion analysis of the luminal surface of the nasal airways *in vitro*. The specific aims of this research were to provide functional and structural data from the rat that characterize the magnitude and consequence of upper respiratory tract injury resulting from prolonged ozone exposure; and to provide morphometric data characterizing the ozone-induced alterations in the nasal epithelium that could be correlated with ozone-induced structural alterations in the airway epithelium lining the lower respiratory tract of the same or similarly exposed rats.

METHODS

ANIMALS, MAINTENANCE, AND EXPOSURES

Male and female F344/N rats (Simonsen Laboratories, Gilroy, CA) were randomly assigned at four to five weeks of age to control or ozone-exposed groups after a 10- to 14-day quarantine period. The rats were housed during the exposures in individual wire cages within 2.0-m³ inhalation exposure chambers (H2000, Hazleton Systems, Aberdeen, MD). The animal maintenance and observation procedures were standard for an NTP-sponsored inhalation bioassay. In brief, the chambers were maintained at approximately 24°C, 59% relative humidity, and a flow rate providing 10 air changes per hour. The exposure rooms were lighted on a 12-hour cycle (from 0600 to 1800). Untreated paper cage board beneath the cages was changed twice daily, and chambers were washed weekly. The rats were provided with a pelleted ration (NIH-07, Zeigler Bros., Gardner, PA) *ad libitum* outside exposure hours and with water *ad libitum* at all times.

Groups of rats were exposed six hours per day (beginning at approximately 0730), five days per week (Monday through Friday), for 20 months, to ozone at 0.12, 0.5, or 1.0 ppm, or to filtered air as unexposed controls. Charcoal and high-efficiency particulate air filters were used to purify ambient air, and ambient ozone was removed using potassium permanganate filters. Ozone was generated from 100% oxygen by corona discharge (OREC Model 03V5-0, Ozone Research and Equipment Corp., Phoenix, AZ). Ozone concentrations were measured with multiplexed ultraviolet spectrophotometric analyzers (Model 1003-AH, Dasibi Environmental Corp., Glendale, CA) calibrated by a chemical method using neutral-buffered potassium iodide. Ozone in the control atmosphere was below the limit of detection (0.002 ppm).

ASSESSMENT OF NASAL MUCOCILIARY FUNCTION

The nasal mucociliary apparatus of 21 male and 26 female F344/N rats was examined by video microscopy between July 15 and September 26, 1991, at Battelle Pacific Northwest Laboratories in Richland, WA (See Appendix A, Table A.1 Functional (Mucous Flow) and Histochemical (Mucous Morphometry) Studies, for the identification numbers of rats used in this study). These rats were killed seven or eight days after the end of the 20-month exposure. Mucociliary function was assessed as rapidly as possible after the time of death, as mucus continues to flow for only 20 to 30 minutes after death in rats (Morgan et al. 1984a). The nasal passages were dissected according to the procedure outlined by Morgan and coworkers (1986a) to yield undamaged mucosal surfaces of selected regions of the nose. The mucosal surfaces examined were the lateral walls of the right lateral meatuses; the lateral aspect of the right nasoturbinates; the medial aspects of the right and left maxilloturbinates, the distal lateral wall, and the ethmoid turbinates; the nasopharyngeal meatus; the nasal septum (most complete side); and the medial aspect of the left nasoturbinates. Figure 1 shows these areas diagrammatically.

After rapid dissection of the nose, the tissue samples were immediately placed on medical gauze soaked in physiologic saline, lying on the stage of a viewing chamber with a controlled environment designed for studies of mucociliary function in small tissue preparations (Morgan et al. 1984b). The chamber temperature was maintained at 37° to 38°C with 100% relative humidity. Temperature was determined with a thermistor probe in the chamber (Linear Thermistor Probe, Omega Engineering, Inc., Stamford, CT). High humidity was achieved by placing about 1 cm of distilled water in the chamber beneath the stage. The high

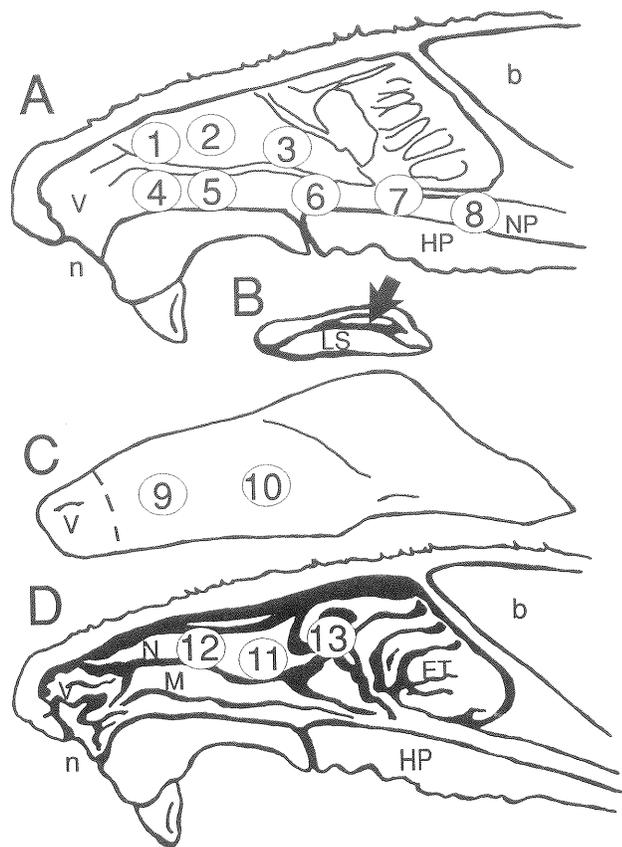


Figure 1. Diagram of the F344/N rat nasal passages opened near the midline with the septum removed to reveal the turbinates. (A) The turbinates have been removed to reveal the lateral wall. (B) The lateral aspect of the nasoturbinates shows the lateral scroll (LS) and lateral ridge (arrow). (C) The septum. (D) The turbinates. The numbers indicate areas used for video motion analysis of mucous flow. Areas 5, 9, and 12 were also morphometrically analyzed for intraepithelial mucosubstances. NP = nasopharynx; n = naris; V = vestibule; HP = hard palate; M = maxilloturbinates; N = nasoturbinates; ET = ethmoid turbinates; b = brain.

humidity was demonstrated by condensation of water on the chamber window and by good preservation of mucociliary function, which is very sensitive to drying. The chamber window was dehumidified as needed with a warm (40° to 44°C) airstream from a commercial hair dryer. The chamber was placed in the circular stage mount of a microscope (Nikon Labophot, Nikon, Tokyo, Japan) equipped with 4× and 10×, long-working-distance objectives. The surface of the epithelium was lighted with a fiber-optic light source; using this requires considerable practice and a systematic approach to tissue placement in the chamber, with the angle of placement being critical.

The patterns and any abnormal features of mucous flow, such as altered mucous opacity, direction of flow, whirling flow, bubbling, or dissection artifacts (e.g., physical tears), were recorded on maps of the tissue preparations, accord-

ing to the system outlined by Morgan and colleagues (1986a). Cessation of mucous flow (mucostasis) in areas where flow is expected in untreated animals was recorded on the maps with a pink marker, and cessation of ciliary beating (ciliastasis) with a yellow marker, to give a combined color of orange for areas with complete cessation of mucociliary activity. The frequency of ciliary beats was not analyzed. Only the presence or absence of ciliary beating in each of the selected areas was recorded.

After collection of subjective data on mucociliary function, video recordings were made of 13 selected areas in the nose (Figure 1) with a video camera (Panasonic CCTV Camera WV-BL200, Division of Matsushita Electric Corporation of America, Secaucus, NJ) and a video recorder (JVC Hi-Fi Stereo VCR, HR-D960W, JVC Company of America, Elmwood Park, NJ). A recording of the minimum length required to provide three suitable particles in the mucous epiphase for flow rate analysis was obtained for each area. Prerecorded images of a slide micrometer, combined with a time-date-generated image on the screen (Morgan et al. 1984b), permitted the determination of mucous flow rates from the recorded images using a computer software package (RS/1, BBN Research System, Cambridge, MA). Recordings for flow rate analysis were made systematically, following the order of sample area numbers (Figure 1). Consistency of approach is also critical for this phase of data collection.

Three of the 13 areas in the nose selected for the functional analyses of mucous flow also were morphometrically analyzed for the amount of stored mucosubstances in the surface epithelium (see Morphometry of Intraepithelial Mucosubstances, Results section). These three regions were the proximal lateral wall (area 5), the proximal septum (area 9), and the medial aspect of the nasoturbinates (area 12) (see Figure 1). In addition, histopathologic examinations of tissues from the remaining 10 sites were conducted by light microscopy (see Histopathology of the Nasal Mucosa, Results section).

STATISTICS FOR MUCOUS FLOW DATA

Mucous flow rates were determined, with a mean of three determinations for each of the 13 areas, whenever this was possible. Flow rate data missing from Tables 1 and 2 are attributable to dissection damage, drying as a result of collecting samples too slowly (a common problem for the thin nasal septum; Morgan et al. 1984b), absence of suitable particles in the recording, and absence of mucous flow due to ozone exposure or unknown causes. For areas where no mucus was flowing, a zero was entered into the data set.

For areas where any of the other three cases (described above) applied, no value was placed in the data set. For each area and exposure group, mean and standard error of the mean (SEM) were determined, and statistical analyses were performed using the RS/1 software. Mean flow rates were assessed using only values of zero or greater for both the numerator and the denominator. The statistical significance of flow rate data was assessed using a linear regression analysis of trends and Student's *t* test comparing each ozone-exposed group to controls. Dunnett's criterion for comparing several exposure groups to controls was used to account for multiple comparisons. Flow incidence data were analyzed using Fisher's exact test on all exposure groups and controls simultaneously and separately in pairwise comparisons of each exposure concentration versus controls. Bonferroni adjustments were used to allow for multiple comparisons in the pairwise incidence tests. An overall value of $p < 0.05$ was used to determine statistical significance in all tests.

MORPHOLOGIC AND MORPHOMETRIC ASSESSMENTS OF THE NASAL MUCOSA

Immediately after video motion analysis (20 minutes after death), nasal tissues from each rat were fixed in 10% neutral-buffered formalin for at least two weeks. Then they were decalcified in 13% formic acid for four days. After decalcification, the septum, lateral wall, and turbinates of half of the nasal cavity (tissues from nasal passage that were not used in the video motion analysis) were transversely sectioned at four specific anatomic locations, using the following gross dental and palatine landmarks: immediately distal to the upper incisor tooth (tissue block 1); at the incisive papilla (tissue block 2); at the second palatal ridge (tissue block 3); in the middle of the front upper molar tooth (tissue block 4) (Figure 2). These four tissue blocks were embedded in glycol methacrylate. Sections 1 μm thick were cut from the proximal surface of all tissue blocks and stained with toluidine blue for morphologic demonstration of surface epithelial cells, or with Alcian blue (pH 2.5)/periodic acid-Schiff (AB/PAS) to demonstrate acidic (blue) and neutral (red) mucosubstances.

Only the 1 μm nasal tissue sections from tissue blocks 1 and 2 (proximal and middle nasal airways) were used for morphometric analyses. The volume density of AB/PAS-stained mucosubstances in the mucosal surface epithelium was determined using a semiautomatic image analysis system. Histochemically stained slides were imaged with a 40 \times planapo objective and a 1.25 \times intermediate lens (Olympus Optical Co., Tokyo, Japan), a CCD camera (TM-840,

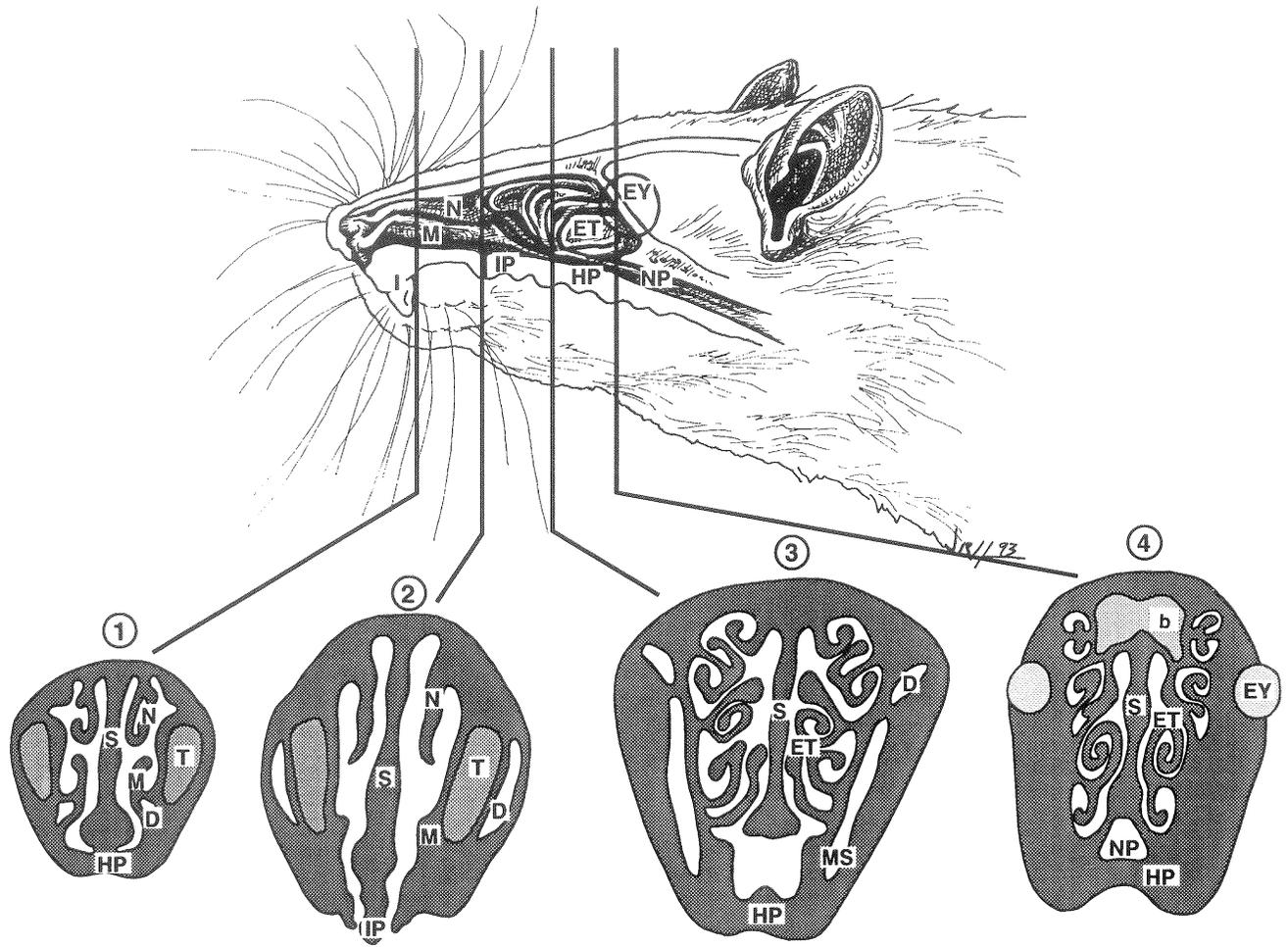


Figure 2. Diagram of the exposed mucosal surface of the nasal lateral wall and turbinates of the F344/N rat. The four vertical lines represent levels of the proximal surfaces of the transverse tissue blocks (1-4) that were microscopically examined. N = nasoturbinate; M = maxilloturbinate; S = septum; ET = ethmoid turbinates; HP = hard palate; IP = incisive papilla; EY = eye; NP = nasopharynx; D = nasolacrimal duct; T = root of the incisor tooth; MS = maxillary sinus; b = brain.

Pulnix America, Sunnyvale, CA), an FG-100 digital image processing board (Imaging Technology, Inc., Woburn, MA), and a color monitor.

The area of stored AB/PAS-stained mucosubstances within the surface epithelium lining the maxilloturbinate, the mid-septum, the lateral wall, the lateral surface of the nasoturbinate, and the medial aspect of the nasoturbinate (Figure 3) in the proximal nasal airway (tissue block 1) was calculated using an image analysis software program (ImageMeasure, Microscience, Inc., Federal Way, WA) developed for morphometric analyses, and the manually or automatically circumscribed perimeter of the stained material. Similar morphometry was conducted on the nasoturbinate, the lateral wall, and the maxilloturbinate in the middle nasal

airway (tissue block 2). Not all of these specific intranasal regions could be examined in each rat because of occasional loss of epithelium resulting from traumatic damage sustained at the time of the tissue dissection or during further tissue processing for light microscopy. This procedurally induced damage to the surface epithelium occurred most often in the nasal septum. The linear length of the basal lamina under each analyzed region of epithelium was determined by tracing the contour of the digitized image of the basal lamina that was projected on a video screen. The computer software program automatically determined the length of the digitized tracing. The basal lamina measured 0.5 to 4 mm in length, depending on the specific site. The method we used to estimate the volume of stored mucosub-

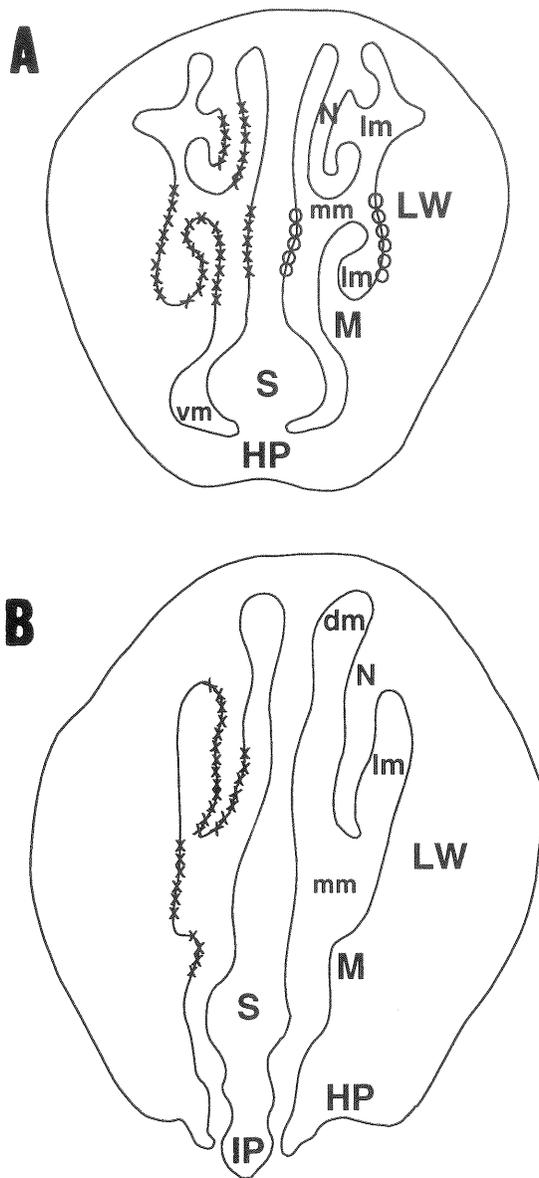


Figure 3. Diagram of the proximal surface of (A) tissue block 1 (proximal nasal passage) and (B) tissue block 2 (middle nasal passage). The marked areas were morphometrically analyzed (x = light microscopy; o = TEM). The numbers indicate areas used for video motion analysis of mucous flow. N = nasoturbinates; M = maxilloturbinates; S = septum; LW = lateral wall; HP = hard palate; dm = dorsal meatus; mm = middle meatus; lm = lateral meatus; vm = ventral meatus; IP = incisive papilla.

stances per unit surface area of epithelial basal lamina has been described (Harkema et al. 1987a). The data were expressed as the mean (\pm SEM) volume density (in nanoliters of intraepithelial mucosubstances per square millimeter of basal lamina) of AB/PAS-positive mucosubstances within the epithelium.

Another 18 rats (4 to 5 per group) were killed seven or eight days after the end of the 20-month exposures (see Appendix A, Table A.I, Structural Studies [with Electron Microscopy] for identification numbers of rats used in this study. Nasal tissues from these animals were fixed in 2% glutaraldehyde for ultrastructural investigations of the nasal surface epithelium by transmission electron microscopy (TEM) and for additional light microscopic analyses to determine the intranasal distribution of the ozone-induced changes to the nasal mucosa. In the ultrastructural investigations, we used a block selection method previously described by Monteiro-Riviere and Popp (1984) that was specifically designed for sampling nasal epithelial tissue from decalcified rat head for TEM. Therefore, epithelial sites of interest around one nasal passage were first detected by light microscopy, then complementary sites around the other nasal passage at the same level of the airway were sampled for analysis by TEM. All of these noses were decalcified with 10% ethylenediaminetetraacetate in 0.1 M cacodylate buffer (pH 7.4) at 4°C for four to five weeks. Transverse tissue sections from the same locations in the nose as described above were selected for further processing for light microscopy or TEM or both (Figure 2).

Sampled tissues for light microscopy were embedded in paraffin, and 6 μ m-thick sections were cut from the proximal surfaces of the paraffin-embedded blocks. These sections were stained with AB/PAS for identification of intraepithelial mucosubstances or with AB (pH 2.5) and hematoxylin and eosin for histopathologic assessment of the tissue.

Sample tissues for TEM were postfixated in 1% osmium tetroxide, dehydrated in a graded series of alcohols, infiltrated with propylene oxide solutions, embedded in epon araldite, and thin-sectioned with a diamond knife on an Ultracut E microtome (Leica, Inc., Deerfield, IL). These sections were mounted on Formvar-coated, slotted grids, stained with uranyl acetate and lead citrate, and examined with a Hitachi H7000 scanning TEM (Hitachi Instruments, Inc., Mountain View, CA). Montages obtained by TEM of continuous nasal epithelium lining the lateral wall and mid-septum in a level of the nasal airway corresponding to the most proximal tissue section examined by light microscopy, as described above, were prepared at a final magnification of 3,000 \times . Differential cell counts were based on counts of all nuclear profiles within the intact epithelium visible on these montages. The numbers of cells per millimeter of nasal airway were determined by counting the nuclear profiles per basal lamina length. Approximately 200 to 500 cells per montage, representing basal lamina lengths of 300 to 1,000 μ m per montage, were counted. Types of nasal epithelial cells within the montaged epithelium

lium included nonciliated cuboidal cells, mucous cells, serous cells, mucoserous cells, ciliated cells, brush cells, and basal cells. Cells were identified on the basis of morphologic criteria given in the Results section.

STATISTICS FOR MORPHOMETRIC DATA

The natural logarithms of the morphometric data were used for statistical analyses. Data were tested for equality of group means by using an unpaired Student's *t* test with Bonferroni adjustment for multiple comparisons. The criterion for statistical significance was set at $p \leq 0.05$.

RESULTS

FUNCTION OF THE NASAL MUCOCILIARY APPARATUS

Mucociliary function studies were performed with no previous knowledge of animal allocation to the exposure groups during data collection. When the data were uncoded, clear exposure-related patterns were observed, with some interanimal variation. The maps were examined subjectively for exposure group characteristics, which were then compared with the mucous flow rate data derived for individual animals and exposure groups.

Control Rats

The structure of the nasal passages of control rats (exposed to 0.0 ppm ozone) resembled that reported for 2-year-old F344 rats, with the characteristic downward tilt of the large ethmoid turbinates and a ventral projection of the distal portion of the nasoturbinate (Gross et al. 1987). With the exception of one animal that had fairly extensive defects of nasal mucociliary function, nasal mucus in control animals had the clear refractile appearance characteristic of nasal mucus in healthy animals, with an underlying vigorous ciliary beat and regional beat characteristics resembling those described previously for F344 (CDF[F344]/Cr1BR) rats (Morgan et al. 1984a). Mucous flow patterns in untreated control animals were similar to those reported for F344 rats (Morgan et al. 1984a,b, 1986a,b; Gross et al. 1987) with the exception of flow patterns in the lateral meatus in some animals. In the present study, the lateral meatus was examined with care because of the frequency of ozone-induced pathology in this region (see the Histopathology of Nasal Mucosa section).

Mucous flow in the lateral meatus of control rats exhibited two characteristic patterns, and one animal exhibited anomalous flow in the distal lateral meatus. In most control

animals (8 out of 15), mucous flow patterns resembled those reported previously (Morgan et al. 1986a). The majority of flow was directed proximally with passage of mucus into the ventral lateral meatus ventrally and into the dorsal recess dorsally (Figure 4A). In three rats, there was apparent reversal of flow in the proximal lateral meatus, with a prominent vortex (zone of circular flow) (Figure 4B). One rat exhibited reversal of flow in the distal lateral meatus (Figure 4C), but flow in other regions resembled the most frequently observed pattern.

Analysis of the 13 areas selected for determinations demonstrated region-specific mucous flow rates (Table 1) resembling those reported previously for F344 rats (Morgan et al. 1984a). The present study provides the first report of mucous flow rates in the lateral meatus. Flow in this location (areas 1, 2, and 3) was faster in the more distal area (area 3) for both males and females. A similar, though more pronounced, proximal-to-distal increase in flow rate was seen on the medial aspect of the maxilloturbinate (areas 4, 5, and 6). In previous reports of mucous flow rates in F344 rats, the fastest flow was consistently observed over the distal lateral wall (Morgan et al. 1984a). In contrast, in the present study the fastest flow occurred over the distal region of the medial maxilloturbinate (area 6), which was not previously examined for mucous flow rate. In one female control rat, a flow rate of 30 mm/minute was observed in area 6. In the nasopharyngeal region, mean flow rates were close to or exceeded 10 mm/minute, while flow over the ethmoid turbinates (area 13) was characteristically slow. Flow rates on the septum (areas 9 and 10) were also slow; however, these data probably do not reflect *in vivo* flow rates, as there was considerable slowing of flow during the 10 minutes (approximately) between first observation and video recording. The nasal septum is susceptible to drying (Morgan et al. 1984a), and ideally flow rate data should be collected from this site as early as possible; however, this was not possible owing to the need to focus on the lateral meatus, a major target for ozone toxicity (Harkema et al. 1989).

In the one abnormal control female referred to above, mucostasis and ciliastasis indicating defective mucociliary function were recorded for the proximal half of the lateral wall (areas 1 and 2), the proximal third of the medial maxilloturbinate (area 4), and the medial aspect of the proximal extremity of the nasoturbinate (proximal to area 12). Elsewhere in the nose of this rat, mucous flow was apparently normal, and the mucus did not exhibit the opacity found in ozone-exposed animals (see the next section). This abnormal control rat also exhibited severe congestion of nasal vasculature, giving the mucosa a dark

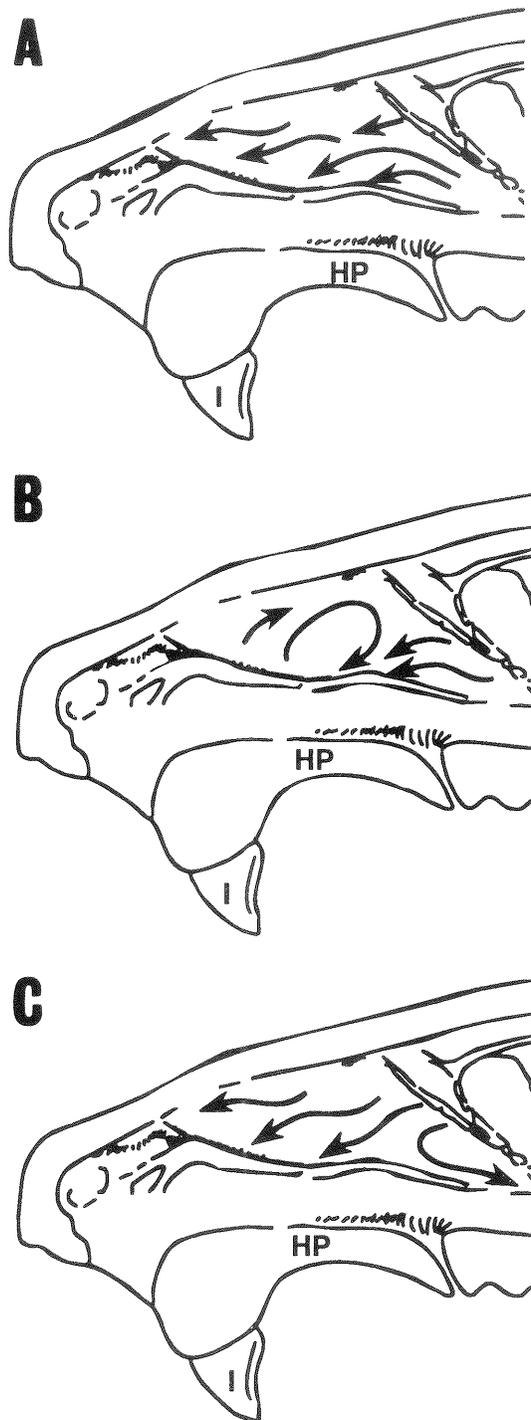


Figure 4. Mucous flow patterns observed on the mucosal surfaces in the lateral wall bordering the lateral meatus of control rats. (A) In the majority of control rats, mucus flowed proximally with ventral portions of these streams passing ventrally into the ventral lateral meatus, and with dorsal lateral portions of these streams passing into the lateral recess of the lateral meatus. (B) In three control rats, proximally directed flow in the distal lateral meatus joined a large vortex or zone of circular flow in the direction opposite of the flow of A above. (C) In one rat, mucus in the distal lateral meatus flowed in the direction opposite of that in all other control rats. I = incisor tooth; HP = hard palate.

reddish color. The data from the defective areas of this animal were therefore not included in the study. With the latter exception, the control data, combined with previously published information (Lucas and Douglas 1934; Morgan et al. 1984a,b, 1986a,b; Gross et al. 1987), were considered suitable as a baseline for assessment of exposure-related effects.

Ozone-Exposed Rats

In ozone-exposed rats there were clear exposure-associated changes in nasal mucociliary function, with some interanimal variation, especially in the group exposed to 0.5 ppm ozone. The maps indicating the extent and location of mucostasis and ciliastasis revealed distinct, concentration-related inhibition of mucociliary function in specific sites in the nose (Figure 5), with the most consistent changes occurring in the lateral meatuses (areas 1, 2, and 3) and the medial maxilloturbinates (areas 4, 5, and 6). These changes were most extensive at 1.0 ppm, more variable in extent at 0.5 ppm, and not observed in the animals exposed to 0.12 ppm ozone. Mucostasis was generally more extensive than ciliastasis, with a zone of active ciliary beating but no mucous flow usually occurring in regions directly distal to areas of ciliastasis (Figure 5). Table 2 presents the incidence of nasal mucostasis in affected intranasal areas.

Animals exposed to 1.0 or 0.5 ppm ozone, in addition to ciliastasis and mucostasis in some areas, had distinct exposure-associated changes in areas where flow was maintained. These effects included altered character of the mucus, which was frequently opaque or "milky"; more copious mucus, in some cases with "strings" of viscid mucus adhering to fixed tissues and extending along the line of mucous flow streams; altered direction of mucous flow adjacent to areas of mucostasis; and whirling or "vortex-like" flow. These effects occurred in all areas of the nose in which mucus was flowing, except on the nasal septum. Flow of mucus over the septum appeared generally unaltered by ozone exposure, with little or no effect on the character of the mucus or the flow patterns.

Statistical Analysis of Mucous Flow Rates

Video motion analysis of mucous flow in ozone-treated animals generated a complex set of data, summarized in Table 1. A trend test was applied to the data using linear regression analysis. Pairwise *t* tests were performed to compare each exposure group with the controls.

Areas 1, 2, 4, 5, 6, and 8 (and to a lesser extent areas 3 and 7) (Figure 1) showed statistically significant decreases in mucous flow rate in ozone-exposed rats. In the other, generally more distal areas of the nose, no statistically significant trends were observed, even though in some cases flow changes were observed with ozone exposure. Changes at the 0.12 ppm level of exposure were less certain;

Table 1. Mucous Flow Rates in 13 Areas of the Nasal Airway After Ozone Exposure^a

| Area ^b | Ozone Exposure Group | | | | | | | |
|-------------------|----------------------|--------------|----------|---------------------------|----------|--------------------------|----------|--------------------------|
| | <i>n</i> | 0.0 ppm | <i>n</i> | 0.12 ppm | <i>n</i> | 0.5 ppm | <i>n</i> | 1.0 ppm |
| 1 | 13 (6/7) | 3.46 ± 0.58 | 5 (3/2) | 5.41 ± 1.57 | 12 (5/7) | 1.64 ± 0.77 | 14 (5/9) | 0.38 ± 0.18 ^c |
| 2 | 14 (7/7) | 3.78 ± 0.70 | 5 (3/2) | 3.81 ± 0.92 | 12 (5/7) | 2.3 ± 0.72 | 14 (6/8) | 0.53 ± 0.24 ^c |
| 3 | 13 (6/7) | 10.57 ± 1.96 | 5 (3/2) | 12.71 ± 4.89 | 12 (5/7) | 7.01 ± 1.50 | 14 (6/8) | 6.14 ± 1.84 |
| 4 | 13 (6/7) | 2.40 ± 0.64 | 4 (2/2) | 1.93 ± 1.27 | 10 (3/7) | 0.33 ± 0.24 ^d | 15 (6/9) | 0.14 ± 0.10 |
| 5 | 13 (6/7) | 7.15 ± 1.25 | 5 (3/2) | 6.18 ± 2.42 | 10 (3/7) | 0.78 ± 0.43 ^d | 13 (4/9) | 0.14 ± 0.14 ^c |
| 6 | 12 (6/6) | 15.95 ± 2.31 | 5 (3/2) | 11.63 ± 5.47 | 11 (4/7) | 4.10 ± 1.20 ^d | 14 (5/9) | 1.64 ± 0.88 ^c |
| 7 | 12 (6/6) | 10.44 ± 1.12 | 4 (2/2) | 16.76 ± 1.11 ^d | 12 (5/7) | 8.32 ± 1.04 | 14 (5/9) | 7.97 ± 1.80 |
| 8 | 12 (6/6) | 9.68 ± 1.38 | 5 (3/2) | 12.25 ± 1.52 | 12 (5/7) | 4.42 ± 0.84 ^d | 10 (4/6) | 3.33 ± 1.16 ^d |
| 9 | 9 (5/4) | 1.15 ± 0.27 | 5 (3/2) | 1.60 ± 0.74 | 8 (4/4) | 2.07 ± 0.65 | 13 (5/8) | 1.83 ± 0.33 |
| 10 | 7 (3/4) | 0.91 ± 0.26 | 5 (3/2) | 2.06 ± 1.01 | 8 (3/5) | 1.13 ± 0.23 | 13 (5/8) | 2.13 ± 0.44 |
| 11 | 12 (6/6) | 6.63 ± 1.40 | 5 (3/2) | 7.37 ± 3.55 | 12 (5/7) | 8.74 ± 2.64 | 14 (5/9) | 7.79 ± 2.42 |
| 12 | 11 (5/6) | 3.02 ± 0.70 | 5 (3/2) | 4.75 ± 1.13 | 11 (4/7) | 4.63 ± 1.21 | 14 (5/9) | 1.91 ± 0.54 |
| 13 | 12 (6/6) | 1.23 ± 0.28 | 5 (3/2) | 2.86 ± 1.62 | 12 (5/7) | 0.59 ± 0.14 | 14 (5/9) | 1.81 ± 0.48 |

^a Mucous flow rate values are means ± SEM given in millimeters per minute. The *n* value for each exposure group is given on the first line with the number of males/females given in parentheses.

^b See Figure 1 for area locations.

^c Significantly different ($p < 0.001$) from control group.

^d Significantly different ($p < 0.05$) from control group.

changes that tended to follow generally decreasing concentration-related flow rates were not statistically significant when compared with control values, probably as a consequence of the small sample sizes. However, in area 7, where there was a marginally significant trend for flow rate to decrease at the higher ozone concentration, the values at 0.12 ppm ozone tended to increase ($p = 0.0086$). A similar pattern of increasing mucous flow rate in the groups exposed to 0.12 ppm ozone was observed in areas 1, 3, 8, 10, and 13, but was not confirmed statistically. Taken together, these results indicate that although the higher concentrations of ozone inhibit mucous flow, 0.12 ppm ozone exposure may induce modest increases in mucous flow rates.

Additional analyses were conducted taking into account the gender and terminal body weight of rats and the day they were killed. In areas 4 and 5, flow increased in males; otherwise, there were no significant differences between males and females. In areas 1, 10, 11, and 12, statistical analysis of the flow rate as a function of terminal body weight indicated a decreased flow rate with increasing body weight after accounting for the effects of ozone and gender. Table 2 shows the incidence of flow (number of rats in which flow is absent versus number in which flow is present) for areas where there was a significant association between the presence of flow and ozone exposure. Analysis of these data by Fisher's exact test indicated that

areas where the number of zero flow values was large (no flow, but sample area suitable for analysis; see the Methods section) tended to occur more frequently at the higher ozone concentrations. The incidence of flow was also examined with respect to the gender of the rats and the day they were killed, but no significant associations were found.

HISTOPATHOLOGY OF THE NASAL MUCOSA

Both male and female rats exposed to 1.0 ppm ozone had significant morphologic alterations of the nasal mucosa that were detected by both light microscopy and TEM. Similar but less severe alterations were evident in rats exposed to 0.5 ppm ozone. No exposure-related lesions were evident in the nasal airways of rats exposed to 0.12 ppm ozone. There were no microscopically detectable differences in the nasal mucosa of rats exposed to 0.0 ppm ozone (controls) and those exposed to 0.12 ppm ozone.

The most severe alterations induced by 0.5 or 1.0 ppm ozone occurred in the nasal mucosa of the lateral wall, the nasoturbinates, and the maxilloturbinates lining the lateral meatus of the proximal and middle regions of the nasal passages. Conspicuous, but less severe, ozone-induced changes were also evident in the respiratory epithelium lining the mid-septum and lateral meatus in the middle nasal airways, the respiratory and olfactory epithelium lining the ethmoid turbinates in the distal nasal cavity, and the respiratory

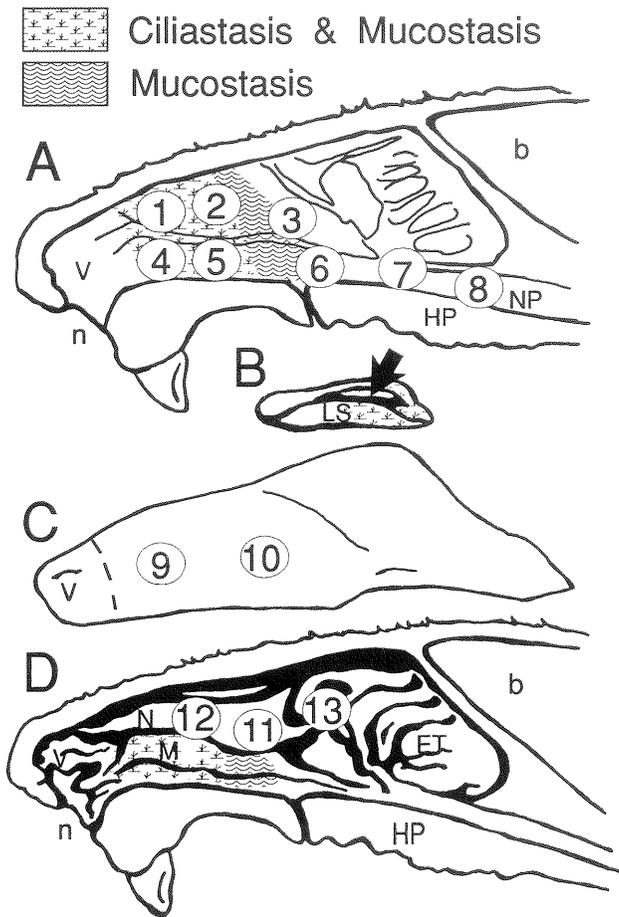


Figure 5. Diagram of rat nasal passages opened near the midline with the septum removed to reveal the turbinates. Areas of ciliastasis and mucostasis and areas of mucostasis only in the nasal mucosa after 0.5 or 1.0 ppm ozone are indicated. Numbers represent the areas that were analyzed by video motion. (A) The turbinates have been removed to reveal the lateral wall. (B) The lateral aspect of the nasoturbinate shows the lateral scroll (LS) and lateral ridge (arrow). (C) The septum. (D) The turbinates. V = vestibule; b = brain; HP = hard palate; n = naris; N = nasoturbinate; M = maxilloturbinate; ET = ethmoid turbinates; NP = nasopharynx.

epithelium lining the proximal nasopharynx. No significant histologic alterations were identified in the nasal respiratory epithelium lining the nasal septum in the proximal nasal airways (tissue block 1) of rats exposed to 0.5 or 1.0 ppm ozone.

Ozone-induced lesions in the proximal regions (tissue block 1) of the nasal airways included marked mucous cell metaplasia and epithelial hyperplasia in the nasal transitional epithelium lining the entire surface of the lateral meatus (that is, surface epithelium covering the lateral wall, maxilloturbinates, and lateral surfaces of the nasoturbi-

nates in the proximal nasal airways). Similar lesions were present in the surface epithelium lining the lateral wall, maxilloturbinate, and nasoturbinate in the middle region of the nose (tissue block 2) and in the respiratory epithelium lining the maxillary sinus.

In rats exposed only to filtered air (0 ppm ozone), the nasal transitional epithelium was only one to two cells thick and was composed predominantly of nonciliated cuboidal or columnar cells with little histochemically detectable mucosubstances. In contrast, the nasal transitional epithelium exposed to 1.0 ppm ozone was four to six cells thick and contained numerous columnar mucous cells filled with copious histochemically detectable mucosubstances (both acidic AB-staining and neutral PAS-staining mucosubstances) (Figure 6). Mucous cell metaplasia with increased intraepithelial mucosubstances was present to a lesser degree in rats exposed 0.5 ppm ozone. Epithelial cell hyperplasia was not a consistent feature in this latter group of rats.

Figure 7 illustrates the intranasal distribution of this ozone-induced mucous cell metaplasia in the surface epithelium throughout the nasal airways of rats exposed to 0.5 or 1.0 ppm ozone. Mucous cell metaplasia was also present in some subepithelial ducts and glands within the lamina propria of the proximo-lateral aspects of the nasoturbinates. This glandular lesion was variable among ozone-exposed rats, but was usually more severe in rats exposed to 1.0 ppm ozone. Mucous cell metaplasia in the nasal transitional epithelium was accompanied by varying numbers of intraepithelial glands composed of several circumscribing mucous cells whose apical surfaces were directed to a common central lumen within the surface epithelium. Intraepithelial gland formation did not accompany the ozone-induced mucous cell metaplasia in the respiratory epithelium lining the maxillary sinus.

A mild to moderate mixed inflammatory cell influx of lymphocytes, plasma cells, and neutrophils was present in the nasal mucosa of the lateral walls, maxilloturbinates, and lateral aspects of the nasoturbinates in the proximal half of the nasal airways. Surface or glandular epithelial cell necrosis did not accompany the chronic rhinitis in the ozone-exposed rats. However, a bony atrophy of the maxilloturbinates and the lateral ridges of the nasoturbinates was conspicuous in the proximal nasal airways of rats exposed to 0.5 or 1.0 ppm ozone (Figure 8). Areas of bone resorption (that is, Howship's lacunae) with numerous associated mononuclear leukocytes, osteoclasts, and osteoblasts were present in the atrophic bone of the affected turbinates. Though both male and female rats exposed to 0.5 or 1.0 ppm ozone had bony atrophy, this alteration was more conspicuous in the turbinates of male rats.

Table 2. Incidence of Nasal Mucostasis in Affected Intranasal Areas After Ozone Exposure^a

| Area ^b | Mucous Flow | Ozone Exposure Group | | | |
|-------------------|-------------|----------------------|----------|----------------|-----------------|
| | | 0.0 ppm | 0.12 ppm | 0.5 ppm | 1.0 ppm |
| 1 | Absent | 2 | 1 | 7 | 10 ^c |
| | Present | 11 | 4 | 5 | 4 ^c |
| | Total | 13 | 5 | 12 | 14 |
| 2 | Absent | 1 | 0 | 3 | 9 ^c |
| | Present | 13 | 5 | 9 | 5 ^c |
| | Total | 14 | 5 | 12 | 14 |
| 4 | Absent | 3 | 2 | 8 ^c | 13 ^c |
| | Present | 10 | 2 | 2 ^c | 2 ^c |
| | Total | 13 | 4 | 10 | 15 |
| 5 | Absent | 0 | 0 | 6 ^c | 12 ^d |
| | Present | 13 | 5 | 4 ^c | 1 ^d |
| | Total | 13 | 5 | 10 | 13 |
| 6 | Absent | 0 | 1 | 1 | 7 ^c |
| | Present | 12 | 4 | 10 | 7 ^c |
| | Total | 12 | 5 | 11 | 14 |

^a Values given are the numbers of rats in which mucous flow is absent or present and the total number of rats.

^b See Figure 1 for area locations.

^c Significantly different ($p < 0.05$) from control group (0 ppm ozone).

^d Significantly different ($p < 0.001$) from control group (0 ppm ozone).

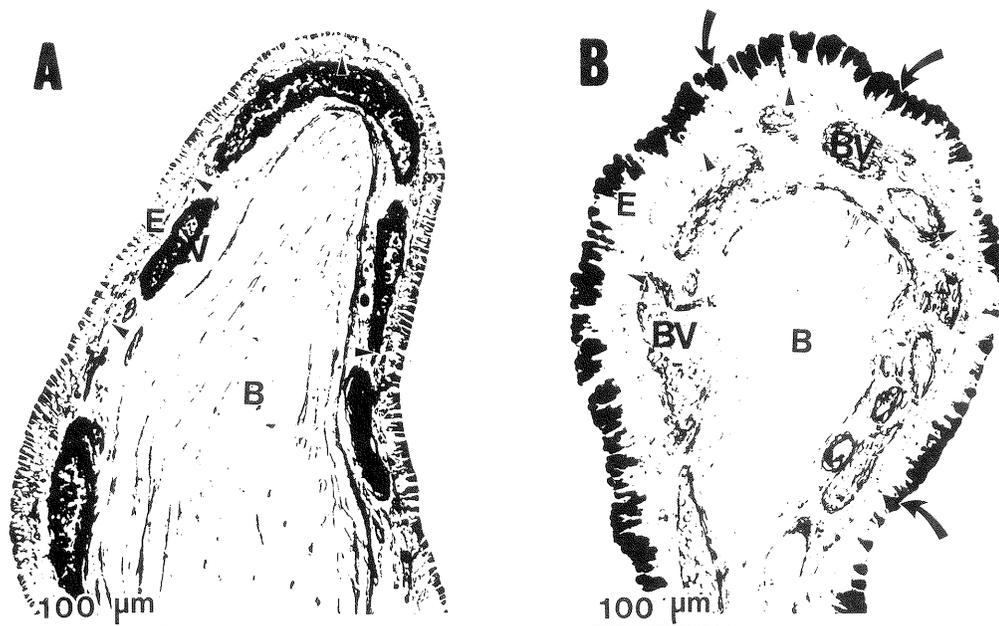


Figure 6. Light photomicrographs of maxilloturbinates from the proximal nasal passages of rats exposed to (A) 0.0 ppm ozone or (B) 1 ppm ozone. Intraepithelial mucosubstances (arrows), stained with AB/PAS, are present in the rat exposed to 1.0 ppm ozone, but not in the control rat (0 ppm ozone). E = surface epithelium; B = bone; BV = blood vessel; arrowheads = basal lamina.

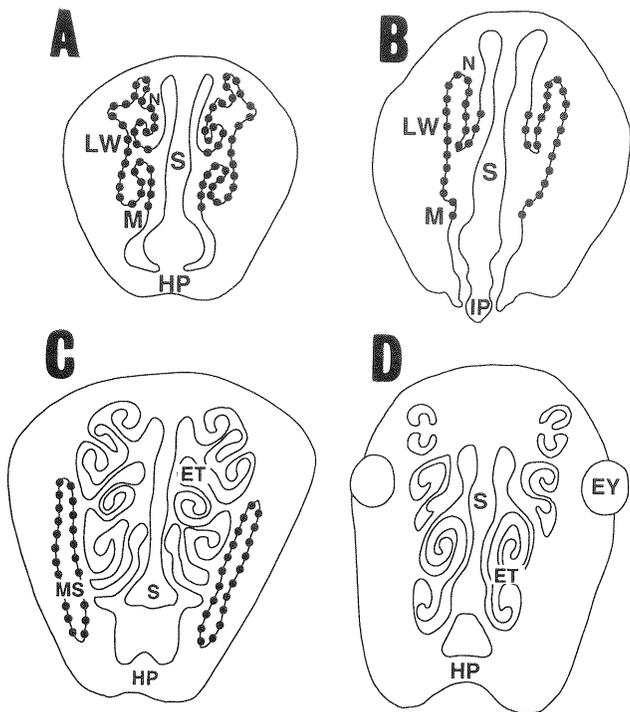


Figure 7. Diagram of the distribution of mucous cell metaplasia (closed circles) in the surface epithelia lining the nasal passages of rats exposed to 0.5 or 1.0 ppm ozone. Proximal surfaces of the four transverse tissue blocks (A–D moving from proximal to distal nasal airways). S = septum; N = nasoturbinate; M = maxilloturbinate; ET = ethmoid turbinates; HP = hard palate; IP = incisive papilla; LW = lateral wall; EY = eye.

In addition to the ozone-induced histopathology already described above, numerous eosinophilic globules were scattered throughout the respiratory and olfactory epithelium lining the ethmoid turbinates (that is, endoturbinates and ectoturbinates) in distal nasal passages (tissue blocks 3 and 4) and in the respiratory epithelium lining the nasoturbinates in the middle region of the nasal passages (tissue block 2) of rats exposed to 0.5 or 1.0 ppm ozone. This alteration was present in both males and females. These eosinophilic globules were identified, by TEM, as dilated cisternae of smooth endoplasmic reticulum in the cytoplasm of sustentacular cells in the olfactory epithelium and in the cytoplasm of nonciliated secretory, columnar cells in the respiratory epithelium. The enlarged cisternae were distended by copious proteinaceous material with a moderately electron-dense matrix. Only a few widely scattered eosinophilic globules were present in the similar nasal epithelia of rats exposed to 0.0 or 0.12 ppm ozone.

MORPHOMETRY OF INTRAEPITHELIAL MUCOSUBSTANCES

The effects of ozone exposure on the amount of intraepithelial mucosubstances in surface epithelium in various

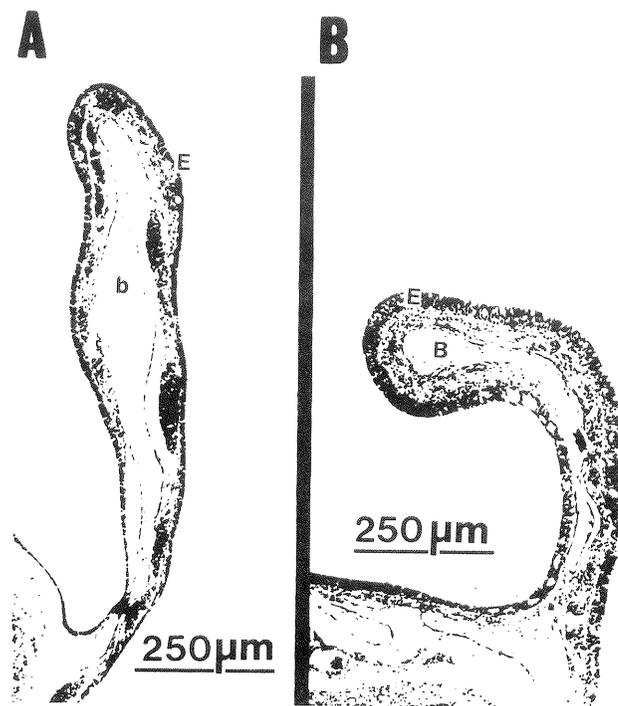


Figure 8. Maxilloturbinate from male F344/N rats exposed to (A) 0.0 ppm ozone or (B) 1.0 ppm ozone for 20 months. Marked bony atrophy with reduction of size and alteration of the shape of the turbinate in the rat exposed to 1.0 ppm ozone (B). E = surface epithelium lining the maxilloturbinate; B = bone.

regions of the proximal and middle aspects (tissue blocks 1 and 2) of the rat nasal airways are presented in Tables 3 and 4. Exposure to 1.0 ppm ozone induced increases in intraepithelial mucosubstances in the nasal transitional epithelium lining the lateral aspect of the nasoturbinate (317 times the amount in controls), the maxilloturbinate (171 times the amount in controls), and the lateral wall (27 times the amount in controls). Ozone-induced increases in intraepithelial mucosubstances were slightly smaller in the surface epithelium lining the nasoturbinate, maxilloturbinate, and lateral wall in the middle nasal airway (tissue block 2). No significant differences were found between the amounts of intraepithelial mucosubstances in the respiratory epithelium lining the nasal septum and the medial aspect of the nasoturbinate in the proximal nasal passage of these ozone-exposed rats and the amounts in controls.

Mucous cell metaplasia with increases in intraepithelial mucosubstances was also present in both male and female rats exposed to 0.5 ppm ozone (Tables 3 and 4). There were no significant gender-related differences in these increases. As in the rats exposed to 1.0 ppm ozone, in the rats exposed to 0.5 ppm ozone, there were dramatic increases in the stored intraepithelial mucosubstances in the nasal transi-

Table 3. Amount of Stored Mucosubstances in Surface Epithelia Lining Various Regions in the Proximal Nasal Airways (Tissue Block 1)

| Ozone Concentration (ppm ozone) | n (males/females) | Volume/Surface Area ^a (nL/mm ² of basal lamina × 10 ⁻²) |
|---|-------------------|---|
| Lateral Aspect of Nasoturbinates | | |
| 0.0 (control) | 14 (7/7) | 1.1 ± 0.3 |
| 0.12 | 4 (3/1) | 1.0 ± 0.1 |
| 0.5 | 12 (5/7) | 108.1 ± 16.3 ^b |
| 1.0 | 15 (6/9) | 348.7 ± 39.4 ^b |
| Medial Aspect of Nasoturbinates | | |
| 0.0 (control) | 14 (7/7) | 52.1 ± 10.3 |
| 0.12 | 3 (2/1) | 90.8 ± 72.5 |
| 0.5 | 12 (5/7) | 74.3 ± 21.6 |
| 1.0 | 15 (6/9) | 102.2 ± 21.9 |
| Lateral Wall | | |
| 0.0 (control) | 15 (7/7) | 14.0 ± 2.8 |
| 0.12 | 4 (3/1) | 4.0 ± 1.2 |
| 0.5 | 12 (5/7) | 186.0 ± 17.1 ^b |
| 1.0 | 15 (6/9) | 373.3 ± 30.6 ^b |
| Maxilloturbinates | | |
| 0.0 (control) | 15 (7/8) | 1.9 ± 0.6 |
| 0.12 | 4 (3/1) | 0.5 ± 0.3 |
| 0.5 | 12 (5/7) | 148.0 ± 30.8 ^b |
| 1.0 | 15 (6/9) | 324.9 ± 30.2 ^b |
| Septum | | |
| 0.0 (control) | 5 (3/2) | 558.3 ± 109.1 |
| 0.12 | 4 (3/1) | 736.3 ± 101.4 |
| 0.5 | 5 (2/3) | 423.3 ± 87.7 |
| 1.0 | 5 (2/3) | 723.1 ± 186.7 |

^a Values are given as means ± SEM.

^b Significantly different (*p* < 0.05) from control group.

tional epithelium lining the nasoturbinates (108 times the amount in controls), maxilloturbinates (78 times the amount in controls), and lateral wall (14 times the amount in controls) in the proximal nasal passage. These increases, however, were approximately 50% to 70% less than in rats exposed to 1.0 ppm ozone, depending on the specific intranasal region examined. In addition, there were significant, but smaller increases in the amount of mucosubstances within the surface epithelium lining these structures in the middle nasal passage (Table 4). As in the rats exposed to 1.0 ppm ozone, in the rats exposed to 0.5 ppm ozone, there were no detectable differences between the amounts of mucosubstances in the respiratory epithelium lining the septum and the medial aspect of the nasoturbinates in the proximal nasal passage and the amounts in controls.

Table 4. Amount of Stored Mucosubstances in Surface Epithelia Lining Various Regions in the Middle Nasal Airways (Tissue Block 2)

| Ozone Concentration (ppm) | n (males/females) | Volume/Surface Area ^a (nL/mm ² of basal lamina × 10 ⁻²) |
|---------------------------|-------------------|---|
| Nasoturbinates | | |
| 0.0 (control) | 15 (7/8) | 2.7 ± 1.3 |
| 0.12 | 4 (3/1) | 1.5 ± 1.5 |
| 0.5 | 12 (5/7) | 166.1 ± 25.1 ^b |
| 1.0 | 14 (5/9) | 380.1 ± 31.0 ^b |
| Maxilloturbinates | | |
| 0.0 (control) | 15 (7/8) | 13.4 ± 10.3 |
| 0.12 | 4 (3/1) | 24.4 ± 20.6 |
| 0.5 | 12 (5/7) | 158.2 ± 56.3 |
| 1.0 | 14 (5/9) | 325.3 ± 58.4 |
| Lateral Wall | | |
| 0.0 (control) | 15 (7/8) | 0.9 ± 0.4 |
| 0.12 | 4 (3/1) | 0.0 |
| 0.5 | 12 (5/7) | 87.0 ± 17.9 ^b |
| 1.0 | 14 (5/9) | 252.0 ± 35.6 ^b |

^a Values are given as means ± SEM.

^b Significantly different (*p* < 0.05) from control group.

No significant differences in the amounts of intraepithelial mucosubstances were detected between rats exposed to 0.12 ppm ozone and controls exposed to 0.0 ppm ozone in any of the regions examined.

ULTRASTRUCTURAL MORPHOLOGY IN NASAL TRANSITIONAL EPITHELIUM

The principal ultrastructural difference in the nasal transitional epithelium of rats exposed to 1.0 or 0.5 ppm ozone and that of air-exposed controls was a marked increase in the number of luminal nonciliated cells with varying amounts of secretory granules (Figure 9). These secretory cells were classified as either mucous cells or nonciliated cuboidal cells with small numbers of secretory granules. The mucous cells were tall cuboidal to low columnar in shape with a microvillar luminal surface and abundant secretory granules filling most of the cytoplasm between the nucleus and the luminal surface. In contrast, nonciliated cuboidal cells with secretory granules were cuboidal to low columnar in shape with microvillar luminal surfaces and only a few secretory granules in the very apical portion of the cell. Both of these cells extended from the basal lamina to the luminal surface. The secretory granules in both cells were membrane-bound with a homogeneous electron-lucent matrix. The granules were either individ-

Table 5. Abundance of Cells in the Nasal Transitional Epithelium Lining the Lateral Walls of Proximal Nasal Airways^a

| Cell Type | Ozone Exposure Group | | | |
|------------------------|----------------------|---------------------|---------------------|-----------------------|
| | 0.0 ppm (n = 4) | 0.12 ppm (n = 4) | 0.5 ppm (n = 4) | 1.0 ppm (n = 5) |
| Nonciliated | 175 ± 10 | 189 ± 12 | 124 ± 12 | 178 ± 19 |
| Secretory | 0 | 0 | 71 ± 7 ^b | 94 ± 10 ^b |
| Ciliated | 5 ± 3 | 2 ± 2 | 1 ± 1 | 0 |
| Brush | 1 ± 1 | 3 ± 1 | 5 ± 2 | 3 ± 3 |
| Basal | 57 ± 3 | 56 ± 2 | 68 ± 15 | 65 ± 2 |
| Total epithelial cells | 238 ± 14 | 249 ± 13 | 267 ± 15 | 341 ± 23 ^b |

^a Values are given as the mean (± SEM) numbers of cells/mm of basal lamina.

^b Significantly different ($p < 0.05$) from control group (0 ppm ozone).

ual or coalescing. Neither mucous cells nor nonciliated cells with secretory granules were present in control rats exposed to 0.0 ppm ozone.

Nonciliated cuboidal cells were the most common cell type in the nasal transitional epithelium of all rats. These cells were cuboidal to low columnar in shape and extended from the basal lamina to the luminal surface. These cells had microvillar luminal surfaces with abundant smooth endoplasmic reticulum and numerous mitochondria in their apical cytoplasm. These cells did not contain mucous secretory granules.

Basal cells in the nasal transitional epithelium of all rats were elongated to an oval shape with basal surfaces attached to the basal lamina. The nucleus was central, and the cytoplasm contained few organelles. These cells in the nasal transitional epithelium of ozone-exposed rats were not morphologically different from those in controls.

Brush cells had distinct ultrastructural characteristics. These cells were often pear-shaped with a wide base containing the nucleus and a narrow apical tip projecting into the airway lumen. The apical tip had long, dense, non-branching microvilli and numerous microfilaments and microtubules. These cells were not altered by any of the ozone exposures.

MORPHOMETRY OF NASAL TRANSITIONAL EPITHELIUM

Table 5 compares the differences in total and differential epithelial cells per millimeter of basal lamina in the nasal transitional epithelium lining the lateral wall in the proximal nasal passages of rats in the four experimental groups.

Total epithelial cells were significantly increased in rats exposed to 1.0 ppm ozone, compared with controls, but not in rats exposed to 0.5 or 0.12 ppm ozone. This increase in epithelial cells within the nasal transitional epithelium of rats exposed to 1.0 ppm ozone was due to significant increases in secretory cells (that is, in mucous cells with abundant secretory granules and in nonciliated cuboidal cells with few secretory granules). There were 94 ± 10 secretory cells per millimeter of basal lamina in rats exposed to 1.0 ppm ozone compared with 0 secretory cells per millimeter of basal lamina in controls. The numbers of ciliated cells, nonciliated cuboidal cells without secretory granules, brush cells, and basal cells were statistically similar in controls and rats exposed to 1.0 ppm ozone.

Although there was no significant increase in total epithelial cells (that is, hyperplasia) in the rats exposed to 0.5 ppm ozone, the number increased to 71 ± 7 secretory cells per millimeter of basal lamina in ozone-exposed rats compared with 0 secretory cells per millimeter of basal lamina in controls (Table 5). As in the rats exposed to 1.0 ppm ozone, the increase in secretory cells in the nasal transitional epithelium of rats exposed to 0.5 ppm ozone was due to increases in both mucous cells and nonciliated cuboidal cells with small numbers of secretory granules. There was a concurrent decrease in the number of nonciliated cells without secretory granules in these ozone-exposed rats, suggesting that some of the secretory cells in the ozone-exposed animals may have been derived from nonciliated cuboidal cells without secretory granules. There were no significant differences in the abundance of ciliated, brush, or basal cells between rats exposed to 0.5 ppm ozone and rats exposed to 0.0 ppm ozone (controls).

A

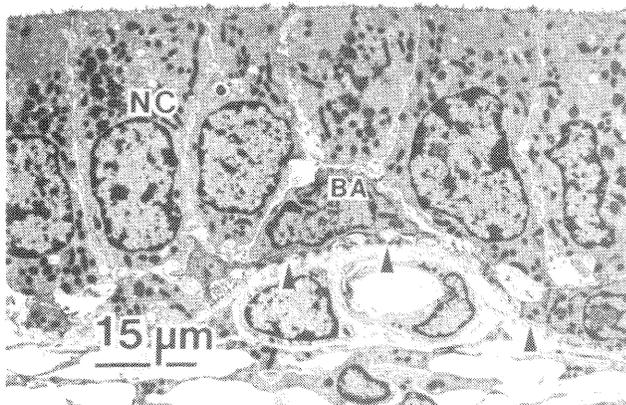


Figure 9A. Transmission electron photomicrographs of nasal transitional epithelium lining the lateral walls of rats exposed to 0.0 ppm ozone for 20 months. BA = basal cell; NC = nonciliated cuboidal cell; arrowheads = basal lamina.

B

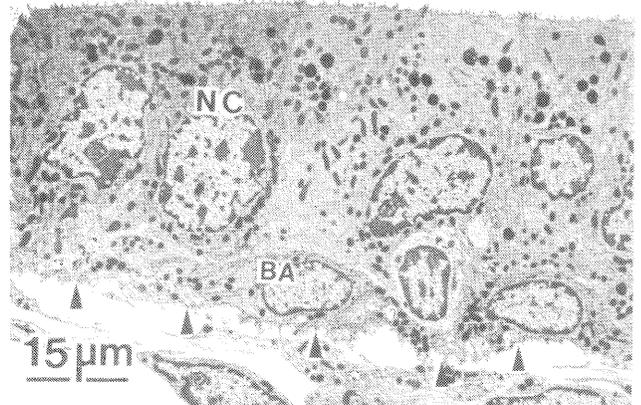


Figure 9B. Transmission electron photomicrographs of nasal transitional epithelium lining the lateral walls of rats exposed to 0.12 ppm ozone for 20 months. BA = basal cell; NC = nonciliated cuboidal cell; arrowheads = basal lamina.

Rats exposed to 0.12 ppm ozone had epithelial cell densities in the nasal transitional epithelium of the lateral wall that were similar to those in controls.

ULTRASTRUCTURAL MORPHOLOGY OF RESPIRATORY EPITHELIUM IN THE NASAL SEPTUM

The nasal respiratory epithelium in all the rats was composed of basal cells, secretory cells (that is, mucous cells, serous cells, and mucoserous cells), and ciliated cells. Only the respiratory epithelium from rats exposed to 1.0 ppm ozone had morphologic differences from that of controls. The principal ozone-induced alteration was a shift in the number of serous, mucoserous, and mucous cells. The animals exposed to 1.0 ppm ozone had more mucous cells

and fewer mucoserous and serous cells than did controls. All of these secretory cells were columnar with microvillar luminal surfaces and basally located oval nuclei. Serous cells had numerous small, membrane-bound individual secretory granules with an electron-dense matrix. Granules were in the apical third of the cell. In contrast, mucous cells were characterized by numerous, large, electron-lucent secretory granules in the apical one-half to three-quarters of the cell. The mucoserous cell contained individual granules with some electron-lucent and some electron-dense granules. In addition, these cells usually contained secretory granules with a varying-sized electron-dense core surrounded by an electron-lucent border.

Ciliated cells, characterized by luminal cilia with basal bodies and numerous mitochondria in the apical portions

C

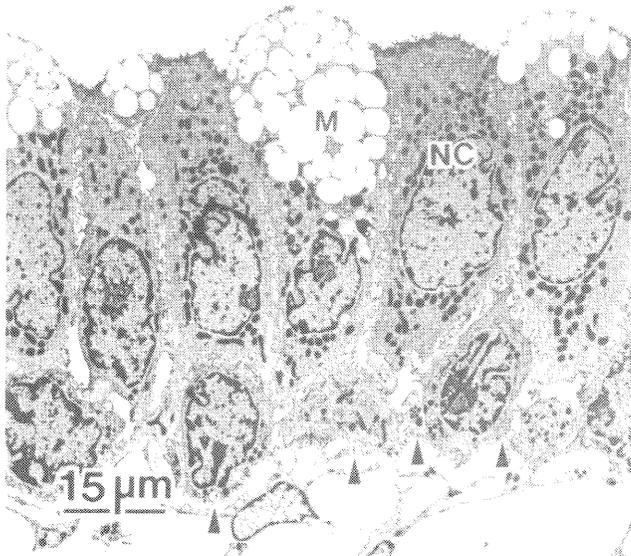


Figure 9C. Transmission electron photomicrographs of nasal transitional epithelium lining the lateral walls of rats exposed to 0.5 ppm ozone for 20 months. M = mucous cell; BA = basal cell; NC = nonciliated cuboidal cell; arrowheads = basal lamina.

of the cells, were not morphologically different among the four experimental groups. No ultrastructural features of basal or brush cells were different from those of controls after any ozone exposure, except for mild to moderate basal cell hyperplasia in the respiratory epithelium of rats exposed to 1.0 ppm ozone.

MORPHOMETRY OF RESPIRATORY EPITHELIUM OF THE NASAL SEPTUM

The effects of ozone on the abundance and differential densities of cells in the respiratory epithelium lining the proximal nasal septum are summarized in Table 6. The only exposure group that differed significantly from controls in the abundance of these epithelial cells was the group exposed to 1.0 ppm ozone. These rats had a 21%

D

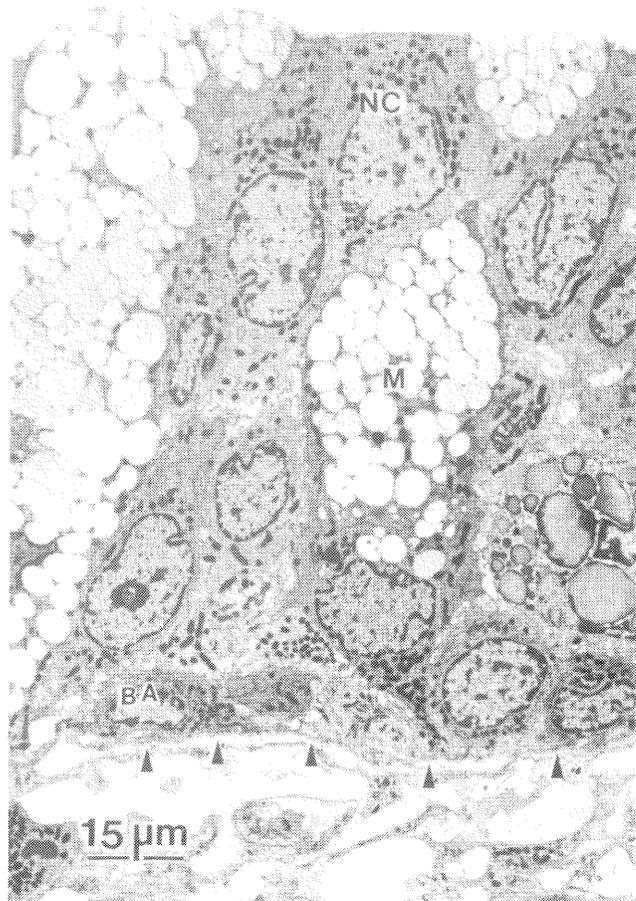


Figure 9D. Transmission electron photomicrographs of nasal transitional epithelium lining the lateral walls of rats exposed to 1.0 ppm ozone for 20 months. M = mucous cell; BA = basal cell; NC = nonciliated cuboidal cell; arrowheads = basal lamina.

increase, compared with controls, in total epithelial cells. This hyperplastic response was due primarily to an increase in basal cells. Though total secretory cell numbers did not increase after exposure to 1.0 ppm ozone in this epithelium, there was a noticeable decline in the numbers of serous and mucoserous cells and a concomitant increase in mucous cells (twice as many mucous cells in ozone-exposed respiratory epithelium as in that of controls).

DISCUSSION AND CONCLUSIONS

This was the first study to demonstrate that chronic inhalation of ozone can cause significant alterations in nasal mucociliary function of specific regions of the rat nose, with clear concentration-response relations. The

Table 6. Abundance of Cells in the Respiratory Epithelium Lining the Midseptum of Proximal Nasal Airways^a

| Cell Type | Ozone Exposure Group | | | |
|------------------------|----------------------|---------------------|--------------------|-----------------------|
| | 0.0 ppm (n = 5) | 0.12 ppm (n = 4) | 0.5 ppm (n = 5) | 1.0 ppm (n = 4) |
| Ciliated | 100 ± 9 | 90 ± 8 | 94 ± 7 | 112 ± 8 |
| Mucous | 39 ± 6 | 29 ± 5 | 23 ± 6 | 68 ± 8 ^b |
| Serous | 3 ± 1 | 3 ± 2 | 10 ± 4 | 1 ± 1 |
| Mucoserous | 17 ± 4 | 18 ± 3 | 13 ± 5 | 1 ± 1 |
| Brush | 1 ± 1 | 1 ± 1 | 1 ± 1 | 2 ± 1 |
| Basal | 44 ± 5 | 50 ± 6 | 56 ± 5 | 66 ± 7 ^b |
| Total epithelial cells | 206 ± 10 | 192 ± 9 | 198 ± 11 | 249 ± 13 ^b |

^a Values are given as mean (± SEM) numbers of cells/mm of basal lamina.

^b Significantly different ($p < 0.05$) from control group (0 ppm ozone).

proximal location of these alterations in the nose resembled the distribution of alterations induced by the gaseous irritants dimethylamine (Gross et al. 1987) and formaldehyde (Morgan et al. 1986a,b). Extensive involvement of the mucociliary lining of the lateral meatus observed in the present study was also reported for formaldehyde exposure (Morgan et al. 1986a). In contrast to formaldehyde, ozone did not disrupt mucociliary clearance over the nasal septum. Alteration of mucous flow patterns with formation of whirlpool-like flows was observed with both ozone and dimethylamine (Gross et al. 1987). However, each of these gases exhibited characteristic features with respect to distribution, relation of mucostasis to ciliastasis, alterations of flow patterns, and visual characteristics of the surface secretions. Future studies specifically designed to investigate factors responsible for these similarities and differences may shed light upon underlying mechanisms involved in the toxicant-induced alterations.

The specific causes for the ozone-induced decreases in nasal mucous flow rates and mucostasis could not be determined from the results of this study; however, some reasonable speculations can be made. The decrease in mucous flow of the lateral meatus may have resulted from the concurrent ciliastasis that was also observed by video microscopy. The reason for the ozone-induced ciliastasis is unknown. Analyses by light microscopy and TEM did not reveal any structural alterations in the number or character of cilia in these affected regions from rats exposed to 0.5 or 1.0 ppm ozone. Ozone may have directly altered the function of the cilia without changing their morphology.

The absence of structural alterations in nasal cilia of rats after chronic exposure to ozone in the present study is markedly different from the previously reported effects of

both short- and long-term exposures of ozone on the nasal cilia of monkeys (Harkema et al. 1987a). There was marked attenuation and loss of nasal cilia in the proximal nasal passages of bonnet monkeys exposed to 0.15 or 0.3 ppm ozone for 6 or 90 days (eight hours per day). This suggests that there are species differences in the response of nasal cilia to ozone, with rats being much less sensitive than nonhuman primates. Because rodents are obligate nose breathers and primates are oronasal breathers, one would assume that the nasal epithelium of rats would be exposed to a greater amount of ozone than the nasal epithelium of primates if they were subjected to similar ozone exposures. The previous reports that monkeys had significant alterations of nasal cilia along with other mucosal alterations (i.e., epithelial hyperplasia and inflammation) after a shorter exposure to smaller concentrations of ozone, compared with the regimen to which our rats were exposed, suggests that nonhuman primates are more sensitive to ozone toxicity than rats. In addition, this implies that rats may have better protective mechanisms (e.g., more antioxidant enzymes) in their nasal tissues to make them more resistant to ozone injury. However, significant strain-related differences in the response of nasal airway tissue to ozone exposure in rats have been reported (Harkema et al. 1994). Therefore, it is possible that if another strain of rat (e.g., Wistar) had been used in the present study, nasal injury may have been observed at a lower concentration.

Alterations to the mucous flow rates in rats exposed to 0.5 or 1.0 ppm ozone also may have been partially due to an ozone-induced change in the chemical or mechanical character of the mucus lining the surface epithelium. Ozone may have altered the viscosity and elasticity of the mucus, which could have caused the decrease in flow.

Histochemical analyses revealed tremendous increases in both acidic and neutral mucosubstances in the nasal transitional epithelium. Increases in the amount and character of the mucus after 0.5 and 1.0 ppm ozone exposures may have also contributed to the ciliastasis.

Ozone did not alter mucous flow rates in the septum of the proximal nasal passages. This tissue normally contains copious intraepithelial mucosubstances that supply the overlying luminal mucus covering the respiratory epithelium. Mucous flow was conspicuously slower on the nasal septum than on the surfaces lining the lateral meatus in controls. This inherent regional difference in mucous flows also may have contributed in some way to the differences in response to ozone.

The distribution of toxic responses to inhaled gases may be attributable to regional dosimetry, local tissue susceptibility, or a combination of these factors. For formaldehyde, it was concluded, on the basis of studies of nasal airflow (Morgan et al. 1991) and computational fluid dynamics (Kimbell et al. 1993a), that airflow-driven dosimetry plays a major role in lesion distribution for this chemical. On the basis of similar studies with ozone, combined with studies of regional dosimetry of inhaled ^{18}O -ozone, it was concluded that both regional dosimetry and site-specific tissue factors, including surface secretions, influence regional toxicity of ozone in the rat (Kimbell et al. 1993b). The proposal is supported by a recent consideration of the kinetics of interactions between ozone and the nasal surface secretions (Pryor 1992), which indicate that little, if any, ozone absorbed by the mucous epiphase reaches the underlying epithelium. Future studies of ozone dosimetry for interspecies extrapolations will require consideration of interactions between the complex flow fields of air (Morgan et al. 1991) and mucus (Morgan et al. 1984a).

Although there was no decrease in mucous flow rates in any nasal region examined in rats exposed to 0.12 ppm ozone, there was a statistically significant increase in mucous flow rates in one region examined (area 7) and a trend toward an increase in mucous flow rates (though not statistically confirmed) in several other intranasal regions. No histopathology was evident in these regions after exposure to 0.12 ppm ozone, which suggests that the increase in mucous flow rates was a physiologic response of the mucociliary apparatus to the low concentration of this irritating agent, rather than a pathologic response to injury.

The mucous cell metaplasia in the nasal transitional epithelium of rats chronically exposed to 0.5 or 1.0 ppm ozone was similar to the mucous cell metaplasia previously described in rats acutely exposed to 0.8 ppm ozone (six hours per day for seven days) (Harkema et al. 1989). The major differences between the metaplasia in the nasal tran-

sitional epithelium of rats acutely exposed to 0.8 ppm ozone and the metaplasia in the nasal transitional epithelium of rats exposed for 20 months to 0.5 or 1.0 ppm ozone in the present study were in the distribution and severity of lesions. The rats chronically exposed to 0.5 or 1.0 ppm ozone had a greater distribution of mucous cell metaplasia in the nasal passages than those acutely exposed to 0.8 ppm ozone. Metaplasia of nasal transitional epithelium was limited to the most proximal aspect of the nasal airways (only tissue block 1) in rats exposed for seven days. In contrast, mucous cell metaplasia was present in proximal, middle, and distal aspects of the nasal passages (tissue blocks 1, 2, and 3) after the 20-month exposure to 0.5 or 1.0 ppm ozone. In addition, the amount of intraepithelial mucosubstances in similar regions of nasal transitional epithelium in the proximal airways was 1.5 and 3 times greater in rats chronically exposed to 0.5 and 1.0 ppm ozone, respectively, in the present study compared with rats acutely exposed to 0.8 ppm ozone for seven days (six hours per day) in the previously reported study.

A similar morphologic change has been reported to occur in the nasal transitional epithelium of monkeys repeatedly exposed to ambient concentrations of ozone (0.15 and 0.3 ppm ozone; Harkema et al. 1987a). As in the rats exposed to 0.5 or 1.0 ppm ozone in the present study, there was a marked increase in mucous cells with increased amounts of intraepithelial mucosubstances in the nasal transitional epithelium of ozone-exposed monkeys (Harkema et al. 1987b), a species whose nasal anatomy resembles, at gross and microscopic levels, that of humans. The results of these animal studies suggest that a similar change could occur in the human nose after repeated exposure to ambient concentrations of ozone. Whether or not a similar morphologic change in the human nasal mucosa would result in functional abnormalities, as was documented in the ozone-exposed rats of our study, is unknown. More well-designed human and animal studies using similar exposure conditions and analyzing techniques are needed to better understand the pathogenesis of ozone nasal toxicity, and to develop good methods for establishing, on the basis of animal data, the risk ozone poses to humans. Advances in this area could be made if methods were developed to determine the dosimetry of ozone to specific nasal epithelia within human and animal airways.

The mucous cell metaplasia, along with the increase in intraepithelial mucosubstances, is probably an adaptive mechanism of the animal in an attempt to protect the underlying nasal tissues and more distal pulmonary tissues from further oxidant injury. It has been reported that air-

way mucus is an effective antioxidant (Cross et al. 1984). Therefore, more mucous cells contributing increased amounts of luminal mucous could significantly reduce the direct or indirect toxic effects of ozone on airway tissues.

Though mucous cell metaplasia in the nasal airways of rats exposed to 0.5 or 1.0 ppm ozone was extensive, additional histopathologic changes in the underlying lamina propria and bone suggest that the metaplasia was not fully protective. The moderate inflammatory cell influx (rhinitis) in the lamina propria of rats chronically exposed to 0.5 or 1.0 ppm ozone indicated ongoing cell injury. Another indicator of ongoing ozone injury in subepithelial tissues was the bony atrophy in maxilloturbinates and nasoturbinates of these same ozone-exposed rats. Loss of turbinate bone could lead to marked and permanent losses in the surface area of turbinates and subsequently alter nasal airway flow patterns, air filtration, and humidification of the inhaled air. To our knowledge this is the first study that has demonstrated that ozone exposures can alter the bony tissue of the nose. The reasons for this atrophy are not known, but may be related to the chronic inflammation in the adjacent lamina propria. Inflammatory mediators (prostaglandins, leukotrienes, and cytokines) from mononuclear inflammatory cells have been implicated in the pathogenesis of bone resorption (Robinson 1989).

Furthermore, the negative effects of the two higher concentrations of ozone on mucous flow rates indicate that the concomitant mucous cell metaplasia in the nasal transitional epithelium could not protect the rat from functional alterations and may have even contributed to this specific nasal dysfunction. Ozone was previously shown to decrease mucociliary function in the trachea after short-term exposures (Frager et al. 1979; Kenoyer et al. 1981), but this is the first study to demonstrate the detrimental effects of chronic ozone exposure on nasal mucociliary function. Marked decreases in mucous flow, as demonstrated in this study, could lead to significant alterations in nasal mucociliary clearance, an important upper respiratory tract mechanism that defends the lung from excessive burdens of harmful agents. It has been suggested that even minimal impairment of normal nasal air modification could increase toxic insult to lower airways and subsequently lead to chronic small airways diseases such as chronic bronchitis or emphysema (Proctor et al. 1978). The nasal damage induced by chronic ozone exposure could also be a factor in increasing susceptibility to acute infections of the upper respiratory tract. More studies comparing morphologic and physiologic responses of upper and lower respiratory tract epithelium to common inhaled pollutants are needed in order to evaluate and understand properly the impact of air pollution on human health.

The increase in eosinophilic globules (that is, dilated cisternae of agranular endoplasmic reticulum) in respiratory and olfactory epithelium has also been reported in mice and rats exposed to other irritating chemicals, like dimethylamine (Buckley et al. 1985). This alteration has not been previously reported in rats exposed to ozone, but has been observed in the nasal transitional epithelium and respiratory epithelium of monkeys exposed to 0.15 or 0.3 ppm ozone for 90 days, eight hours per day (Harkema et al. 1987a). The exact composition of the material within the cisternae has not yet been identified, but may be proteinaceous secretory material that accumulates in the cell. These globules have been observed in secretory cells of nasal respiratory epithelium (e.g., mucous cells) and olfactory epithelium (e.g., sustentacular cells) of monkeys after long-term repeated exposures to ozone. Harkema and co-workers (1987a) have suggested that these alterations in the endoplasmic reticulum may reflect a dysfunction in the biosynthetic or storage activity that could take the form of either a defect in transport from the rough endoplasmic reticulum to the Golgi complex or excessive protein production by the endoplasmic reticulum leading to increased cisternal storage. In addition, Lewis and associates (1994) recently reported that eosinophilic globules in the olfactory epithelium of F344 rats exposed to cigarette smoke contain at least one form of a xenobiotic metabolizing enzyme, carboxylesterase. Therefore, the induced increase in the number of eosinophilic globules in the olfactory epithelium of ozone-exposed rats may also reflect increased production of nasal enzymes used in metabolism of xenobiotics. Studies specifically designed to examine the content of the proteinaceous material within the eosinophilic globules induced by ozone and other inhaled toxicants are needed to elucidate the nature and significance of these structures.

In summary, chronic exposure of rats to 0.5 or 1.0 ppm ozone, but not to 0.12 ppm ozone, induced significant functional and structural alterations in the nasal mucociliary apparatus of both male and female F344/N rats. The nasal lesions had a regional distribution with the most severe alterations occurring in the more proximal aspects of the nasal passages, but also involving distal regions. The ozone-induced changes were concentration dependent with the most severe alterations occurring in rats exposed to 1.0 ppm ozone. Ozone-exposed animals had significant decreases in nasal mucous flow rates in the proximal nasal airways, along with histopathology in both males and females that included chronic rhinitis, mucous cell metaplasia, epithelial hyperplasia, and increases in intraepithelial eosinophilic globules. In addition, ozone-induced bony atrophy of turbinates was observed in rats exposed to 0.5 or 1.0 ppm ozone.

ACKNOWLEDGMENTS

The authors thank Ms. Lois Herrera and Ms. Pat Cossey of the Inhalation Toxicology Research Institute (ITRI) for their excellent technical assistance in the morphometric analyses and Mr. Donald Joyner of the Chemical Industry Institute of Toxicology (CIIT) for his assistance in the analysis of mucous flow rates. We also thank Ms. Paula Bradley for her editorial assistance in the preparation of this manuscript. In addition, we thank many of our colleagues at ITRI and CIIT for reviewing this report and supplying helpful comments and suggestions. This work was part of the NTP/HEI Collaborative Ozone Project and was supported by the HEI under the Funds-in-Agreement DE-FI04-91AL75007, and by the U.S. Department of Energy/Office of Health and Environmental Research under Contract No. DE-AC04-76EV01013.

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ABOUT THE AUTHORS

Jack R. Harkema, D.V.M., Ph.D., is presently Professor of Pathology at Michigan State University, College of Veterinary Medicine, in East Lansing, MI. Dr. Harkema was an experimental pathologist and manager of the Pathogenesis Program at the Inhalation Toxicology Research Institute in Albuquerque, NM, from 1985 to 1994. He received his Ph.D. in comparative pathology from the University of California at Davis. He is a diplomate of the American College of Veterinary Pathologists.

Kevin T. Morgan, B.V.Sc., Ph.D., is an experimental pathologist at the Chemical Industry Institute of Toxicology in Research Triangle Park, NC. He received his Ph.D. in veterinary pathology from the University of Edinburgh,

APPENDIX A. Identification of Specific Animals in Exposure Groups

Table A.1. Specific Animals Studied

| Ozone Exposure (ppm) | Gender | Identification Numbers for Specific Animals | |
|----------------------|--------|---|---|
| | | Functional (Mucous Flow) and Histochemical (Mucous Morphometry) Studies | Structural Studies (with Electron Microscopy) |
| 0 | Male | H16, H28, H34, H85, H133, H149, H161 | H37, H117, H125 |
| 0.12 | Male | H134, H150, H162 | H118, H126 |
| 0.5 | Male | H17, H23, H29, H35, H87 | H119, H127 |
| 1.0 | Male | H18, H24, H30, H36, H88, H164 | H120, H128 |
| 0 | Female | H4, H10, H19, H31, H89, H113, H137, H138 | H121, H129 |
| 0.12 | Female | H38, H154 | H122, H130 |
| 0.5 | Female | H5, H11, H32, H91, H111, H115, H155 | H43, H123, H131 |
| 1.0 | Female | H6, H12, H21, H33, H92, H112, H116, H140, H156 | H124, H132 |

Scotland. He is a diplomate of the American College of Veterinary Pathologists, and a member of the Royal College of Pathologists.

Elizabeth A. Gross is a senior research associate at the Chemical Industry Institute of Toxicology in Research Triangle Park, NC, and a Laboratory Supervisor in the Department of Experimental Pathology and Toxicology at CIIT.

Paul J. Catalano, Ph.D., is an Assistant Professor of Biostatistics at the Harvard School of Public Health and the Dana-Farber Cancer Institute. He received a doctoral degree in biostatistics from the Harvard School of Public Health in 1991. His research interests focus on development and application of statistical methodology to environmental problems including multivariate modeling, analysis of animal bioassay data, and methods for risk assessment. Dr. Catalano is also involved in the statistical design and analysis of cancer clinical trials through the Eastern Cooperative Oncology Group.

Willim C. Griffith, M.S. is a biomathematician and biostatistician at the Inhalation Toxicology Research Institute in Albuquerque, NM.

PUBLICATIONS RESULTING FROM THIS RESEARCH

Harkema JR, Hotchkiss JA. 1994. Ozone-induced proliferative and metaplastic lesions in nasal transitional and respiratory epithelium: Comparative pathology. *Inhalation Toxicol* 6(Suppl):187-204.

ABBREVIATIONS

| | |
|-----------------|---|
| AB/PAS | Alcian blue (pH 2.5)/periodic acid-Schiff |
| CIIT | Chemical Industry Institute of Toxicology |
| ITRI | Inhalation Toxicology Research Institute |
| NAAQS | National Ambient Air Quality Standards |
| NTP | National Toxicology Program |
| ¹⁸ O | oxygen-18 |
| ppm | parts per million |
| SEM | standard error of the mean |
| TEM | transmission electron microscopy |

INTRODUCTION

Ozone is a highly reactive gas that is found in two regions of the atmosphere. In the stratosphere, miles above the earth's surface, ozone provides a protective shield by filtering out the sun's harmful ultraviolet radiation. In the troposphere, the atmosphere immediately surrounding the earth's surface, ozone is an ambient air pollutant to which people, agricultural crops, forests, and ecosystems are exposed (McKee 1993).

Whether prolonged exposure to ozone contributes to or exacerbates chronic lung diseases is uncertain (reviewed by Lippmann 1992; U.S. Environmental Protection Agency 1993). The widespread exposure of the population to this pollutant in many areas of the United States led the Health Effects Institute (HEI) and the National Toxicology Program (NTP)* to enter into a collaborative agreement (described in HEI Research Report Number 65 Part I) to evaluate how laboratory rats may be affected by long-term exposure to low, medium, and high doses of ozone.

The investigators participating in the HEI-funded research were selected through a competitive, peer-review process in response to RFA 90-1, "Effects of Chronic Ozone Inhalation: Collaborative National Toxicology Program-Health Effects Institute Studies." In response to Part B of this RFA, "Structural, Biochemical, and Other Alterations", Dr. Jack Harkema (then at the Inhalation Toxicology Research Institute, Lovelace Biomedical and Environmental Institute, Albuquerque, NM, and presently at Michigan State University) submitted a proposal entitled "Effects of Chronic Ozone Exposure on the Nasal Mucociliary Apparatus in the Rat." Because there is little information about the effects of prolonged ozone exposure on the nose, this application addressed an important health concern. The nasal region of the respiratory system is the first line of defense against inhaled pathogens, dusts, or irritant gases; therefore, changes in its normal defense capabilities could lead to increased nasal infections and increased susceptibility to lower respiratory diseases.

Dr. Harkema's one-year study began on September 18, 1991. Total expenditures were \$82,036. The Investigators' Report was received at HEI in August 1993; a Revised Report, received in June 1994, was accepted by the Health Review Committee in July 1994. During the review of the Investigators' Report, the Review Committee and the inves-

tigators had the opportunity to exchange comments and to clarify issues in the Investigators' Report and in the Health Review Committee's Commentary. The following Commentary is intended to aid the sponsors of HEI and the public by highlighting both the strengths and limitations of the study and by placing the Investigators' Report in scientific and regulatory perspective.

REGULATORY BACKGROUND

The U.S. Environmental Protection Agency (EPA) sets standards for oxidants (and other pollutants) under Section 202 of the Clean Air Act, as amended in 1990. Section 202(a)(1) directs the Administrator to "prescribe (and from time to time revise) . . . standards applicable to the emission of any air pollutant from any class or classes of new motor vehicles or new motor vehicle engines, which in his judgment cause, or contribute to, air pollution which may reasonably be anticipated to endanger public health or welfare." Sections 202(a), (b)(1), (g), and (h) and Sections 207(c)(4)-(6) impose specific requirements for reductions in motor vehicle emissions of certain oxidants and other pollutants. In some cases they provide the EPA with limited discretion to modify those requirements.

Section 109 of the Clean Air Act provides for the establishment of National Ambient Air Quality Standards (NAAQS) to protect the public health. Ozone's potentially harmful effects on respiratory function led the United States EPA to promulgate an NAAQS for ozone of 0.12 parts per million (ppm), a level not to be exceeded for more than one hour once per year. Section 181 of the Act classifies the 1989 nonattainment areas according to the degree that they exceed the NAAQS, and assigns a primary standard attainment date for each classification.

The current ozone standard relies heavily on data derived from controlled exposure studies in which human subjects experienced lung dysfunction after a short-term exposure to ozone while exercising. These studies did not address the possibility that long-term exposure to ozone may cause health effects. Determining the appropriate standards for emissions of oxidants and their precursors depends, in part, on an assessment of their risks to health. Research studies of the effects of long-term exposure of the airways to ozone, such as those supported by the NTP/HEI collaborative agreement, are therefore essential to the informed regulatory decision-making required by the Clean Air Act.

* A list of abbreviations appears at the end of the Investigators' Report for your reference.

SCIENTIFIC BACKGROUND

Ozone is a highly reactive oxidant gas that is created in polluted air by photochemical processes. In laboratory animals, inhaling ozone can cause injury in at least three target areas: the nasal cavity, the trachea, and the centriacinar region of the lung (the junction between the tracheobronchial conducting airways and the alveolar gas exchange area) (Plopper et al. 1991; Lippmann 1992, 1993).

Although the nasal cavity absorbs a major fraction of the inhaled ozone (Yokoyama and Frank 1972; Miller et al. 1979; Ultman et al. 1994), ozone's effects on lung structure and function have been more extensively studied than its effects on the nasal cavity. Dr. Harkema and colleagues provided important information by assessing the effects of ozone on the cells that line the nasal cavity and on the mucociliary clearance process. This section describes these two nasal defense mechanisms against inhaled toxicants and discusses what is known about ozone's effects on the nasal passages from studies of acute and subchronic exposure to ozone.

PROTECTIVE ROLE OF MUCUS

The epithelial cells that line the luminal surface of the nasal cavity are damaged by inhaled toxicants (Harkema 1991). Four distinct nasal epithelial cell populations are contained in the luminal surface: squamous epithelium, respiratory epithelium, transitional epithelium, and olfactory epithelium. They differ in their structure and in the types of cells within each population. Much of the surface epithelium is covered with a thin layer of mucus, which protects it from inhaled microorganisms, other particulate matter, and gaseous air pollutants. Microorganisms and other particles are trapped in the mucous layer and soluble gases dissolve in the mucus (reviewed by Hatch 1991).

Mucus is a complex mixture of water, glycoconjugates (glycoproteins and proteoglycans that contain carbohydrates), proteins, lipids, inorganic ions, low-molecular-weight organic compounds, and, in some cases, DNA (Sleigh et al. 1988). Mucus is produced by epithelial secretory (serous and mucous) cells.

Mucus operates through several mechanisms to protect the underlying epithelium from injury induced by toxicants. First, mucous proteins can inactivate certain bacterial, viral, and fungal pathogens. Second, mucous constituents can react with inhaled pollutants and detoxify them before they reach the underlying epithelium. For example, antioxidants can scavenge inhaled oxidants. Third, inhaled toxicants, trapped or dissolved in mucus, can be removed by the flow of mucus caused by the beating cilia, a mechanical process called

mucociliary transport. Cilia, which are hair-like projections on the surface of certain epithelial cells, provide a cyclic back-and-forth force that propels mucus through the nasopharynx, after which it is swallowed.

The viscous and elastic properties of the mucus overlying the cilia contribute to effective mucociliary transport (Sleigh et al. 1988; Wolff 1991; Samet and Cheng 1994). The viscosity of mucus is essential for its ability to trap and retain foreign particles; elasticity contributes to the efficiency of the rate of mucous transport. Researchers believe that glycoproteins are the most important components that confer viscoelastic properties to mucus (Sleigh et al. 1988; Rose 1992; Samet and Cheng 1994). (The term "viscoelasticity" denotes a viscous material that also exhibits elastic properties, such as the ability to store energy when it becomes deformed.) The viscosity of mucus decreases as force is applied (a property called non-Newtonian viscosity). Therefore, the more forcefully the cilia beat, the more easily mucus flows. The force generated by ciliary beating causes mucus to deform (stretch); however, its elasticity allows it to store the energy transferred from the beating cilia and use it to recoil to its original shape.

Although mucus is essential for proper nasal function, its overproduction can alter nasal properties. The hypersecretion of mucus can be induced by inhaling ammonia, ozone, and certain dusts (reviewed by Samet and Cheng 1994). One possible mechanism for this response is an increase in the number of mucus-producing cells, which can be caused by (1) the existing mucus-producing cells proliferating abnormally, or (2) the nonsecreting cells transforming into mucus-secreting cells, or both. For example, inhaling elevated concentrations of sulfur dioxide, nitrogen dioxide, ozone, or cigarette smoke can cause an increase in the number of airway cells. Other mechanisms for hypersecretion of mucus include an increased release of existing intracellular glycoproteins and an elevated rate of glycoprotein biosynthesis by cells in the submucosal glands (reviewed by Samet and Cheng 1994).

EFFECTS OF OZONE ON NASAL STRUCTURE AND FUNCTION

Until recently there had been few studies of ozone's effects on the nasal passages. Dr. Harkema and colleagues (1987a,b) previously reported that monkeys exposed to 0.15 ppm or 0.3 ppm ozone for 6 or 90 days showed cell damage in the transitional and respiratory epithelia. Both types of epithelia showed a marked increase in the number of mucous cells; however, the amount of intraepithelial mucous components increased only in the transitional epithelium. Interestingly, the increase in mucous cell number depended

on the duration of exposure, rather than the ozone level. The investigators also observed some ciliary cell necrosis, and many remaining cilia that were shortened after both 6 and 90 days of ozone exposure. Thus, in the primate model, ozone exposure altered two components (mucus and cilia) that are essential for the proper functioning of the mucociliary clearance system. Dr. Harkema and colleagues (1989) later demonstrated that rats exposed for one week to 0.8 ppm ozone for six hours per day showed an increased number of mucous cells in the transitional epithelium, but not in the respiratory epithelium.

Recently, Calderon-Garcidueñas and associates (1992) provided evidence for an effect of ozone exposure on human nasal epithelial tissue. These investigators reported that residents of the southwestern section of Mexico City (where ozone levels range from 0.1 ppm to 0.4 ppm for several hours a day all year long) showed marked changes in their nasal epithelium compared with residents of Veracruz, a Mexican city with low ozone levels. The changes included mild to moderate epithelial dysplasia (abnormal tissue development), a severe loss of normal respiratory epithelium, an increased number of basal cells, and abnormal changes in squamous cells. Ozone is the major oxidant gas in Mexico City's photochemical smog; however, high levels of sulfur dioxide and elevated levels of nitrate- and sulfate-containing suspended particles are also present. The present study by Dr. Harkema and his associates provides information on structural and chemical changes in the rat nasal cavity caused by prolonged exposure to ozone. It also describes, for the first time, ozone's effect on mucociliary function.

JUSTIFICATION FOR THE STUDY

The Health Effects Institute's primary objectives for RFA 90-1 were to support biochemical, structural, and functional studies to determine whether prolonged inhalation of ozone would cause changes in the respiratory system of rats that might potentially be related to chronic lung disease in humans.

Dr. Jack Harkema had extensive experience in morphometric and structural analyses of nasal tissue. Dr. Kevin Morgan (of the Chemical Industry Institute of Toxicology), had developed sensitive methods to assess nasal mucociliary function. Thus, these two investigators had the appropriate skills and experience to conduct the proposed studies. Although it had been established that acute and subchronic exposures to ozone caused changes in the nasal epithelium of laboratory animals, it was not known whether these changes progressed over time, or if they correlated with changes in mucociliary clearance. Thus, the HEI Research Committee thought that

Dr. Harkema's proposal met the goals of the RFA and would complement the other studies in the NTP/HEI Collaborative Ozone Project.

TECHNICAL EVALUATION

STUDY OBJECTIVES

The objectives of the study were:

1. To determine the nature and magnitude of the structural changes in the rat nasal mucosa after prolonged inhalation of ozone, and to correlate these findings with ozone's effects on mucociliary function.
2. To characterize the alterations induced by ozone in the nasal epithelium, using morphometric techniques, and to compare them with alterations in the epithelium that lines the lower respiratory tract, which other investigators in the NTP/HEI Collaborative Ozone Project would be analyzing.

The investigators successfully accomplished each objective. Because of methodological problems (described in the Investigators' Report), data collection for the mucous flow studies was incomplete; however, the investigators obtained sufficient data to allow a correlation to be established between the functional changes and the structural changes caused by ozone exposure. A comparison of the nasal changes with the changes found in the lower airways by other investigators will be described in the Integrative Summary that forms Part X of Research Report Number 65.

METHODS AND STUDY DESIGN

The study was carried out on male and female rats killed approximately one week after a 20-month exposure to 0.0, 0.12, 0.5, or 1.0 ppm ozone. Details of the exposure protocol can be found in Part VI of Research Report Number 65. The one-week waiting period allowed transient effects to disappear, thereby emphasizing the long-term changes. Control animals breathed filtered air. The inhalation component of this project was conducted at the Battelle Pacific Northwest Laboratory in compliance with the NTP laboratory health and safety regulations, and with the Food and Drug Administration Good Laboratory Practice Regulations. Animal care and use were in accordance with the Public Health Service Policy on Humane Care and Use of Animals.

A limiting feature of the study design, which was not under the investigators' control, was the requirement that ozone exposures be carried out during the day when rats are inactive. The possible ramifications of this design aspect are discussed below in the section on Results and Interpretations.

Functional Studies

Dr. Morgan and his associates used in vitro video motion analysis to map and quantify changes induced by ozone in the mucous flow rates at 13 sites in the nasal passages. The location of these sites (see Figure 1 in the Investigators' Report) and the video microscopic technique are described by the Investigators. They also examined changes in mucous flow patterns and the presence or absence of ciliary beating. Dr. Morgan and his colleagues have been leaders in developing the use of in vitro video microscopy to quantitatively analyze mucous flow rates by timing the movement of particles in the mucus over a calibrated distance (Morgan et al. 1984). Because mucus continues to flow in rats for only 20 to 30 minutes after death, the investigators assessed mucociliary function as rapidly as possible after the rats were killed.

Structural Studies

Dr. Harkema and colleagues used a combination of standard and state-of-the-art methods to assess structural changes in the nasal mucosa. The morphometric and structural analyses were done thoroughly. Of the 13 sites selected for functional analysis of mucous flow rates, Dr. Harkema and colleagues selected three for morphometric measurements of the amount of mucosubstances stored in the epithelial cells. They used specific histochemical stains to detect intraepithelial cell mucosubstances, and image analysis to quantify their levels. For the remaining 10 sites, the investigators used histopathologic techniques and light microscopy to examine the nasal mucosa for structural alterations. The investigators successfully used quantitative morphometric techniques to determine the precise changes in the cell populations in the transitional and respiratory epithelia caused by ozone exposure.

STATISTICAL METHODS

The investigators used linear regression for analysis of trends and *t* tests to compare each ozone exposure group to the control group to assess the statistical significance of the differences in the mucous flow rates. Dunnett's criterion for comparing several exposure groups to the control group was used to account for multiple comparisons. Fisher's exact test was used to analyze flow incidence data for all exposure groups and the control group both simultaneously and separately in pair-wise comparisons of each exposure concentration versus 0.0 ppm ozone. Bonferroni adjustments were used to allow for multiple comparisons in the pair-wise incidence tests.

The investigators used natural logarithms of the morphometric data for statistical analyses. Data were tested for equality of group means by an unpaired Student's *t* test with the Bonferroni correction for multiple analyses.

The statistical methods selected were, in general, appropriate for the hypotheses to be tested. The basic format for all analyses was to compare the measurements of changes in nasal parameters in response to increasing concentrations of ozone exposure with the measurements of the same parameters in the control group of animals exposed to clean air. The Dunnett criterion, the Fisher Exact test, and the Bonferroni adjustment were each used appropriately, but not consistently. For example, it is not clear why the investigators applied linear regression to analyze mucous flow rates but not morphometric changes. In addition, the morphometric analyses consisted of paired *t* tests with the Bonferroni adjustment instead of the Dunnett criterion that was used to analyze mucous flow rates. The investigators used multiple testing to analyze their data on ozone-induced changes in mucous flow rates at 13 areas of the nasal passages. When they analyzed their data for mucous flow at each region, they controlled for Type I errors by using Dunnett's procedure; however, there was no statistical control across the regions. Thus, the possibility arises that spurious results could appear to be statistically significant. For example, the investigators state that the increase in mucous flow in one region in rats exposed to 0.12 ppm ozone was statistically significant. Although this may be true, the result also could be a chance occurrence in the context of multiple testing.

RESULTS AND INTERPRETATIONS

This section highlights the major findings of the functional and structural studies. For a full discussion of detailed structural and ultrastructural findings at various anatomical sites within the nasal passages, refer to the Investigators' Report.

Functional Studies

Rats exposed to 0.5 and 1.0 ppm ozone showed consistent dose-dependent decreases in mucous flow rates at 6 of 13 sites tested in the nasal passages. Differences were also noted in the character of the mucus and in the increases in the amount of mucus in the nasal tissue prepared from rats exposed to 0.5 and 1.0 ppm ozone compared with tissues from control animals.

The impairment of mucous flow varied according to anatomic location; the most pronounced changes occurred in the anterior parts of the nasal lateral wall. For example, after exposure to 1.0 ppm ozone, flow rates at two regions in this area were markedly reduced by 11%-14% of the flow

rates in control rats. At some sites, both mucous flow and ciliary activity decreased; however, sites with reduced mucous flow were more extensive than sites with decreased ciliary activity. Reduced mucous flow rates at sites with an active ciliary beat may have been caused by the ozone-induced elevation in intraepithelial mucosubstances (discussed below), which could have increased the viscoelastic properties of the mucus that lines the surface epithelium of the nasal airways. Because the investigators did not find structural alterations in the cilia, changes in the composition of the mucus in rats exposed to 0.5 ppm or 1.0 ppm ozone also may have contributed to decreased ciliary activity. Possible consequences of impaired nasal mucociliary clearance are an increase in nasal infections and an elevated burden of airborne pollutants in the lower airways.

In contrast to the findings at the two highest ozone concentrations, no changes were noted in the mucous flow rates in nasal tissue from rats exposed to 0.12 ppm ozone, except for a small increase in mucous flow rate in one region of the nasal passages. The relevance of this observation is questionable because the sample size was small, the observed change was opposite to that found at the two higher ozone concentrations, and a statistically significant increase occurred at only 1 of the 13 regions examined.

Structural Studies

Marked structural alterations were found in the nasal mucosa at three sites in the proximal and middle regions of the nasal passages of male and female rats exposed to 1.0 ppm ozone compared with the same sites in rats breathing clean air. Similar but less severe alterations were seen in rats exposed to 0.5 ppm ozone. No structural changes were seen in nasal tissue from rats exposed to 0.12 ppm ozone for 20 months.

The most severe structural alterations appeared in the transitional epithelium of rats exposed to 1.0 ppm ozone. These alterations included mucous cell metaplasia (the transformation of an epithelium with no mucous cells into an epithelium with numerous mucous cells) and epithelial hyperplasia (an increase in the total number of epithelial cells). Histochemical and image analysis indicated that the levels of mucosubstances in cells of the transitional epithelium were much higher than in the same cells from control rats. Epithelial cell hyperplasia was not a consistent feature of the transitional epithelium of rats exposed to 0.5 ppm ozone.

Dr. Harkema and colleagues reported that the changes induced by ozone in the respiratory epithelium were less severe than those in the transitional epithelium. The major change in the respiratory epithelial layer was hyperplasia at sites in the proximal nasal passages of rats exposed to 1.0 ppm ozone.

The investigators observed a mixture of inflammatory cells at sites in the proximal half of the nasal airways in rats exposed to 0.5 or 1.0 ppm ozone. Adjacent sites also showed bony atrophy, characterized by bone resorption, which was attributed to the chronic inflammation in this area. Bone resorption could alter nasal airway flow patterns, air filtration, and the humidification of inhaled air.

Mucous hypersecretion is considered to be an adaptive mechanism that protects the underlying epithelium from ozone. However, mucous hypersecretion clearly did not protect the structure and function of the rat nose, as evidenced by reduced mucous flow rates, injury to the transitional and respiratory epithelia, and the appearance of bony atrophy. Instead, mucous hypersecretion may have contributed to these conditions by altering the properties of the mucus produced in response to ozone exposure.

In summary, the results of the functional and structural studies indicate that the cells lining the nasal passages are sensitive to injury induced by ozone. In fact, of the eight studies comprising the NTP/HEI Collaborative Ozone Project, the most dramatic effects of prolonged ozone exposure were observed in the nose. Furthermore, the results suggest that a threshold of ozone toxicity for the nasal region of F344/N rats may lie between 0.12 ppm ozone (where no effects were evident) and 0.5 ppm ozone.

Although there were clear dose-related changes in nasal function and structure in rats exposed to 0.5 ppm and 1.0 ppm ozone, extrapolation of these findings to humans is difficult. First, the gross architecture of rodent and primate nasal passages is different, and is chiefly related to the structure and number of nasal turbinates (reviewed by Harkema 1991). (Turbinates are structures that comprise the primary air filtration network in the nasal cavity and project from the nasal walls). The number of turbinates, their extent within the nasal cavity, the number of branches, and their overall complexity all vary. Rats have a complex turbinate structure; in contrast, the turbinate structure is minimal in primates (Plopper et al. 1991). Thus, the amount of ozone removed from incoming air can differ markedly between rodent and primate nasal passages. Second, the distribution of the various epithelial cell populations that line the nasal passages differs among species (Harkema 1991). Because ozone can injure epithelial populations to different degrees (for example, transitional and respiratory epithelium in this study), it is important to identify the extent of the various epithelial populations in the nose of both species. Third, measurements of mucous flow in healthy humans is complicated by a high degree of variability among individual subjects (D. Proctor, personal communication). Finally, extrapolating the study's results from rats to humans is complicated by the fact that the ozone exposures were

carried out when the rats, which are nocturnal animals, were relatively inactive. Thus, these results may not apply to people exercising in ambient air polluted with ozone, because exercise increases the effective dose to the respiratory tract.

IMPLICATIONS FOR FUTURE RESEARCH

The finding that the nasal region of the rat is sensitive to prolonged ozone exposure raises questions about human sensitivity. At the present time we lack sufficient information to predict whether the rat is more or less sensitive than humans. Because rodents are obligate nose breathers and primates are oronasal breathers, exposing both species to similar levels of ozone might result in a greater dose to rodents. However, the results of Plopper and colleagues (1991) suggest that primates, such as bonnet monkeys, are more sensitive than rodents to ozone's toxic effects on nasal epithelia. One explanation is the possibility that rodents may have evolved better defense mechanisms (for example, an increased level of mucous antioxidants) than other species in order to resist exposure to oxidants or other toxic gases. Possible differences in species susceptibility to ozone indicate the need to determine the extent and severity of injury that prolonged exposure to ambient levels of ozone may cause to the primate nose. Studies comparing ozone's effects on primate and rodent nasal tissue using similar experimental protocols, combined with mechanistic studies, could provide information that would allow us to draw inferences about human health effects of ozone exposure from the results of this study.

Another issue that should be addressed in future studies is whether a different ozone exposure profile would have altered the results. Because ambient concentrations of ozone fluctuate, recovery of mucociliary function could occur when ozone levels decrease. For example, it is known that ciliary and epithelial functions, as well as local defense mechanisms, are restored after epithelial injury. Thus, it is possible that using an exposure protocol that simulates ambient urban ozone time and dose profiles may lead to different results. Finally, improved methods for measuring the actual dose received by the respiratory tract are needed.

CONCLUSIONS

This study by Drs. Harkema, Morgan, and colleagues provides important data on the effects of prolonged ozone exposure on nasal function and structure in the laboratory rat, an area that previously lacked critical study. A striking

observation was the greater number of abnormalities found in the nose compared with those found in the lower airways of this rat strain.

Prolonged inhalation of 0.12 ppm ozone did not affect nasal function or structure in this strain of rat. However, there were clear dose-dependent reductions in mucous flow rate in rats exposed to 0.5 ppm or 1.0 ppm ozone for 20 months, compared with control rats breathing filtered air. Ciliary beat also decreased; however, the number of sites with reduced ciliary beat were fewer than those with reduced mucous flow rates. Certain anatomic sites in the rat nasal cavity were more susceptible to ozone-induced injury than others; thus, dose and susceptibility at specific sites contributed to the changes that caused reduced mucous flow rates.

Exposures for 20 months to 0.5 or 1.0 ppm ozone induced structural alterations in the proximal and middle regions of rat nasal passages that were more evident in the transitional epithelium than in the respiratory epithelium. An increased number of cells that produce mucus and the elevated amount of intraepithelial mucosubstances could explain the decreases in both mucous flow rate and ciliary beat that were observed in the functional analyses. Thus, the cost of protecting nasal tissue from ozone injury by increasing mucous production may have been decreased mucous flow.

Rats exposed to 0.5 ppm or 1.0 ppm ozone developed a mild to moderate inflammatory cell influx (rhinitis) in the proximal and middle nasal passages, indicating ongoing cell injury. Chronic inflammation may have caused the bony atrophy seen in adjacent regions of the nasal passages of rats exposed to 0.5 ppm and 1.0 ppm ozone. No neoplastic changes were reported at any of the ozone exposure levels.

The concordance of levels of ozone injury by both functional and structural assessments strengthens the conclusion that the threshold of toxicity in the nasal mucosa of the F344/N rat after prolonged exposure lies between 0.12 ppm and 0.50 ppm ozone. Furthermore, these data will allow direct comparison of ozone's effects on the nasal mucosa with those found in lower respiratory mucosa (Chang et al. 1994; Pinkerton et al. 1994; Integrative Summary Report 1994).

It is difficult to extrapolate the results of this study to humans because of species differences in nasal architecture and possible differences in susceptible epithelial cell populations. However, the fact that ozone can damage epithelial cells lining the rat nasal passages and cause a decrease in mucous flow rates has implications for mucociliary clearance. This is an important defense mechanism that protects the upper respiratory tract against infection and aids in protecting the lung from harmful agents.

ACKNOWLEDGMENTS

The Review Committee would like to thank the ad hoc reviewers for their help in evaluating the scientific merit of the Investigators' Report, and Dr. Bernard Jacobson for assisting the Committee in preparing its Commentary. The Committee also acknowledges Virgi Hepner and Valerie Carr for overseeing the publication of the report and Diane Foster and Mary Stilwell for their editorial and administrative support.

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